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Differential Regulation of Basic Protein Phosphorylation by Calcium Phospholipid and Cyclic-AMP-Dependent Protein Kinases

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Myelin basic protein, an 80-kilodalton (kDa) protein in rat oligodendrocytes, and an 80-kDa basic protein in neuroblastoma × neonatal Chinese hamster brain explant hybrids were phosphorylated extensively when the cells were treated with either phorbol esters (TPA) or diacylglycerols (e.g., oleyoyl-acetyl-glycerol). TPA-stimulated phosphorylation was inhibited by pre-incubation with 50 μM psychosine (galactosyl-sphingosine), confirming that it is mediated through the phospholipid-dependent protein kinase C (PK-C). Surprisingly, phosphorylation of these proteins was inhibited by incubation of cells with agents which result in activation of cyclic-AMP-dependent protein kinase (dibutyryl cyclic AMP or forskolin). In contrast, phosphorylation of other nonbasic proteins, for example, the oligodendrocyte-specific 2′,3′-cyclic nucleotide phosphohydrolase, was stimulated under these conditions (Vartanian et al.: *Proceedings of the National Academy of Sciences of the United States of America* 85:939, 1988) [1]. The possible role of cyclic AMP in activating specific phosphatases or restricting the availability of diacylglycerol for PK-C activation is discussed.

Key words: oligodendrocytes, myelin basic proteins, neurotumor cell lines, protein kinase C, protein kinase A, protein phosphorylation

Protein Kinase C (PK-C) has a broad substrate specificity extending to membrane-associated proteins (e.g., the nicotinic acetylcholine receptor, β-adrenergic receptor, and epidermal growth factor receptor), cytosolic proteins (e.g., glycogen synthase and myelin basic protein), and nuclear-endoplasmic-reticulum-associated proteins such as phospholamban, histone H1, ribosomal protein S6, and eukaryotic initiation factor ($e^{IF\text{-}2}$) [2]. Cyclic-AMP-dependent protein kinase will also phosphorylate serine and threonine residues in many of these proteins, but the nervous system has a number of specific substrates such as synapsin I, DARPP-32, and both voltage-sensitive calcium channels and potassium channels [3]. Basic proteins such as histone H1

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and myelin basic protein (MBP) are not in vivo substrates for PK-A, although MBP can be phosphorylated in vitro using purified PK-A and purified MBP [4,5]. We have previously shown that in cultured sheep oligodendrocytes, MBP is phosphorylated upon attachment of cells to polylysine or when PK-C is activated by phorbol esters (TPA) or Diacyglycerol (DAG). However, addition of forskolin inhibited the in vivo phosphorylation of MBP [6].

Greengard et al. have also identified an 87-kDa acidic protein as a major substrate for PK-C in brain [7]. This protein probably corresponds to the ubiquitous 80-kDa protein in 3T3 cells, or mouse epidermal JB6 cells [8]. Recent studies have linked the 80-kDa protein PK-C substrate with tumor-promoter-induced signal transduction, phosphorylation being highest in promotion-insensitive cells and lowest in transformed cells. However, none of these investigators have studied an 80-kDa protein with basic rather than acidic characteristics which we have identified as a major PK-C substrate in both normal (oligodendrocytes) and transformed (NCB-20) cells. This protein resembles another basic protein (MBP) [9,10] in that it is phosphorylated by TPA and dephosphorylated by cyclic AMP, and is the subject of this study.

MATERIALS AND METHODS

Reagents

Acid-free [^{32}P]-orthophosphate (8 mCi/ml) was obtained from Amersham (Arlington Heights, IL). Pansorbin and forskolin were purchased from Calbiochem Behring (La Jolla, CA). Dibutyryl cyclic AMP, psychosine (galactosylsphingosine), bradykinin, and TPA (12-O-tetradecanoyl phorbol 13-acetate) were obtained from Sigma Chemical (St. Louis, MO). Rabbit antirat myelin basic protein antiserum was obtained courtesy of Dr. A.T. Campagnoni, UCLA.

Cell Culture Conditions

Mouse neuroblastoma × 18-day Chinese hamster embryo brain explant hybrid cell line (NCB-20) was obtained from Dr. J. Minna (Veterans Administration Hospital, Washington, DC). NCB-20 cells were grown on 100-mm Falcon plastic tissue culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn (bobby) calf serum as described by Dawson et al. [11].

Oligodendrocytes were isolated from newborn rat white matter and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn (bobby) calf serum as described by Dawson et al. [11].

Protein Phosphorylation Experiments

Monolayer cultures growing on 100-mm tissue culture dishes (NCB-20 cells) or 60-mm tissue culture dishes (rat oligodendrocytes) were washed three times with 2.5 or 1.5 ml phosphate-free DMEM with high glucose and buffered with phosphate free HEPES pH 7.0 (with 25 mM HEPES and 25 mM glucose), then preincubated in either 2.5 ml or 1.5 ml (100-mm or 60-mm plates, respectively) of this phosphate-free medium (PFM). After preincubation in PFM for 1.5 h carrier-free [^{32}P]orthophosphate (0.060 mCi/plate) was added to the PFM in each plate for 30 min. After 30 min, PK-C and PK-A modulators were added for an additional 30 min (Table 1). Following completion of this incubation period, reactions were stopped by aspiration

TABLE 1. Protein Kinase Modulators Used

Metabolites	Concentrations used
Bradykinin	1×10^{-6} M
Dibutyryl cAMP	20×10^{-6} M
Forskolin	20×10^{-6} M
Prostaglandin E_1	1×10^{-6} M
Psychosine	50×10^{-6} M
TPA	1×10^{-7} M

of the media and the addition of either 5 mM zinc acetate or radioimmunoassay buffer as described below. Cells were harvested mechanically with a rubber policeman into 1.5-ml microfuge tubes in an ice bath.

Acid Extraction of Proteins

Monolayer cultures were preincubated with PFM and incubated with drugs and [^{32}P] orthophosphate as described above but were harvested in 1.2 ml of 5 mM zinc acetate, homogenized in a ground glass homogenizer, and pelleted at 10,000*g* for 5 min as described previously by Dolphin and Greengard [12]. Pellets were washed in 1.2 ml of cold 5 mM zinc acetate and repelleted at 10,000*g* for 5 min. Pellets were resuspended in 200 μl of 10 mM citric acid and the pH was adjusted to 3.0 with 0.1 M HCl. Tubes were vortex-mixed, and the pH 3.0 insoluble fraction was pelleted at 23,500*g* for 15 min. Pellets were saved from each sample and supernatants were decanted off, adjusted to pH 6.0 with 0.2 M Na_2HPO_4, and pH 6.0 insoluble material was pelleted at 10,000*g* for 5 min. Pellets and supernatants were separated. Proteins were solubilized as follows: pH 3.0 and pH 6.0 insoluble pellets were solubilized in 300 μl of 2% SDS per sample and pH 3.0, pH 6.0 soluble supernatants were added to 1/5 volume of 10% SDS. Aliquots of these solutions were then subjected to SDS-PAGE as described in Berry-Kravis et al. [10].

Quantitative determination of dephosphorylation of 80-kDa protein in NCB-20 cells. NCB-20 cells were labeled with [^{32}P]orthophosphate as described followed by treatment with 1×10^{-7} M TPA for 30 min. Cultures were then washed with phosphate-free DMEM and incubated with indicated protein kinase A activators for the indicated times in Figure 2. The cultures were then pH extracted as described, and aliquots of the pH 3.0/6.0 soluble fractions were subjected to 10% SDS-PAGE. Following development of the autoradiogram, the 80-kDa bands were quantitatively removed and counted by Cherenkov counting in the tritium window of a Packard Tri-Carb Scintillation Spectrophotometer. Figure 2 represents the mean values of the experiment performed in triplicate.

Immunoprecipitation with antimyelin basic protein antiserum. The procedure employed was essentially that of Goelz et al. [13]. In brief, samples were dissolved in radioimmunoassay buffer (200 mM NaCl, 10 mM EDTA, 10 mM Na_2HPO_4, 0.1% SDS, 0.5% NP-40 at pH 7.4) plus 1 mM phenylmethylsulfonyl fluoride (PMSF) to a final volume of 100 μl. Ten percent Pansorbin (wt/vol) was pelleted, resuspended in NET buffer (140 mM NaCl; 5mM EDTA; 50 mM Tris, pH 7.4; 0.02% NaN_3; 1% NP-40), and incubated at 23°C for 15 min. The Pansorbin was washed once with NET buffer, then resuspended to original concentration NET

buffer plus 25 mg/ml bovine serum albumin (BSA). Forty microliters of the prepared Pansorbin was added to each sample, followed by incubation at 4°C for 30 min. Samples were microfuged for 3 min to yield a "precleared supernatant." This process was repeated following the overnight incubation of samples with normal rabbit serum. Antiserum to rat myelin basic protein was added at 1:50 dilution, and samples were incubated overnight at 4°C. Afterwards, 20 μl of Pansorbin was added, and the incubation was continued for an additional 1 h at the same temperature, at which time the samples were microfuged for 5 min. The resultant pellets were washed with NET buffer and dissolved in 2% SDS before being subjected to PAGE and autoradiography.

RESULTS

Regulation of Protein Phosphorylation in NCB-20 Cells

Protein phosphorylation experiments were carried out in NCB-20 cells as described above. [^{32}P]Orthophosphate was found to equilibrate rapidly with the cell phosphate pool in NCB-20 cells, as demonstrated by phosphorylation time courses which showed identical relative amounts of protein labeling and no differences in proteins labeled over [^{32}P]orthophosphate incubation times of 10 min to 2 h (data not shown).

Incubation of NCB-20 cells for 30 min with forskolin (which is known to increase cyclic AMP levels in these cells and thus give PK-A stimulation) resulted in clearly evident increased phosphorylation of a 16-kDa protein in the pH 3.0, pH 6.0 soluble basic protein fraction. (Fig. 1, lane 7). However, it was also noted that the presence of forskolin resulted in the decrease in phosphorylation of an 80-kDa protein in the pH 3.0, pH 6.0 soluble fraction (Also Fig. 1, lane 7).

Psychosine (galactosyl-sphingosine), which is known to compete with diacylglycerol for binding to protein kinase C, did not appear to have a significant effect upon the phosphorylation state of any of the proteins in pH 3.0, pH 6.0 soluble basic protein fractions (Fig. 1, lane 6).

TPA stimulated a dramatic increase in the phosphorylation of the 80-kDa protein in the pH 3.0, pH 6.0 soluble fraction; this was accompanied by a noticeable increase in the phosphorylation state of a 50-kDa protein (Fig. 1, lane 5).

Forskolin, in combination with TPA, yielded a decrease in phosphorylation of the 80-kDa protein as compared to the results presented when using TPA alone. (Fig. 1, lane 3) However, the increased phosphorylation of the 16-kDa protein was still apparent. Forskolin, in combination with psychosine (Fig. 1, lane 2), yielded an augmented decrease in phosphorylation of the 80-kDa and also a decrease in the phosphorylation of the 16-kDa protein.

Psychosine, in combination with TPA, resulted in a decreased state of phosphorylation of the 80-kDa and 50-kDa bands, thus confirming their role as protein kinase C substrates (Fig. 1, lane 1).

Measurement of dephosphorylation rates showed that addition of protein kinase A activators to TPA-stimulated NCB-20 cells resulted in rapid dephosphorylation of the 80-kDa protein (Fig. 2). Phosphorylation of 80-kDa was particularly rapid when TPA-pretreated cells were exposed to either dibutyryl cAMP or forskolin. Addition of prostaglandin E1 also resulted in rapid dephosphorylation of the 80-kDa protein although not as marked. The 80-kDa protein was completely dephosphorylated within

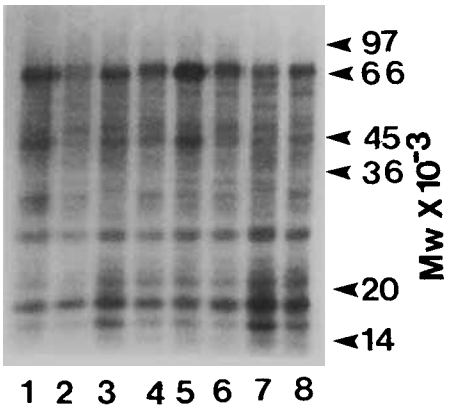

Fig. 1. Autoradiograph of [^{32}P]-labeled NCB-20 cells following treatment with various drugs, fractionation into a pH 3.0/pH 6.0 soluble (basic protein) fraction, and 10% SDS-PAGE. **Lane 1:** TPA + psychosine. **Lane 2:** Psychosine + forskolin. **Lane 3:** TPA + forskolin. **Lane 4:** Untreated. **Lane 5:** TPA alone. **Lane 6:** Psychosine alone. **Lane 7:** Forskolin. **Lane 8:** Untreated. Molecular weight markers are indicated.

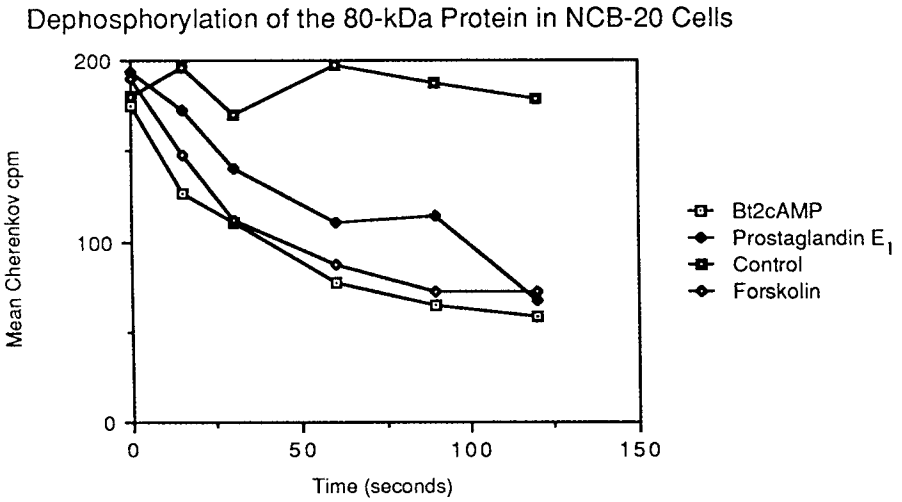

Fig. 2. Cherenkov counts of excised 80-kDa bands from [^{32}P]-labeled NCB-20 cells following treatment with various drugs for the indicated time periods and fractionation into a pH 3.0/pH 6.0 soluble (basic protein) fraction and SDS-PAGE. Time points represent the mean values of experiments completed in triplicate.

5 min following addition of protein kinase A activators, while the TPA, treated controls which were not exposed to protein kinase A activators retained their level of phosphorylation for up to 90 min following TPA treatment (data not shown).

Regulation of Protein Phosphorylation in Rat Oligodendrocytes

Similar pH extraction studies were performed in rat oligodendrocytes and an 80-kDa protein with properties similar to the 80-kDa protein of NCB-20 cells was found (Fig. 3).

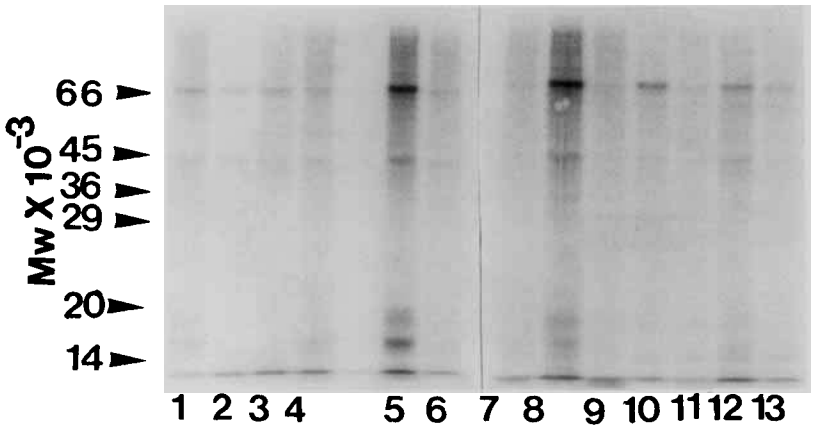

Fig. 3. Autoradiograph of [^{32}P]-labeled rat oligodendrocytes following treatment with various drugs and fractionation into a pH 3.0/pH 6.0 soluble (basic protein) fraction and 10% SDS-PAGE. **Lane 1:** Untreated. **Lane 2:** Bradykinin. **Lane 3:** Bt_2cAMP. **Lane 4:** Forskolin. **Lane 5:** TPA. **Lane 6:** Bradykinin + forskolin. **Lane 7:** Bradykinin + psychosine. **Lane 8:** Bradykinin + TPA. **Lane 9:** Bt_2cAMP + psychosine. **Lane 10:** Bt_2cAMP + TPA. **Lane 11:** Forskolin + psychosine.**Lane 12:** Forskolin + TPA. **Lane 13:** Psychosine + TPA. Molecular weight markers are indicated.

Bradykinin was also used as a metabolite in this study, as bradykinin is known to stimulate the production of second messengers in a variety of neural cell lines and tissues [14]. Therefore, it was of interest to see if bradykinin treatment resulted in enhanced or decreased phosphorylation of basic proteins in the cultured oligodendrocytes. However, upon addition of physiological amounts of bradykinin, no effect on phosphorylation was observed (Fig. 3, lane 2). It should be noted that bradykinin also appeared to have no effect upon the phosphorylation state of basic proteins in NCB-20 cells (data not shown).

Dibutyryl cyclic AMP and forskolin, as expected, gave similar effects in that the 80-kDa protein was "dephosphorylated" following incubation with these compounds (Fig. 3, lanes 3,4, respectively). TPA produced a dramatic increase in the phosphorylation state of the 80-kDa, and an increase, although to a lesser extent, in the phosphorylation of a 43-kDa protein (Fig. 3, Lane 5). As with the NCB-20 cells, TPA in combination with forskolin or psychosine resulted in a noticeable decrease in the phosphorylation of the 80-kDa substrate (Fig. 3, lanes 12,13 respectively). When dibutyryl cyclic AMP was substituted for forskolin, similar effects were noted (Fig. 3, lane 10). An intriguing aspect of these fractionations was that proteins similar in molecular weight to myelin basic proteins which appeared in the pH 3.0, pH 6.0 soluble fractions appeared to exhibit similar patterns of regulation of phosphorylation. Previous studies by Vartanian et al. [6] in the ovine system have shown that MBP phosphorylation is indeed regulated by protein kinases A and C. Therefore, immunoprecipitation of labeled MBP was performed, and the samples subjected to SDS-PAGE (Fig. 4). As with the 80-kDa protein(s), TPA treatment resulted in increased phosphorylation of MBP (lane 5), whereas forskolin and dibutyryl cyclic AMP resulted in decreased phosphorylation of MBP (lanes 2,3, respectively). Although the combination of forskolin or dibutyryl cyclic AMP and TPA did not show the same degree of competition

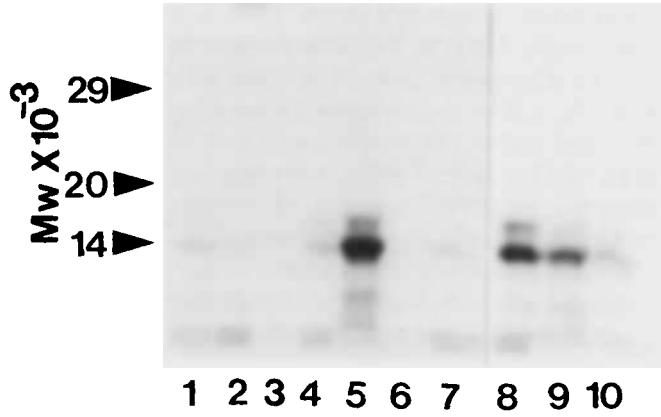

Fig. 4. Autoradiograph of [^{32}P]-labeled rat oligodendrocytes following treatment with various drugs and immunoprecipitation of myelin basic protein and 12% SDS-PAGE. **Lane 1:** Untreated. **Lane 2:** Bt_2cAMP. **Lane 3:** Forskolin. **Lane 4:** Psychosine. **Lane 5:** TPA. **Lane 6:** Bt_2cAMP + psychosine. **Lane 7:** Forskolin + psychosine. **Lane 8:** Bt_2cAMP + TPA. **Lane 9:** Forskolin + TPA. **Lane 10:** Psychosine + TPA. Molecular weight markers are indicated.

of phosphorylation seen with the 80-kDa protein(s), the protein kinase A mediators did appear to facilitate dephosphorylation of the MBP in a similar manner to the results seen with the 80-kDa protein(s) (lanes 8, 9, respectively). Once again, psychosine appeared to compete with TPA for the activation of protein kinase C (lane 10).

DISCUSSION

Of particular interest in the study of the regulation of phosphorylation of basic proteins by protein kinase C is the finding that one of the PK-C substrate threonines in MBP is encompassed in a sequence homologous to SV-40 T antigen [15]. Synthetic peptides from this region have been found to be phosphorylated in vitro by purified rat brain protein kinase C. Recent experiments have shown that "molecular mimicry" of the 8–10 amino acid encephalitogenic site of MBP by a hepatitis B virus polymerase protein determinant can generate an autoimmune response leading to tissue injury in rabbits [16]. Other shared viral determinants with MBP are those of a 9 amino acid sequence of measles virus P3 [17] and a 12 amino acid sequence from visna virus polymerase [18]. Little is known of the physiological role of MBP phosphorylation, but it is conceivable that alteration in the phosphorylation pattern of MBP could facilitate the exposure and hence recognition of the autoimmune determinant to T cell clones specific for MBP. This might induce experimental allergic encephalomyelitis, which has long been considered to be one of the best animal models for multiple sclerosis [19]. MBP is not expressed on the surface of oligodendrocytes, but it should be noted that viral antigens do not necessarily have to be expressed on the surface of a cell to elicit a response from specific T cell subsets [20].

We have previously shown that the major 18.5-kDa basic protein of myelin (MBP) in sheep oligodendrocytes (OLG) is an excellent substrate for PK-C [6]. However, in these same cells the phosphorylation of MBP is inhibited by the addition of forskolin or dibutyryl cyclic AMP in the same manner as the 80-kDa protein from NCB-20 cells [10]. The inhibition appears MBP-specific since another myelin protein expressed in these OLG, 2′,3′-cyclic nucleotide phosphohydrolase is an in vivo substrate for both PK-C and cyclic-AMP-dependent PK-A [1]. Other laboratories have recently described an 80-kDa protein which was phosphorylated in response to phorbol esters and other activators of PK-C in cultured fibroblasts as well as other cultured cell types [8]. However, these latter 80-kDa proteins all seem to have acidic pI values, as opposed to our 80-kDa protein(s), which appear to have pI values of greater than 6.0. We also note that our pH fractionation of oligodendrocyte cultures results in the cofractionation of certain histone and myelin basic proteins with our 80-kDa protein, confirming its basic nature.

There are several possible explanations for the mechanism of dephosphorylation of protein kinase C substrates by the activation of the protein kinase A pathway. Bidirectional control systems in which the activation of protein kinase A by cyclic AMP may result in the inhibition of inositol phospholipid breakdown to diacyglycerol and hence suppression of protein kinase C activity have been reported [2,14]. Data from this laboratory suggest that forskolin mediates the inhibition of MBP phosphorylation by lowering the effective concentration of the free DAG pool accessible to PK-C. Thus, treatment of oligodendrocyte cultures with forskolin has been shown to reduce the levels of diacylglycerol as assessed by 1-h incubation of oligodendrocyte cultures with 1-[^{14}C] arachidonic acid and resolution of isolated lipids by thin layer chromatography. This infers that the mechanism of DAG release is the key event in the inactivation of protein kinase C by protein kinase A-mediated events. However, TPA activates protein kinase C directly both in vitro and in vivo [21] without involving phosphoinositide intermediates by virtue of having a molecular structure similar to the 1,2-*sn* binding configuration of diacylglycerol. Despite this, forskolin or dibutyryl cyclic AMP activation of PK-A still results in the dephosphorylation of the TPA-induced phosphorylation events of the 80-kDa protein. Thus it may be presumptuous to assume that failure to release DAG is directly responsible for the dephosphorylation of the 80-kDa protein(s).

While a specific cyclic-AMP-inducible phosphatase has not been reported, the possibility does exist [9,22]. An ATP/Mg-dependent protein phosphatase is present in pig brain and has been shown to dephosphorylate MBP *in vitro* [23]. Cyclic-AMP-dependent kinase activity has been shown to inhibit the phorbol ester-induced phosphorylation of several proteins in S49 mouse lymphoma cells [24]. While the authors present no data to support a PK-A-stimulated phosphatase activity, they suggest the existence of one. Several studies have shown that thrombin-induced phosphorylation of a 40-kDa protein in platelets could be inhibited by elevation of cAMP levels [25].

One other potential mechanism for dephosphorylation of PK-C substrates by PK-A activation is the possible direct inactivation of PK-C by activation of PK-A. A well-documented example of direct inactivation of PK-C is the action of psychosine and lysosphingolipids in competition with DAG or TPA for correct binding configuration with PK-C [26]. It is doubtful that forskolin-mediated dephosphorylation is mediated by a similar mechanism as addition of TPA can overcome the block by psychosine. However, addition of TPA to forskolin-treated samples appears to only par-

tially restore PK-C-mediated phosphorylation. Furthermore, TPA itself does not inhibit the generation of cyclic AMP by forskolin in ovine oligodendrocytes (data not shown).

One of the lingering questions regarding the interaction between PK-C and PK-A pathways in our system centers upon the intriguing preference for basic proteins as both PK-C and PK-A substrates. We have previously shown that nonbasic proteins may be phosphorylated by both PK-C and PK-A. Therefore, we are assuming that this particular constraint linking the putative PK-C and PK-A pathways is not fortuitous in nature. Future investigation will center around the discrete mechanism of dephosphorylation and characterization of the purpose surrounding the bidirectional as opposed to the unidirectional interactions between PK-C and PK-A. Further, it remains to be seen as to whether the 80-kDa basic protein(s) in rat oligodendrocytes and NCB-20 cell lines are the same protein and what their putative functions may be, not to mention of course their relationship to the ubiquitous acidic 80-kDa protein reported by other laboratories [27].

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