Activation of Protein Kinase C Induces Neurofilament Fragmentation, Hyperphosphorylation of Perikaryal Neurofilaments and Proximal Dendritic Swellings in Cultured Motor Neurons

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Abstract. Characteristic responses of motor neurons to injury include an apparent increase in the phosphorylation of C-terminal domains of neurofilament proteins in the perikaryal and dendritic compartments. This change was induced in dissociated cultures of embryonic spinal cord by activation of protein kinase C (PKC). PKC was activated by: (a) exposure of cultures to 10 mM 12-O-tetradecanoyl phorbol 13-acetate (TPA); (b) microinjection of 1 mM dioctanoylglycerol (diC2) directly into perikarya of motor neurons; (c) addition of 10 μM diC2 to the culture medium. Activation of PKC led to different immediate and long term effects on neurofilaments of motor neurons. After 30 minutes (min), fragmentation of the neurofilament network was observed by labeling with antibodies to low and high molecular weight neurofilament proteins; glial filaments were disassembled after 10 min and reassembled by 1 hour (h). From 4 to 24 h, motor neurons were observed with extensions of perikaryal cytoplasm or massive enlargements of proximal dendritic processes, both containing intact neurofilament networks. Over 1 to 12 days, there was a gradual increase in the number of motor neuronal perikarya immunoreactive with antibodies to neurofilament proteins phosphorylated at KSP sites on the C-terminal domains (SM131, SM134). It is proposed that activation of PKC secondary to other injurious events may contribute to the changes in phosphorylations observed in motor neuron diseases.

Key Words: Amyotrophic lateral sclerosis; Glial fibrillary acidic protein; Motor neuron disease; Motor neurons; Neurofilaments; Phosphorylation; Protein kinase C.

INTRODUCTION

Spinal motor neurons contain large numbers of neurofilaments (NFs), the intermediate filaments of neurons (1). NFs in mature neurons are composed of three protein subunits: low-(NF-L), medium-(NF-M), and high-(NF-H) molecular weight neurofilament proteins (NFs) (2). The NFP subunits are encoded on different genes, but all contain a central, alpha-helical rod domain that is conserved through the family of intermediate filaments; an N-terminal head domain, believed to be involved in regulation of filament assembly; and a hypervariable C-terminal tail domain that extends from the filament core (3–5).

The N-terminal domains of NFs contain serine residues that may be phosphorylated by several kinases including the second messenger-dependent kinases, protein kinase A (PKA), and protein kinase C (PKC) (6–11). Phosphorylation of these sites results in loss of the ability to polymerize into filaments in vitro or in disassembly of already formed filaments, indicating a role for PKA or PKC in regulation of NF assembly (7, 8, 11).

NF-M and NF-H subunits have long C-terminal extensions that contain numerous repeats of the amino acid sequences KSP that are sites for phosphorylation (12–14). Several second messenger-independent kinases have been identified that phosphorylate these serine residues in vitro such as cdc2 or cdc2-like kinases (15–20), calcium/calmodulin-dependent protein kinase (21), protein kinase Fα (22, 23), and tau protein kinases (24, 25). Phosphorylation of multiple serine residues generates multiple phosphorylated variants of NF-M and NF-H that are differentially distributed in different neuronal compartments. The most highly phosphorylated forms are found exclusively in axons, whereas poorly phosphorylated variants or intermediate forms are detected within perikarya, dendrites and at nodes of Ranvier (14, 26–29), C-terminal phosphorylation may modulate the interaction between NFs and other cytoskeletal elements and the rate of NF transport (30).

Abnormal accumulation and/or phosphorylation of NFs are features of several neurodegenerative diseases, chemically-induced neuropathies and mechanical injury. Accumulation of NFs in perikarya and proximal processes has been observed in spinal cords and certain brain stem motor nuclei in familial and sporadic amyotrophic lateral sclerosis (ALS) (31–34), in infantile spinal muscular atrophy (35), and in transgenic mouse models overexpressing NFs (36, 37) or mutant Cu/Zn-superoxide dismutase (SOD-1) linked to familial ALS (38). Enhanced immunoreactivity of motor neuron perikarya with antibodies recognizing hyperphosphorylated forms of NFs has been demonstrated in a number of motor neuron diseases (39–46), following axotomy or other physical trauma (47–50), in mutant SOD-1 transgenic mice (38), and in experimental intoxication with neurotoxic...
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We are investigating the role that various protein kinases may play in initiating these modifications of the cytoskeleton and how they might contribute to the selective vulnerability of motor neurons and loss of motor neuron function in diseases. In the present study, we investigated the effect of PKC activation on the cytoskeleton of cultured primary motor neurons. Although PKC directly phosphorylates N-terminal, not C-terminal, residues of NFPs in vitro, experimental activation of PKC in intact cells has been reported to increase phosphorylation of C-terminal residues of NF-M and NF-H in cultured chromaffin cells (55), in PC12 cells (56) and in SH-SY5Y cells (57), and of NFp in giant interneurons of the lamprey (50). In dissociated cultures of murine spinal cord, we found that activation of PKC by exposure to diC8 or TPA in the medium or microinjection of diC8 into the perikarya of motor neurons resulted in: (a) initial fragmentation of the neurofilament network lasting 4 hours (h); (b) appearance of massive swellings of proximal dendrites of motor neurons after 4 to 24 h; and (c) over a period of 1 to 12 days, motor neuronal perikarya became immunoreactive with antibodies that recognize the C-terminal domains of NFPs in the hyperphosphorylated state.

METHODS

Cell Culture

Primary cultures of dissociated spinal cord and dorsal root ganglia (DRG) were prepared from E13 CD1 mouse embryos as previously described (58). Cells were plated at a density of 180,000 or 200,000 cells per well in 4-well Nuncel culture dishes containing round glass 13 mm coverslips (Fisher Scientific, Montreal, Quebec) coated with poly-D-lysine (Sigma Chemical Co., St Louis, MO) plus Matrigel® basement membrane matrix (Collaborative Research, Inc., Bedford, MA). For some experiments, cells were plated in coated 60 mm culture dishes at a density of 2,000,000 per dish. The culture medium was minimum essential medium enriched with 5 g glucose (EMEM) and supplemented with 2% horse serum, 9 µg/ml bovine serum albumin, 26 µg/ml selenium, 29 µg/ml triiodothyronine, 9 µg/ml insulin, 180 µg/ml transferrin, 29 µg/ml putrescine, 8.2 ng/ml hydrocortisone, 11 ng/ml progesterone, 10 ng/ml dexamethasone, and 10 ng/ml nerve growth factor. Triiodothyronine was purchased from Calbiochem (San Diego, CA); all other growth factors and hormones were purchased from Sigma. On day 4 to 6, cultures were treated with 1.4 µg/ml cytosine-β-D-arabinoside (Calbiochem) to minimize growth of non-neuronal cells. The cultures were maintained at 37°C in 5% CO2. Cultures were used in experiments 6 to 8 weeks (w) following dissociation.

PKC Activation

PKC was activated by 3 methods: (a) Exposure of cultures to 10 nM 12-o-tetradecanoyl-phorbol 13-acetate (TPA) (Sigma, St Louis, MO) for 10 minutes (min); (b) Microinjection of 1 mM sn-1,2-dioctanoylglycerol (diC8) (ICN Biomedicals, Aurora, OH) directly into perikarya of single motor neurons; (c) Addition of 10 µM diC8 for 10 min to culture medium. TPA and diC8 were dissolved in dimethyl sulfoxide (DMSO) (BDH, Montreal, Quebec) at a concentration of 0.003% for dissolution in culture medium or 2.5% for microinjection. Control cultures received equivalent concentrations of DMSO.

Assay of PKC Activity

Purification and assay of PKC in membrane and cytosolic fractions was performed as described by Azarani et al. (59). Cells grown in 60 mm dishes were rinsed twice with cold PBS, scraped into 800 µl of homogenization buffer (250 mM sucrose, 5 mM ethylenebis-(oxyethyl)tetraoxaethane acid [EGTA], 2 mM Tris-HCl, 20 mM ethylenediamine-tetraoxaethane acid [EDTA], 10 mM dithiothreitol [DTT], 50 mM phenylmethylsulfonyl fluoride [PMSF] and 2.5 mg/ml leupeptin). The homogenate was sonicated for 10 seconds (s) at low density over ice. After sonication, the homogenate was centrifuged at 100,000 x g for 1 h. The supernatant was collected and designated the cytosolic fraction. The pellet was resuspended in 776 µl of the above buffer containing 25 µl of 10% Triton X-100 and shaken slowly for 1 h at 4°C. The suspension was centrifuged at 100,000 x g for 1 h, and the supernatant collected as the particulate (membrane) PKC fraction. The crude cytosolic and membrane extracts were applied to DEAE columns (0.3 ml bed volume) pre-equilibrated in buffer A (2 mM Tris HCl, 1 mM EDTA, 1 mM EGTA, 10 mM DTT, 50 mM PMSF and 2.5 mg/ml leupeptin). Columns were washed with 2 ml buffer A and bound PKC was stepwisely eluted with 0.3 ml of 130 mM NaCl and 0.3 ml of 150 mM NaCl. PKC activity was measured by phosphorylation of the peptide FKKSKFL-NH2. Reactions were carried out at 30°C for 5 min in 25 µl of 20 mM Tris buffer, pH 7.5 containing 0.5 M magnesium acetate, 0.10 mM [32P]ATP, 0.4 mM CaCl2, 9 mM peptide, 10 mg/ml phosphatidyl serine and 5 mg/ml diolein, 0.5 mg/ml leupeptin and enzyme preparations (50 µl of cytosol and membrane fraction, normalized by the amount of protein present). The phospholipid-independent activity was measured under the same condition without phosphatidyl serine, but containing 0.02 M Tris. At the end of 5 min, 125 µl of acetic acid was added to the mixture and 100 µl of the reaction mixture was placed on 3 cm × 3 cm Whatman filter paper. The papers were washed with 2 ml/paper of 75 mM phosphoric acid 3 times for 10 min and once with methanol. [32P] was counted in a Branson 450 scintillation counter. PKC activity was calculated by subtracting the non-specific kinase activity (counts obtained in the absence of phosphatidyl serine) from counts in the presence of phosphatidyl serine. Each enzyme preparation was analyzed in triplicate and results expressed as pmol of ATP transferred/min/mg of protein.

Microinjection

Microinjection into motor neuron perikarya was performed as previously described (60). The injection solution was 5 mM KCl containing 1 mM diC8 and 25 mg/ml formalin-fixable fluorescent-dextran (FITC-dextran) (Molecular Probes Inc., Eugene, OR) as a marker of injected neurons. Neurons in control cultures were injected with 2.5% DMSO and FITC-dextran.


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without diC₆. Solutions for microinjection were clarified by centrifugation at 11,000 × g for 10 min just prior to use. During the procedure, cultures were bathed in EMEM without sodium bicarbonate titrated to pH 7.2. Large motor neurons were identified by size and morphology (28). After completion of injection, cultures were returned to the incubator in culture medium plus 0.75% gentamycin (GibcoBRL). Twenty to 40 motor neurons were microinjected on each coverslip. On average, 75% of cells survived the injection procedure. Three different cultures were evaluated at each condition and time of observation.

**Immunocytochemistry**

At various times after treatment or microinjection (0 min to 12 days), the organization of the NF network in motor neurons was visualized by indirect immunocytochemistry using antibodies against phosphorylation-independent epitopes of NF-H (mouse monoclonal antibody N52 or rabbit polyclonal antibody N4142 from Sigma; diluted 1:100) or NF-L (ICN, diluted 1:400). Also, phosphorylation of multiple serine residues on the C-terminal domain of NF-H was examined using the antibodies SM131 or SM134 (Sternberger Monoclonals Inc., Baltimore, MD; diluted 1:1,000) which recognize these extensions in intermediate and highly phosphorylated states, respectively. In some experiments double labeling was performed using rabbit anti-GFAP (Dakopatts, Glostrup, Denmark; diluted 1:100) or mouse anti-MAP2 (Amersham; diluted 1:100). Cells on coverslips were fixed in methanol for 4 min followed by acetone for 2 min, both at −20°C. Fixation of injected cells included 3 min in paraformaldehyde, 4 min in methanol and 2 min in acetone. Nonspecific binding was blocked by 20 min incubation in 3% skim milk dissolved in phosphate buffered saline (PBS), or 0.05 M Tris buffer in experiments with SM131 or SM134 antibodies. Cells were reacted for 30 min with primary antibody. Two methods were used to visualize the distribution of antibody: (a) epifluorescence microscopy following incubation with anti-mouse IgG conjugated to Texas Red or anti-rabbit IgG conjugated to FITC (Jackson Laboratories, Bar Harbor, ME; diluted 1:75) and (b) bright field or phase microscopy after incubation with biotinylated secondary antibody and Vectastain (avidin-biotin-HRP complex) (ABC kit, Vector Laboratories, Burlingame, CA). All antibody incubations were followed by three 5 min washes in PBS or Tris buffer. Coverslips were mounted with 50% glycerol in PBS. Cells were visualized using a Leitz Orthoplan microscope equipped with epifluorescence optics and photographed with a Nikon FX-35 camera using Kodak Tmax3200 film. To evaluate the extent of phosphorylation of KSP sites on perikaryal NFs, the number of motor neurons labeled and unlabeled by SM131 or SM134 were counted on each coverslip. Microinjected neurons were identified by the presence of FITC-dextran.

**RESULTS**

Fragmentation/Disassembly of NF and Glial Filaments

10 nM TPA or 10 μM diC₆ in the culture medium induced translocation of PKC from cytosolic to membrane fractions (Fig. 1). Two min exposure to diC₆ or TPA induced a 5-fold and 10-fold increase, respectively, in membrane/cytosolic PKC relative to the DMSO or untreated controls. Ten min exposure to 10 μM diC₆ resulted in rearrangement of the NF network in motor neurons and glial intermediate filaments in astrocytes, visualized by immunolabeling with mouse anti-NF-H (N52) and rabbit anti-GFAP, respectively (Fig. 2). In motor neuron perikarya and proximal dendrites, the NF network appeared fragmented, with loss of the continuous filamentous arrays characteristically found in control neurons. NF in axons and distal dendrites appeared less disrupted. Similar disruption of neurofilaments was observed by labeling with antibody to NF-L (not shown). Fragmentation of NF was observed 30 min after addition of activating agent and lasted for approximately 4 h, after which an intact network appeared. Similar results were obtained after addition of 10 nM TPA to the medium or microinjection of 1 mM diC₆ into perikarya (not shown). The percentage of motor neurons with a disrupted NF network was determined and results for each of the 3 methods of activating PKC are presented in Figure 3. Disruption of glial filaments was observed at 10 min,
Fig. 2. Disruption of NF and glial intermediate filaments after activation of PKC. Cultures were exposed to (A, C) 0.003% DMSO (control) or (B, D) 10 μM diC₈ for 10 min. After 1 h (A, B) or 30 min (C, D), cultures were fixed and immunolabeled with (A, B) mouse anti-NF-H or (C, D) rabbit anti-GFAP. Scale bar = 11 μm (A, B), 20 μm (C, D).

lasted for approximately 1 h and was characterized by punctate, rather than filamentous, anti-GFAP labeling (Fig. 2D).

Focal Swelling of Proximal Processes and Extension of Perikaryal Cytoplasm in Motor Neurons

From 4 to 24 h after addition of diC₈ to the culture medium, some motor neurons were observed with extensions of perikaryal cytoplasm (15.9 ± 1.2% compared to 3.6 ± 0.9% in control cultures) or massive swellings of proximal processes (18.6 ± 0.9% compared to zero in control cultures), both containing intact NF networks (Fig. 4). These swellings were only observed in large motor neurons in treated cultures, not in DRG or small neurons. The morphology of processes with focal swelling indicated that they were dendrites. This was confirmed by double-labeling cultures with rabbit anti-NF-H and mouse anti-MAP2. The latter labels dendrites and cell bodies, but not axons. Swellings were located in anti-MAP2 positive processes (not shown).

Hyperphosphorylation of Perikaryal and Dendritic Neurofilaments

Over a period of 1 to 12 days following microinjection of diC₈ or exposure to diC₈ or TPA in the medium, a gradual increase in the number of motor neuronal perikarya immunoreactive with SMI31 and SMI34 antibodies was observed (Fig. 5). Although this effect occurred with all three methods of PKC activation, the time course was slightly different. The maximum number of antibody-labeled perikarya occurred at 12 days following microinjection of diC₈, at 3 days after exposure to diC₈, and at 6 days after exposure to TPA. Examples of SMI31- or SMI34-positive motor neuronal perikarya following activation of PKC are illustrated in the micrographs of Figure 6 and Figure 7. Immunoreactivity of motor neuron processes also appeared stronger in cultures exposed to PKC activators. The specific inhibitor of PKC, calphostin C, added to the medium at the same time as TPA or diC₈ prevented the increase in perikaryal immunoreactivity with SMI34 induced by diC₈ or TPA (Fig. 8).
Fig. 3. Fragmentation of perikaryal neurofilament networks in motor neurons exposed to activators of PKC. Motor neurons were exposed for 10 min to 10 nM TPA or 10 μM diC₄ in the culture medium or microinjected with 1 mM diC₄. One, 4, or 24 h later, cultures were fixed and immunolabeled with mouse anti-NF-H. Control = exposure to 0.003% DMSO or microinjection of 2.5% DMSO. Results are expressed as mean ± SEM.

DISCUSSION

PKC exists in several isoforms. Of those that are activated by diacylglycerol (DAG), Ca²⁺-dependent forms α, Β, βΙΙ, and γ as well as the Ca²⁺-independent forms δ and ε have been isolated from nervous tissue (reviewed in 61). Each isoform has a particular distribution within the cell; PKC has been found localized in the cytoplasm, nucleoplasm, nucleoli, microfilament bundles, focal contacts, cell membranes and membranous organelles, and with intermediate filaments and to undergo translocation to membrane and cytoskeletal compartments upon activation (reviewed in 61). PKC-β, in particular, has been associated with intermediate filaments and stress fibers (62–64). In the present study, activation of PKC by the phorbol ester, TPA, or by the synthetic DAG, diC₄, had different short-term and long-term effects on cultured motor neurons.

Fragmentation/“Disassembly” of NF and GFAP-Filaments

The fragmentation of the NF network in motor neurons and “disassembly” of GFAP-filaments in astrocytes that occurred shortly after activation of PKC presumably resulted from phosphorylation of amino-terminal domains of NF proteins and GFAP. N-terminal serine residues in the three NF subunits and in GFAP are phosphorylated by PKC in vitro (6, 7, 9, 65) and analysis of these proteins isolated from nervous tissue has demonstrated that these sites are phosphorylated in vivo (6, 9, 66). In vitro, filaments composed of NF-L or GFAP, as well as other intermediate filament proteins, are disassembled or subunits are prevented from assembling when phosphorylated by PKC (7, 8, 65).
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Fig. 5. Percentage of motor neuronal perikarya immunoreactive with antibodies (A) SMI31 or (B, C, D) SMI34, 1 to 12 days following PKC activation. PKC was activated by (A, B) microinjection of 1 mM diC₈ into perikarya, (C) 10 min exposure to 10 μM diC₈ in the medium, or (D) 10 min exposure to 10 nM TPA in the medium. Data are presented as mean ± SEM, n = 3.

Disassembly of intermediate filaments in intact cells by PKC has been more difficult to demonstrate. In astrocytes, exposure to phorbol ester has been reported to promote extension of thin processes containing intermediate filament bundles, not filament disassembly (67). The effect observed may depend on the concentration of phorbol ester and the time of observation. In this study, GFAP-filament “disassembly” and fragmentation of NF were transient and not as evident when concentrations of TPA higher than 10 nM were used. Disruption of the NF network similar to that induced by PKC activators has been observed in DRG neurons exposed to the phosphatase inhibitor, okadaic acid (68). Increased phosphorylation of N-terminal domains of NFP was associated with redistribution of these proteins to the detergent-soluble fraction from the detergent-insoluble cytoskeleton, indicating an ordered disassembly of NF (68, 69).

To what extent PKC normally regulates neurofilament dynamics in vivo is not known. It is likely that multiple kinases regulate phosphorylation and assembly of NFP and that the consequences of activating specific kinases may vary with the cell type and the cellular environment. For example, in this study NF in perikarya and proximal dendrites appeared more sensitive to disruption by PKC activators than NF in axons and distal dendrites. This supports the contention that NF in perikaryal and dendritic compartments are more dynamic than axonal NF (30).

Swelling of Proximal Dendrites of Motor Neurons

An unusual finding was large, focal swelling of dendrites and extensions of perikaryal cytoplasm of motor neurons in cultures exposed to diC₈ in the medium. Given the multiple physiological processes in which PKC is involved, including control of cell volume (70) and neurite extension (reviewed in 61), further experimentation will be required to determine the mechanism by which these swellings occur.

Hyperphosphorylation of C-Terminal Domains of Perikaryal NF

In addition to the immediate, short-term effects of PKC activation on NF of motor neurons, activation of PKC by three different methods resulted in a gradual increase in the number of perikarya reactive with SMI31 or SMI34, antibodies against hyperphosphorylated NF. SMI31 has been characterized by Lee et al (14) as a P⁺ antibody, reacting with the conformation of the KSP multiphosphorylation sites on C-terminal projections of NF-M and NF-H when these sites are in an intermediate state of
Fig. 6. SMI31 labels perikarya of motor neurons microinjected with dIC₆. Motor neurons from cultures microinjected with (A) 2.5% DMSO (control) or (B, C) 1 mM dIC₆. After 12 days, cultures were fixed and immunolabeled with SMI31. Note the increased labeling of perikaryal neurofilaments in B and C. Scale bar = 20 µm.

phosphorylation. This antibody recognizes conformation rather than specific amino acid sequence and its binding is fixation-dependent (14, 28). The pattern of SMI34 labeling is typical of a P°°° antibody which only reacts with highly phosphorylated NF typically found only in axons (14) and is fixation-independent (Durham, unpublished results). The requirement for higher levels of phosphorylation to obtain binding by SMI34 could explain the lower percentage of perikaryal labeled by this antibody than by SMI31 after microinjection of dIC₆. Increased immunoreactivity with such antibodies is gener-
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Two factors indicate the apparent hyperphosphorylation of NF to be an indirect rather than a direct effect of PKC activation: (a) PKC does not directly phosphorylate KSP sites of NFPs (9, 15); (b) the effect appeared over 3 to 12 days, whereas changes in NF and GFAP-filament organization, thought to be due to direct N-terminal phosphorylation, were evident almost immediately. The hyperphosphorylation of perikaryal NF observed after axotomy in vivo is also delayed, even when the injury is close to the cell body (47, 50, 73–75). It is particularly relevant that hyperphosphorylation of somatodendritic NF in axotomized lamprey neurons was prevented by microinjection of PKA and PKC inhibitors into the neuronal cell bodies and that microinjection of PKA catalytic subunit, DAG, or okadaic acid induced phosphorylation of somatodendritic NF with the same time course as close axotomy (50). Hyperphosphorylation of somatodendritic NF in cultured motor neurons reported in this paper occurred with a similar delay following activation of PKC, indicating these mechanisms operate in mammalian neurons. Our findings support the hypothesis of Hall and Kosik (1993) that axotomy, activation of PKC, or inhibition of phosphatases might cause a persistent alteration in the somatodendritic environment including activation of an NF kinase or phosphatase that acts directly on the multiphosphorylation site. Given the multitude of known cellular processes mediated by PKC, further studies will be required to identify the cascade of events that ultimately result in phosphorylation of KSP sites.

A question raised by the present study is whether the NF fragmentation observed immediately after exposure to PKC activating agents is a prerequisite for delayed phosphorylation or is an independent event. It is possible that early loss of integrity of the NF network results in delayed transport of some NF or subunits into the axon, providing increased opportunity for phosphorylation of C-terminal KSP sites. Alternatively, C-terminal phosphorylation could result from an independent effect of PKC on gene expression or activation of kinase cascades.

Significance for Motor Neuron Diseases

Brief exposure to activators of PKC in cultured spinal motor neurons reproduced one common feature of motor neuron diseases, i.e. hyperphosphorylation of perikaryal and dendritic NF. How might PKC play a role in motor neuron diseases? Loss of intracellular Ca\(^{2+}\) homeostasis, abnormalities of glutamate metabolism leading to excitotoxic damage, and free radical mechanisms have been implicated in the etiology of these diseases (see 76–79). PKC activation may occur with all of these mechanisms (see 61, 80–82) and, theoretically, PKC-mediated phosphorylation of multiple proteins could contribute to loss of homeostasis and cell death. This is supported by several studies showing that downregulation of PKC in neu-

Fig. 8. The PKC inhibitor, calphostin C, prevented (A) TPA- or (B) diC\(_8\)-induced increase in perikaryal immunoreactivity with SM34. Cultures were exposed to 10 nM TPA or 10 μM diC\(_8\) ± 100 nM calphostin C or calphostin C alone for 10 min. After 6 days or 3 days, respectively, cultures were fixed, immunolabeled with SM34 and the number of labeled motor neuronal perikarya counted.
rons is neuroprotective (83). The only direct evidence that PKC is involved in ALS is a report by Lanius et al. (84) that PKC activity is increased in ALS spinal cords.

How might PKC-mediated phosphorylation of NFP contribute to motor neuron disease? Abnormalities of NF phosphorylation and expression are not unique to motor neurons; in fact, such changes induced by axotomy are more pronounced in DRG neurons (47). However, evidence is accumulating that the high NF content in dendritic, perikaryal and axonal compartments and the organization of NF in motor neurons may make these cells more vulnerable in certain diseases. Motor neurons contain large numbers of NF that contribute to the axonal caliber and dendritic form suited to their function (1, 85) and are particularly susceptible to disorders of NF transport (37, 86). Accumulation of NF occurs preferentially in motor neurons of transgenic mice overexpressing NFPs (36, 37). Variants in the sequences encoding the C-terminal extensions of NF-H, the portion of the molecule involved in crossbridge formation, have been associated with ALS (87). Previous studies in our laboratory suggest that the organization of perikaryal and dendritic NF in continuous bundles may predispose them to aggregation (60). The morphological features of ALS are mimicked in transgenic mice overexpressing NFPs, demonstrating that maldistribution of NF is harmful to motor neurons and can contribute to their demise, whether a primary or secondary event in the disease (36, 37). In this study, we have demonstrated that activation of PKC leads to changes in NF commonly observed after injury.

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