Journal of Neurochemistry
Raven Press, Ltd., New York

Neurofilament Phosphorylation in Cultured Bovine Adrenal Chromaffin Cells Is Stimulated by Phorbol Ester

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Abstract: Primary cultures of bovine adrenal chromaffin cells contain neurofilament proteins that are hypophosphorylated. When the cells were grown in medium containing $^{32}P_i$ and 0.1 μM 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA), ^{32}P-labelling of the three neurofilament subunits was increased 6- to 20-fold relative to controls, the highest level of stimulation occurring for the mid-sized subunit. Addition of the protease inhibitor leupeptin to the growth medium had no effect on TPA-stimulated phosphorylation. The increased ^{32}P incorporation was accompanied by a marked reduction in the gel electrophoretic mobilities of the two largest subunits. The augmented phosphorylation was observed 10 min after addition of TPA to a concentration of 0.1 μM or after 1 h of incubation in the presence of 0.01 μM TPA. One-dimensional peptide mapping and phosphoamino acid analysis indicated that TPA stimulated the phosphorylation of seryl residues at new sites in the mid-sized subunit. All of the latter subunit contained in the cytoskeletal fraction of chromaffin cells was converted to a more highly phosphorylated state after the cells were grown in the presence of TPA for 1 h. **Key Words:** Neurofilaments—Phosphorylation—Protein kinase—Phorbol ester. **Georges E. et al.** Neurofilament phosphorylation in cultured bovine adrenal chromaffin cells is stimulated by phorbol ester. *J. Neurochem.* **52,** 1156–1161 (1989).

The low (NF-L), middle (NF-M), and high (NF-H) molecular weight subunits of mammalian axonal neurofilaments (NFs) contain about 3, 8, and 13 mol of phosphate/mol of polypeptide, respectively, the latter values being based on the actual molecular weights of the subunits (Georges et al., 1986). These high phosphorylation levels may be attained through the actions of more than one enzyme, as a number of protein kinases have been shown to phosphorylate NFs in vitro. These include the casein kinase I (Julien et al., 1983) and NF-specific protein kinase (Toru-Delbauffe et al., 1986) activities that copurify with NFs, cyclic AMP-dependent protein kinase (protein kinase A) activities associated with microtubule-associated protein-2 (Leterrier et al., 1981) and calcium-activated neutral protease (Zimmerman and Schlaepfer, 1985), Ca^{2+}/calmodulin-dependent protein kinase (Vallano et al., 1985), and Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C) (Sihag et al., 1988). Whether these various enzymes phosphorylate NFs in situ is still a matter of conjecture due to the lack of studies involving whole cell systems perturbed by agents with known specificities for different protein kinases.

We have been studying NF phosphorylation in primary cultures of bovine adrenal chromaffin cells, which display many neuron-like properties (Fujita, 1977; Trifaró, 1982). The NFs in these cells are restricted to the perikaryon (Bader et al., 1984) and NF-M and NF-H occur in a hypophosphorylated state compared to their axonal counterparts (Georges et al., 1987). Since protein kinase C has been shown to phosphorylate a variety of chromaffin cell proteins (Pocotte and Holz, 1986), it was of interest to determine whether NF proteins were among its substrates. The present results show that the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Nishizuka, 1984) stimulated NF phosphorylation in chromaffin cells. The augmented ^{32}P uptake was not due to increased synthesis of NF proteins and, in the case of NF-M, appeared to involve phosphorylation at new sites. A preliminary report of this work has been presented previously (Mushynski, 1987).

Received May 5, 1988; revised manuscript received September 15, 1988; accepted September 23, 1988.

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Abbreviations used: DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; NF, neurofilament; NF-L, NF-M, NF-H refer to the low, middle and high molecular weight NF subunits, respectively; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; protein kinase A, cyclic AMP-dependent protein kinase; protein kinase C, Ca^{2+}/phospholipid dependent protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Triton, Triton X-100.

MATERIALS AND METHODS

Cell culture, ^{32}P labelling, and immunoprecipitation

Bovine adrenal glands were obtained from a local slaughterhouse and chromaffin cells were isolated, processed, and cultured as previously described by Bader et al. (1984) and Trifaró and Lee (1980). Chromaffin cells plated at a concentration of 5×10^6 cells/85 mm dish were incubated in 5 ml of P_i-free Dulbecco's modified Eagle medium (DMEM) containing 10% dialyzed fetal calf serum and 200 μCi/ml of carrier-free $^{32}P_i$ [Dupont (NEN) Products] for 2 h. TPA was added to a final concentration of 0.1 μM [2 μl of a 250 μM stock solution in dimethyl sulfoxide (DMSO)] and labelling of control and TPA-treated cells was continued for another 1 h. The controls were exposed to the same volume (2 μl) of DMSO alone or containing an equivalent amount of 4α-phorbol 12,13-didecanoate, a phorbol ester that does not activate protein kinase C (Castagna et al., 1982). The effect of leupeptin on stimulation of NF phosphorylation by TPA was determined by adding the protease inhibitor to a final concentration of 1 mM either 10 min before addition of phorbol ester or at the same time. Plates were washed with ice-cold phosphate-buffered saline (PBS) containing 2 mM EGTA and 2 mM phenylmethylsulfonyl fluoride (PMSF). The cells were detached with a rubber policeman in 1.0 ml of lysis buffer containing 150 mM KF; 10 mM Tris-HCl, pH 7.2; 5 mM $MgCl_2$; 2 mM EGTA; 0.25 mM dithiothreitol (DTT); 2 mM PMSF; 2 mM leupeptin; and 1.0% Triton X-100 (Triton). The lysate was centrifuged for 30 min at 100,000 g in a Beckman Air-Fuge and both supernatant and pellet fractions were processed for immunoprecipitation with anti-NF-L, anti-NF-M, and anti-NF-H combined, as previously described by Georges et al. (1987). The polyclonal antibodies were prepared by injection of gel-purified subunits into male New Zealand white rabbits and were purified by affinity chromatography. These antibodies have been previously shown to bind equally well to both untreated and in vitro dephosphorylated NF subunits (Georges et al., 1987; Lindenbaum et al., 1987).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were solubilized in SDS-containing sample buffer and separated on 6% or 7.5% SDS-polyacrylamide slab gels according to the method of Laemmli (1970). Protein bands were visualized by Coomassie blue staining (Fairbanks et al., 1971) and gels were subsequently dried under vacuum and exposed to Kodak XAR-5 film with Lightning Plus intensifying screens (E. I. Dupont) (Swanstrom and Shank, 1978).

Electrophoretic blotting and immunological detection

Polypeptides from soluble and cytoskeletal fractions separated by SDS-PAGE were electrophoretically transferred to nitrocellulose paper for 6 h at 1 A using a transfer buffer for basic proteins (Szewczyk and Kozloff, 1985). The immunoblots were incubated overnight with 0.5 μg/ml each of affinity-purified antibodies against the three NF subunits combined. The blots were washed several times and incubated for 2 h with 0.1 μCi/ml of ^{125}I-protein A [10 μCi/μg, Dupont (NEN) Products] as previously described by Georges et al. (1987). NF subunits were visualized by a 6-h exposure to Kodak XAR-5 film.

Peptide mapping and phosphoamino acid analysis

^{32}P-labelled polypeptide bands were excised from 7.5% SDS-polyacrylamide gels, hydrated, and incubated for 30 min in 0.125 mM Tris-HCl, pH 6.8, containing 0.1% SDS and 1 mM EDTA. The gel slices were then digested with 0.5, 1, or 5 μg of *Staphylococcus aureus* V-8 protease per slice according to the method of Cleveland et al. (1977). The slab gels were dried under vacuum and phosphopeptide bands were visualized by 3-day exposure to Kodak XAR-5 film. Analysis of phosphoamino acids in ^{32}P-labelled NF-M excised from SDS-slab gels was carried out as described previously by Cooper et al. (1983).

RESULTS

The decrease in the apparent molecular weights of NF-H and NF-M on SDS-PAGE after dephosphorylation reflects the marked effect of phosphate moieties on the gel electrophoretic mobilities of these polypeptides (Julien and Mushynski, 1982; Georges et al., 1986). However, the subunits still contain significant levels of phosphate after extensive in vitro dephosphorylation (Georges et al., 1986). The results in Fig. 1 demonstrated that NF-M and NF-H in cultured chromaffin cells (lane 3) were present in a hypophosphorylated state when cells were incubated for 3 h in the presence of 200 μCi/ml of carrier-free $^{32}P_i$, as their mobilities were similar to those of in vitro dephosphorylated NF-H and NF-M standards from bovine brain (lane 2). However, when cells were treated with TPA for the final 1 h of the 3-h labelling period a marked increase in the phosphorylation of NF proteins was observed (Fig. 1, lane 4). Densitometric analysis of the autoradiogram revealed a 6- to 20-fold increase in phosphorylation of the various subunits, the highest level of stimulation being observed for NF-M and the lowest for NF-L (not shown). In addition, the mobilities of NF-M and NF-H decreased markedly after TPA treatment, indicating that they had attained higher phosphorylation levels (Julien and Mushynski, 1982;

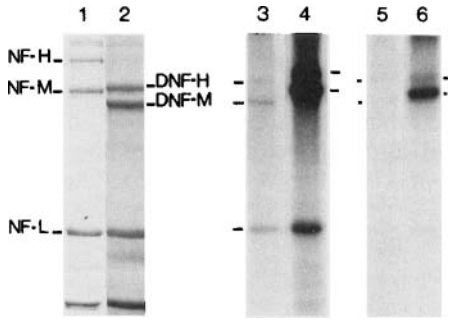

FIG. 1. Effect of TPA on the phosphorylation of NF proteins in chromaffin cells. Lanes 1 and 2 show Coomassie Blue-stained gels of isolated and dephosphorylated bovine brain NF subunits, respectively. Lanes 3 and 4 show an autoradiogram of ^{32}P-labelled NF subunits immunoprecipitated from the cytoskeleton extract of control chromaffin cells and cells treated with 0.1 μM TPA for 1 h, respectively. Lanes 5 and 6 are immunoprecipitates from the Triton-soluble fractions of control and TPA-treated cells, respectively. The cells were grown in the presence of $^{32}P_i$ for 3 h and immunoprecipitation, SDS-PAGE, and autoradiography were carried out as described in Materials and Methods. NF-L, NF-M, and NF-H denote the low, middle, and high molecular weight NF subunits, and DNF-M and DNF-H refer to the corresponding in vitro dephosphorylated subunits. The bars to the left of lanes 3 and 4 and lanes 5 and 6 mark the positions of NF subunits in control cells and the bars on the right mark the positions after TPA treatment.

Bennett and Dilullo, 1985). The results in Fig. 1 (lanes 5 and 6) show that TPA also stimulated the phosphorylation of Triton-soluble forms of NF-M and NF-H (Georges et al., 1987). Again, a decrease in the electrophoretic mobility of soluble NF-M was noted, although there was only a small change in the mobility of soluble NF-H compared to that of NF-H in the cytoskeleton fraction.

To rule out the possibility that apparent TPA-stimulated increases in NF phosphorylation were due to increased protein synthesis (Siebert and Fukuda, 1985), chromaffin cells were incubated in the presence and absence of 0.1 μM TPA for 1 h and cell extracts were analyzed by immunoblotting. The results in Fig. 2 show that approximately equal amounts of NF-M and NF-L were present before (lane 2) and after (lane 3) TPA treatment. It was also noted that TPA treatment reduced the electrophoretic mobility of all the NF-M in the cytoskeletal fraction (lane 3), as well as that of all Triton-soluble NF-M (not shown). The reduced levels of NF-L in Fig. 2 reflect the loss of this subunit during the prolonged electrophoretic blotting required to effect the transfer of the much larger NF-M, as more NF-L than NF-M was seen after 3 h of blotting (not shown). Failure to detect NF-H on the immunoblot was due to the small amount of this subunit present in primary cultures of chromaffin cells (Georges et al., 1987), the levels in some of our preparations being virtually undetectable (also see Fig. 3, below). In addition, it is difficult to transfer high molecular weight polypeptides such as hypophosphorylated NF-H, which has a slightly basic isoelectric point (Julien and Mushynski, 1982), to nitrocellulose (Gershoni and Palade, 1983; Szewczyk and Kozloff, 1985).

The time course of TPA-stimulated NF phosphorylation shown in Fig. 3 indicates that the response to phorbol ester was rapid, as increased phosphorylation levels were observed after only 10 min of incubation with TPA (Fig. 3, lane 2). Moreover, a gradual decrease in the electrophoretic mobility of NF-M on SDS-PAGE was noted during growth of chromaffin cells in the presence of TPA (Fig. 3, lanes 1–5). The amount of

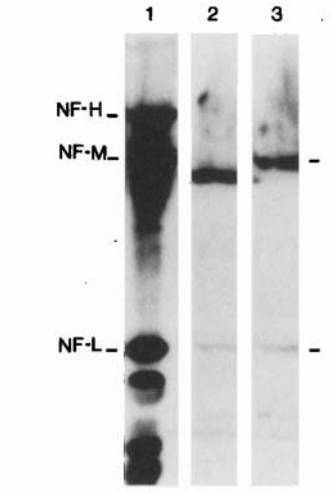

FIG. 2. Immunoblot analysis of NF proteins from control and TPA-treated cells. Cytoskeleton fractions from control cells (lane 2) and cells treated with 0.1 μM TPA for 1 h (lane 3) were resolved by SDS-PAGE and immunoblotting was carried out as described in Materials and Methods. Lane 1 contains NF standard from bovine brain. The bands are labelled as described in the legend to Fig. 1.

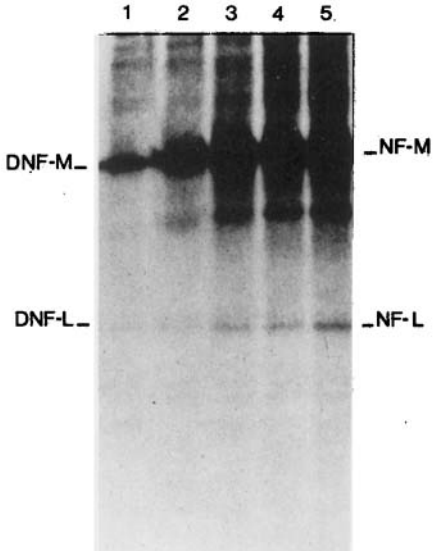

FIG. 3. Gel electrophoretic analysis of the time course of incubation of chromaffin cells with phorbol ester. Cells were incubated with $^{32}P_i$ for a total of 4 h. TPA was present in the incubation medium at a concentration of 0.1 μM for 0 h (lane 1) or 4 h (lane 5), or for the final 10 min (lane 2), 30 min (lane 3), or 60 min (lane 4) of the incubation period. The NF subunits were then immunoprecipitated and resolved by SDS-PAGE as described in Materials and Methods. The various bands are labelled according to the legend to Fig. 1.

NF-H in the chromaffin cell cultures used in this experiment was too low to permit an assessment of its phosphorylation time course. The identity of the prominent ^{32}P-labelled band running between NF-L and NF-M is unknown but its presence in the immunoprecipitate suggests it may be a proteolytic fragment of one of the NF subunits (Julien and Mushynski, 1983).

The effect of varying TPA concentration on NF-M phosphorylation is shown in Fig. 4A. A shift in the mobility of NF-M was first seen at 0.01 μM TPA and a plateau in the level of ^{32}P incorporation was reached at between 0.05 and 0.1 μM TPA. Figure 4B shows that the increased ^{32}P incorporation in NF-M was not due to an increased uptake of $^{32}P_i$ by chromaffin cells grown in the presence of TPA, as similar changes were seen when $^{32}P_i$ was removed from the growth medium prior to addition of the phorbol ester (compare lanes 2 and 3). The shift in electrophoretic mobility of NF-M was not seen when 4α-phorbol 12,13-didecanoate, an analog of TPA that does not activate protein kinase C (Castagna et al., 1982), was added to the culture medium (Fig. 4B, lane 4), although an apparent slight increase in ^{32}P incorporation was noted. Leupeptin, which has been shown in other systems to prevent the proteolytic conversion of protein kinase C to a soluble form (Tapley and Murray, 1985; Melloni et al., 1986), had no effect on the appearance of more highly phosphorylated NF-M when added at the same time as TPA (Fig. 4B, compare lanes 5 and 6) or when cells were preincubated with the inhibitor for 10 min prior to addition of TPA (not shown). However, leupeptin

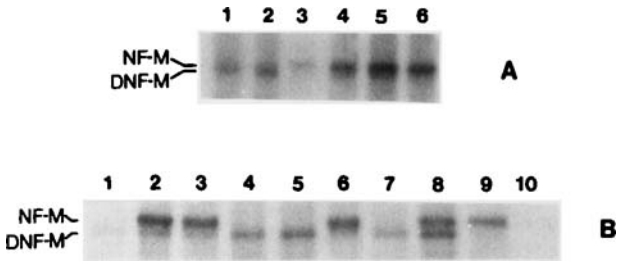

FIG. 4. Effect of varying TPA concentration (**A**) and of different treatments (**B**) on phosphorylation of NF-M. In A, chromaffin cells were incubated for the final 1 h of the 3-h labelling period with 0 (lane 1), 0.001 μM (lane 2), 0.01 μM (lane 3), 0.05 μM (lane 4), 0.1 μM (lane 5), and 0.5 μM (lane 6) TPA, and NF-M was immunoprecipitated and analyzed by SDS-PAGE. The TPA was always added to the cultures in the same volume (2 μl) of DMSO. In B, chromaffin cells were prelabelled with ^{32}P for 2 h, and cells were incubated for an additional 1 h in the same medium (lane 1), in medium containing 0.1 μM TPA (lane 2), or in $^{32}P_i$-free medium containing 0.1 μM TPA (lane 3). The inactive phorbol ester, 4α-phorbol 12,13-didecanoate, did not induce a shift in the mobility of NF-M when added at 0.1 μM in the final 1 h of the 3-h labelling period (lane 4). Lanes 5, 6, and 7 show NF-M from cells treated with 1 mM leupeptin, 1 mM leupeptin and 0.1 μM TPA, or 2 μl of DMSO, respectively, during the final 30 min of a 2.5-h labelling period. The turnover of ^{32}P-labelled NF-M was determined by treating cells with 0.1 μM TPA for the final 1 h of a 3-h labelling period, washing the cells, and growing them in DMEM devoid of $^{32}P_i$ or TPA for an additional 1 h (lane 8), 2 h (lane 9), or 16 h (lane 10).

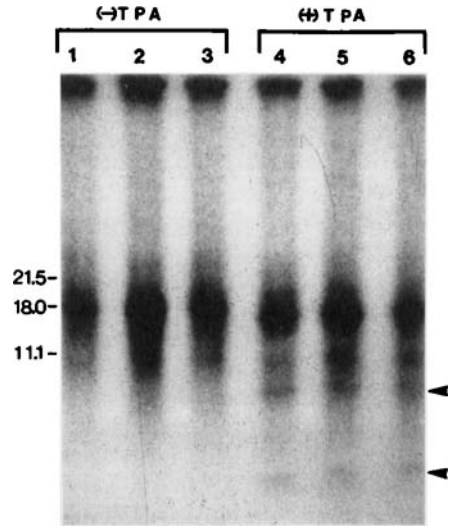

FIG. 5. One-dimensional phosphopeptide maps of ^{32}P-labelled NF-M from control and TPA-treated chromaffin cells. ^{32}P-labelled NF-M from the cytoskeletal fractions of control (lanes 1–3) and TPA-treated (lanes 4–6) chromaffin cells was immunoprecipitated; purified by SDS-PAGE; digested with 0.5 (lanes 1 and 4), 1.0 (lanes 2 and 5), or 5.0 μg (lanes 3 and 6) of *S. aureus* V8 protease, respectively; and processed as described in Materials and Methods. The positions of molecular weight markers ($\times 10^3$) are indicated to the left of lane 1, and the arrowheads at the right of lane 6 indicate phosphopeptides unique to NF-M from TPA-treated cells.

alone did appear to cause a slight increase in ^{32}P incorporation (lane 5). The ^{32}P incorporated into NF-M in the presence of TPA appeared to turn over relatively slowly as the more highly phosphorylated, slowly migrating form of NF-M persisted for 2 h following removal of TPA from the growth medium (Fig. 4B, lanes 8 and 9). The prominent band seen at the position of dephosphorylated NF-M (DNF-M) after 1 h of chase (Fig. 4B, lane 8) probably represented NF-M that was in the process of being dephosphorylated as it disappeared by 2 h. After a 16-h chase period a barely visible band was seen at a position between those of dephosphorylated and highly phosphorylated forms of NF-M (Fig. 4B, lane 10).

One-dimensional phosphopeptide mapping of NF-M from control cells and TPA-stimulated cells revealed the presence of two new bands in the latter over a broad range of protease concentrations (Fig. 5). Phosphoamino acid analysis showed that [^{32}P]phosphoserine was the only species formed in the presence of TPA (Fig. 6).

DISCUSSION

The increased level of NF phosphorylation in cultured bovine adrenal chromaffin cells incubated with TPA indicates that the subunits are substrates of protein kinase C (Nishizuka, 1984), as the phorbol ester does not increase cyclic AMP levels in these cells (Pocotte and Holz, 1986), 4α-phorbol 12,13-didecanoate does not stimulate phosphorylation, and purified mouse brain protein kinase C phosphorylates NF proteins in vitro (Sihag et al., 1988). Our earlier demonstration that the NF proteins in chromaffin cells are in a hypophosphorylated state was based on the comigration of NF-M and NF-H with in vitro dephosphorylated bovine brain NF subunits on SDS-PAGE (Georges et al., 1987). However, TPA treatment decreased the electrophoretic mobilities of NF-M, and to a lesser extent, of NF-H in both cytoskeletal and Triton-soluble fractions, indicating a marked increase in phosphate content (Julien and Mushynski, 1982; Bennett and Di-

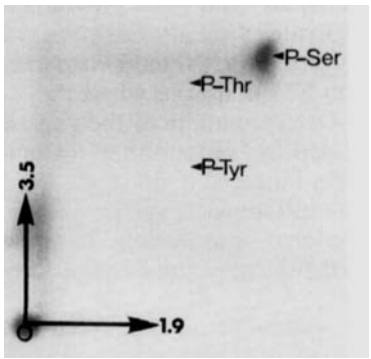

FIG. 6. Analysis of phosphoamino acids in NF-M from TPA-treated cells. NF-M was immunoprecipitated from extracts of TPA-treated cells and purified by SDS-PAGE. The polypeptide was electroeluted from gel slices, hydrolyzed in acid, and the hydrolysate electrophoresed as described previously (Cooper et al., 1983). The numbers 3.5 and 1.9 represent the pH values of the buffers used for the two-dimensional electrophoresis. P-Ser, P-Thr, and P-Tyr represent the positions of the respective phosphorylated amino acids used as standards.

lullo, 1985). Since about half of the phosphate is removed by in vitro dephosphorylation of NF-M in axonal NFs (Georges et al., 1986), protein kinase C may add up to four additional phosphates to each molecule of NF-M in TPA-treated cells. The latter is likely an exaggeration of the normal situation in chromaffin cells due to the stability of TPA in comparison to diacylglycerol, the physiological activator of protein kinase C (Blumberg, 1988).

In addition to stimulating the phosphorylation of many cellular proteins, TPA can also cause the de novo induction of cytoskeletal proteins such as actin and vimentin (Siebert and Fukuda, 1985). However, immunoblotting indicated that 1 h of TPA treatment did not significantly increase the amount of NF protein in chromaffin cells (Fig. 2). The latter experiment also demonstrated that all cytoskeleton-associated NF-M had attained a higher phosphorylation level after 1 h of exposure to 0.1 μM TPA. This result was unexpected because protein kinase C activity may be expressed only in the immediate vicinity of the plasma membrane under physiological conditions (Kraft and Anderson, 1983; Wooten and Wrenn, 1984; Woodgett and Hunter, 1987), whereas the NFs in cultured chromaffin cells are distributed throughout the perikaryon (Bader et al., 1984). Assuming that leupeptin had an effect on chromaffin cells similar to that observed in other cell systems (Tapley and Murray, 1985; Melloni et al., 1986), it is unlikely that the quantitative conversion of NF-M to a more highly phosphorylated state was due to a TPA-stimulated formation of the soluble, proteolytic fragment of protein kinase C (Murray et al., 1987) because leupeptin did not prevent the phorbol ester-stimulated phosphorylation of NF-M (Fig. 4B). Perhaps TPA caused a redistribution of protein kinase C to intracellular membrane systems that appear to be closely associated with NFs (Burton and Laveri, 1985). The recent reports that NF proteins bind phospholipids (Traub et al., 1986) and that peripheral cytoskeletal membrane proteins bind both protein kinase C and phosphatidyl serine (Wolf and Sahyoun, 1986) suggest the possibility that the activated kinase may be directly associated with NFs in regions where they interact with membranes. Determination of the subcellular distribution and functional properties of the many different types of protein kinase C (Ono et al., 1987) may help elucidate these questions.

One-dimensional phosphopeptide mapping of NF-M demonstrated the appearance of two new phosphopeptides after TPA treatment, indicating that the subunit is phosphorylated by more than one type of protein kinase. As mentioned above, in vitro studies have shown that NF proteins are phosphorylated by a variety of protein kinases, including protein kinase A (Leterrier et al., 1981; Zimmerman and Schlaepfer, 1985) and protein kinase C (Sihag et al., 1988). Protein kinase A and protein kinase C have different substrate specificities as they phosphorylate seryl and threonyl residues proximal to an arginyl or a lysyl residue on the amino-terminal or carboxyl-terminal side, respectively (Kikkawa and Nishizuka, 1986). On the other hand, seryl and threonyl residues that are phosphorylated by both enzymes have basic amino acid residues on both sides (Kishimoto et al., 1985). The latter arrangement is seen in the carboxyl-terminal domains of NF-M and NF-H, where repetitive degenerate repeats containing the triplet Lys-Ser-Pro are located (Myers et al., 1987; Geisler et al., 1987; Julien et al., 1988; Lees et al., 1988). The predominance of [^{32}P]phosphoserine in NF-M from TPA-treated cells (Fig. 6) may be due to phosphorylation at such clustered sites.

The NFs in chromaffin cells are restricted to the perikaryon (Bader et al., 1984) and do not show the regional variation in phosphorylation state that distinguishes axonal NFs from perikaryal and dendritic NFs in neurons (Sternberger and Sternberger, 1983). The indication that protein kinases in addition to protein kinase C are involved in NF phosphorylation could explain the complex regional and developmental patterns of NF phosphorylation (Nixon and Lewis, 1986; Carden et al., 1987) as well as the presence in NF proteins of phosphate moieties with different characteristics (Ksiezak-Reding and Yen, 1987).

Acknowledgment: This work was supported by grants from the Medical Research Council of Canada to W.E.M. (MT-5159) and J.-M.T. (PG-20). The secretarial aid of Margaret Licorish and photographic work of Kathy Teng are gratefully acknowledged. The authors would also like to thank L. Potvin and R. Tang for their excellent technical assistance in the preparation of cell cultures.

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