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Dephosphorylation of B-50 in Synaptic Plasma Membranes

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DOKAS, L. A., M. R. PISANO, L. H. SCHRAMA, H. ZWIERS AND W. H. GISPEN. *Dephosphorylation of B-50 in synaptic plasma membranes*. BRAIN RES BULL **24**(3) 321–329, 1990.—Synaptic plasma membranes from rat brain cortex possess intrinsic ability to dephosphorylate the endogenous protein B-50. At low concentrations of [γ-^{32}P]ATP, B-50 phosphorylation in synaptic membranes is maximal at 30 seconds, followed by dephosphorylation for an additional 60 minutes. The dephosphorylation of ^{32}P-labeled B-50 is not sensitive to the protease inhibitor leupeptin and not correlated with a loss of the B-50 content of synaptic membranes as measured with immunoblot analysis. Dephosphorylation of membrane-associated B-50 is stimulated to a small extent by Mg^{2+} but not by Ca^{2+}. Heat-stable protein phosphatase inhibitors prevent dephosphorylation of ^{32}P-labeled B-50. Dephosphorylation of B-50 in synaptic membranes is stimulated by ATP, ADP, or adenosine 5′-O-thiotriphosphate, but not by adenine, adenosine, other adenine or guanine nucleotides, nonhydrolyzable analogs of ATP or GTP, nor by adenosine 5′-O-(2-thiodiphosphate). B-50, phosphorylated by exogenous protein kinase C and purified to homogeneity, has been used as a substrate to follow the purification of B-50 phosphatase activity. B-50 phosphatase activity can be solubilized from synaptic membranes with 0.5% Triton X-100 and 75 mM KCl. Chromatography of the extract on DEAE-cellulose yields enhanced B-50 phosphatase activity.

B-50 Protein phosphatase Synaptic plasma membranes

PRESYNAPTIC membranes contain a prominent phosphoprotein, B-50, which is functionally related to several aspects of neuronal growth and synaptic plasticity. B-50 is a substrate for protein kinase C or a closely related enzyme in the central nervous system (2,5). Phosphorylation of B-50 by protein kinase C determines its ability to modulate phosphoinositide metabolism, at the level of phosphatidylinositol-4-phosphate kinase (19). Furthermore, phosphorylation of B-50 (F1) is increased during establishment of long-term potentiation in the hippocampus (23,32). Independent, but convergent, studies from several other laboratories have shown B-50 to be identical to one of the growth-associated proteins—GAP-43 (6, 28, 40). Synthesis of GAP-43 is increased 20- to 100-fold during development or axon regeneration and lack of GAP-43 induction is synonymous with the failure of neuronal regenerative responses (18,39). The sequence of GAP-43/B-50 is virtually identical to that of P-57, a calmodulin-binding protein with the unusual characteristic that it binds calmodulin with less affinity in the presence of Ca^{2+} (4). Phosphorylation by protein kinase C of P-57 prevents binding of calmodulin to this protein (1).

Since phosphorylation is related to B-50-mediated effects, it is important to characterize not only the enzyme responsible for its phosphorylation, but also that catalyzing the reverse process. The classes of soluble phosphatases are well-defined (16). However, it has not yet been established that the same classification can be applied to forms that exist in association with cellular membranes nor how membrane localization modulates characteristics of these enzymes. The relationships that exist between soluble and membrane-bound phosphatases will be delineated only as detailed characterizations of the latter become available.

The cyclic AMP-dependent system which phosphorylates protein II (the regulatory subunit of cyclic AMP-dependent protein kinase) has been shown to be a membrane-bound complex of substrate, protein kinase and protein phosphatase (42). The same arrangement may pertain to the other major (calcium/calmodulin-dependent; protein kinase C) phosphorylation systems of the brain. B-50 and protein kinase C do exist in close association within synaptic plasma membranes (2). This study reports characteristics of a protein phosphatase activity found in synaptic plasma membranes that dephosphorylates B-50. In addition, homogeneous ^{32}P-labeled B-50 has been prepared and used as a substrate to measure protein phosphatase activity solubilized from synaptic plasma membranes.

METHOD

Materials

Male Sprague-Dawley rats were used for all experiments. [γ-^{32}P]ATP (10-40 Ci/mmole) was from Dupont-NEN Research

Products, Boston, MA. Kodak panoramic dental x-ray film (DF-85) was used for autoradiography. Zeta probe nylon membrane used for immunoblot analysis was from Biorad Laboratories, Richmond, CA. Adenine and guanine nucleotides were obtained from Sigma Chemical Company, St. Louis, MO. Diethylaminoethyl (DEAE)-cellulose was purchased from the Anspec Company, Ann Arbor, MI. Prepacked Sephadex G-25 columns (PD-10) used for desalting B-50 samples, ampholines and Sephadex IEF were purchased from Pharmacia, Inc., Piscataway, NJ. Heat-stable protein phosphatase inhibitor-1 was purified as previously described (29). Heat-stable inhibitor-2 was prepared, using methodology described by Yang *et al.* (45). One unit of activity for either inhibitor produces a 10% inhibition of phosphorylase phosphatase activity in a standard assay (29).

Preparation of Synaptic Plasma Membranes

Synaptic plasma membranes were prepared from rat brain cortex, as described by Dokas *et al.* (8). Briefly, a mitochondrial-synaptosomal pellet (P2), prepared from approximately 0.5 g of cortex, was lysed in double-distilled water, centrifuged at 10,000 × g and the supernatant applied to a discontinuous sucrose gradient, consisting of 4.0 ml of 0.4 M sucrose, overlaid on 8 ml of 1.0 M sucrose. The gradient was centrifuged at 100,000 × g for 80 minutes in a Beckman SW 27 rotor. The synaptic plasma membranes were collected by aspiration from the interface of the 0.4 M and 1.0 M layers, then diluted with acetate buffer (10 mM sodium acetate, 10 mM magnesium acetate and 1 mM calcium acetate, pH 6.5) and centrifuged in the SW 27 rotor at 100,000 × g for 30 minutes. The final pellet was gently resuspended in 250 μl of acetate buffer, pH 6.5. This fraction is highly enriched in presynaptic membranes of low buoyant density (41) and electron microscopic analysis does not show significant contamination with mitochondria or myelin (3). Protein content was estimated by the method of Lowry *et al.* (24), with bovine serum albumin as a standard.

Phosphorylation and Dephosphorylation Assays

When utilizing synaptic plasma membranes, the standard protein phosphorylation assay, in a total volume of 25 μl, contained 25 μg of protein and 2 μCi of [γ-^{32}P]ATP adjusted with cold ATP to a final concentration of 7.5 μM. All components of the assay were dissolved in acetate buffer, pH 6.5. Samples were preincubated at 30°C for 5 minutes and the reaction was started by the addition of labeled ATP. After 30 seconds, 2000–4000 cpm are incorporated into phosphorylated B-50. Reactions were stopped and prepared for SDS-polyacrylamide slab gel electrophoresis by the addition of one-half volume of a solubilization mixture (stop mix) to bring the final concentration to 62.5 mM Tris-HCl pH 6.5, 2% SDS, 10% glycerol, 0.001% bromphenol blue and 5% 2-mercaptoethanol. Samples not immediately applied to a gel were stored at −20°C.

The time course of synaptic plasma membrane protein phosphorylation was determined. Samples were preincubated at 30°C for 5 min, the reaction was started by addition of [γ-^{32}P]ATP and stopped at increasing periods of time. To analyze the effects of various agents on dephosphorylation of B-50, synaptic plasma membranes were labeled with [γ-^{32}P]ATP for 30 seconds and incubated for an additional 30 minutes in the presence of each compound. Such assays discriminate protein kinase from protein phosphatase activity since the endogenous ATPase activity of synaptic plasma membranes hydrolyzes all of the unincorporated [γ-^{32}P]ATP within 30 seconds at the concentrations of ATP and membranes used in these experiments (9,43). Therefore, after 30 seconds of labeling, only dephosphorylation of B-50 determines the amount of label recovered in B-50 following SDS-polyacrylamide slab gel electrophoresis and autoradiography.

To determine the cation sensitivity of B-50 dephosphorylation, synaptic plasma membranes were prepared and labeled with [γ-^{32}P]ATP under the standard assay conditions, except that the reaction volume and the amount of membranes were increased 5-fold. After 30 seconds at 30°C, the synaptic plasma membranes were pelleted in 1.5-ml conical tubes at 10,000 rpm for 10 min. The supernatant was removed and the membranes resuspended in 200 μl of 10-mM sodium acetate—0.1 mM EDTA, pH 6.5. The synaptic plasma membranes were repelleted under the same conditions and resuspended in 100 μl of the same buffer. Twenty μl of ^{32}P-labeled synaptic plasma membranes were added to assay tubes containing various concentrations of Mg^{2+} or Ca^{2+} or both. Duplicate samples of each type were immediately prepared for electrophoresis by addition of stop mix. A second set of duplicate samples were incubated at 30°C for 30 minutes and then processed as described above. Proteins were separated on SDS-polyacrylamide gels, using a 3% stacking gel and a 11% running gel, as previously described (8). Following autoradiography, the amount of labeled B-50 was quantitated by densitometry, and in most cases, by liquid scintillation counting of the excised band. Dephosphorylation of B-50 was measured as the loss of ^{32}P-labeled B-50 in the incubated, as compared to the nonincubated, samples.

Immunoblot (Western Blot) Analysis

The procedure for immunoblotting was adapted from that of Schrama *et al.* (34). To determine whether there was degradation of B-50 by protease activity during the dephosphorylation reaction, synaptic plasma membranes were phosphorylated under the standard assay conditions. Reactions were terminated after 30 seconds or 30 minutes with addition of stop mix. Samples were analyzed by SDS-polyacrylamide gel electrophoresis on a 15% running gel. Six samples from each time point were dried for autoradiography. A second set of identical samples were transferred to a nylon membrane. The completeness of the transfer was checked by autoradiography. After inactivation of unoccupied binding sites, the blot was incubated with anti-B-50 immunoglobulins at a 1:4000 dilution. The immunoreactive proteins were detected with a second antibody conjugated to alkaline phosphatase, using the protocol of Promega Corporation, Madison, WI.

Purification and Phosphorylation of B-50

A procedure involving alkaline extraction of the protein from synaptic plasma membranes was used as described by Zwiers *et al.* (49). Briefly, rat brain was homogenized in distilled water (4 ml per gram of rat brain) and centrifuged at 48,000 × g for 20 minutes in an SS 34 rotor. After addition of magnesium acetate (1 mM final concentration), the pellet was extracted with sodium hydroxide at pH 11.5 and centrifuged as above. Following neutralization to pH 5.5 and centrifugation, the sample was heated at 70°C and recentrifuged, yielding a supernatant that was enriched in B-50. Further fractionation was accomplished by collecting the proteins precipitating between 57–82% ammonium sulfate. The final purification consisted of preparative isoelectric focusing on a Sephadex-IEF flatbed. The yield from 90 rat brains with this procedure is approximately 1–3 mg of B-50 protein.

Purified B-50 was phosphorylated with protein kinase C, prepared through the DEAE-Sephadex chromatography step, as described by Sahyoun *et al.* (31). B-50 (275 μg) was incubated at 30°C in a 2.5-ml assay mixture, containing 0.2 mM $CaCl_2$, 0.064

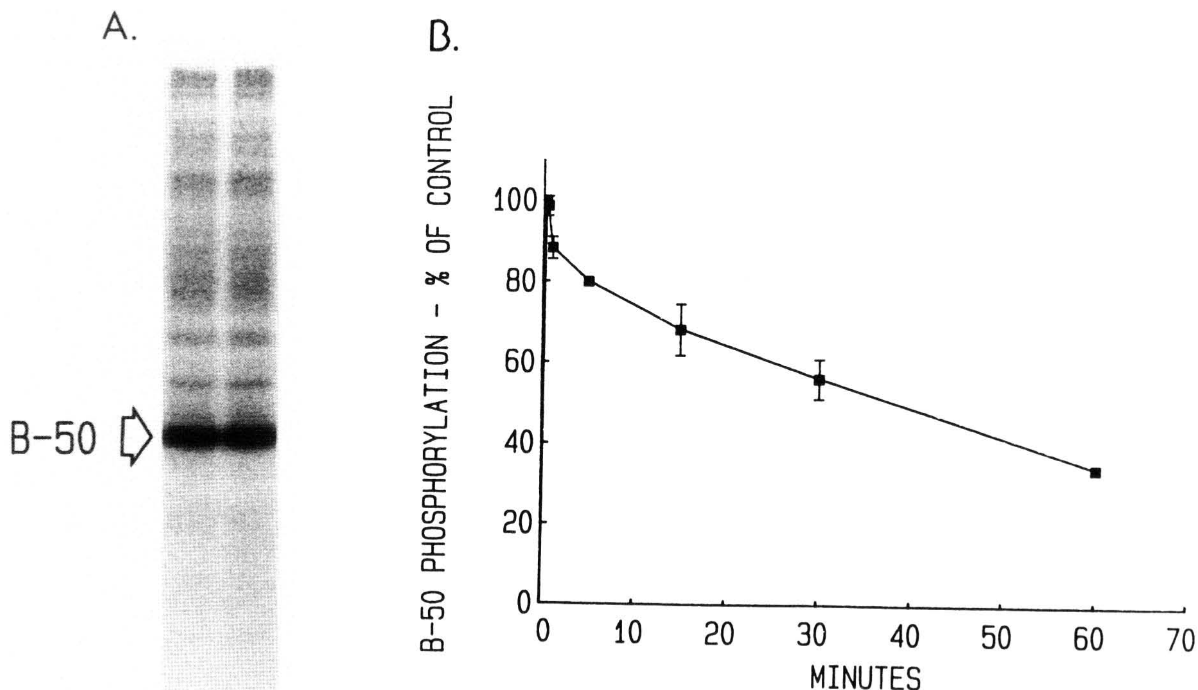

FIG. 1. Time course of the phosphorylation of B-50 in cortical synaptic plasma membranes. After a 5-minute preincubation at 30°C, 25 μg of synaptic membrane protein were phosphorylated in the standard assay, but for increasing periods of time with 2 μCi of [γ-^{32}P]ATP. The phosphorylation of B-50 was measured by SDS-polyacrylamide gel electrophoresis and autoradiography. (A) An autoradiogram showing levels of B-50 phosphorylation after labeling for 30 sec with [γ-^{32}P]ATP. The position of B-50, with an apparent M_r of 48,000, is shown. (B) Quantitation of B-50 phosphorylation. The labeled B-50 band was excised from gels and counted. Each value is represented as a % of the 20-second labeling, which is set at 100%. Each point represents the mean of 3–5 experiments ± SEM.

μM 12-O-tetradecanoylphorbol 13-acetate (TPA), 20 μg/ml of phosphatidylserine and approximately 4000 units of protein kinase C. The reaction was started by the addition of a mixture containing magnesium acetate and [γ-^{32}P]ATP to give final concentrations of 10 mM magnesium acetate and 0.2 mM ATP (700–2000 cpm/pmole). After 60 minutes, the phosphorylation was stopped by placing the reaction vessel on ice. The sample was then subjected to flatbed isoelectric focusing a second time to separate B-50 and protein kinase C. The final yield of B-50 was quantitated by densitometric scanning of fast green-stained B-50 on SDS-polyacrylamide gels, as compared to standard amounts of bovine serum albumin. Radioactivity incorporated into B-50 was determined by liquid scintillation counting. For calculation of the specific activity of B-50, a molecular weight of 24,000 was assumed, based on the amino acid composition as deduced from the nucleotide sequence of the cDNA for B-50 (20). After 60 minutes, the reaction was complete with approximately 0.6 to 1.0 pmoles of phosphate incorporated per pmole of B-50.

Solubilization and Partial Purification of Synaptic Plasma Membrane-Associated Protein Phosphatase

The methodology is an adaptation of that used to purify B-50 and B-50 kinase (47). All procedures were performed at 4°C. A mitochondrial-synaptosomal pellet was prepared in 0.32 M sucrose from 10 rat forebrains. The pellet was lysed in 80 ml of double-distilled water. The lysate was centrifuged at 12,000 × g in an SS34 rotor for 20 minutes and the resultant supernatant at 45,800 × g for 30 minutes to collect light synaptic plasma membrane fragments. The membrane pellet was extracted with 0.5% Triton X-100 and 75 mM KCl and the extract chromatographed on DEAE-cellulose, as previously described.

The absorbance at 280 nm was read for each column fraction and the chloride ion concentration was measured with a Corning chloride meter-920M. Twenty-five μl aliquots of each protein-containing fraction were run on a SDS-polyacrylamide slab gel and stained with fast green. Protein content of the fractions was measured by the method of Lowry *et al.* (24) with bovine serum albumin as a standard. Protein phosphatase-containing fractions were identified (after dialysis against Buffer A at 4°C for at least 5 hours) by their ability to dephosphorylate purified B-50. To measure protein phosphatase activity, 10 μl of the dialyzed DEAE-cellulose column fractions were incubated at 30°C in a 20-μl assay mixture containing 125 mM Tris-HCl, pH 7.4 and 10 mM $MgCl_2$. The reaction was started by the addition of ^{32}P-labeled B-50 (approximately 7 μg and 2000 cpm) and after 10

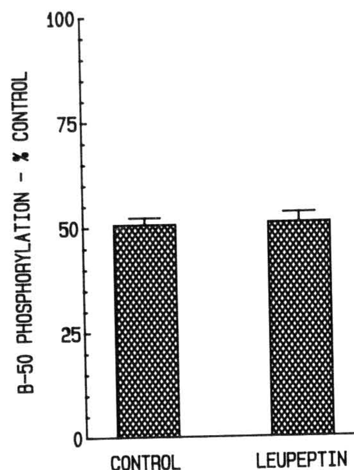

FIG. 2. Effect of leupeptin on B-50 dephosphorylation. Cortical synaptic plasma membranes were incubated in the standard dephosphorylation assay in the presence or absence of 25 μg/ml of leupeptin. The dephosphorylation of B-50 was compared after 30 minutes of incubation under the two conditions to the labeling of B-50 at 30 seconds, which was set at 100%. The results are the mean ± SEM for three separate experiments.

minutes, terminated by the addition of stop mix. DEAE-cellulose column fractions containing B-50 phosphatase activity were pooled and reassayed. The activity of this pool was compared to that of samples from the whole homogenate and Triton-KCl extract which had been dialyzed at 4°C for at least 5 hours against buffer A and assayed in the same manner. Samples were analyzed with SDS-polyacrylamide slab gel electrophoresis and autoradiography.

RESULTS

Time Course of B-50 Phosphorylation

When light synaptic plasma membranes (1 μg/μl) from rat brain cortex are incubated at 30°C with 7.5 μM [γ-^{32}P]ATP in acetate buffer, pH 6.5, containing 10 mM Mg^{2+}, 10 mM Na^{+} and 1 mM Ca^{2+}, the most prominent phosphoprotein is B-50, in the terminology of Gispen *et al.* (12), with an apparent molecular weight of 48,000 on 11% SDS-polyacrylamide gels (Fig. 1A). Maximal phosphorylation of B-50 is seen at 20 or 30 seconds of labeling. Net phosphorylation stops at this point due to breakdown of the relatively low concentration of ATP in the assay by the endogenous ATPase activity of the synaptic plasma membranes (9,43). Comparison of phosphorylation values at later times to that seen at 30 seconds shows a gradual loss of label from B-50 which is continuous through 60 minutes, at which point the labeling of B-50 is 34 ± 1.0% that of the 30 second value (Fig. 1B).

B-50 Dephosphorylation and Synaptic Plasma Membrane-Associated Protease Activity

Loss of ^{32}P-labeled B-50 following prolonged incubation times could result from protease-mediated breakdown of B-50 to small fragments that would not be apparent on 11% SDS-polyacrylamide gels. To examine this possibility, two experiments were performed. In the first of these, synaptic plasma membranes were incubated with [γ-^{32}P]ATP for 30 seconds followed by an additional 30 minutes in the presence or absence of 25 μg/ml of leupeptin. This concentration of leupeptin will maximally inhibit Ca^{2+}-dependent proteases (27), which will degrade B-50 (48). Addition of leupeptin did not alter the dephosphorylation of B-50 after 30 minutes (Fig. 2). At 30 minutes the labeling of B-50 was found to be 50.6 ± 1.6% of the 30 second value in the absence of leupeptin and 51.5 ± 1.9% in the presence of leupeptin.

Secondly, the membrane content of B-50 was measured with immunoblot analysis before and after dephosphorylation. Synaptic plasma membranes were phosphorylated under standard assay conditions for 30 seconds or 30 minutes and proteins were separated on 11% SDS-polyacrylamide gels. One set of samples on half of the gel were dried for autoradiography (data not shown). Another set of replicate samples were analyzed by Western blotting. Completeness of the transfer to nitrocellulose was verified by autoradiography of the membrane (Fig. 3A). The transferred proteins were reacted with anti-B-50 antibodies after inactivation of unoccupied binding sites. Comparison of ^{32}P-labeling of B-50 (Fig. 3A) with the immunoblot of B-50 (Fig. 3B) demonstrates, that while there is a considerable loss of radioactivity from the B-50 protein after incubation for 30 minutes, there is no loss of B-50 protein content. Moreover, there are no detectable phosphorylated fragments of B-50 formed during the incubation. Thus, protease activity has been excluded as a significant factor in B-50 dephosphorylation in synaptic plasma membranes.

Properties of Synaptic Plasma Membrane-Bound B-50 Phosphatase Activity

A charcteristic of type-1 protein phosphatase is that it can be inhibited by two heat-stable proteins, termed inhibitor-1 and inhibitor-2 (16). To determine if B-50 dephosphorylation in synaptic plasma membranes was due to such an enzyme, prelabeled synaptic plasma membranes were incubated in the presence and absence of these inhibitors during the dephosphorylation reaction. Both inhibitors almost completely blocked B-50 dephosphorylation, relative to the control sample (Table 1). Addition of an equal volume of the solutions in which the inhibitors had been stored (50 mM imidazole, 5 mM EGTA and 1 mM dithiothreitol, pH 7.4, for inhibitor-1; double-distilled water for inhibitor-2) had no effect on B-50 dephosphorylation, relative to control values. The sensitivity of B-50 dephosphorylation to the protein inhibitors is another indication that protein phosphatase activity is being measured and suggests that B-50 is dephosphorylated in synaptic plasma membranes by a type-1 protein phosphatase.

Dephosphorylation of B-50 is increased in the presence of excess cold ATP (Table 2). Addition of 0.5 mM ATP to the 30-minute incubation of prelabeled synaptic plasma membranes caused about a 2-fold increase in the dephosphorylation of B-50 as compared to samples incubated without the nucleotide. When concentrations of ATP from 0.1 to 1.0 mM were compared, 0.5 mM was found to be optimal for the apparent stimulation of B-50 phosphatase activity (data not shown). An equal concentration of ADP or adenosine 5-O-thiotriphosphate (ATP-γ-S) was as effec-

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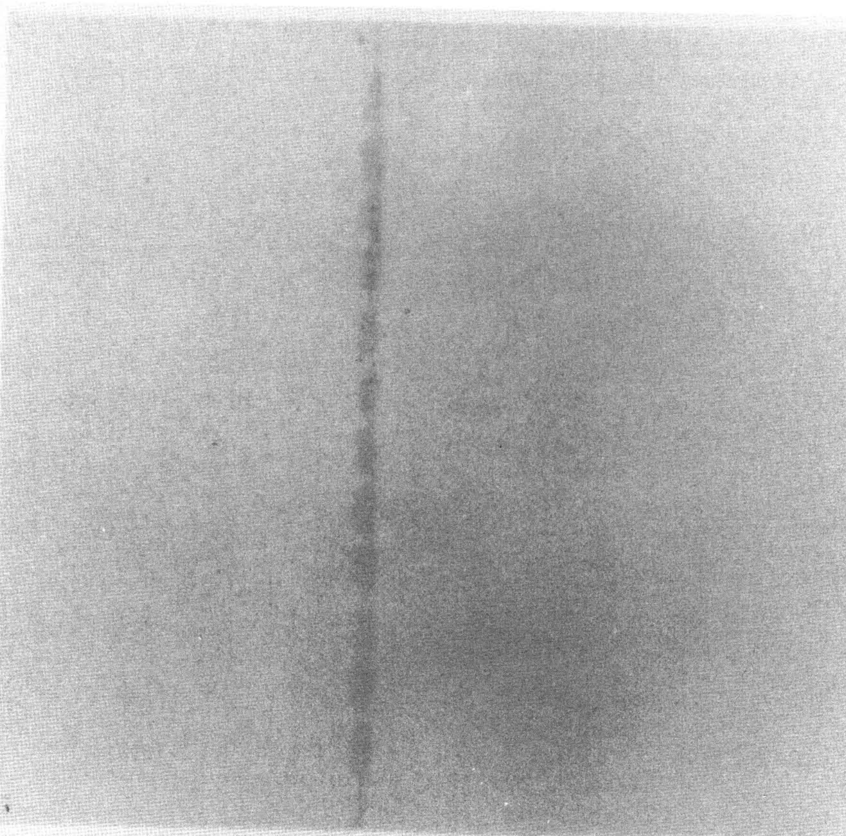
FIG. 3. Immunochemical analysis of phosphorylated and dephosphorylated B-50 in synaptic plasma membranes. Cortical synaptic plasma membranes were incubated with [γ-^{32}P]ATP as described in Fig. 1 for 30 seconds or 30 minutes and labeled proteins were transferred to nitrocellulose membranes. Phosphorylation of the proteins was analyzed by autoradiography (A). Immunostaining of the nitrocellulose membrane was performed with anti-B-50 immunoglobulins at a 1:4000 dilution and the immunoreactive proteins were detected with a second antibody conjugated to alkaline phosphatase (B).


B.
30sec
30min

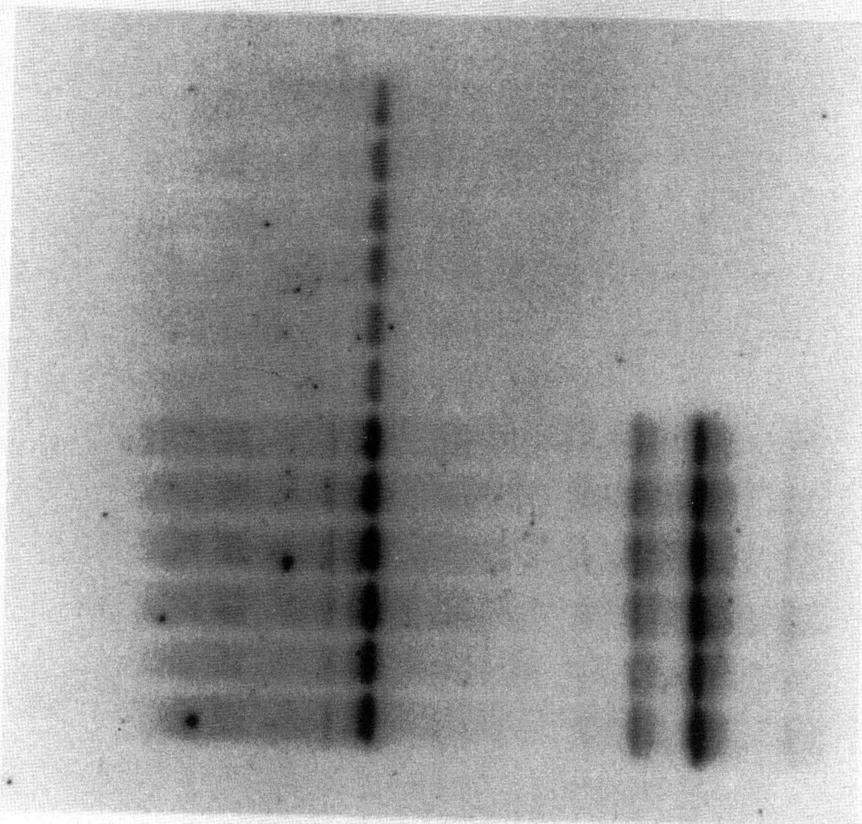

A.
30 sec
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TABLE 1

EFFECT OF PROTEIN PHOSPHATASE INHIBITOR-1 AND INHIBITOR-2 ON B-50 DEPHOSPHORYLATION IN SYNAPTIC PLASMA MEMBRANES

Condition	B-50 Dephosphorylation (% of 30 second cpm)
Control	64.0 ± 7.2
Inhibitor-1	96.4 ± 5.5*
Inhibitor-2	92.7 ± 2.7†

*Statistically different from the control value at the $p<0.05$ level.
†Statistically significant from the control value at the $p<0.005$ level.
Cortical synaptic plasma membranes (5 μg) were incubated with 2 μCi of [γ-^{32}P]ATP for 30 seconds and then for an additional 30 minutes in the presence or absence of 100 units of inhibitor-1 or 30 units of inhibitor-2. Labeling of B-50 at the end of 30 minutes is compared to that seen at 30 seconds, which is set at 100%. All values are the mean ± SEM from three or four experiments.

tive as ATP. Adenine, adenosine, AMP, adenosine 5′-O-(2-thiodiphosphate) or ADP-β-S, and the nonhydrolyzable ATP analogs, adenosine 5′-(β,γ-imino)triphosphate (AMP-PNP) and adenosine 5′-(β,γ-methylene)triphosphate (AMP-PCP) had no effect on B-50 dephosphorylation. None of the guanine derivatives tested—GDP, GTP and guanosine 5′-(β,γ-imino)triphosphate (GMP-PNP)—altered the dephosphorylation of B-50 (Table 2).

Addition of 20 mM EDTA to prelabeled synaptic plasma membranes inhibited the dephosphorylation of B-50 by approximately 20% (data not shown). But because this relatively high concentration of EDTA could alter association of Ca^{2+}-dependent modulators to the membranes, this effect might not be indicative of a cation requirement for this synaptic plasma membrane-associated phosphatase. To more directly determine the cation sensitivity of this enzyme, synaptic plasma membranes were phosphorylated under standard assay conditions and immediately washed with 10 mM sodium acetate—0.1 mM EDTA, pH 6.5.

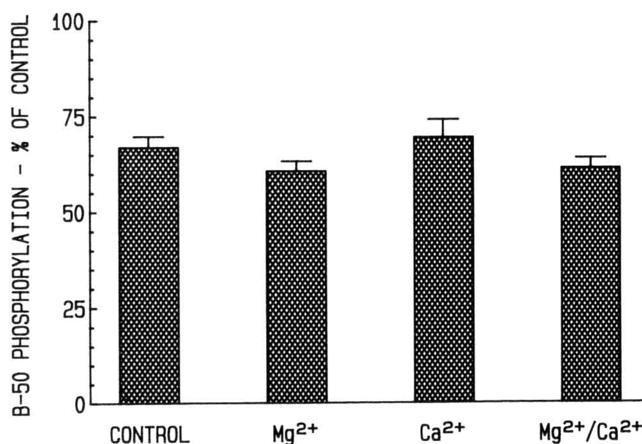

FIG. 4. Effect of cations on B-50 dephosphorylation in synaptic plasma membranes. Cortical synaptic plasma membranes were labeled under the standard conditions with [γ-^{32}P]ATP for 30 seconds to label B-50. Membranes were washed and suspended in 10 mM sodium acetate—0.1 mM EDTA, pH 6.5. Dephosphorylation assays were then performed for 30 minutes in the presence of 10 mM Mg^{2+}, 1 mM Ca^{2+} or both. Each value is the mean ± SEM from 4 or 5 separate experiments, expressed as a % of the 30 second value which is set at 100%.

TABLE 2

DEPHOSPHORYLATION OF SYNAPTIC PLASMA MEMBRANE-ASSOCIATED B-50 IN THE PRESENCE OF ADENINE AND GUANINE DERIVATIVES

Addition	Labeling of B-50 (% of 30 second cpm)
Control	56.3 ± 2.4
Adenine	55.9 ± 3.1
Adenosine	50.8 ± 5.4
AMP	49.0 ± 2.9
ADP	22.2 ± 3.2*
ADP-β-S	60.2 ± 1.9
ATP	30.5 ± 2.3*
ATP-γ-S	34.2 ± 3.9†
AMP-PCP	62.9 ± 5.7
AMP-PNP	62.9 ± 5.6
GDP	52.2 ± 1.2
GTP	57.0 ± 3.5
GMP-PNP	55.1 ± 2.5

*Statistically significant at the $p<0.005$ level.
†Statistically significant at the $p<0.01$ level.
Synaptic plasma membranes (25 μg) were incubated with 2 μCi [γ-^{32}P]ATP for 30 seconds and then for an additional 30 minutes in the presence or absence of each of the indicated adenine or guanine derivatives (0.5 mM). Labeling of B-50 at the end of 30 minutes is compared to that seen at 30 seconds, which is set at 100%. All values are the same ± SEM from 3–10 experiments.

Dephosphorylation assays were then carried out for 30 minutes in the presence of 10 mM magnesium acetate, 1 mM calcium acetate or both. As shown in Fig. 4, Mg^{2+} produced a small, but statistically significant ($p<0.01$) stimulation of B-50 dephosphorylation. Higher concentrations of Mg^{2+} had no additional effect. Calcium had no effect on the dephosphorylation of B-50. The presence of both Mg^{2+} and Ca^{2+} had no effect beyond that seen with Mg^{2+} alone (Fig. 4). Calcium had no effect in either the presence or absence of 25 units (1 μM final concentration) calmodulin (data not shown).

Solubilization of Synaptic Plasma Membrane-Associated Protein Phosphatase Activity and Assay with Purified B-50 as Substrate

The methodology is an adaptation of that used by Zwiers *et al.*

TABLE 3

PARTIAL PURIFICATION OF B-50 PROTEIN PHOSPHATASE ACTIVITY FROM RAT BRAIN SYNAPTIC PLASMA MEMBRANES

Specific Activity	Fraction (units/mg protein)
Whole homogenate	0.43
Extract	3.41
DEAE-cellulose column pool	6.50

A synaptic membrane fraction from rat brain was prepared and extracted with 0.5% Triton X-100—75 mM KCl. The extract was chromatographed on a DEAE-cellulose column. The protein phosphatase activity of the whole homogenate, extract and DEAE-cellulose column fractions was measured with purified ^{32}P-labeled B-50 as substrate. One unit of activity is defined as the amount of protein phosphatase activity which removes 1 pmole of phosphate from B-50 in 1 minute at 30°C. The results are representative of 4 separate experiments.

(47) to purify B-50 and protein kinase C in which membranes were extracted with 0.5% Triton X-100 and 75 mM KCl. The extract was chromatographed on DEAE-cellulose and column fractions were assayed for protein phosphatase activity using purified ^{32}P-labeled B-50 as substrate. The B-50 preparation had no apparent endogenous protein phosphatase activity. A peak of B-50 phosphatase activity was eluted from the column at approximately 260 mM NaCl. Column fractions containing B-50 phosphatase activity were pooled. The addition of leupeptin had no effect on the loss of ^{32}P-labeling of B-50, indicating the lack of protease activity in the DEAE-cellulose column fractions (data not shown). The specific activity of the B-50 phosphatase activity in the DEAE-cellulose pool was determined and compared to that of the whole homogenate and the Triton-KCl extract. Partial purification of B-50 phosphatase with these procedures resulted in a 15-fold enrichment in activity (Table 3).

DISCUSSION

The major classes of cellular phosphatases were first characterized with regard to their involvement in glycogen metabolism (16). However, the brain, which has little capacity for glycogen deposition and no significant reliance upon its metabolism (21), possesses a relatively high content of protein phosphatase activity. This is particularly true for the fraction of protein phosphatase activity which is associated with synaptic and particulate fractions (17,25). Consistent with multiple indications of a regulatory role for protein phosphorylation in synaptic function, the same fractions contain high concentrations of protein kinase activities and available substrates (15,26).

B-50 is a prominent synaptic membrane-bound substrate for protein kinase C in the brain (2). A close association between this substrate and enzyme is indicated by the coextraction and purification of both through multiple steps (47). Assuming that the synaptic functions of B-50 depend upon its phosphorylated state, reversibility of phosphorylation is mandatory, a condition which would be met most efficiently if a protein phosphatase were also membrane-bound in close proximity to B-50 and protein kinase C. These studies report the characteristics and partial purification of such an enzyme. Since B-50 has been used as a substrate, in both synaptic plasma membrane-bound and purified forms, this phosphatase is referred to in this discussion as B-50 phosphatase.

Incubation of synaptic plasma membranes with a low concentration of [γ-^{32}P]ATP (7.5 μM) shows net phosphorylation of B-50 for only very short periods of time. This has previously been shown to result from the rapid degradation of the labeled ATP by endogenous ATPase activity of these membranes (43). The loss of B-50 labeling which follows can best be interpreted as an indication of synaptic plasma membrane-bound protein phosphatase activity.

The product of the B-50 dephosphorylation reaction was not characterized in these studies as ^{32}Pi because of the simultaneous presence of excess ^{32}Pi derived from hydrolysis of exogenous-labled ATP and multiple membrane-associated phosphoproteins and phospholipids. However, several alternate controls indicate that a protein phosphatase, rather than a protease, activity is being measured in the synaptic plasma membranes. This preparation of membranes does contain a Ca^{2+}-dependent protease with a high affinity for B-50 (48). However, leupeptin, which inhibits such enzyme activity (27), has no effect on the dephosphorylation of B-50 in synaptic plasma membranes. Western blot analysis shows no loss of B-50 immunoreactivity from synaptic plasma membranes comparable to the loss of ^{32}P-labeling. Moreover, no breakdown products of B-50 are seen on the Western blot, most particularly the fragment B-60, which is readily generated by Ca^{2+}-dependent protease activity. Finally, the loss of phosphorylated B-50 is sensitive to two heat-stable protein phosphatase inhibitors, directly implicating activity of a synaptic membrane-bound protein phosphatase, presumably the type-1 form.

Although inhibition of B-50 dephosphorylation by either inhibitor-1 or inhibitor-2 is virtually complete, the concentrations needed to produce this effect are considerably above those needed to maximally inhibit phosphorylase phosphatase activity (29). The contrast is especially striking given the small amount of B-50 phosphatase which would be present in the 5 μg of synaptic plasma membranes used in these assays. Given the possibility of nonspecific effects with a relatively high ratio of inhibitor to phosphatase, the interpretation that the major fraction of synaptic membrane-bound B-50 phosphatase is phosphatase-1 is made with some caution.

However, when definition of units of activity are made comparable (11,29), the amounts of inhibitors found to block B-50 phosphatase are similar to those used by Shields *et al.* (35), who demonstrated, based on its sensitivity to inhibitor-1 and inhibitor-2, that the major form of protein phosphatase in postsynaptic junctions was type-1. This equivalence of inhibitor sensitivity and the fact that, in both cases, inhibitor-1 and inhibitor-2 produced close to complete inhibition of enzyme activity would suggest that protein phosphatase-1 is the predominant form associated with both pre- and postsynaptic membranes. This would be in accord with other reports that a significant fraction of protein phosphatase-1 activity is membrane-bound and that the brain has a high content of type-1 phosphatase (17,46).

The lack of a cation requirement does eliminate coidentity of this B-50 phosphatase and several known types of phosphatases. Protein phosphatase-2B (calcineurin) has a strict requirement for Ca^{2+} and calmodulin (16). Since Ca^{2+} has no effect on the dephosphorylation of B-50 in synaptic plasma membranes, calcineurin is unlikely to be associated with B-50 and protein kinase C as a functional complex within the membranes. However, the recent demonstrations (22,33) that B-50 is a substrate for purified calcineurin suggest that distinct cytoplasmic and membrane-bound protein phosphatases may contribute to its dephosphorylation. The fact that calcineurin accounts for only 60% of the total B-50 phoshatase activity in a bovine brain extract (33) supports such a conjecture. The attachment of B-50 by its N-terminal region to the cytoplasmic face of the synaptic membrane (38) and its ability to bind calmodulin (4) make this an intriguing possibility. The small, but significant, stimulation of B-50 phosphatase activity by Mg^{2+} suggests the presence of a minor form of enzyme in the membranes with a requirement for this cation. Of the known phosphatases, this would be the 2-C or Mg^{2+}/ATP-dependent phosphatases (16).

Dephosphorylation of synaptic plasma membrane-bound B-50 is stimulated in the presence of ATP. The most direct interpretation of this effect is that B-50 phosphatase or some factor regulating its activity is altered by phosphorylation. The ability of ADP to produce a similar effect would be explained by the reverse reaction of ATPase in the presence of excess dinucleotide to generate ATP for a protein kinase reaction. The inactivity of less phosphorylated adenine derivatives, the nonhydrolyzable ATP analogs and any guanine derivative would be consistent with this hypothesis. In addition, ADP-β-S would not be effective since it cannot accept a third phosphate to form ATP (13). However, although the stimulation of B-50 dephosphorylation by ATP or ADP is consistent with a phosphorylation/activation step, other alternatives must be considered.

Addition of excess unlabeled ATP will cause an exchange of nonradioactive for radioactive phosphate groups on B-50, but it is not clear how this would lead to an increase in the rate or extent of apparent B-50 dephosphorylation. Addition of ATP to the membranes would increase the content of phosphorylated B-50, thereby

increasing the amount of substrate for, and the activity of, B-50 phosphatase. However, ATP-γ-S, which adds a thiophosphate group onto proteins that cannot be removed by protein phosphatases (10) has the same effect. Other major forms of protein kinases (cAMP-dependent, phosphorylase kinase) have been shown to be active in the reverse direction under appropriate assay conditions (36,37). A similar reaction involving protein kinase C would remove phosphate groups from labeled B-50 and appear as apparent protein phosphatase activity. But since ATP-γ-S also is a relatively poor substrate for ATPase (10), the possibility that the excess ADP or ATP (after conversion to ADP by ATPase) added to the dephosphorylation reaction could be promoting the reverse reaction of protein kinase C is unlikely. Finally, B-50 phosphatase may be similar to the ATP/ADP-dependent form of phosphatase, found by Pauloin and Jolles (30) to be associated with coated vesicles in brain. Although classification of this enzyme is not complete, its stimulation by ADP, in equal potency to ATP, is similar to that reported here.

Further characterization of solubilized B-50 phosphatase will be necessary to establish its identity and relationship to other forms. Given the lack of a detailed classification for membrane-associated protein phosphatases, it remains a distinct possibility that B-50 phosphatase is a novel form. Moreover, its regulation may depend upon its association with the membrane environment. As purification proceeds, care will need to be taken in comparing the properties of the solubilized enzyme to those of the membrane-associated form.

As is the case for cyclic AMP-dependent protein kinase, protein phosphatase-1 and the dopaminoceptive cell-specific form of inhibitor-1 called DARPP-32 (14,44), it is anticipated that regulation of B-50 phosphatase will be coordinated and interactive with other components of the protein kinase C-associated complex. In this regard it should be noted that the same solubilization protocol which yields enhanced B-50 phosphatase activity is that used for the coextraction and purification of B-50 and protein kinase C (47). The literature accumulating on the properties of the substrate protein B-50 (GAP-43, pp46, F-1, P-57) suggests that the biochemical actions of this system will be functionally relevant to presynaptic processes regulating neurotransmitter release (7) and the mechanisms of synaptic growth and plasticity (38).

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REFERENCES

1. Alexander, K. A.; Cimler, B. M.; Meiri, K. E.; Storm, D. R. Regulation of calmodulin binding to P-57. A neurospecific calmodulin binding protein. J. Biol. Chem. 262:6108–6113; 1987.
2. Aloyo, V. J.; Zwiers, H.; Gispen, W. H. Phosphorylation of B-50 protein by calcium-activated, phospholipid-dependent protein kinase and B-50 kinase. J. Neurochem. 41:649–653; 1983.
3. Burbach, J. P. H.; DeKloet, E. R.; Schotman, P.; DeWied, D. Proteolytic conversion of β-endorphin by brain synaptic membranes. J. Biol. Chem. 256:12463–12469; 1981.
4. Cimler, B. M.; Giebelhaus, D. H.; Wakim, B. T.; Storm, D. R.; Moon, R. T. Characterization of murine cDNAs encoding P-57, a neural-specific calmodulin-binding protein. J. Biol. Chem. 262: 12158–12163; 1987.
5. Coussens, L.; Parker, P. J.; Rhee, L.; Yang-Feng, T. L.; Chen, E.; Waterfield, M. D.; Fracke, U.; Ullrich, A. Multiple distinct forms of bovine and human protein kinase C suggest diversity in cellular signalling pathways. Science 233:859–866; 1986.
6. DeGraan, P. N. E.; VanHooff, C. O. M.; Tilly, B. C.; Oestreicher, A. B.; Schotman, P.; Gispen, W. H. Phosphoprotein B-50 in nerve growth cones from fetal rat brain. Neurosci. Lett. 61:235–241; 1985.
7. Dekker, L. V.; DeGraan, P. N. E.; Versteeg, D. H. G.; Oestreicher, A. B.; Gispen, W. H. Phosphorylation of B-50 (GAP-43) is correlated with neurotransmitter release in rat hippocampal slices. J. Neurochem. 52:24–30; 1989.
8. Dokas, L. A.; Zwiers, H.; Coy, D. H.; Gispen, W. H. Somatostatin and analogs inhibit endogenous synaptic plasma membrane protein phosphorylation *in vitro*. Eur. J. Pharmacol. 88:185–193; 1983.
9. Dokas, L. A.; Klis, M.; Liauw, A.; Coy, D. H. Characteristics of [D-Trp^8]-somatostatin-sensitive B-50 phosphorylation. Peptides 6: 1101–1107; 1985.
10. Eckstein, F. Nucleoside phosphorothioates. Annu. Rev. Biochem. 54:367–402; 1985.
11. Foulkes, J. G.; Cohen, P. The regulation of glycogen metabolism. Purification and properties of protein phosphatase inhibitor-2 from rabbit skeletal muscle. Eur. J. Biochem. 105:195–203; 1980.
12. Gispen, W. H.; VanDongen, C. J.; DeGraan, P. N. E.; Oestreicher, A. B.; Zwiers, H. The role of the phosphoprotein B-50 in phosphoinositide metabolism in brain synaptic plasma membranes. In: Bleasedale, J. E.; Hauser, G.; Eichberg, J., eds. Inositol and phosphoinositides. Dallas: Humana Press; 1985:399–414.
13. Goody, R. S.; Eckstein, F.; Schirmer, R. H. The enzymatic synthesis of thiophosphate analogs of nucleotides. Biochim. Biophys. Acta 276:155–161; 1972.
14. Hemmings, H. C.; Greengard, P.; Lim Tung, H. Y.; Cohen, P. DARPP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. Nature 310:503–508; 1984.
15. Hemmings, H. C.; Nairn, A. C.; Greengard, P. Protein kinases and phosphoproteins in the nervous system. In: Martin, J. B.; Barchas, J. D., eds. Neuropeptides in neurologic and psychiatric disease. New York: Raven Press; 1986:47–69.
16. Ingebritsen, T. S.; Cohen, P. Protein phosphatases: Properties and role in cellular regulation. Science 221:331–338; 1983.
17. Ingebritsen, T. S.; Stewart, A. A.; Cohen, P. The protein phosphatases involved in cellular regulation. 6. Measurement of type-1 and type-2 protein phosphatases in extracts of mammalian tissues; an assessment of their physiological roles. Eur. J. Biochem. 132: 297–307; 1983.
18. Jacobsen, R.D.; Virag, I.; Skene, J. H. P. A protein associated with axonal growth, GAP-43, is widely distributed and developmentally regulated in rat CNS. J. Neurosci. 6:1843–1855; 1986.
19. Jolles, J.; Zwiers, H.; VanDongen, C.; Schotman, P.; Wirtz, K. W. A.; Gispen, W. H. Modulation of brain polyphosphoinositide metabolism by ACTH-sensitive protein phosphorylation. Nature 286:623–625; 1980.
20. Karns, L. R.; Ng, S.-C.; Freeman, J. A.; Fishman, M. C. Cloning of complementary DNA for GAP-43, a neuronal growth-related protein. Science 236:597–600; 1987.
21. Lajtha, A. L.; Maker, H. S.; Clarke, D. D. Metabolism and transport of carbohydrates and amino acids. In: Seigel, G. J.; Albers, R. W.; Agranoff, B. W. and Katzman, R., eds. Basic neurochemistry. 3rd ed. Boston: Little, Brown and Company; 1981:329–353.
22. Liu, Y.; Storm, D. R. Dephosphorylation of neuromodulin by calcineurin. J. Biol. Chem. 264:12800–12804; 1989.
23. Lovinger, D. M.; Colley, P. A.; Akers, R. F.; Nelson, R. B.; Routtenberg, A. Direct relation of long-term synaptic potentiation to phosphorylation of membrane protein F1, a substrate for membrane protein kinase C. Brain Res. 399:205–211; 1986.
24. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265–275; 1951.
25. Maeno, H.; Greengard, P. Phosphoprotein phosphatases from rat cerebral cortex. Subcellular distribution and characterization. J. Biol.

Chem. 247:3269–3277; 1972.
26. Mahler, H. R.; Kleine, L. P.; Ratner, N.; Sorensen, R. G. Identification and topography of synaptic phosphoproteins. Prog. Brain Res. 56:27–48; 1982.
27. Malik, M. N.; Fenko, M. D.; Iqbal, K.; Wisniewski, H. Purification and characterization of two forms of Ca^{3+}-activated neutral protease from calf brain. J. Biol. Chem. 258:8955–8962; 1983.
28. Meiri, K. F.; Pfenninger, K. H.; Willard, M. B. Growth-associated protein, GAP-43, a polypeptide that is induced when neurons extend axons, is a component of growth cones and corresponds to pp46, a major polyeptpide of a subcellular fraction enriched in growth cones. Proc. Natl. Acad. Sci. USA 83:3537–3541; 1986.
29. Mellgren, R. M.; Schlender, K. K. Rabbit kidney cortex phosphorylase phosphatases: Evidence for complexes between high molecular weight forms and heat-stable inhibitor proteins. J. Cyclic Nuc. Res. 8:27–37; 1982.
30. Pauloin, A.; Jolles, P. Presence of a MgATP/ADP-dependent pp50 phosphatase in bovine brain coated vesicles. J. Biol. Chem. 261: 12568–12573; 1986.
31. Sahyoun, N.; LeVine, H., III; McConnell, R.; Bronson, D.; Cuatracasas, P. A specific phosphoprotein phosphatase acts on histone H1 phosphorylated by protein kinase C. Proc. Natl. Acad. Sci. USA 80:6760–6764; 1983.
32. Schrama, L. H.; DeGraan, P. N. E.; Wadman, W. J.; Lopes da Silva, F. H.; Gispen, W. H. Long-term potentiation and 4-aminopyridine-induced changes in protein and lipid phosphorylation in the hippocampal slice. Prog. Brain Res. 69:245–257; 1986.
33. Schrama, L. H.; Heemskerk, F. M. J.; DeGraan, P. N. E. Dephosphorylation of protein kinase C—phosphorylated B-50/GAP-43 by the calmodulin-dependent phosphatase calcineurin. Neurosci. Res. Commun. 5:141–147; 1989.
34. Schrama, L. H.; Weeda, G.; Edwards, P. M.; Oestreicher, A. B.; Schotman, P. Multiple phosphorylation of pp30, a rat brain polyribosomal protein sensitive to polyamines and corticotropin. Biochem. J. 224:747–753; 1989.
35. Shields, S. M.; Ingebritsen, T. S.; Kelly, P. T. Identification of protein phosphatase-1 in synaptic junctions: Dephosphorylation of endogenous calmodulin-dependent kinase II and synapse-enriched phosphoproteins. J. Neurosci. 5:3414–3422; 1985.
36. Shizuta,Y.; Beavo, J. A.; Bechtel, P. J.; Hofman, F.; Krebs, E. G. Reversibility of adenosine 3′,:5′-monophosphate-dependent protein kinase reactions. J. Biol. Chem. 250:6891–6896; 1975.
37. Shizuta, Y.; Khandelwal, R. L.; Maller, J. L.; Vandeheede, J. R.; Krebs, E. G. Reversibility of phosphorylase kinase reaction. J. Biol. Chem. 252:3408–3413; 1977.
38. Skene, J. H. P. Axonal growth-associated proteins. Annu. Rev. Neurosci. 12:127–156; 1989.
39. Skene, J. H. P.; Willard, M. Axonally-transported proteins associated with axon growth in rabbit central and peripheral nervous system. J. Cell. Biol. 89:96–103; 1981.
40. Skene, J. H. P.; Jacobson, R. D.; Snipes, G. J.; McGuire, C. B.; Norden, J. J.; Freeman, J. A. A protein induced during nerve growth (GAP-43) is a major component of growth cone membranes. Science 233:783–786; 1986.
41. Sorenson, R. G.; Kleine, L. P.; Mahler, H. R. Presynaptic localization of phosphoprotein B-50. Brain Res. Bull. 7:57–61; 1981.
42. Ueda, T.; Rudolf, S. A.; Greengard, P. Solubilization of a phosphoprotein and its associated cyclic AMP-dependent protein kinase and phosphoprotein phosphatase from synaptic plasma membranes and some kinetic evidence for their existence as a complex. Arch. Biochem. Biophys. 170:492–503; 1975.
43. Weigant, V. M.; Zwiers, H.; Schotman, P.; Gispen, W. H. Endogenous phosphorylation of rat brain synaptosomal membranes *in vitro:* Some methodological aspects. Neurochem. Res. 3:443–453; 1978.
44. Williams, K. R.; Hemmings, H. C.; LoPresti, M. B.; Konigsberg, W. H.; Greengard, P. DARPP-32, a dopamine and cyclic AMP-regulated neuronal phosphoprotein. J. Biol. Chem. 261:1890–1903; 1986.
45. Yang, S-D.; Vandeheede, J. R.; Merleverde, W. A simplified procedure for the purification of the protein phosphatase modulator (inhibitor-2) from rabbit skeletal muscle. FEBS Lett. 132:293–295; 1981.
46. Yang, S-D.; Fong, Y-L. Identification and characterization of an ATP.Mg-dependent protein phosphatase from pig brain. J. Biol. Chem. 260:13464–13470; 1985.
47. Zwiers, H.; Schotman, P.; Gispen, W. H. Purification and some characteristics of an ACTH-sensitive protein kinase and its substrate protein in rat brain membranes. J. Neurochem. 34:1689–1699; 1980.
48. Zwiers, H.; Gispen, W. H.; Kleine, L.; Mahler, H. Specific proteolysis of a brain membrane phosphoprotein (B-50): Effects of calcium and calmodulin. Neurochem. Res. 7:127–137; 1982.
49. Zwiers, H.; Verhaagen, J.; VanDongen; DeGraan, P. N. E.; Gispen, W. H. Resolution of rat brain synaptic phosphoprotein B-50 into multiple forms by two-dimensional electrophoresis: evidence for multisite phosphorylation. J. Neurochem. 44:1083–1090; 1985.