Biochemical Pharmacology, Vol. 46, No. 4, pp. 677–681, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

EFFECTS OF CALCIUM CHANNEL ANTAGONISTS ON THE PHOSPHORYLATION OF MAJOR PROTEIN KINASE C SUBSTRATES IN THE RAT HIPPOCAMPUS

FREDERICK J. HOFFMAN, JR. and RONALD A. JANIS*
Miles Research Center, Miles Inc., West Haven, CT 06516, U.S.A.

(*Received* 21 *December* 1992; *accepted* 23 *April* 1993)

Abstract—K^+-induced depolarization of rat hippocampal slices resulted in significant increases in the phosphorylation state of myristoylated, alanine-rich C kinase substrate (MARCKS; also known as 87K, pp80) and neuromodulin [also known as growth associated protein 43 (GAP43), B50, F1] as determined by back-phosphorylation using protein kinase C. The effect of organic and inorganic Ca^{2+} antagonists on the phosphorylation of these major protein kinase C substrates in the rat hippocampus was studied to determine whether Ca^{2+} influx through L- or N-type voltage-sensitive Ca^{2+} channels was required for the phosphorylation changes observed. The depolarization-induced changes appeared to be dependent on extracellular Ca^{2+}, based on evidence indicating that the chelation of extracellular Ca^{2+} with ethylene glycol-bis (β-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) inhibited these changes. In addition, pretreatment of the slices with 500 μM Cd^{2+}, but not 300 nM nimodipine, 10 μM ω-conotoxin GVIA or 10 μM MK-801, blocked the K^+-induced change in phosphorylation. These results suggest that K^+-induced changes in the phosphorylation of MARCKS and neuromodulin are mediated by Ca^{2+}-dependent mechanisms other than, or in addition to, those sensitive to the organic Ca^{2+} channel antagonists employed.

Nimodipine, a 1,4-dihydropyridine Ca^{2+} channel antagonist, has a variety of effects on the brain that include neuroprotection, accelerated neural regeneration and cognition enhancement [1, 2]. In neuronal systems, Ca^{2+} and various protein kinases are implicated in mechanisms of ischemic cell damage, neuroplasticity and in electrophysiological models of imprinting [3–8]. In the present study, the effects of nimodipine and other Ca^{2+} blockers were investigated to determine whether these treatments modulated depolarization- and extracellular Ca^{2+}-dependent phosphorylation of hippocampal proteins by protein kinase C.

Different methods have been used to study drug effects on Ca^{2+}-dependent protein phosphorylation. The use of tissue homogenates incubated with drug, cofactors and [γ-^{32}P]ATP relies on endogenous kinase and phosphatase activity that may not be representative of the *in situ* state. An alternative *in vivo* method labels the intracellular ATP pool of intact tissue or cells with radioactive $^{32}PO_4$ prior to drug treatment. In both methods the substrates of interest are isolated using immunoprecipitation techniques or electrophoretic separation. As critically discussed by Nestler and Greengard [9], the *in vivo* studies are dependent upon the consistency of labeling the ATP pool, and the high background, due to the use of millicurie amounts of ^{32}P, may make direct quantitation difficult. In using either method, the comigration on gel electrophoresis of numerous phosphorylated proteins may make identification difficult. Back-phosphorylation techniques, properly conducted, can determine the state of phosphorylation of discrete substrates for a specific kinase [9]. We report here for the first time a back-phosphorylation procedure using protein kinase C to study the mechanism of action of Ca^{2+} channel antagonists.

MATERIALS AND METHODS

Materials. Drugs, chemicals and protein kinase C were obtained as follows: nimodipine (Miles Inc.); ω-conotoxin GVIA (Peninsula Laboratories Inc.); (±) MK-801 (Research Biochemicals Inc.); Na_2HPO_4, KH_2PO_4, NaOH, KCl, $CdCl_2$, NaCl, $CaCl_2$ (Baker); acrylamide, bis-acrylamide, piperazine diacrylamide (PDA†), urea, sodium dodecyl sulfate (SDS, Ultrapure grade), glycine, 2-mercaptoethanol, *N*,*N*,*N*′,*N*′-tetra-methylethyldiamine (Bio-Rad); *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (HEPES) (Calbiochem); phosphoric acid (H_3PO_4) (EM Science); ampholines (40%) pH 5–7, pH 5–10 (LKB Pharmacia); [γ-^{32}P]ATP (10–30 Ci/mmol) (New England Nuclear); aprotinin, leupeptin, dithiothreitol, Nonidet P-40, sodium phosphatidylserine, 1,2-dioleoyl-rac-glycerol, magnesium acetate, citric acid, ethylene glycol-bis-(β-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), $CaCl_2$, Trizma Base and prestained molecular weight markers "SDS 7B" (Sigma Chemical).

The protein kinase C was purified from rat

* Corresponding author: Dr. R. A. Janis, Miles Research Center, Miles Inc., 400 Morgan Lane, West Haven, CT 06156.

† Abbreviations: EGTA, ethylene glycol-bis (β-amino-ethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MARCKS, myristoylated, alanine-rich C kinase substrate; PDA, piperazine diacrylamide; and SDS, sodium dodecyl sulfate.

forebrain [10] and was supplied by Drs. A. C. Nairn and P. Greengard of Rockefeller University, New York, NY.

Equipment. The Bio-Rad model 175 tube cell and Protean II 20-cm multicell 2D Vertical Electrophoresis Systems were used for protein separation and the AMBIS Mark II Radioanalytic Imaging System for quantitative 2D gel analysis.

Methods. Phosphorylation changes were determined using a modification of the direct back-phosphorylation technique of Guitart and Nestler [11], which measures the dephospho form of the protein. The general procedure was as follows: (1) treatment of hippocampal slices; (2) homogenization and extraction of slices with acidification (pH 2.9) to inactivate endogenous kinases and phosphatases, thereby maintaining the phosphorylation state of the proteins; (3) centrifugation of homogenates resulting in an acid extract composed of soluble and membrane-bound proteins; (4) neutralization of the extract followed by phosphorylation with purified protein kinase C and [γ-^{32}P]ATP; and (5) electrophoretic separation of the phosphorylated proteins using two-dimensional polyacrylamide gel electrophoresis. Since only the dephospho form of the protein is phosphorylated in this system, a decrease in phosphorylation generally reflects an increase in the phosphorylation state of the protein [11, 12].

Experiments were performed *ex vivo* using hippocampal slices (400 μm) obtained from 3- to 9-month-old male Long-Evans Hooded rats. The slices were placed, two per tube, in 5 mL of a low Ca^{2+} Krebs-bicarbonate buffer (pH 7.33) of the following composition (in mM); NaCl, 124; KCl, 3.33; $CaCl_2$, 0.01; KH_2PO_4, 1.25; $MgSO_4$, 1.33; $NaHCO_3$, 25.7; D-glucose, 10; HEPES, 20. The slices were equilibrated for 60 min at 33–34° with water-saturated oxygenation (95% O_2, 5% CO_2). After 60 min the solution was replaced with Krebs buffer containing a physiological level of Ca^{2+} (1.3 mM) and incubated for an additional 90 min [13]. After the pre-equilibration period, the slices were pretreated for 15 min with either vehicle or drug. The tissue was depolarized by increasing the K^+ concentration 50 mM by the addition of 250 μL of a 1 M KCl solution, and the slices were incubated for an additional 5 min. The buffer was removed and the slices sonicated in 300 μL of acidified homogenization buffer (pH 2.9) containing 20 mM citric acid, 0.1% Nonidet P-40, 10 mM Tris, 1 mM EGTA, 1 mM dithiothreitol, 0.1 trypsin inhibitor units/mL aprotinin, and 10 μg/mL leupeptin [modified from Ref. 11].

Acidified homogenates (pH 2.9) were centrifuged at 10,000 *g* for 15 min at 4°. Aliquots of the supernatant, containing acid-soluble proteins, were neutralized with the addition of 200 mM Na_2HPO_4 (20%, v/v) [11, 14]. Protein content was determined by the method of Bradford [15], using bovine serum albumin as the standard. An aliquot (≈15–30 μL) containing 10 μg protein was back-phosphorylated in a reaction solution (total volume 80 μL) containing 50 mM Tris, pH 7.4, 10 mM magnesium acetate, 1 mM EGTA, 10 mM dithiothreitol, 0.5% Nonidet P-40, 10 μg phosphatidylserine, 0.4 μg dioleoyl-rac-glycerol, 1.8 mM Ca^{2+}, 0.01% bovine serum albumin, 10 μg leupeptin, 60 ng protein kinase C and 8 μM [γ-^{32}P]ATP (10–30 Ci/mmol) [modified from Ref. 16]. The reaction was initiated by the addition of [γ-^{32}P]ATP and incubated for 30 min at 30°. The reaction was terminated and proteins were denatured with 47 mg urea and 8 μL stop solution [final concentration: 9 M urea, 2% Nonidet P-40, 2% ampholines (ratio of pH 3–10 to pH 5–7 1:1), 5% 2-mercaptoethanol] [11].

Samples were subjected to two-dimensional electrophoretic separation using a modification of the method of O'Farrell [17]. Isoelectric focusing tube gels (2 mM) containing 9 M urea, 4% acrylamide (ratio of acrylamide to PDA, a cross-linker, was 25:1), 2% Nonidet P-40, and 4% ampholines (ratio of pH 3–10 to pH 5–7 was 1:1) were prepared and polymerized for 4–6 hr prior to use. Eighty microliters of the reaction mixture was loaded onto the tube gels and overlaid with 20 μL of sample overlay buffer (6.5 M urea, 0.4% ampholine, pH 3–10). Tube gels were electrophoresed for 20–24 hr at 400 V (constant). The anode solution was 10 mM H_3PO_4 and the cathode solution was 20 mM NaOH. The tube gels were extruded and soaked in 3 mL SDS stop solution (50 mM Tris, pH 6.7, 4% glycerol, 2% SDS, 2% 2-mercaptoethanol, 0.01% bromophenol blue) for 40–60 min at room temperature.

Second-dimensional gels were run by layering the tube gel on an SDS–polyacrylamide slab gel [18]. The acrylamide concentration of the upper stacking gel was 3.6% and that of the lower resolving gel was 7.5% with 4% *N,N'*-methylene-bis-acrylamide. Gels were electrophoresed at 50 V (constant) overnight until the dye front had traveled 11–12 cm from the bottom of the stacking gel. The electrophoretic separation was calibrated for molecular weight by use of prestained markers run in the second dimension (Sigma "SDS 7B"), and the pH gradient of the focusing gel was determined by homogenizing 1-cm sections of an equilibrated tube gel in 200 μL of deionized water and measuring the pH. The gradient was linear from pH 3.8 to 7.0.

The two-dimensional gels were dried under vacuum at 80° and major proteins were directly quantitated in counts per minute on an AMBIS Radioanalytic Imaging System (San Diego, CA). Instrument sensitivity and linearity were verified for ^{32}P from 30 to 30,000 dpm (≈30% efficiency) using a 1-hr scan. Autoradiographic exposures on Kodak X-OMAT 8 × 10 film were used for comparative purposes. Statistical analysis was performed using a two-way analysis of variance with a Bonferroni *t*-test to determine the level of significance.

RESULTS

Substrates for protein kinase C were analyzed in rat hippocampal CA1 slices. Approximately ten major phosphoproteins were detected by back-phosphorylation and separation using two-dimensional electrophoretic techniques. Two of the prominent phosphoproteins were identified as myristolated, alanine-rich C kinase substrate (MARCKS) and neuromodulin based on their electrophoretic properties (Fig. 1). Using increasing

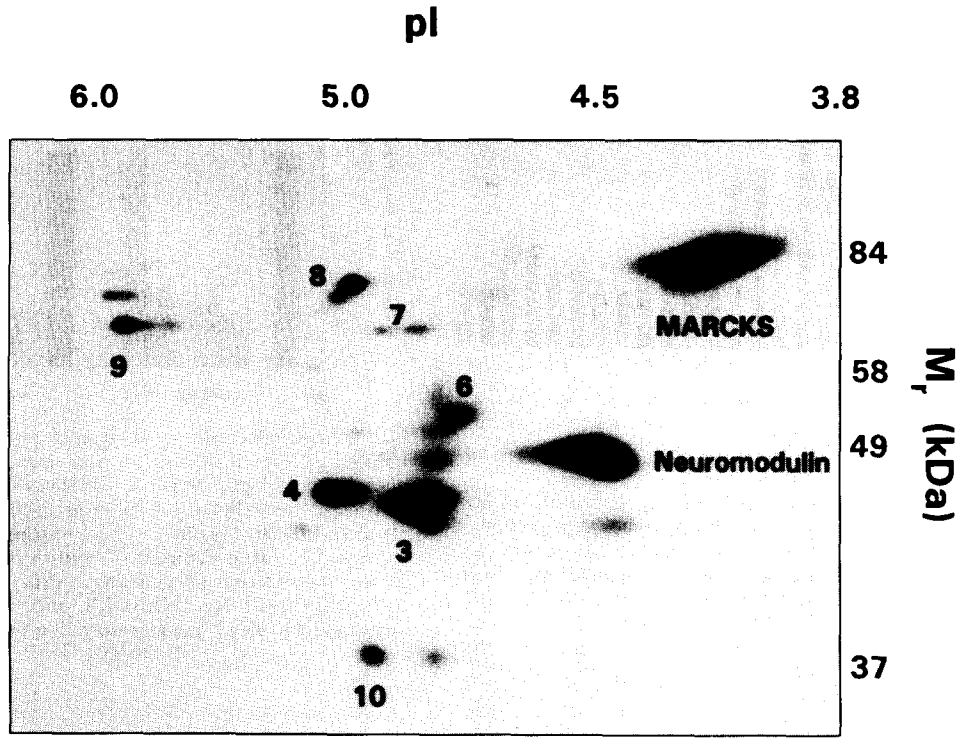

Fig. 1. Autoradiogram of a two-dimensional gel showing ^{32}P incorporation into hippocampal slice proteins back-phosphorylated by protein kinase C. Aliquots of neutralized acid extracts containing 10 μg protein were incubated at 30° with 60 ng protein kinase C (sp. act. 0.3 nmol phosphate/min/μg using histone III S as substrate), 8 μM [γ-^{32}P]ATP in a volume of 80 μL.

concentrations of protein kinase C, it was demonstrated that 60 ng was necessary to achieve maximal phosphorylation (Fig. 2A) of MARCKS and neuromodulin. Analysis of the time course of phosphorylation indicated that 30 min was an appropriate incubation time for back-phosphorylation (Fig. 2B). The acid extraction procedure recovered >95% of MARCKS, ≈80% of neuromodulin and ≈50% of the remaining phosphoproteins as determined by quantitation of the total yield of each phosphoprotein present in the supernatant and pelleted material.

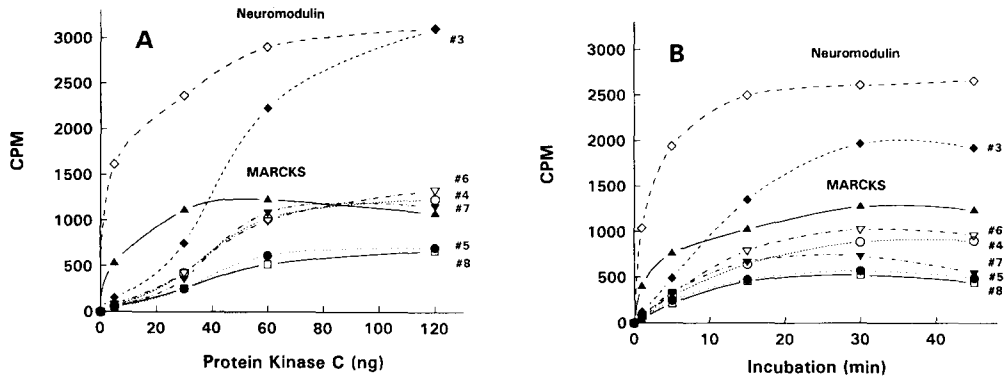

Fig. 2. (A) Effect of increasing protein kinase C concentration on the back-phosphorylation of protein substrates in acid extracts from hippocampal CA1 slices. Samples were incubated for 30 min at 30°. (B) Time course of phosphorylation of protein substrates in acid extracts from the CA1 region of rat hippocampus. Aliquots of neutralized acid extracts were incubated at 30° with 60 ng protein kinase C. Incorporation was terminated at selected time points and radioactivity was quantitated as described in Materials and Methods.

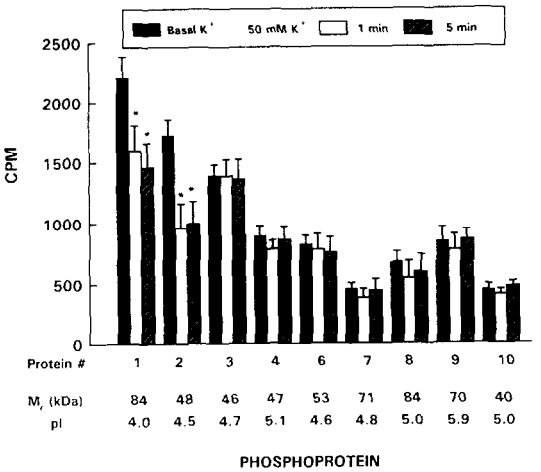

Fig. 3. Effects of K^+-induced depolarization of intact hippocampal slices on the phosphorylation of proteins by protein kinase C. Results are expressed as mean cpm ± SEM per protein; N = 4 per group. Key: (*) significantly different from control, $P < 0.05$.

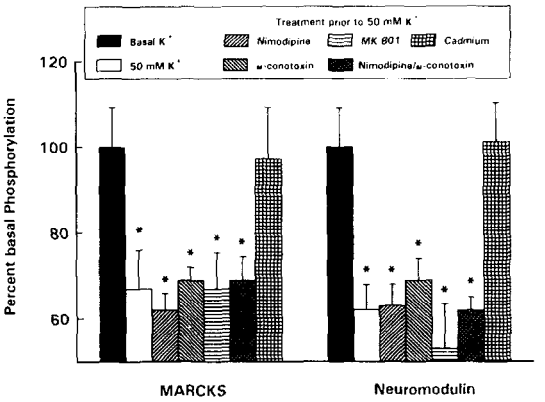

Fig. 5. Acute effect of Ca^{2+} antagonists on depolarization-induced changes in MARCKS and neuromodulin phosphorylation in hippocampal slices. Slices were pretreated with either nimodipine (300 nM) or ω-conotoxin GVIA (10 μM), MK-801 (10 μM), or Cd^{2+} (500 μM). Results are expressed as mean per cent of control SEM ± (N = 4). Basal values in cpm for cumulative experiments (N = 7) were 2820 ± 410 for MARCKS and 1940 ± 190 for neuromodulin. Key (*) significantly different from control, $P < 0.05$.

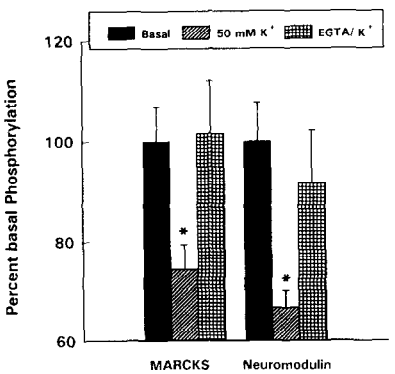

Fig. 4. Effect of low extracellular Ca^{2+} on K^+-induced depolarization changes. Hippocampal slices were pretreated for 15 min in low Ca^{2+} (10 μM) Krebs buffer with 100 μM EGTA prior to K^+ depolarization for 5 min. Data represent the mean per cent ± SEM of 5–6 determinations obtained in two independent experiments. Basal values (N = 5) in cpm were 2130 ± 130 for MARCKS and 1150 ± 90 for neuromodulin. Key: (*) significantly different from control, $P < 0.05$.

Depolarization of intact hippocampal slices with 50 mM K^+ for 5 min produced a significant change in phosphorylation of the two major protein kinase C substrates identified as MARCKS (#1) and neuromodulin (#2) (Fig. 3). The addition of EGTA (100 μM) using low Ca^{2+} (10 μM) buffer prevented the depolarization-induced changes (Fig. 4). Pretreatment with nimodipine (300 nM) and/or ω-conotoxin GVIA (10 μM), or MK-801 (10 μM) for 15 min prior to depolarization had no effect on the K^+-induced phosphorylation change. Cd^{2+} (500 μM), a non-specific inhibitor of cation channels [19], abolished the depolarization-induced changes in phosphorylation (Fig. 5).

DISCUSSION

This study demonstrates for the first time the application of a back-phosphorylation procedure using purified protein kinase C to determine changes in the phosphorylation state of major endogenous substrate proteins. Previous investigations employing back-phosphorylation techniques have studied substrates of the cyclic AMP-dependent kinase in neuronal preparations using the purified catalytic subunit [11, 14]. Procedures to examine substrates of protein kinase C have relied upon front or endogenous phosphorylation techniques to quantitate changes to proteins.

The method used here quantitated phosphorylation changes in MARCKS and neuromodulin induced by K^+ depolarization of hippocampal slices with results similar to those obtained with immunoprecipitation techniques used in the study of neuromodulin [5]. These changes were dependent on extracellular Ca^{2+} as shown by experiments using low Ca^{2+} conditions. This result is consistent with those of other studies [5, 20]. The finding that Cd^{2+} (500 μM) inhibited depolarization-induced changes in phosphorylation has been reported recently in studies of neuromodulin in synaptosomes [21].

Neuromodulin is localized presynaptically and its phosphorylation is correlated with neurotransmitter release in hippocampal slices [3, 5]. The lack of effect of combined treatment with nimodipine (300 nM) and ω-conotoxin (10 μM) in the present study may indicate that Ca^{2+} influx through neither L- nor N-type channels is rate-limiting for phosphorylation changes and/or neurotransmitter

release induced by K^+ depolarization. Electrophysiological studies in hippocampal CA1 neurons indicate that there is a large component (≈50%) of voltage-sensitive calcium current that is insensitive to both nimodipine and ω-conotoxin, but blocked by Cd^{2+} [22]. Ca^{2+} influx through this "P-type" Ca^{2+} channel [23] may contribute to the depolarization-induced changes in phosphorylation observed in the present study. In addition, the lack of effect of MK-801 (10 μM), a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist [24], suggests that calcium influx through the NMDA ion channel is not solely responsible for these changes in phosphorylation. It is likely that multiple pathways contribute to the increase in intracellular Ca^{2+} that activates protein kinase C under the conditions of these experiments.

Acknowledgements—We wish to thank Drs. E. J. Nestler and D. B. Beitner-Johnson (Yale University Laboratory of Molecular Psychiatry) for their valuable technical assistance on back-phosphorylation and two-dimensional gel electrophoresis procedures, and Drs. A. C. Nairn and P. Greengard (Rockefeller University) for supplying protein kinase C and for their comments on this manuscript.

REFERENCES

1. Scriabine A, Schuurman T and Traber J, Pharmacological basis for the use of nimodipine in central nervous system disorders. *FASEB J* **3**: 1799–1806, 1989.
2. Janis RA and Triggle DJ, Drugs acting on Ca^{2+} channels. In: *Ca^{2+} Channels: Their Properties, Functions, Regulation, and Clinical Relevance* (Eds. Hurwitz L, Partridge LD and Leach JK), pp. 195–249. CRC Press, Boca Raton, 1991.
3. Walaas SI and Greengard P, Protein phosphorylation and neuronal function. *Pharmacol Rev* **43**: 299–348, 1991.
4. Taft WC, Tennes-Rees KA, Blair RE, Clifton GL and De Lorenzo RJ, Cerebral ischemia decreases endogenous calcium-dependent protein phosphorylation in gerbil brain. *Brain Res* **447**: 159–163, 1988.
5. Dekker LV, De Graan PNE, Versteeg DHG, Oestreicher AB and Gispen WH, Phosphorylation of B-50 (GAP43) is correlated with neurotransmitter release in rat hippocampal slices. *J Neurochem* **52**: 24–30, 1989.
6. Linden DJ and Routtenberg A, The role of protein kinase C in long-term potentiation: A testable model. *Brain Res Rev* **14**: 279–296, 1989.
7. Routtenberg A, Protein kinase C activation leading to protein F1 phosphorylation may regulate synaptic plasticity by presynaptic terminal growth. *Behav Neural Biol* **44**: 186–200, 1985.
8. Nelson RB, Linden DJ and Routtenberg A, Phosphoproteins localized to presynaptic terminal linked to persistence of long-term potentiation (LTP): Quantitative analysis of two-dimensional gels. *Brain Res* **497**: 30–42, 1989.
9. Nestler EJ and Greengard P, *Protein Phosphorylation in the Nervous System*. John Wiley, New York, 1984.
10. Woodgett JR and Hunter T, Isolation and characterization of two distinct forms of protein kinase C. *J Biol Chem* **262**: 4836–4843, 1987.
11. Guitart X and Nestler EJ, Identification of morphine- and cyclic AMP-regulated phosphoproteins (MARPPs) in the *locus coeruleus* and other regions of rat brain: Regulation by acute and chronic morphine. *J Neurosci* **9**: 4371–4387, 1989.
12. Kirschenbaum B and Pulsinelli WA, Posthoc phosphorylation of proteins derived from ischemic rat hippocampus, striatum and neocortex. *Brain Res* **511**: 21–29, 1990.
13. Halpain S and Greengard P, Activation of NMDA receptors induces rapid dephosphorylation of the cytoskeletal protein MAP2. *Neuron* **5**: 237–246, 1990.
14. Walaas SI, Sedvall G and Greengard P, Dopamine-regulated phosphorylation of synaptic vesicle-associated proteins in rat neostriatum and substantia nigra. *Neuroscience* **29**: 9–19, 1989.
15. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–253, 1976.
16. Albert KA, Nairn AC and Greengard P, The 87-kDa protein, major specific substrate for protein kinase C: Purification from bovine brain and characterization. *Proc Natl Acad Sci USA* **84**: 7046–7050, 1987.
17. O'Farrell PH, High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* **250**: 4007–4021, 1975.
18. Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685, 1970.
19. Edmonds B, Klein M, Dale N and Kandel ER, Contributions of two types of Ca^{2+} channels to synaptic transmission and plasticity. *Science* **250**: 1142–1147, 1990.
20. Heemskerk FMJ, Schrama LH, DeGraan PNE and Gispen WH, 4-Aminopyridine stimulates B-50/GAP-43 phosphorylation in synaptosomes. *J Mol Neurosci* **2**: 11–17, 1991.
21. Heemskerk FMJ, Schrama LH, Ghijsen WEJM, DeGraan PNE, Lopes da Silva FH and Gispen WH, Presynaptic mechanism of action of 4-aminopyridine: Changes in intracellular free Ca^{2+} concentration and its relationship to B-50 (GAP-43) phosphorylation. *J Neurochem* **56**: 1827–1835, 1991.
22. Regan LJ, Sah DWY and Bean BP, Ca^{2+} channels in rat central and peripheral neurons: High threshold current resistant to dihydropyridine blockers and ω-conotoxin. *Neuron* **6**: 269–280, 1991.
23. Mintz IM, Venema VJ, Swiderek KM, Lee TD, Bean BP and Adams ME, P-type Ca^{2+} channels blocked by the spider toxin ω-Aga IVA. *Nature* **355**: 827–829, 1992.
24. Monaghan DT, Bridges RJ and Cotman CW, The excitatory amino acid receptors: Their classes, pharmacology, and distinct properties in the function of the central nervous system. *Annu Rev Pharmacol Toxicol* **29**: 365–402, 1989.