Phorbol Ester Receptors and Protein Kinase C in Primary Neuronal Cultures: Development and Stimulation of Endogenous Phosphorylation

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Abstract. Embryonic rat neurons cultured in defined medium, essentially in the absence of glia, were highly enriched in phorbol ester receptors. The neurons displayed a single class of phorbol 12,13-dibutyrate binding sites with a maximum binding capacity, after 10 d in culture, of 18.6 pmol/mg protein and an apparent dissociation constant of 7.1 nM. Phorbol ester binding sites were associated with protein kinase C, which represented a major protein kinase activity in primary neuronal cultures. Ca\(^{2+}\)-phosphatidylserine-sensitive phosphorylation of endogenous substrates was more marked than that observed in the presence of cyclic AMP or Ca\(^{2+}\) and calmodulin.

Phorbol ester receptors and protein kinase C levels were critically dependent on the culture age. Thus, about a 20-fold increase in binding sites occurred during the first week in culture and was accompanied by a corresponding increase in Ca\(^{2+}\)-phosphatidylserine-sensitive protein phosphorylation in soluble neuronal extracts. These changes largely paralleled a similar rise in phorbol ester binding during fetal development in vivo. The apparent induction of phorbol ester receptors was specific relative to other cellular proteins and could be inhibited by cycloheximide or Actinomycin D.

Phosphorylation of endogenous substrates in intact cultured neurons paralleled the age-dependent increase in protein kinase C. Furthermore, \(^{32}\)P incorporation into several major phosphoproteins was markedly augmented by treating the neuronal cultures with phorbol esters. Such phosphorylation events may provide a clue to the significance of protein kinase C in developing neurons.

Brain tissue is highly enriched in protein kinase C and associated phorbol ester binding sites (6, 16, 17, 26, 28). Moreover, brain tissue is particularly active in metabolizing phosphatidylinositol and its phosphorylated derivatives, leading to the formation of diacylglycerol (20, 22), an endogenous protein kinase C activator (27, 29, 36, 37). The regulatory role of protein kinase C in brain remains to be determined; however, several conjectures may be entertained. Especially relevant to neuronal tissue is the synergistic relationship between protein kinase C stimulation and increased Ca\(^{2+}\) levels. Concerted activation of protein kinase C and increased intracellular Ca\(^{2+}\) concentration have been implicated in stimulus-secretion coupling, metabolic regulation, and cell division (27, 29); neuronal processes including neurotransmitter release from synaptic vesicles may be regulated by a similar mechanism (31). In addition, stimulation of protein kinase C by phorbol esters appears to mediate the down-regulation of several types of receptors in cultured cell lines (7, 13, 14, 21), and may play such a role in the brain. The regulation of ionic fluxes by protein kinase C may also be critical to neuronal function (3, 8). The ability of protein kinase C to phosphorylate cytoskeletal components (12, 15, 24, 40) may also be relevant to a neuronal regulatory role. In addition to its actions in mature cells, protein kinase C has been implicated in molecular processes leading to cellular differentiation (27, 29, 37); the association of phorbol ester binding sites with cells that are elaborating neurites in fetal brain, and with synaptic zones in adult brain (23) suggests a role for protein kinase C in neuronal differentiation as well. Consistent with these possibilities is the presynaptic and perinuclear immunolocalization of the enzyme by electron microscopy (38), and the rise in rat brain protein kinase C activity during the first postnatal month (10), which is a period of extensive synaptogenesis.

Accordingly, we undertook to characterize phorbol ester binding and protein kinase C activity in primary neuronal cultures. Data presented here indicate that these cultures are rich in phorbol ester receptors and in protein kinase C activity. The level of receptors and of the associated enzyme activity increased markedly during the first week in culture. The prevention of this increase by transcription and translation inhibitors implies regulation of protein kinase C levels at the

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stage of RNA transcription. The induction of this enzyme during late embryogenesis in vivo and in embryonic neurons in vitro denotes a dependence of protein kinase C on neuronal differentiation. Enhanced phosphorylation of several cellular polypeptides in the presence of phorbol esters was observed, and the identification of these substrates may help unravel the role of protein kinase C in developing neurons.

Experimental Procedures

Materials

Phorbol 12,13-dibutyrate (PDBu), 12-O-tetradecanoyl-phorbol-13-acetate (TPA), 4a-phorbol dibutyrate, phosphatidyserine (PS), leupeptin, histone H1 (Sigma type III), johnson 2A187, and calmodulin were purchased from Sigma Chemical Co. (St. Louis, MO). (3H)35S]methionine, HI, 3H]PDBu, [3H]PDBu, 20% (NH4)2SO4)-PDBu (6.5 or 13.2 Ci/mmol), and Autofluor were obtained from New England Nuclear (Boston, MA). Rabbit anti-gal fibrillary acidic protein (GFAP) was obtained from Accurate Chemical & Scientific Corp. (Westbury, NY). Fluorescein-conjugated goat anti–mouse immunoglobulins and fluorescein-conjugated goat anti–rabbit IgG. A2B5 hybridoma cells were obtained from the American Type Culture Collection (Bethesda, MD). Monoclonal antibodies to neurofilament protein were obtained from J. Wood (Harvard University).

Methods

Protein kinase C in soluble extracts of brain tissue or cultured cells was assayed in 100 µl of 50 mM Tris-HCl, pH 7.7, containing 50 µM ATP, 1 µCi [3H]ATP, 50 µg histone HI, 10 mM MgCl2, 100 µg/ml CaCl2, 20 µg/ml PS, and 2 mM dithiothreitol (DTT). The assay was performed at 30°C for 10 min, stopped by 20% trichloroacetic acid, and 3H-histone was retrieved by filtration over 0.45 µm HAWP Millipore filters (Millipore Co., Bedford, MA). Protein kinase C purification from rat brain was achieved as described earlier (41). [3H]PDBu binding to aliquots of the above extracts was carried out in 100 µl of 50 mM Tris-HCl, pH 7.7, 2 mM DTT, 100 µg/ml CaCl2, 20 µg/ml PS, and 10 nM [3H]PDBu. After 1 h at 23°C, PDBu bound to its receptor was separated from unbound PDBu by gel-permeation chromatography on 2-ml G-50 Sephadex columns. Bound [3H]PDBu appeared in the void volume, while free [3H]PDBu eluted at total column volume. Nonspecific binding, determined in the absence of PS and Ca++ or in the presence of 1 µM unlabeled PDBu, was <10% of the total binding. Proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) using 10% acrylamide gels (19). Two-dimensional (2-D) separation of polypeptides was performed using isoelectric focusing using SDS PAGE (30). For autoradiography, gels were dried and exposed to Kodak DEF-5 film, or to Kodak Blue Brand film in the presence of a fluorescent enhancer screen. Protein concentration was determined by the Coomassie Blue R-250 method (4). Cultured cells were categorized according to cell type (5) by immunofluorescent staining using antibodies against GFAP (astroglia), fibronectin (fibroblasts), or monoclonal antibodies to either A2B5 or neurofilament protein (neurons). Specificity of staining was demonstrated by the lack of fluorescence with non-immune sera and by the characteristic staining pattern of each antibody.

Enriched cultures of rat central nervous system neurons were prepared from brains aseptically removed from 16-d embryos. Mid- and hind-brain regions were dissected, freed of meninges, and collected in saline 1 (138 mM NaCl, 5.4 mM KCl, 1.1 mM NaHCO3, and 22 mM glucose; pH 7.1). The tissue was mechanically dissociated by trituration. The cells that remained in suspension after 5 min were collected, pelleted by centrifugation, and resuspended in a defined medium consisting of Eagle's basal medium with Earle's salts/Hams F12 (1:1) with 100 µg/ml glutamine, 600 µg/ml glucose, 50 IU/ml of penicillin, 50 IU/ml of streptomycin, 5 µg/ml insulin, 50 µg/ml transferrin, and in some experiments 100 µg/ml fatty acid free bovine serum albumin (BSA). Cells were plated on poly-D-lysine coated (1), 24-well tissue culture dishes (at a density of 2-5 × 104 trypan blue–excluding cells/cm²) and grown in the medium at 37°C with 5% CO2. The great majority of cells were neuronal in appearance and stained, by indirect immunofluorescence, with neuron-selective monoclonal antibodies to A2B5 or neurofilament protein. Astroglia and fibroblasts (cells positive for GFAP and fibronectin staining, respectively) did not proliferate in this medium: counting of stained cells showed that these cell types represented <5% of the total cell population. The GFAP* cells were of the process-bearing type. This same preparation supplemented at the time of plating with 5% fetal calf serum supported a limited proliferation of glial, but not neuronal, cells, and yielded, after 5 d, a mixed population of neurons (80% A2B5+) and glia; a non-confluent bed layer enriched in astroglia and fibroblasts was overlaid with neuronal cells as well as other process-bearing cells.

To determine whether the small number of glial cells present in these mixed cultures might contribute a disproportionately large number of phorbol receptors (as is the case with beta-adrenergic receptors [5]), PDBu binding to purified monolayer cultures of glial cells was measured. These astroglial cultures were prepared by plating dissociated cortical cells of the same brains in medium containing 10% fetal calf serum into an uncoated 60-mm tissue culture dish, then removing cells that were non-adherent after 24 h. The remaining flattened cells were harvested with trypsin after 7 d and replated in the same medium in 24-well, poly-D-lysine coated plates. These cells formed a confluent layer of flattened cells at the time of assay (total of 13 d in vitro). The majority of these cells appeared to be polygonal astroglia (60% GFAP +) or fibroblasts (25% fibronectin +) with minimal neuronal contamination (1% A2B5+); the remaining cells were negative for any of these three immunocytochemical markers.

Phorbol ester binding to intact cells was performed in tissue culture wells containing 20-50 µg of protein in enriched neuronal cultures and mixed cultures, or 25-30 µg of protein in purified glial cultures. To measure phorbol ester binding, growth medium was replaced with Eagle's basal medium buffered with 25 mM Hepes, pH 7.2, at room temperature; this buffer was then treated with 0.5 ml of this medium containing 7 nM [3H]PDBu (0.1 µCi/ml) and varying concentrations of unlabeled PDBu, TPA, or 4a-phorbol. The incubation was carried out at room temperature for 40 min; cells were then washed twice with ice-cold PBS and harvested for scintillation counting and protein determination. Nonspecific binding was defined as that observed in the presence of 1 µM PDBu. Assays were done in duplicate or in triplicate wells. To measure developmental changes in phorbol ester binding sites, binding of [3H]PDBu was measured as above on succeeding days to cells taken from a single neuronal culture preparation. To some wells, 5% fetal calf serum had been added either at the time of plating (day 0) or on day 3.

Overall protein synthesis during each of the first 7 d in culture was estimated by measuring the incorporation of [3S]methionine into primary neuronal cultures. The [3S]methionine (5 µCi/well) was added to the normal culture medium on days 0-6. After 24 h, the intact cells were rinsed once with 1 ml tracer-free medium, then 200 µl of SDS buffer with DTT was used to harvest the cells. Total incorporation of [35S]methionine was measured by scintillation counting of aliquots. The samples were subjected to SDS PAGE, the gels were impregnated with Autoradiograph, and then dried and autoradiographed. At day 3, some cells were treated with Actinomycin D (1 µg/ml) or with cycloheximide (10 µg/ml) just before addition of [35S]methionine. These were harvested the following day as above. Additional cells treated at this time with these drugs or untreated were used to measure levels of [3H]PDBu binding on days 3 and 4.

Phosphorylation of intact cells was studied by incubating the cells in their usual growth medium for 1 h (37°C, 5% CO2) with 1.4 µCi/ml32PO4 (for 2-D gels) or with 0.3 µCi/ml (for SDS PAGE gels). Incubation was continued at room temperature for an additional 10 min in this medium to be phosphorylated with 250 nM PDBu. Cells were washed with 1 ml fresh medium and were harvested with urea buffer for 2-D analysis, or with SDS sample buffer for one-dimensional analysis.

Results

PDBu Binding to Primary Cultures of Neurons and Glia

Phorbol ester receptors were characterized in three types of cultures, consisting of enriched neurons, purified glia, and mixed cultures. Binding of [3H]PDBu (7 nM) to enriched primary neuronal cultures grown in defined medium reached a half-maximal value within 5 min and was essentially complete by 1 h at 23°C (Fig. 1A). Nonspecific binding measured in the presence of 1 µM PDBu amounted to 2-7% of the total binding. Fig. 1B demonstrates that bound [3H]PDBu was displaced by unlabeled PDBu but not by the inactive analogue 4a-phorbol dibutyrate; TPA was more potent than PDBu itself in displacing PDBu. Binding of [3H]PDBu to enriched...
neuronal cultures 10 d in culture was saturable, revealing a single class of binding sites with a $K_d$ value of 7.1 nM (Fig. 2) and a $B_{max}$ of 18.6 pmol/mg. Analogous experiments with purified glial cultures resulted in a $K_d$ value comparable to that obtained for neurons, but with 40% of the number of binding sites per milligram protein. Glial contamination of enriched neuronal cultures in defined medium was assessed at <5% immunocytochemically, as indicated in Materials and Methods. This suggests that glia accounted for <2% of the binding in enriched neuronal cultures. Furthermore, even in mixed (80% neuronal cells) cultures, the majority of PDBu receptors are contributed by neurons, assuming that the maximum number of glial cells in a mixed culture cannot exceed that of a confluent monolayer. No change in affinity and a slight decrease in number of binding sites were observed in cultures of ages varying from 10 d to 4 wk.

Phorbol ester receptor levels in primary neuronal cultures seem to be subject to regulation by increased extracellular K+ levels. Increasing the concentration of K+ for at least 4 d from 3 to 23 mM, in four different experiments, was accompanied by a consistent decrease (26.3% ± 1.8) in the number of PDBu binding sites, with no significant changes in $K_d$ values. The addition of 5% fetal calf serum to the culture medium allowed proliferation of astroglia, fibroblasts, and other non-neuronal cells to nearly 20% of total cell number. In these cultures there was an increase (36.0% ± 12.7; n = 5 experiments) in phorbol ester binding without an accompanying change in $K_d$ and without a general change in the SDS PAGE polypeptide pattern of treated cells. The addition of 0.5% BSA to the defined medium did not alter the number of PDBu binding sites (data not shown), indicating that the serum effect is due to factors other than serum protein.

**Activity of Protein Kinase C and Other Protein Kinases in Extracts of Primary Neuronal Cultures**

Protein kinase C has been designated as the major phorbol ester receptor (6, 17, 26); consequently, protein kinase C was assayed in soluble extracts derived from enriched neuronal preparations. Fig. 3 depicts the gel-permeation chromatographic profile of protein-phosphorylating activity in neuronal extracts, revealing a major Ca²⁺-PS-sensitive protein kinase peak (Fig. 3A) that co-migrates with purified protein kinase C isolated from rat brain (41). [³H]PDBu binding coincided with protein kinase C catalytic activity (Fig. 3B). Furthermore, the phosphorylation of several endogenous substrates ($M_r$ values 87,000; 78,000; 76,000; 43,000; 41,000; 33,000; and several low molecular weight polypeptides ~20,000) was markedly enhanced in the combined presence of Ca²⁺ and PS (Fig. 4). The activity of other protein kinases was also measured using endogenous and exogenous substrates. Phosphorylation of exogenous histone H1 by soluble extracts from primary neuronal cultures was examined, in fractions collected from AcA-34 chromatography columns, in the presence of 20 μM 8-bromo cyclic AMP, or 20 μM cAMP and 0.5 mM 3-isobutyl-1-methylxanthine, or in the presence of 200 μM Ca²⁺ and 10 μg/ml calmodulin, or with 100 μM Ca²⁺ and 40 μg/ml PS. The specific activity of protein kinase C was 720 pmol ³²P incorporated per milligram protein of soluble extract. The cyclic AMP- and Ca²⁺-calmodulin-stimulated phosphorylation was ~30% and 20% of Ca²⁺-PS-dependent phosphorylation, respectively. More significantly, cyclic AMP or
Figure 3. [3H]PDBu binding and protein kinase C activity in soluble extracts from primary neuronal cultures. Neurons from day 8 primary cultures (150 μl packed cells) were homogenized in 3 vol of 50 mM Tris-HCl, pH 7.7, 5 mM DTT, 2 mM EDTA, and 5 mM EGTA. 0.4 ml of the 200,000 g (×1 h) supernatant was applied to a 10 ml Ultrogel AcA-34 column that was eluted with a buffer containing 50 mM Tris-HCl, pH 7.7, 1 mM EDTA, and 0.1% BSA. 0.5-ml fractions were collected and assayed for [3H]PDBu binding and protein kinase C activity. (A) Protein kinase activity was measured using histone H1 as substrate in the presence of 100 μM CaCl2 with (○) or without (□) PS; the PS effect was not observed when 1 mM EGTA was substituted for CaCl2. The elution peak of standard purified protein kinase C is indicated by an arrow. (B) In a separate experiment, Ca2+-PS-sensitive protein kinase (○; 720 pmol/mg protein per min) and Ca2+-PS-dependent [3H]PDBu binding (□; units, at right, are pmol bound per 100-μl aliquot) were assessed for 8-d cultures in the presence of 100 μM CaCl2 and 20 μg/ml PS. [3H]PDBu binding was diminished by 92% when PS and Ca2+ were omitted. Ca2+-PS-sensitive protein kinase activity obtained from day 1 cultures is also shown (△). [3H]PDBu binding in column fractions from extracts of cells 1 d in culture was no greater than background values measured.

Ca2+-calmodulin-dependent phosphorylation of endogenous substrates was much less evident than that observed in the presence of Ca2+ and PS (Fig. 4).

Induction of Phorbol Ester Receptors in Primary Neuronal Cultures

Receptor levels underwent a remarkable increase between the third and eighth day after plating of the dissociated neurons from 16-d-old embryos. This trend was preceded by a slower change during the first three culture days. The total increase in receptor levels (Fig. 5) was about 18-fold in the presence of insulin and transferrin; in contrast, total cellular protein increased by <30%. Fetal calf serum, 5%, further enhanced the receptor levels by 30-60% (Fig. 5). This latter effect was discernible only after 3 d following the addition of serum, even if the serum was added after 7 d in culture. The Kd for PDBu binding (Fig. 2; 7.1 nM on day 10) did not vary from day 1 to day 21 in vitro, indicating that the altered binding reflects changes in Bmax. For example, the Bmax increased from 2.4 pmol/mg protein on day 2 to 16.2 pmol/mg on day 9. The relatively low content of phorbol ester receptors in freshly cultured neurons did not arise from the release or shedding of cellular components during the dissociation or plating procedures. The binding of [3H]PDBu to intact, freshly dissociated cells from neonatal rats was 4-5 times as great as that observed on an equivalent preparation (same number of cells) from embryos 7 d younger. Thus, similar if not identical developmental increases in PDBu receptors occurred in vivo and in vitro. However, a larger proportion of PDBu binding sites was recovered in the supernatant of the dissociating buffer with neonatal than embryonic cells (28% versus 17% of total binding, respectively) possibly representing protein kinase C associated with elaborated neurites that were sheared from the older neurons by the dissociation process. As expected, a striking elevation in protein kinase C levels accompanied the alteration in phorbol ester binding. Enzyme activity, assayed by the phosphorylation of histone H1, at day 1 after culture was barely detectable, while much higher levels were achieved at day 8 (Fig. 3). The absolute number of binding sites appears to be related to the time since conception, rather than the time in culture. We found equal values...
Cellular proliferation was minimal under these conditions; increase was prevented by the addition of 1 µg/ml Actinomycin D (Table 1) and on days 1, 3, 4, 5, 6, 8, and 12 in triplicate wells of cells grown in defined medium alone (G) or with 5% fetal calf serum (F). Each point represents the mean ± SEM for three values obtained in the same experiment; three separate experiments yielded the same time course. Data are expressed as specific binding of [3H]PDBu (fmol) bound per culture well. Each well was plated with 5 × 10^5 cells. Cell proliferation was minimal under these conditions; increase in protein values from 22 to 28 µg per well reflected extensive neurite outgrowth and increased cell mass occurring between day 1 and day 12 in culture.

(about one-half the maximal levels attained) for phorbol binding on the expected day of birth, embryonic day 22, whether cultures were begun at embryonic day 16 and grown for 6 d, or at embryonic day 13 and grown for 9 d. However, this apparent shift in the developmental time course may be due to different populations of cells being selected in cultures of brains derived from different gestational ages.

The rise in phorbol ester receptors was completely blocked by treatment with 10 µg/ml cycloheximide, and was partially prevented by the addition of 1 µg/ml Actinomycin D (Table 1). Similar effects were noted in the presence or absence of 5% serum. [35S]Methionine incorporation into total proteins or into individual polypeptides was likewise inhibited by these reagents. However, the increase in protein kinase C and phorbol ester receptor was not a trivial component of a general concomitant increase in polypeptide biosynthesis. The pattern of [35S]methionine incorporation into individual neuronal polypeptides was similar during the pre-induction phase (day 1–2) and the induction phase; furthermore, total protein content rose only ~30% during this time without an appreciable change in polypeptide composition.

**Endogenous Neuronal Protein Phosphorylation by Protein Kinase C**

The relevance of the previous findings to the phosphorylation of native neuronal polypeptides was also investigated. Intact day 1 and day 8 neurons were labeled with inorganic PPI-phosphate, and polypeptide phosphorylation was examined in the presence or absence of PDBu. Basal polypeptide phosphorylation was much higher in day 8 than in day 1 neurons; the nature of PDBu-sensitive phosphorylation was further, analyzed by two-dimensional isoelectrofocusing/SDS PAGE (Fig. 6). A PDBu effect was detected in day 1 cultures, becoming more prominent in day 8 cultures. The major polypeptide targets for PDBu-induced phosphorylation were located on two-dimensional gels at the following Mr, 10^{-3}/pl values: 83/4.3, 75/4.5, 43/4.5, and 47/4.8. In addition, a group of low molecular weight substrates (Mr, ~21,000, pl 5.5–6.2, and one basic polypeptide, pl > 7.5) was phosphorylated in PDBu-treated cells. These substrates were all minor proteins; none were prominent by Coomassie Blue staining. Comparison of these data with a 2-D autoradiogram of autophosphorylated purified protein kinase C indicates that none of these major phosphoproteins is protein kinase C itself. However, several polypeptides that were phosphorylated by protein kinase C in soluble extracts (Fig. 4) displayed similar Mr, values to polypeptides phosphorylated after treatment of intact cells with PDBu.

**Discussion**

A number of factors have been reported to affect neuronal survival, growth, and elaboration of processes. However, the molecular identity and mechanism of action of these factors remain largely unclear. The present study attempts to identify intracellular regulatory processes that may pertain to neuronal differentiation. Activation of protein kinase C by phorbol esters appears to initiate differentiation in transformed cell lines such as leukemic HL-60 cells (39). In other cells, exposure to phorbol esters leads to increased DNA synthesis rather than differentiation (9). Hormones, growth factors, and neurotransmitters (28) seem to enhance the production of diacylglycerol, a protein kinase C activator and the physiological analogue of phorbol esters (27, 29, 36, 37). Accordingly, phorbol ester-sensitive protein kinase C seems to be intimately involved in regulatory mechanisms leading to cell division or to cell differentiation. Thus, the study of this enzyme system in primary neuronal cultures may prove germane to neuronal growth and differentiation.

Phorbol ester receptors were expressed at low levels in fetal rat brains, with a marked increase in receptor levels during the last week in gestation. A postnatal increase in protein kinase C activity, of two- to threefold from neonate to adult levels, had previously been reported (38). Cultured neurons taken from 16-d-old rat embryos appeared to be already

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**Table I. Developmental Increase in Phorbol Ester Binding Blocked by Protein Synthesis Inhibitors**

|                | PDBu binding (% change from previous day) | Methionine incorporation |
|----------------|------------------------------------------|--------------------------|
| Control        | +52 ± 6%                                  | 100                      |
| Cycloheximide  | −1 ± 3%                                   | 14                       |
| Actinomycin D  | +15 ± 7%                                  | 34                       |

Neuronal cultures grown with 5% fetal calf serum were treated with 10 µg/ml cycloheximide or 1 µg/ml Actinomycin D on day 3. After 18 h, triplicate wells were used to measure [3H]PDBu binding; single wells (to which [35S]methionine, 5 µCi/well, was added at the time of drug treatment) were used to analyze polypeptide synthesis. The developmental increase in PDBu binding from day 3 to day 4 was prevented completely by cycloheximide and attenuated by Actinomycin D. Incorporation of [35S]methionine was blocked by cycloheximide and Actinomycin D without altering the Coomassie Blue staining pattern noticeably.
Figure 6. Phorbol ester-stimulated labeling of phosphoproteins in 8-d neuronal cultures. Neuronal cultures grown in defined medium for 8 d were incubated for 1 h at 37°C with 1.4 mCi/ml \(^{32}P\)O\(_4\) before 10-min incubation at room temperature with or without 250 nM PDBu. 30 \(\mu\)g of total cellular protein for each sample were analyzed by 2-D isoelectrofocusing SDS PAGE. The gels were dried and autoradiographed with Kodak Direct Exposure Film (DEF-5). (A) Control; (B) with PDBu. Autoradiograms were exposed for 2 d. pH values are across top of gels; \(M_r \times 10^{-3}\) values are to the left. Proteins whose phosphorylation increases with PDBu are indicated by arrowheads (individual spots) or an asterisk (cluster of low molecular weight spots).
committed to the induction of phorbol ester binding sites and protein kinase C, which occurred between days 3 and 8 in culture. The induction process did not require the presence of exogenous growth factors other than insulin and transferrin, which are needed for neuronal survival; the mechanism of transcriptional induction of protein kinase C remains to be determined.

Furthermore, the physiological function of protein kinase C in developing neurons is not yet known, but it may be related to the expression of differentiated properties of neurons such as neurite outgrowth (23). This possibility is supported by the recent report that addition of low concentrations of TPA (1.6–16 nM) to serum-free growth medium promoted neurite plexus formation in neuronal cultures at the same time the neurite plexus is being extensively elaborated by these cells is consistent with enrichment of newly synthesized enzyme in these neurites. Subcellular localization of protein kinase C on cultured neurons, using polyclonal antiserum developed in this laboratory, may help answer this question. Protein kinase C actions may be clarified by identifying its specific neuronal substrates. Several polypeptides in intact cultured neurons served as substrates for PDBu-stimulated protein kinase C. For example, an M, 83,000 (pl 4.3) protein was hyperphosphorylated in PDBu-treated cells and might correspond to an acidic M, 87,000 substrate for protein kinase C found in rat brain synaptosomes (42). The proteins at M, 43,000 (pl 4.5) and M, 47,000 (pl 4.8), which were specifically phosphorylated after PDBu treatment, might be related to protein kinase C substrates in synaptosomes (42) and in synaptic plasma membranes (M, 48,000, pl 4.5; reference 2). The latter protein was identified as B-50 protein (also known as F1 protein), which has been implicated in synaptic plasticity (33). Another group of six phosphoproteins at M, 70,000/pl 6.5–6.8 appear to correspond to a similar group of proteins in sympathetic neurons that are regulated by nerve growth factor and depolarization (32), but were not strongly affected by PDBu treatment. Although autophosphorylation of protein kinase C is prominent in purified preparations, this enzyme did not appear to autophosphorylate in intact cells.

Relevant protein kinases may serve as convenient molecular landmarks in the pathway of neuronal differentiation. For example, calmodulin-dependent protein kinase type II is highly enriched in brain and appears to mediate the effects of Ca2+ in several subcellular compartments (25). We have recently shown that this enzyme is mostly produced during the first three postnatal weeks, during late stages of differentiation and synaptogenesis (34). The present work reveals that the prenatal synthesis of protein kinase C clearly precedes that of calmodulin-dependent protein kinase, and may be involved with earlier stages of differentiation. The early production of other protein kinases in mammalian brain is suggested by recent evidence that the c-src gene product, which has tyrosine kinase activity, is expressed more abundantly in fetal brain than in adult human and rat brain (18, 35). The genetic mechanisms underlying the expression of these protein kinases may play an important role in neuronal differentiation. The authors are grateful to Marlene Wolf for providing purified protein kinase C used in this study.

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