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The synthesis of ATP by glycolytic enzymes in the postsynaptic density and the effect of endogenously generated nitric oxide

(glycolysis)

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ABSTRACT **The major contribution of this paper is the finding of a glycolytic source of ATP in the isolated postsynaptic density (PSD). The enzymes involved in the generation of ATP are glyceraldehyde-3-phosphate dehydrogenase (G3PD) and phosphoglycerate kinase (PGK). Lactate dehydrogenase (LDH) is available for the regeneration of NAD^+, as well as aldolase for the regeneration of glyceraldehyde-3-phosphate (G3P). The ATP was shown to be used by the PSD Ca^{2+}/calmodulin-dependent protein kinase and can probably be used by two other PSD kinases, protein kinase A and protein kinase C. We confirmed by immunocytochemistry the presence of G3PD in the PSD and its binding to actin. Also present in the PSD is NO synthase, the source of NO. NO increases the binding of NAD, a G3PD cofactor, to G3PD and inhibits its activity as also found by others. The increased NAD binding resulted in an increase in G3PD binding to actin. We confirmed the autophosphorylation of G3PD by ATP, and further found that this procedure also increased the binding of G3PD to actin. ATP and NO are connected in that the formation of NO from NOS at the PSD resulted, in the presence of NAD, in a decrease of ATP formation in the PSD. In the discussion, we raise the possible roles of G3PD and of ATP in protein synthesis at the PSD, the regulation by NO, as well as the overall regulatory role of the PSD complex in synaptic transmission.**

The experiments in this paper are a continuation of our work on postsynaptic density (PSD) and an elaboration of the possible role of the PSD in synaptic function. The PSD organelle is a dense concentration of proteins attached to the cellular surface of the postsynaptic membrane in dendritic spine heads; it contains receptors for some, and possibly all, of the neurotransmitters, plus channels for Ca^{2+}, K^+, and possibly other relevant ions, plus three protein kinases and their substrates (for reviews, see refs. 1 and 2). The particular set of experiments described here were generated by three papers: first and second, the discovery by Walsh and Kuruc (3) by using gel electrophoresis and Rogalski-Wilk and Cohen (4) by using specific antibody that glyceraldehyde-3-phosphate dehydrogenase (G3PD) is found in the isolated PSD and that it is bound to the PSD protein, actin (4); and third, the finding by Aoki *et al.* (5) using immunocytochemistry that NO synthase (NOS), the enzyme producing NO from L-arginine, is also present in the PSD as well as other neuronal sites. Furthermore, a linkage between G3PD and NOS was shown by the publication in 1992 of three papers wherein NO was found to stimulate a so-called ADP ribosylation of G3PD in brain (6), platelets (7), and erythrocytes (8) (see ref. 9), though later papers (10, 11) indicated that NO stimulates the linkage of NAD to purified G3PD, probably to a cysteine residue in G3PD (12), and not the ADP ribosylation *per se*. In this paper, we confirm the presence of the G3PD in the PSD, show that its response to NO is similar to that found from other sources, and indicate factors that regulate G3PD binding to actin. Furthermore, we demonstrate that G3PD and other glycolytic enzymes are present in the PSDs and are capable of glycolytic metabolism and synthesis of ATP.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (6000 Ci/mmol; 1 Ci = 37 GBq), [adenylate-^{32}P]NAD (30 Ci/mmol), and [^{32}P]orthophosphate (^{32}Pi) (2000 Ci/mmol) were purchased from Amersham. The following compounds were products of Sigma: G-actin; porcine muscle G3PD; sodium nitroprusside (SNP), a source of NO (13); calmodulin (CaM); L-arginine; *N*-*L*-arginine-methyl ester (*N*-LAME), specific inhibitor of NOS (14); microcystin-LR, a potent inhibitor of protein phosphatase type 1 and type 2A (15); and leupeptin, inhibitor of Ca^{2+}-activated proteases (16, 17). *S*,*S*′-dinitrosodithiol (SND), another source of NO (18), was obtained from Calbiochem. Affinity purified rabbit anti-G3PD antibodies were prepared and characterized (19) by injecting purified human red cell G3PD into rabbits. LDH, hexokinase and phosphoglycerate kinase (PGK) were products of Worthington. Other chemicals were of reagent grade from commercial sources.

Electrophoresis. In the following experiments, SDS/PAGE was carried out according to ref. 20. All experiments were performed three times. Inter-experimental variations in relative band intensities were less than 10%.

Subcellular Fractions. Adult porcine brain, obtained from a local slaughter house, was dissected, and the cerebral cortex was employed for the studies illustrated in the figures. In some cases, rat cerebral cortex was also used. Synaptosome, synaptic membrane, PSD fractions (21), and a crude synaptic vesicle (CSV) fraction (22) were all isolated as described, with the CSV fraction supplied by R. S. Cohen (University of Illinois, Chicago).

Determination of the Activities of Glycolytic Enzymes in the Isolated PSD Fractions. The activities of G3PD (23), lactate dehydrogenase (LDH) (24), PGK (25), and hexokinase (26) were all determined as described. Enzyme levels in the PSD fractions were estimated by comparing the PSD enzyme activities to those of the purified enzymes and converting to specific units or amounts (units or μg per mg PSD).

NO-Stimulated [Adenylate-^{32}P]NAD Incorporation into G3PD in Subcellular Fractions Isolated from Adult Porcine

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Abbreviations: CaM, calmodulin; G3P, glyceraldehyde-3 phosphate; G3PD, glyceraldehyde-3-phosphate dehydrogenase; LDH, lactate dehydrogenase; PGK, phosphoglycerate kinase; NOS, NO synthase; PSD, postsynaptic density; SNP, sodium nitroprusside; *N*-LAME, *N*-*L*-arginine-methyl ester; SND, *S*,*S*′-dinitrosodithiol; mPSDp, major PSD protein.

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Cerebral Cortex. NO-dependent NAD incorporation into G3PD was performed according to ref. 6. Western blot analysis of G3PD was performed as described (27) with the exception that anti-G3PD was the primary antibody (0.5 μg/ml).

Assays of Endogenous Enzymatic NOS Activity in the Porcine Cortical PSD Fractions. The endogenous enzymatic NOS activity in the PSD was determined essentially according to ref. 28 by the ability of the enzyme to catalyze the formation of NO from L-arginine, the NO being measured by the NO-stimulated [adenylate-^{32}P]NAD incorporation into the G3PD (6). PSD (100 μg) in 100 μl final volume were preincubated at 37°C for 3 min (28), and then for 15 min with the addition of radiolabeled NAD, as noted (6). To define enzyme specificity, the reaction mixtures were preincubated at 37°C for 3 min with a specific NOS inhibitor (14), *N*-LAME (0.5 mg/ml), followed by addition of radiolabeled NAD, as above.

Binding of G3PD to Actin. Binding of purified G3PD to purified actin was measured by using the centrifugation procedure of Walsh and Knull (29) with the following modifications: 3.5% instead of 14% poly(ethylene glycol) (PEG8000) and 40-min incubation times for the binding.

Effects of Exogenous NO-Induced NAD Incorporation into G3PD on Subsequent Binding of the G3PD to Actin. G3PD (100 μg) was subjected to NO-stimulated NAD incorporation (6) by using 1 mM SNP or 0.1 mM SND as exogenous sources of NO. The mixtures were then dialyzed at 4°C against 1 liter of double distilled water (three changes, 6 h each). Binding of G3PD to actin was analyzed by the centrifugation method (29).

Autophosphorylation of Purified G3PD. Autophosphorylation of G3PD by ATP was determined according to ref. 30. G3PD (200 μg/ml) was incubated at 30°C for 10 min without (control) and with 1 mM ATP or 1 mM [γ-^{32}P]ATP (50–100 μCi/ml) in a phosphorylation buffer containing 10 mM imidazole (pH 7.0), 10 mM sodium acetate, 1 mM magnesium acetate, 1 mM DTT, and 0.1 mM EGTA. The mixtures were dialyzed against double distilled water. The G3PD phosphorylated by ATP was subsequently used in the actin binding experiments, as given above.

Effects of Autophosphorylation of Purified G3PD on Subsequent Binding to Purified Actin. Five micrograms each of control or phosphorylated G3PD proteins as obtained above were incubated at 4°C for 40 min with or without actin (10 μg) in 100 μl final volume and were used for the binding assays by using the centrifugation method, as mentioned above.

The Endogenous Formation of ATP as Assayed by the Ca^{2+}/CaM-Dependent Phosphorylation of Porcine PSD Proteins. *Endogenous ATP formation and phosphorylation.* PSD samples (100 μg each in 25 μl) were incubated at room temperature for 10 min with 25 μl of a buffer [90 mM triethanolamine, pH 7.8/1.43 mM $MgSO_4$/0.6 mM EDTA/10.5 mM D,L-glyceraldehyde-3 phosphate (G3P)/1 mM phenylmethylsulfonyl fluoride/10 μM leupeptin/50 μM microcystin-LR/270 μM β-NAD/0.1 mM ADP) and 10 μl of 1 mM ^{32}Pi. The mixtures were then processed for substrate phosphorylation in the absence or presence of Ca^{2+}/CaM as described (20). For estimation of the amount of ATP formed endogenously, Ca^{2+}/CaM-dependent phosphorylation of the PSD samples was performed in parallel by using 8.3 fmol, 16.6 fmol, and 33.2 fmol of [γ-^{32}P]ATP added exogenously. After incubation, the reaction mixtures were subjected to SDS/PAGE for autoradiographic analysis.

Quantitation of ATP formation. The intensity of the Ca^{2+}/CaM-stimulated phosphorylation of the major PSD protein (mPSDp) band in the autoradiograph was measured by a scanning densitometer (CliniScan, Helena Laboratories). The mPSDp phosphorylation levels per unit protein were obtained by normalizing arbitrary densitometric data of autoradiographs per unit protein. A standard dose-response curve was constructed by using the intensity of the Ca^{2+}/CaM-enhanced phosphorylated mPSDp treated with the known amount of exogenously added [γ-^{32}P]ATP. The amount of [γ-^{32}P]ATP formed endogenously was calculated from the standard curve by using the intensity of the Ca^{2+}/CaM-enhanced mPSDp treated with endogenously formed [γ-^{32}P]ATP.

Effects of Exogenous and Endogenous NO-Stimulated NAD Incorporation into the PSD Proteins on Subsequent Formation of ATP. PSD samples (1 mg each in a final volume of 200 μl) were subjected to exogenous NO-stimulated (6) NAD incorporation by using SNP as the source of NO, or endogenous NO-stimulated (28) NAD incorporation as described earlier. Aliquots of the PSD proteins (100 μg each), control or NAD-incorporated, were examined for ATP formation, as above.

Immunocytochemistry. The avidin-biotinylated peroxidase complex method of Hsu *et al.* (31) was used to visualize immunoreactive sites within the adult visual cortex of rats by light and electron microscopy as described (32). The dilution of the antibody ranged from 1:500 up to 1:10,000, and the duration of incubation of sections with primary antibodies was from 12 to 18 h. Frequency of immunoreactive synapses was assessed by counting the number of immunoreactive and nonimmunoreactive synapses encountered within 405 μm^2 of randomly sampled areas from layer 1.

RESULTS

Immunostaining of G3PD in Brain Slice. In a previous paper (4), the presence of G3PD in the isolated PSD fraction was demonstrated by enzymatic and immunoblot analyses. Here we confirm this finding by immunocytochemistry on brain slices. Incubation of aldehyde-fixed sections of adult rat visual cortex with the G3PD antibody resulted in immunostaining of all layers of the cerebral cortex. By light microscopy (data not shown), immunoreactivity in fine processes of astrocytes was evident within layer 1, whereas in the remaining layers, perikarya of pyramidal neurons were clearly immunolabeled. The presence of the enzyme in neurons as well as astrocytes was confirmed by electron microscopic inspection (Fig. 1). Within neurons, immunoreactivity occurred in dendritic shafts and spines, and in terminal portions of axons so identified by the clustering of vesicles within the cytoplasm,

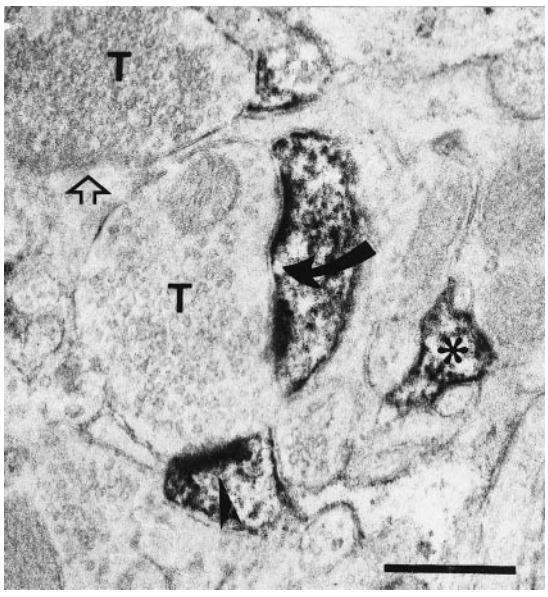

FIG. 1. Electron micrographic localization of rat visual cortex G3PD. Experimental procedures are as stated in the *Materials and Methods* section. The G3PD antibody was diluted 1:2,000. T marks unlabeled terminals whereas arrowheads mark immunoreactive PSD at a dendritic spine. The large curved arrow points to the perforation along one immunoreactive PSD, whereas the open arrow points to an unlabeled PSD. An asterisk denotes an immunoreactive astrocytic process. (Bar = 500 nm.)

and by the parallel alignment of their plasma membranes with that of dendrites exhibiting thick PSDs. Immunoreactivity was also present in portions of axons lacking synaptic specialization but containing vesicles (preterminal axons). As many as 66% of all morphologically identifiable synapses with well-defined PSDs exhibited immunoreactivity, including symmetric and asymmetric synapses and those with perforated PSDs. An unlabeled PSD was detected in the vicinity of a labeled one, indicating that the labeled PSDs were not artifacts resulting from diffusion and nonspecific attachment of the peroxidase reaction product. Also, it is clear that the reaction product was over both portions of the perforated PSD and not on the postsynaptic membrane in the center of the perforation. Immunoreactivity within astrocytic processes was indicated by their distinctively irregular contour that closely followed the round contours of terminals and dendritic spines. Specificity of immunolabeling was assessed by preadsorption controls. Addition at 12 h prior to incubation of 1.2 mg/ml of commercial G3PD purified from porcine muscle greatly reduced the intensity of immunoreactivity over each synapse and reduced the number of immunoreactive synapses to 28%. That the preadsorption was not complete may be due to the fact that the antigen used for antibody production was from human cells (19), whereas the brain sections were from rat.

NO-Stimulated [Adenylate-^{32}P]NAD Incorporation into G3PD in Adult Porcine Cerebral Cortical Subcellular Fractions. Subcellular fractionation was used to localize the NO-stimulated ADP ribosylation of G3PD found in whole brain (6). SNP, an exogenous source of NO, significantly enhanced binding of NAD to a 37-kDa species in all the fractions examined, with the PSD and synaptosomes (which contain PSDs) showing the greatest enhancement (Fig. 2*A*). Western blot analysis showed that the NAD-bound, 37-kDa species comigrated with a band immunoreactive with the specific anti-G3PD antibody (Fig. 2*B*), thus identifying it as G3PD. Unlike porcine cortex PSDs, rat cortex PSDs did not show the enhancement by NO, for reasons unknown. Of interest is the finding that although G3PD was found in all fractions (Fig. 2*B*), the NO-stimulated NAD incorporation was only of significance in the synaptosome and PSD fractions (Fig. 2*A*).

Assay of NOS Catalytic Activity in the Isolated PSD. To biochemically verify the presence of NOS in the PSD determined by immunocytochemistry (5), we measured the ability of the isolated PSD enzyme to catalyze the generation of NO as determined by the NO-mediated incorporation of NAD into G3PD under varying conditions as given in *Materials and Methods*. The results are presented in Fig. 3. Ca^{2+}/CaM, FMN, and FAD, known cofactors of NOS (28), greatly enhances the NAD incorporation into the G3PD. In contrast, *N*-LAME, a specific inhibitor of NOS (14), inhibits the incorporation. Without L-arginine, the substrate of NOS, there was no NAD incorporation. These findings indicate that the isolated PSD retains the enzymatic NOS activity.

Effects of NO-Induced Incorporation of NAD into G3PD on Subsequent Binding of the G3PD to Actin. Because brain G3PD is an actin binding protein (4, 33–36) and NO-dependent NAD incorporation potentially regulates the protein (37), we examined whether NO-mediated NAD incorporation in G3PD may affect association of the enzyme with actin, a structural protein intrinsic to the PSD (1, 2, 4). We used purified G3PD and actin. Because the isolated PSD can bind endogenous G3PD to endogenous actin (4) and can cause a NO-stimulated binding of NAD to PSD G3PD (see above), we thought the results obtained with purified proteins would shed light on how these proteins interact within the PSD. The increased amount of G3PD in the pellet fractions (cf. Figs. 4 and 5) would indicate binding of G3PD to actin. G3PD or actin without (Fig. 4, lanes 1 and 2) and with (Fig. 4, lanes 3–10) preincubation gave similar protein profiles after SDS/PAGE, suggesting that there was no protein degradation during the preincubation and even the subsequent incubation. SNP alone (Fig. 4, lane 7) or NAD alone (Fig. 4, lane 9) had no effect on G3PD binding to actin. However, NO generated by SNP, resulting in an increase of NAD binding to G3PD (see above), increased G3PD binding to actin by 3-fold as determined by densitometric scanning (Fig. 4, compare lane 5 with lanes 3, 7, and 9). Similar results (data not shown) were obtained by using SND (18) instead of SNP as exogenous source of NO.

Effects of Autophosphorylation of Purified G3PD on Subsequent Binding to Purified Actin. Muscle G3PD is reported to be autophosphorylated in the presence of ATP (30). We have verified the phosphorylation (data not shown) and examined whether this phosphorylation might regulate G3PD binding to the actin. Again, we used actin and G3PD for the same reasons as above. G3PD and actin without (Fig. 5, lanes 1 and 2) and with (Fig. 3, lanes 3–8) preincubation in phos-

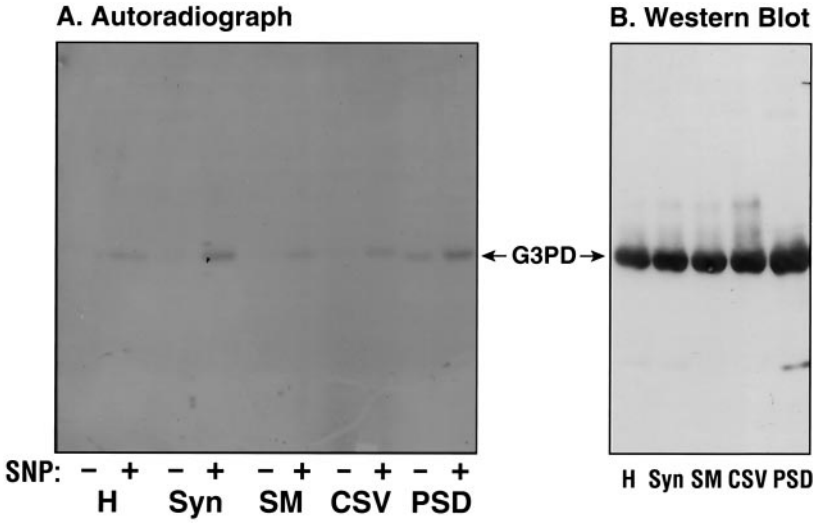

FIG. 2. (*A*) NO-stimulated [adenylate-^{32}P]NAD incorporation into G3PD in subcellular fractions isolated from adult porcine cerebral cortex. Assays are described in *Materials and Methods*. PSD (50 μg) and 100 μg each of the other fractions, in 100 μl final volume, including whole homogenate (H), synaptosomes (Syn), synaptic plasma membranes (SPM), and crude synaptic vesicles (CSV), were incubated at 37°C for 15 min. NAD incorporation was performed in the absence (−) or presence (+) of SNP as exogenous source of NO. The mixtures were subjected to SDS/PAGE and then autoradiography. (*B*) Western blot analysis of the G3PD in the subcellular fractions. To confirm that the radioactive protein in the subcellular fractions was indeed G3PD, Western blot analysis was performed by using specific anti-G3PD antibodies as described.

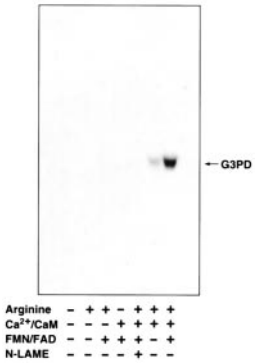

FIG. 3. Assay of NOS catalytic activity in the isolated PSD. NOS activity is assessed as described. The reaction mixtures were subsequently subjected to SDS/PAGE and were analyzed autoradiographically.

phorylation buffer gave protein profiles, after SDS/PAGE, similar to those found in Fig. 4. Phosphorylation of G3PD elicited a 4-fold increase in binding of the G3PD to actin (Fig. 5, compare lane 7 with lanes 3 and 5). Pretreatment with phosphorylation buffer alone (Fig. 4, lane 5) had no effect on the binding. Thus, the autophosphorylation of G3PD increases its binding to actin.

Catalytic Activities of Glycolytic Enzymes in the Isolated PSD Fractions. Because the data so far (refs. 3 and 4, and Figs. 1 and 2*B*, above) indicated that G3PD is an intrinsic component of the PSD, we decided to examine whether the G3PD and the other glycolytic enzymes might be catalytically active in the isolated PSD. Our studies revealed that the PSD fractions contain the glycolytic enzymes with the following specific activities ($n = 6$): G3PD, 1.28 ± 0.04 units/mg PSD (or 12.8 ± 0.39 μg/mg PSD); LDH, 0.66 ± 0.03 units/mg PSD (or 3.07 ± 0.14 μg/mg PSD); PGK, 0.37 ± 0.03 units/mg PSD (or 1.48 ± 0.12 μg/mg PSD); hexokinase, negligible.

It should be noted that there is about 25 μg monomeric actin per mg PSD protein, estimated from the Coomassie brilliant blue staining profile (data not shown), and based on the report (3) that approximately half of the protein at the 43-kDa region is actin. Because the monomeric molecular weights of G3PD and actin are approximately equal, there seems to be one mole of G3PD bound to every 2 mol of actin.

Endogenous Formation of ATP in the PSD. Because G3PD is catalytically active in the isolated PSD as shown above, and G3PD, with its cognate, PGK, is one of the two sources of glycolytically generated ATP, we examined whether PSD fractions might contain the biochemical machinery for the formation of ATP. First, we assayed for the presence of PGK, the enzyme catalyzing ATP synthesis from ADP and the

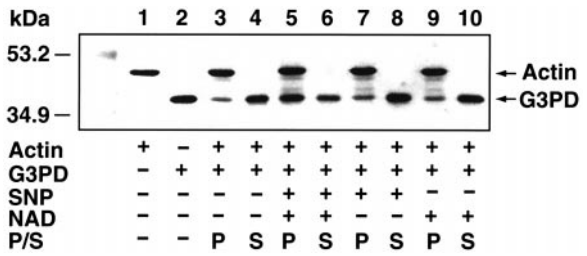

FIG. 4. Effects of NO-induced incorporation of NAD into G3PD on subsequent binding of the G3PD to actin. Analyses of binding of G3PD to actin was performed by using the centrifugation approach, followed by SDS/PAGE as described. Lanes 1 and 2 are standards without incubation. Lanes 3 and 4 are pellet (P) and supernatant (S) of actin incubated with G3PD but without preincubation. Lanes 5 (P) and 6 (S) are G3PD preincubated with SNP and NAD. Lanes 7–10 are controls in the absence of NAD (lanes 7 and 8) or in the absence of SNP (lanes 9 and 10). Proteins are stained with Coomassie brilliant blue. The left lane contains molecular weight standards.

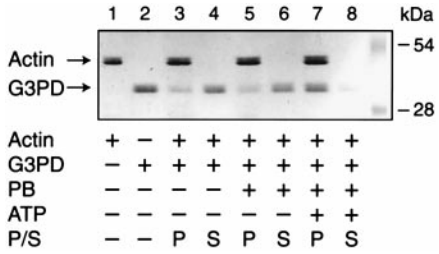

FIG. 5. Effects of autophosphorylation of purified G3PD on subsequent binding to actin. Experiments were performed as described. G3PD was incubated with and without ATP. The mixtures were centrifuged to separate pellets (P) and supernatants (S), subjected to SDS/PAGE and proteins stained with Coomassie brilliant blue. Lanes 1 and 2 are actin and G3PD, respectively. Lanes 3 and 4 are actin and G3PD incubated together. Lanes 5 and 6 are G3PD treated with phosphorylation buffer (PB) without ATP. Lanes 7 and 8 are G3PD treated with the ATP and PB. Molecular weight marker proteins are shown on the right.

substrate for which is 1,3-bisphosphoglycerate, the latter being the immediate metabolic product of G3PD. As noted above, the enzyme was found to be present in the PSD fraction, in contrast to an earlier report (4). Second, the PSD fractions were incubated with ADP, D,L-G3P, NAD, and ^{32}Pi. The amount of ATP formed endogenously was determined by the Ca^{2+}/CaM-dependent phosphorylation of the PSD proteins (Fig. 6, lanes 1 and 2) and then compared with a standard phosphorylation run in parallel by using a known amount of exogenous [γ-^{32}P]ATP (Fig. 6, lanes 3–8). The mPSDp was the reference protein for estimation of the amount of ATP formed endogenously. By using the kinase activity to estimate the ATP amount, our results indicated that, in three independent experiments, the ATP formed endogenously was 369, 360, and 353 fmol ATP per mg PSD, resulting in an average yield of 361 ± 2.1 fmol ATP per mg PSD protein. Endogenous formation of ATP with rat cortex PSD was similar to that using the porcine PSD (data not shown).

In order for ATP production to be physiologically significant, there should be a regeneration of ADP and NAD. We found that to be the case: (*i*) ADP is regenerated by the activity of various protein kinase found in the PSD (1, 2); (*ii*) LDH, which converts NADH to NAD, is, as noted above, found in the PSD fraction. We also observed (data not shown) that

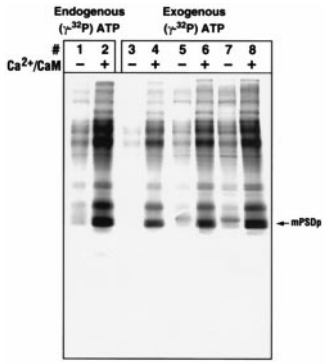

FIG. 6. Assay of endogenously formed ATP in the PSD by Ca^{2+}/CaM-dependent phosphorylation of the PSD proteins. The experiments were performed as described. PSD fractions (100 μg each) were incubated at room temperature for 10 min with ^{32}Pi and ADP (lanes 1 and 2) and the mixtures were processed for phosphorylation in the absence (−) or presence (+) of Ca^{2+}/CaM. Control experiments were examined in parallel in which the Ca^{2+}/CaM-dependent phosphorylation of the PSD samples was performed by using three doses of exogenously added [γ-^{32}P]ATP (lanes 3 and 4, 8.3 fmol each; lanes 5 and 6, 16.6 fmol each; lanes 7 and 8, 33.20 fmol each). The mixtures were then subjected to SDS/PAGE for autoradiographic analysis. mPSDp is the major PSD protein.

fructose-1,6 bisphosphate, but not glucose, glucose-6 phosphate, or fructose-6 phosphate, was found to be the source in the PSD of G3P, indicating the presence of aldolase in the PSD. The latter experiment was performed by adding fructose bisphosphatate and measuring G3PD activity resulting from the conversion of this substrate to G3P. It could be that *in vivo*, glucose, glucose-6-phosphate, or fructose-6-phosphate may be metabolized in the PSD, but the enzymes involved may have been washed out during the PSD preparation.

Effects of NAD Incorporation into PSD G3PD on Subsequent Endogenous ATP Formation. Because NO-stimulated NAD incorporation (NOSNI) into the G3PD resulted in an inhibition of the G3PD activity (7, 38), which we confirmed (about 90% of the activity was inhibited), we examined as suggested (6) whether NOSNI might affect endogenous ATP formation in our PSD fractions. Control experiments indicated that without ADP (Fig. 7 *A* and *B*, lanes 1 and 2) there was no ATP formation. When an exogenous (Fig. 7*A*) or endogenous (Fig. 7*B*) source of NO was used, it was clear that the NO-activated binding of NAD to G3PD decreased the production of ATP and subsequent phosphorylation of proteins by Ca^{2+}/CaM (Fig. 7*A*, compare lane 6 with lane 4; Fig. 7*B*, compare lane 6 with lane 4).

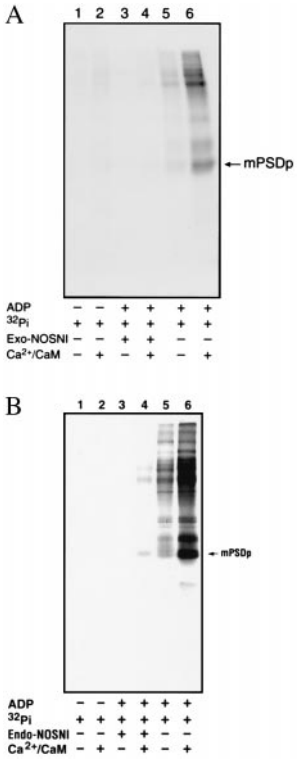

FIG. 7. (*A*) Effects of exogenous NO-stimulated NAD incorporation (Exo-NOSNI) of PSD proteins on subsequent formation of the endogenous ATP. Experiments were performed as described. PSD fractions (100 μg aliquots) pretreated with (lanes 3 and 4) and without (lanes 1, 2, 5, and 6) SNP plus NAD were subsequently incubated at room temperature for 10 min with ^{32}Pi in the absence (lanes 1 and 2) and presence (lanes 3–6) of ADP. The mixtures were examined for phosphorylation with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) Ca^{2+}/CaM and were then subjected to SDS/PAGE for autoradiographic analysis. (*B*) Effects of endogenous NO-stimulated NAD incorporation (Endo-NOSNI) of PSD proteins on subsequent formation of the endogenous ATP. PSD samples (100 μg aliquots) preincubated with (lanes 3 and 4) or without (lanes 1, 2, 5, and 6) NAD, 0.5 mM L-arginine, 1 mM each of FMN/FAD, and 2 mM Ca^{2+}/0.25 mM CaM were subsequently incubated at room temperature for 10 min with ^{32}Pi in the presence (lanes 3–6) or absence (lanes 1 and 2) of ADP. The reaction mixtures were then processed for phosphorylation in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of Ca^{2+}/CaM.

DISCUSSION

The significant findings of this paper are the glycolytic generation of ATP at the PSD, and the potential regulation of glycolysis by NO generated by NOS at the PSD. The importance of this finding is that we, and others (39–42), only rarely have observed mitochondria, the oxidative source of ATP, in cerebral cortex spine-heads, though they are more prevalent in hippocampal tissue (42). It has been assumed by us, and probably by others, that the mitochondria seen in dendritic shafts were the source of spine ATP, possibly by diffusion, but in the case of PSDs this might not be so. The glycolytic activity is not unimportant, because even though most of the energy supply in the brain has an aerobic source, for local activity glycolysis is necessary (refs. 43 and 44, and also see ref. 45). The isolated PSD fraction provides an example of localized generation of ATP via glycolysis. This ATP can be used by three protein kinases found in the PSD: protein kinase A, protein kinase C, and Ca^{2+}/CaM-activated protein kinase plus their substrates (1, 2). The ATP could also play a role in maintaining the ion channels found in the PSD (1). Of relevance is the finding (46) that in brain the K^+ activation of the Na^+–K^+ pump is specifically dependent on glycolytically generated energy, and that in heart a compartmentalized glycolytic system regulates an ATP-sensitive K^+ channel (47).

There is a growing realization that some local protein synthesis (48), which needs ATP, occurs in the spine head. Steward and Levy (49) first recognized polyribosomes in dendritic spines close to the PSD. Garner *et al.* (50) found a mRNA for the PSD MAP_2, and there have been many observations confirming these findings (for reviews, see refs. 51 and 52). Of interest are the findings (53, 54) that some proteins of the PSD are synthesized in the spine head and that there exist many mRNAs in the spine (55–57), including (57) the one for Ca^{2+}/CaM-dependent protein kinase, the major PSD protein. The present finding of locally synthesized ATP at the PSD level thus suggest an energy source for protein synthesis at the spine head.

Our finding of G3PD, PGK, LDH, aldolase, and possibly other glycolytic enzymes in the PSD suggests there exists at the PSD level a rather complete glycolytic complex for the generation of ATP, including the enzymes involved in the regeneration of the key substrate, G3P, and of the cofactors, NAD and ADP. These results, showing localized generation of ATP, should not be too surprising, as noted by Masters (35) and Knull (36), considering the localization of G3PD and other glycolytic enzymes in different mammalian systems. Indeed, earlier suggestions (58, 59) have been made that an association of G3PD and PGK with membranes may denote a separate ATP-generating pool for membrane-bound ATP-dependent processes and that a G3PD and aldolase complex may be involved as initiators of energy production (35). Of importance is the finding that G3PD is a key enzyme involved in control of glycolysis in cerebral cortex slices (60). Recently, Han *et al.* (61) found that the triadic junction of skeletal muscle contains a compartmentalized reaction sequence of four glycolytic enzymes active in the synthesis of ATP. Of further interest to this work is the appearance of G3PD on the surface of group A Streptococci (62), and the recent observations by the same group (V. Pancholi and V. Fischetti, The Rockefeller University, personal communication) that a five-enzyme glycolytic complex is found there, including the G3PD and PGK. Thus, it could be that in various cells a localized need for ATP is supplied not by mitochondria, but by the glycolytic complex at unique sites, such as the PSD.

Our result of the occurrence of NOS in the isolated PSD is strengthened by the recent finding that a PSD protein, PSD-95 or PSD-93 (63, 64), binds to NOS, as well as to the PSD NMDA receptor (refs. 64–67, and also see ref. 68) and to PSD K^+ channels (64). A recent review (69) lists these binding proteins.

It is interesting that NOS activity has been found to modulate NMDA receptors (70). However, in our PSD preparation we found no effect of NO on the binding of radioactive glutamate to the PSD NMDA receptor, for reasons unknown. The PSD involvement in possible regulation of NOS is also shown (71, 72) by the ability of protein kinase A, a PSD enzyme, to phosphorylate NOS, inhibiting its activity. Also, *in vivo* injections of a NO-releasing reagent, as well as NMDA, were found (73) to result in a specific decrease in the mRNA for Ca^{2+}/CaM kinase II. The activity of this kinase toward PSD proteins is increased by the addition of NO (74). However, as shown by the data in this paper, the addition of NAD to this reaction resulted in an inhibition of the G3PD and thus an inhibition of the phosphorylation. There have been many studies on possible neuronal roles for NOS and NO, too many to refer to here. Two recent reviews (75, 76) summarize the literature including, for the context of this paper, its interaction with the NMDA receptor, and its possible involvement in long-term potentiation at the postsynaptic site.

The data in this paper, and the references above, strengthen our premise (1, 77) that the interactions among the proteins in the PSD play important roles in neuronal synaptic transmission. The physiological meaning of the presence (1, 77) of neurotransmitter receptors and ion channels at the PSD is obvious. Whether these functions can be regulated by the protein kinases at the PSD is unknown, but possible. However, we did find that the ATP necessary for the activities of these kinases can be generated at the PSD site, and that the amount of ATP can be regulated by the NOS, generating NO, which can increase the activity of one of the kinases (74), or with the addition of NO plus NAD, decreases ATP production, as shown in this paper. Also, because G3PD is probably attached to the PSD structure via an interaction with actin (4), the strength of this binding may play a regulatory role. Agents such as NO plus the G3PD enzymatic cofactor, NAD, as well as the autophosphorylation of G3PD, increase its binding to actin, concomitantly decrease its activity and thus decrease ATP production. Therefore, any changes in the activity of NOS (75, 76) can result in changes in ATP production at the PSD site.

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