Nerve Growth Cones Isolated from Fetal Rat Brain

III. Calcium-dependent Protein Phosphorylation¹

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Abstract

Calcium-dependent protein kinase activities have been studied in nerve growth cone particles (GCPs) and compared with those of synaptosomes. GCPs contain a set of phosphoproteins qualitatively similar to that of synaptic nerve terminals. However, major quantitative differences appear to exist: whereas synapsin I phosphorylation is relatively weak, the major kinase substrates of GCPs are a 46,000-dalton membrane protein (calcium/calmodulin dependent) and two acidic proteins of 80,000 and 40,000 daltons, phosphorylated by a calcium/phospholipid-dependent protein kinase. The presence of synaptic kinase activities in GCPs is consistent with their neuronal origin. The role of these kinases in GCPs is not understood at present. They may be involved in growth-related functions and/or may prepare the sprouting neuron for synaptic function.

Subcellular fractionation can be used to isolate particles from fetal brain that have the cytological features of nerve growth cones (Pfenninger et al., 1983). We are now using these "growth cone particles" (GCPs) to explore developmentally regulated events in fetal brain, especially the biochemical events accompanying the transition from neuronal sprouts to mature nerve terminals. As an initial step, we have chosen to analyze the phosphoproteins of GCPs and to compare them with the well characterized phosphoproteins of synaptosomes, pinched-off synaptic terminals isolated from adult

brain (Gray and Whittaker, 1962; for review, see Nestler and Greengard, 1983). In the preceding paper, we reported on cAMP-dependent phosphorylation of GCP proteins (Ellis et al., 1985). In the present study, we extend this analysis to include (i) a description of calcium/calmodulin- and calcium/phospholipid-dependent protein kinase activities in GCPs and (ii) the comparison of these activities with those observed in synaptosomes. The characterization of phosphoproteins of GCPs leads to new information on the presence and distribution of calcium-dependent protein kinases and their substrates in fetal brain. A preliminary report of this work has been presented in abstract form (Ellis et al., 1983).

Materials and Methods

Materials. All reagents were as described in the preceding paper (Ellis et al., 1985) with the following additions. Dithiothreitol (DTT), β -mercaptoethanol (BME), bovine serum albumin (BSA), piperazine-*N,N'*-bis[2-ethanesulfonic acid] (PIPES), bovine brain calmodulin (CM), phosphatidylserine (PS), and 1,3-diolefin (D) were from Sigma Chemical Co. Magnesium chloride (crystals, 0.0006% Ca^{2+} contamination) and ethylene diaminetetra-acetic acid (EDTA) were from Fisher. Trifluoperazine (TFP) was from Boehringer-Mannheim. All other chemicals were reagent grade or better.

Preparation of GCPs. GCPs were prepared as described in the preceding paper (Ellis et al., 1985). Except where indicated, GCPs were used fresh immediately after preparation.

Calcium/calmodulin-stimulated phosphorylation. The analysis of calcium-stimulated phosphorylation of GCP proteins (except where indicated) was according to the conditions of Kennedy and Greengard (1981) with the following modifications. The reaction mixture, in a final volume of 50 μl , contained: 50 mM PIPES, pH 7.0, 10 mM MgCl_2 , 1 mM DTT, 0.4 mM EGTA, 30 to 50 μg of GCP protein, and additions as indicated in the figure legends. After a 1-min preincubation at 30°C, reactions were initiated by the addition of γ -³²P-ATP (14 to 22 Ci/mmol) to 5 μM , and were incubated for 15 sec at 30°C. Under these conditions, incorporation of ³²P is dependent on ATP concentration over a range of 2.5 to 80 μM . At 5 μM ATP, incorporation of ³²P is linear over the first 2 min of the reaction (analysis of trichloroacetic acid-precipitable counts per unit of time; data not shown). In all reactions described below (run for 15 sec) we are examining initial rates. Reactions for one-dimensional gel analysis were terminated by the addition of 25 μl of twice-concentrated Laemmli sample buffer (Laemmli, 1970); the samples were heated to 100°C for 3 min and then frozen at -20°C until use. Reactions for two-dimensional analysis (isoelectric focusing (IEF) or non-equilibrium pH gradient gel electrophoresis (NEPHGE); see below) were terminated by the addition of 50 μl of a twice-concentrated stop solution containing 10% BME and 4% Nonidet P-40 (NP-40). Samples were frozen at -20°C until use.

Calcium/phospholipid-stimulated phosphorylation. The analysis of calcium/phospholipid-stimulated phosphorylation of GCP proteins was modified from the procedure of Wu et al. (1982) as follows. The reaction mixture, in a final volume of 50 μl , contained: 20 mM HEPES, pH 7.5, 10 mM MgCl_2 , 1 mM DTT, 0.4 mM EGTA, 17 to 50 μg of GCP protein containing 0.1% Triton X-100 (TX-100), added for a minimum of 30 min on ice before addition of the homogenate to the reaction mixture, and various additions as indicated in the figure legends. The reaction was initiated by the addition of γ -³²P-ATP (22 Ci/mmol) to 5 μM after a 1-min preincubation at 30°C. The reaction proceeded for 15 sec at 30°C and was terminated as above. This is the

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"standard reaction." Phospholipid was prepared according to the method of Kaibuchi et al. (1981), with the following modifications. PS or D in chloroform/methanol was dried under vacuum and resuspended in 20 mM HEPES (pH 7.5) containing 1 mM DTT. The suspension was perfused with N₂ in a capped Eppendorf tube and was vigorously vortexed, sonicated at 4°C for 5 min in a Fisher sonifier, and added to the reaction just before the 1-min preincubation. Free calcium was calculated using a computer program generously provided by Dr. Philip Brandt, based on a calcium/EGTA buffer containing 0.4 mM EGTA. Using a 20 mM HEPES (pH 7.5) buffer, an apparent binding constant of $1.92 \times 10^6 \text{ M}^{-1}$ for calcium/EGTA was used to calculate free calcium concentration (Orentlicher et al., 1977).

Preparation of cytosol and particulate fractions of the GCP. GCPs (which were stored at -70°C and thawed before use) were lysed in 5 mM Tris-HCl (pH 8.1) with 0.5 mM EDTA and stirred for 45 min at 4°C. The lysed material was spun at $200,000 \times g_{\text{max}}$ in an SW55Ti rotor for 60 min at 4°C (Beckman L8-55 ultracentrifuge). The supernatant was recovered and used as the lysate. The pellet was resuspended by homogenization (glass-glass) in double-distilled water and used as the particulate fraction.

Preparation of synaptosomes. Synaptosomes were prepared from adult rat brain as described by Cohen et al. (1977) and used as indicated in the preceding paper (Ellis et al., 1985).

Electrophoresis of GCP proteins was performed as described previously (Ellis et al., 1985). The molecular weight designations indicate the migration of protein standards as follows: 200,000, myosin; 116,250, β -galactosidase; 94,000, phosphorylase b; 67,000, BSA; 43,000, egg white ovalbumin; 30,000 carbonic anhydrase; 20,100, soybean trypsin inhibitor; and 14,400, α -lactalbumin.

Other procedures. Protein was determined by the method of Bradford (1976; Bio-Rad reagent) using bovine plasma gamma globulin as standard. For the quantitation of individual proteins, bands were localized by autoradiography of dried SDS gels, excised, and quantitated by liquid scintillation spectrometry (Huttner et al., 1981), using Omnifluor-toluene scintillation fluid (New England Nuclear).

For peptide mapping, spots were localized by autoradiography of dried SDS gels, excised, reswollen in equilibrium buffer, and loaded on 15% SDS-PAGE gels according to the procedure of Cleveland et al. (1977) with the following modifications: gels of 0.75 mm thickness were used with 3% stacking gels 4.5 cm in length. EDTA was not included in the gel solutions. Slices were overlaid with 10 μg of *Staphylococcus aureus* V8 protease. Electrophoresis was not interrupted at the stacker/running gel interface.

Results

Exogenous calcium stimulates the phosphorylation of GCP proteins. GCPs were incubated with γ -³²P-ATP under various experimental conditions and their phosphoproteins were analyzed by SDS-PAGE and radioautography. The resulting patterns of phosphorylated GCP proteins are shown in Figure 1. The addition of Ca²⁺ to the incubation medium leads to a marked stimulation in the incorporation of ³²P into bands of 40,000, 46,000, 52,000, 56,000, 60,000, and 80,000 daltons, in addition to several minor bands. Phosphorylation of all GCP substrates requires Mg²⁺ and is inhibited by TFP.

When exogenous CM is included with Ca²⁺ in the reaction, phosphorylation of the 46,000-, 56,000-, and 60,000-dalton substrates is stimulated. Based on liquid scintillation spectrometry of excised polypeptide bands, the CM stimulation of phosphorylation is always detectable. However, the extent of stimulation is never greater than 1.5-fold and varies in different experiments, possibly due to variable amounts of endogenous CM in the GCP preparations. In order to ascertain the presence of endogenous CM, we searched for a protein that shows the CM-characteristic shift in apparent molecular weight in the presence versus the absence of Ca²⁺ (cf. Grab et al., 1979). Indeed, when GCPs are incubated in the presence of EGTA and analyzed by SDS-PAGE, a minor band of approximately 18,000 daltons is observed after Coomassie blue staining. In the presence of exogenous Ca²⁺, this band is absent. Instead, we see a new band of approximately 16,000 daltons, which co-migrates with exogenous CM (data not shown).

In the presence of CM, Ca²⁺-stimulated phosphorylation of the 40,000- and 80,000-dalton bands is inhibited (Fig. 1). This finding is reminiscent of a similar inhibition observed in synaptosomes for substrates of protein kinase C (Wu et al., 1982; Albert et al., 1984).

Figure 1. Effect of Ca²⁺, CM, and TFP on phosphorylation of GCP proteins. GCPs (50 μg /reaction) were incubated with γ -³²P-ATP under standard conditions for Ca²⁺/CM phosphorylation with the indicated modifications, followed by SDS-PAGE, protein staining (left lane), and autoradiography (middle and right lanes). Each lane contained 37 μg of protein. The additions indicate: MgCl₂, 10 mM; CaCl₂, 0.7 mM; CM, 5 μg ; and TFP, 0.5 mM. Reactions including 0.5 μg of CM and 0.05 mM TFP, where appropriate, gave identical results. The arrow indicates tubulin (α - and β -tubulin migrate as a single, broad band in this gel system). The autoradiogram was exposed for 4 hr at room temperature.

Therefore, we investigated the effect of exogenous phospholipid on Ca²⁺-stimulated phosphorylation in GCPs.

The GCP contains calcium/phospholipid-dependent protein kinase activity. Nishizuka and his collaborators (Takai et al., 1979) have described a Ca²⁺/phospholipid-activated protein kinase (protein kinase C) which is active at submicromolar concentrations of free Ca²⁺ in the presence of PS and diacylglycerol (diolein). In particulate fractions, however, its activity is masked in the absence of the non-ionic detergent, TX-100 (Kikkawa et al., 1982). Because GCPs contain large amounts of membranes, we first tested the effect of TX-100 on Ca²⁺-activated kinase activity in the presence of PS and diolein (D). In Figure 2 (lanes 1 and 2) a marked effect of TX-100 is evident. TX-100 greatly enhances the phosphorylation of the 40,000- and 80,000-dalton proteins but slightly inhibits the phosphorylation of the 46,000-dalton substrate.

We next examined the effect of the concentration of free Ca²⁺ on phosphorylation of these three substrates. In the presence of PS, D,

Figure 2. Effect of TX-100 and Ca^{2+} concentration on Ca^{2+} /phospholipid-activated phosphorylation of GCP proteins. Lanes 1 and 2, GCPs were incubated with 0.4 mM EGTA, 0.7 mM CaCl_2 , 2.5 μg of PS, and 0.5 μg of D in the standard reaction mixture for Ca^{2+} /phospholipid phosphorylation in the presence (lane 1) or absence (lane 2) of 0.1% TX-100. Lanes 3 to 8, Standard reaction as above except that the concentration of free Ca^{2+} was increased from lane 3 to lane 8 as follows: no calcium, 1×10^{-7} M, 5×10^{-7} M, 1×10^{-6} M, 5×10^{-6} M, and 1×10^{-5} M. Free Ca^{2+} was calculated using a Ca^{2+} /EGTA buffer system as described under "Materials and Methods." Dots indicate the positions of the 80,000-, 46,000-, and 40,000-dalton substrates. Each lane contained 34 μg of protein. Autoradiograms were exposed for 30 min at room temperature.

and TX-100, a stimulation in the phosphorylation of all three substrates at 10^{-7} M free Ca^{2+} is detectable (Fig. 2, lanes 3 and 4). Incorporation of ^{32}P into the 40,000- and 80,000-dalton proteins is roughly linear over the range of 10^{-7} to 10^{-6} M free Ca^{2+} (Fig. 2, lanes 4 to 6; quantitation by liquid scintillation spectrometry, data not shown). However, at 5×10^{-6} M free Ca^{2+} (Fig. 2, lane 7), one observes a slight inhibition of phosphorylation of the 40,000- and 80,000-dalton bands. At higher concentration of free Ca^{2+} (10^{-5} M; Fig. 2, lane 8), this inhibition is overcome, and both substrates are again maximally phosphorylated. By contrast, incorporation of ^{32}P into the 46,000-dalton substrate is saturated only at 5×10^{-6} M free Ca^{2+} .

To examine the effect of phospholipid on the phosphorylation of the 40,000- and 80,000-dalton proteins directly, whole GCP homogenate was incubated in the presence and absence of PS and D (Fig. 3a). At a free Ca^{2+} concentration of 5×10^{-7} M, addition of PS and D results in a marked inhibition of the phosphorylation of the 40,000- and 80,000-dalton proteins but not the 46,000-dalton protein. The addition of phosphatidylcholine and D similarly results in an inhibition of the phosphorylation of the 40,000- and 80,000-dalton

substrates (data not shown). These results might be expected if the kinase is already saturated with endogenous phospholipid and/or if the addition of exogenous lipid in the presence of TX-100 perturbs the microenvironment necessary for kinase activity. Therefore, we decided to isolate soluble components from GCPs and to assay for the presence of phospholipid-dependent kinase activity in the cytosol separately.

When the membrane-depleted lysate of the GCP is assayed for the effect of PS and D on protein phosphorylation, a marked stimulation of ^{32}P incorporation into the 40,000- and 80,000-dalton proteins is evident (Fig. 3b). This is true both in the presence of excess free Ca^{2+} (large plus sign in Fig. 3) and at 5×10^{-7} M free Ca^{2+} (small plus sign in Fig. 3). Ca^{2+} /PS results in less stimulation than Ca^{2+} /PS/D, and Ca^{2+} /D has no effect above Ca^{2+} alone. As in the whole GCP homogenate (see Fig. 1), Ca^{2+} /CM causes an inhibition of phosphorylation of the 40,000- and 80,000-dalton substrates in the cytosol (Fig. 3b).

Soluble and particulate GCP subfractions contain different sets of kinase substrates. The subfractionation of GCPs into cytosol and membranes enables one to examine the relative distribution of the major Ca^{2+} -dependent substrates in these two compartments (Fig. 3, b and c). The 40,000- and 80,000-dalton substrates are present in both fractions, as are the 52,000- and 56,000-dalton proteins. A substrate of 49,000 daltons, stimulated by Ca^{2+} /CM but not previously detected, is also present in both preparations. On the other hand, a 60,000-dalton protein is highly enriched in the cytosol, whereas the 46,000-dalton substrate is detectable only in the particulate fraction, under the experimental conditions used. In this crude membrane preparation, the CM stimulation of protein phosphorylation is more pronounced than in whole GCP homogenate.

Calcium-dependent protein kinase activities of fetal brain can be enriched by subcellular fractionation. GCPs are derived from the low-speed supernatant of fetal brain homogenate. Fractions A, B, and C are removed from the interfaces of a discontinuous sucrose density gradient and contain distinct cytological profiles (Pfenninger et al., 1983). The high-speed supernatant (O) is the soluble protein of the load. The A-fraction is collected at the interface between the load and 0.75 M sucrose, and it contains GCPs and soluble protein. GCPs are freed from soluble protein by dilution of the A-fraction and pelleting. The highly heterogeneous B- and C-fractions contain small glial profiles, subcellular organelles, and (especially the C fraction) lysed GCPs.

To assess the relative abundance of the major Ca^{2+} -dependent kinases and/or their substrates in these subfractions, aliquots containing equal amounts of total protein were taken at each stage of the fractionation and assayed for Ca^{2+} -stimulated phosphorylation. PS and D were included in each reaction to standardize the "phospholipid background" in these highly distinct subcellular fractions. Because the GCP, B-, and C-fractions have a significant membrane component, TX-100 was also added to the incubation mixture. A phosphorylation profile across the fractions is shown in Figure 4. Homogenate (H), low-speed pellet (P), low-speed supernatant (S), and the O-fraction are relatively poor in phosphoproteins, whereas the particulate subfractions of the low-speed supernatant (GCPs, B, and C) are enriched. The GCP fraction (G) is highly enriched in the major substrates relative to the A-fraction, from which it was prepared. None of these substrates, however, are found exclusively in any single fraction. Due to the major differences between these fractions, it is not possible to control the reactions for all variability in the contents of endogenous cofactors. Thus, the patterns in Figure 4 can convey only a rough estimate of the enrichment of kinase activity. When these assays are done in the presence of Ca^{2+} without PS, D, or TX-100, the GCP and A-fractions appear somewhat more enriched in the major substrates. However, the substrates observed are qualitatively similar (data not shown).

GCPs and synaptosomes share major substrates for calcium-dependent protein kinases. To facilitate the characterization of GCP substrates for Ca^{2+} -dependent protein kinases, and to compare the

Figure 3. Effect of Ca²⁺, CM, PS, and D on the phosphorylation of GCP proteins in whole homogenate, cytosol, and particulate fractions. Except where indicated otherwise, the reaction medium included: 0.7 mM CaCl₂ with 0.4 mM EGTA, 2.5 μ g of PS, 0.5 μ g of D, 0.5 μ g of CM, and 0.1% TX-100. *a*, Standard reaction (GCP homogenate; 12 μ g of protein/lane) for Ca²⁺/phospholipid-stimulated phosphorylation with 5 $\times$ 10⁻⁷ M free calcium, in the presence or absence of PS and D. *b*, A cytosol fraction derived from GCP homogenate was incubated under standard conditions as above, with the indicated modifications. *Large plus signs* denote 0.3 mM Ca²⁺; *small plus signs* denote 5 $\times$ 10⁻⁷ M free Ca²⁺ (20 μ g of protein/lane). *c*, A crude membrane fraction of GCP was incubated under standard conditions with the indicated modifications. *Double arrowheads* indicate the positions of the 80,000-, 52,000-, 46,000-, and 40,000-dalton substrates, respectively. The autoradiograms were exposed as follows: *a*, for 2 hr at room temperature; *b*, for 6 hr at -80°C; and *c*, for 1.5 hr at -80°C.

distribution of these substrates in fetal versus adult brain, we have juxtaposed the patterns of GCP and synaptosomal phosphoproteins (Fig. 5). When proteins phosphorylated in the presence of exogenous Ca²⁺ or Ca²⁺/CM are analyzed by one-dimensional SDS-PAGE, both GCP and synaptosomes (SYN) have substrates of 40,000, 46,000, and 80,000 daltons (Fig. 5). The 80,000-dalton substrate of the SYN, however, is a distinct doublet. Bands corresponding in mobility to the major SYN phosphoproteins at 49,000 and 60,000 daltons can be seen in the GCP lanes after long exposure (data not shown) and are visible in the cytosol and particulate GCP subfractions shown in Figure 3.

To further characterize the major substrates for Ca²⁺-dependent protein kinases, GCP and SYN proteins phosphorylated in the presence of exogenous Ca²⁺ or Ca²⁺/CM were analyzed on two-dimensional gels employing acidic IEF in the first dimension (Fig. 6). In the presence of Ca²⁺ alone (Fig. 6, *top*), three major acidic proteins at 40,000, 46,000, and 80,000 daltons with isoelectric points of 4.2, 4.3, and 4.0, respectively, are observed. On the basis of electrophoretic mobility, tubulin is likely to be among a prominent group of spots at 52,000 daltons. When exogenous CM is included in the reaction (Fig. 6, *bottom*), the phosphorylation of the 40,000- and 80,000-dalton acidic substrates is markedly inhibited, as was observed on one-dimensional gels (cf. Fig. 1).

The extent of phosphorylation of these substrates is markedly different in the two preparations. On a per microgram total protein

scale, the GCP 40,000- and 46,000-dalton proteins incorporate 20 times as much ³²P as the SYN 40,000- and 46,000-dalton proteins, and the GCP 80,000-dalton protein (acidic) incorporates 7 times more ³²P than its SYN counterpart (based on liquid scintillation spectrometry of excised spots). The major SYN substrates of 49,000 and 60,000 daltons are not visible on these IEF gels because they are basic proteins (see below).

To compare further the 46,000- and 80,000-dalton substrates of the GCP and SYN, the respective spots were cut out of these two-dimensional gels and subjected to limited proteolysis with *S. aureus* V8 protease. The 46,000-dalton proteins of both the GCP and SYN yield a single 23,000-dalton phosphopeptide, suggesting that these substrates in fetal (GCP) and adult (SYN) brain are identical (Fig. 7, *lanes 1 to 3*). V8 protease cleavage of the GCP's acidic 80,000-dalton protein generates two phosphopeptides of 9,000 and 13,000 daltons (Fig. 7, *lane 4*), which are similar to the phosphopeptides of a previously characterized acidic SYN protein of similar molecular weight (see "Discussion").

To compare basic proteins, which are excluded from the IEF gels, Ca²⁺-stimulated phosphoproteins of GCP and SYN were analyzed on two-dimensional gels using NEPHGE in the first dimension (Fig. 8). The 49,000- and 60,000 (doublet)-dalton SYN major proteins are now included in the gel and occupy positions characteristic of the subunits of synapsin I kinase (Fig. 8, SYN, spots 3 and 4; Bennett et al., 1983; Kennedy et al., 1983b). In addition, a very basic doublet

Figure 4. Phosphorylation of proteins in subcellular fractions of fetal brain. Aliquots of subcellular fractions generated during the preparation of GCPs (stored at -70°C and thawed immediately before use) were phosphorylated in the presence of 10^{-5} M free Ca^{2+} , $2.5\text{ }\mu\text{g}$ of PS, $0.5\text{ }\mu\text{g}$ of D, and 0.1% TX-100 under standard conditions for Ca^{2+} /phospholipid-stimulated phosphorylation. H, homogenate of fetal brain; P, low-speed pellet; S, low-speed supernatant; O, high speed supernatant; A, fraction collected from $0.32/0.75$ M sucrose interface, containing GCPs and soluble protein; G, pelleted GCPs; B, fraction from $0.75/1.0$ M sucrose interface; C, fraction from $1.0/2.66$ M sucrose interface. Equal amounts of protein were loaded in each lane and analyzed by SDS-PAGE and autoradiography. The autoradiogram shown was exposed for 6 hr at room temperature.

of approximately 80,000 daltons is present (Fig. 8, *arrowhead*). This SYN protein has the electrophoretic properties of synapsin I and has an identical counterpart in the GCP. However, autoradiograms of GCP gels, such as the one shown in Figure 8, have to be exposed for extended periods of time to reveal this phosphoprotein doublet. Under these conditions, a series of additional, minor substrates becomes visible. We do not know whether they are present in SYN as well.

Discussion

Two classes of Ca^{2+} -dependent protein kinase activities are well known to occur in adult mammalian brain, one that requires CM as a cofactor and one that requires phospholipid, protein kinase C (for review, see Schulman, 1982; Kennedy, 1983; Nishizuka, 1984). However, little is known about the presence and distribution of these

Figure 5. Comparison of GCP and synaptosomal (Ca^{2+} -stimulated phosphoproteins. GCP and synaptosome (SYN) homogenates were phosphorylated under standard conditions for Ca^{2+} /CM phosphorylation in the presence of 0.7 mM CaCl_2 or 0.7 mM $\text{CaCl}_2/0.5\text{ }\mu\text{g}$ of CM, as indicated, and analyzed by SDS-PAGE and radioautography. GCP lanes contain $15\text{ }\mu\text{g}$ of protein; SYN lanes contain $32\text{ }\mu\text{g}$ of protein. The asterisk indicates the position of tubulin. The autoradiogram was exposed for 2 hr at room temperature.

kinase activities in the developing brain. The only information available heretofore was that protein kinase C activity, which is found at higher levels in brain than in any other tissue (Kuo et al., 1980; Kikkawa et al., 1982), is also present in extracts of cerebral cortex from newborn guinea pig (Kuo et al., 1980). Whereas Ca^{2+} -dependent protein kinase activities are very low in homogenates of fetal brain, our results demonstrate that they are highly concentrated in subfractions of a low speed supernatant.

Calcium/calmodulin-dependent protein kinases and their substrates in GCPs. Several distinct Ca^{2+} /CM-dependent protein kinase activities are now known to occur in the adult mammalian brain (for review, see Schulman, 1982; Kennedy, 1983). The present results demonstrate that various Ca^{2+} /CM-dependent kinase activities are also present in fetal brain, and that the major substrates for these kinases are enriched in the GCP. Stimulation of kinase activity with exogenous CM, relative to Ca^{2+} alone, is only moderate in whole GCP homogenates but is more pronounced in a crude membrane

Figure 6. Two-dimensional IEF analysis of Ca²⁺/CM-stimulated GCP and SYN phosphoproteins. GCP and SYN proteins were phosphorylated under standard conditions for Ca²⁺/CM phosphorylation in the presence of 0.7 mM CaCl₂ (*top*) or 0.7 mM CaCl₂/0.5 μg of CM (*bottom*) and analyzed on two-dimensional IEF gels followed by radioautography. GCP protein (11 μg) and SYN protein (22 μg) were loaded on the respective gels. Exposure times of the autoradiograms were selected so as to provide for optimal comparison of the patterns (the 46,000-dalton spots are about equal in all exposures shown).

Figure 7. V8 protease phosphopeptide analysis of GCP and SYN phosphoproteins. Phosphoproteins were excised from the two-dimensional gels shown in Figure 6 and subjected to proteolysis with *S. aureus* V8 protease. Lanes 1 and 3, acidic 46,000-dalton GCP phosphoprotein; different exposures of the same gel lane. Lane 2, Acidic 46,000-dalton SYN phosphoprotein. Lane 4, Acidic 80,000-dalton GCP phosphoprotein.

subfraction. Thus, the former result may be explained by the presence of significant levels of endogenous CM. Indeed, endogenous CM has been identified in pelleted GCPs, on the basis of its characteristic electrophoretic mobility, and in intact GCPs purified on a column of controlled-pore glass, by immunoblotting (C. Hyman and K. H. Pfenninger, manuscript in preparation). The Ca^{2+} /CM-dependent kinase activities are blocked by the CM-binding drug trifluoperazine (Weiss et al., 1980).

The major substrates for the Ca^{2+} /CM-dependent kinase activity in GCPs include proteins that migrate with apparent $M_r = 46,000$, 52,000, 56,000, 60,000 and 80,000 (basic species). ^{32}P incorporation into these substrates does not seem to be stimulated by Ca^{2+} /phospholipid, nor is it affected by cGMP or cAMP; an exception is the basic 80,000-dalton protein, synapsin I, whose phosphorylation is also cAMP-dependent. The identification of synapsin I is discussed in the preceding paper (Ellis et al., 1985). In GCPs, unlike SYNs, synapsin I is a relatively minor phosphoprotein component.

The phosphorylation of synapsin I suggests the presence in GCPs of its specific Ca^{2+} /CM-dependent kinase. In SYNs, this kinase is comprised of subunits of 50,000 and 60,000 (doublet) daltons, which have a basic isoelectric point and are autophosphorylated (Huttner and Greengard, 1979; Bennett et al., 1983; Kennedy et al., 1983b). In GCPs, especially in their membrane subfraction, we have observed Ca^{2+} /CM-dependent phosphoproteins of 49,000 and 60,000 daltons that may be the subunits of this kinase. However, these proteins are much less prominent than in SYNs.

Burke and DeLorenzo (1981, 1982) have described a Ca^{2+} /CM-dependent kinase in adult brain which is specific for tubulin. In GCPs, Ca^{2+} /CM stimulation of tubulin phosphorylation has been inconsistent. This may be due to the reported extreme lability of the tubulin kinase, the activity of which can only be shown under special conditions (Burke and DeLorenzo, 1981, 1982). However, in the particulate GCP subfraction, we have observed Ca^{2+} /CM-dependent phosphorylation of a polypeptide that co-migrates with tubulin.

Sometimes we observe a minor phosphorylated band or doublet at approximately 300,000 daltons (e.g., Fig. 1). This co-migrates with a cAMP-stimulated phosphoprotein that we have tentatively identified as microtubule-associated protein 2 (MAP 2) (Ellis et al., 1985). MAP 2 is known to be phosphorylated by both Ca^{2+} - and cAMP-dependent kinases (for review, see Nestler and Greengard, 1983).

The most prominent substrate of Ca^{2+} /CM-dependent kinase

activity of the GCP is an acidic ($\text{pI} = 4.2$) protein of 46,000 daltons, which is enriched in the particulate subfraction of GCPs. On the basis of its electrophoretic mobility in two-dimensional gels, the 46,000-dalton phosphoprotein is identical to one of the major polypeptides (visible after Coomassie blue staining) in salt-washed membranes prepared from GCPs (P. Simkowitz, L. Ellis and K. H. Pfenninger, manuscript in preparation). Based on two-dimensional gel analysis and phosphopeptide mapping, this membrane polypeptide, referred to henceforth as pp46, is also present in SYNs, but as a minor component. The identity of the pp46 kinase is unknown at present.

A number of minor phosphoproteins are present in GCPs but seem absent from synaptosomes (Fig. 8). The possibility that some of these represent GCP-specific and/or developmentally regulated phosphoproteins remains to be investigated.

Protein kinase C substrates in GCPs. A second type of Ca^{2+} -dependent protein kinase activity has been demonstrated in GCPs. This kinase is inhibited rather than stimulated by CM (cf. Albert et al., 1984) and operates at lower concentrations of Ca^{2+} than do the CM-activated kinases. In whole GCPs, additional kinase activity can be unmasked by the non-ionic detergent TX-100 (cf. Kikkawa et al., 1982); in cytosol subfractions of the GCP, protein phosphorylation can be stimulated with PS/D. This protein kinase activity is blocked (like its CM-dependent counterpart) by TFP (cf. Mori et al., 1980; Wrenn et al., 1980; Wise et al., 1982). These findings are consistent with the presence of a Ca^{2+} /phospholipid-dependent kinase activity, protein kinase C, or a very similar enzyme. As already pointed out, this kinase and/or its substrates are highly enriched in GCPs relative to the low-speed pellet and supernatant of fetal brain.

Two of the major Ca^{2+} -dependent phosphoproteins in the GCP are the 40,000- and 80,000-dalton substrates for Ca^{2+} /phospholipid-dependent kinase. As analyzed on two-dimensional gels, these proteins are very acidic, with isoelectric points of 4.3 and 4.0, respectively. On the basis of electrophoretic mobility, these proteins have identical counterparts in SYNs (cf. Fig. 6). Our 80,000-dalton substrate is likely to be identical to an 87,000-dalton acidic protein described by Wu et al. (1982). Both proteins are substrates for a Ca^{2+} /phospholipid-stimulated protein kinase and have similar phosphopeptides after V8 protease digestion. The 87,000-dalton protein is apparently located inside presynaptic terminals (Wu et al., 1982). One possibility for the identity of the acidic 80,000-dalton phosphoprotein is protein kinase C itself. This kinase has an $M_r = \sim 77,000$ to 82,000 and is autophosphorylated (Kikkawa et al., 1982). However, its reported isoelectric point of 5.6 (Kikkawa et al., 1982) is significantly more basic than that of our 80,000-dalton substrate ($\text{pI} = 4.0$). In addition, whereas protein kinase C activity has been demonstrated in a variety of tissues (Takai et al., 1979; Kuo et al., 1980; Kaibuchi et al., 1981), the SYN 87,000-dalton protein appears to be brain specific (Wu et al., 1982).

The second major substrate (40,000 daltons) of phospholipid-dependent kinase is very similar in its electrophoretic mobility (in two dimensions) to a SYN phosphoprotein (cf. Fig. 6). Wu et al. (1982) have described a SYN substrate (47,000 daltons according to their calibration) of which the phosphorylation is also Ca^{2+} /phospholipid-dependent and, like our 40,000-dalton GCP protein (data not shown), produces a major phosphopeptide at 11,000 daltons after V8 protease digestion. Zwiers et al. (1979, 1980; see also Sorenson et al., 1981; Aloyo et al., 1982; Kristjansson et al., 1982) have described a further, acidic and nerve-tissue-specific substrate (B50) of phospholipid-dependent kinase between 40,000 and 50,000 daltons.

Protein kinase activities of GCPs and SYNs compared. Initially, the identification of the GCP rested on ultrastructural criteria and on the co-purification with GCPs of growth cones dissected from primary cultures (Pfenninger et al., 1983). In the meantime, we have observed the enrichment in GCP membranes of a 180,000- to 250,000-dalton sialoglycoprotein that is selectively expressed during neurite growth (Wallis et al., 1983). The present study and the preceding paper (Ellis et al., 1985) identify in the GCP fraction

Figure 8. Two-dimensional NEPHGE analysis of Ca²⁺/CM-stimulated GCP and SYN phosphoproteins. GCPs were phosphorylated in the presence of 50 mM HEPES, pH 7.4, 10 mM MgCl₂, 0.2 mM EGTA, and 0.5 mM CaCl₂ but otherwise according to the standard protocol. SYNs were phosphorylated according to the standard procedure for Ca²⁺/CM-stimulated phosphorylation in the presence of 0.7 mM CaCl₂ and 0.5 μ g of CM. Phosphoproteins were analyzed on two-dimensional NEPHGE gels followed by radioautography. Proteins can be identified as follows: *arrowheads*, synapsin I; 1 and 5, tubulin; 2, 40,000-dalton protein of the GCP; 3 and 4, putative subunits of synapsin I kinase (49,000 and 60,000 daltons). The 46,000-dalton protein is represented as a small spot between 1 and 2. This protein often does not completely enter two-dimensional gels and, in these particular gels, is under-represented. Note that exogenous CM was added to the SYN but not the GCP reaction mixture. The exposure times of the two autoradiograms are not comparable but were selected to optimize visualization of synapsin I. This necessitated overexposure of the GCP autoradiogram.

additional biochemical markers, i.e., neuron- and nerve terminal-specific protein kinases and their substrates. As pointed out in the previous paper (Ellis et al., 1985), a problem inherent to biochemical studies on subcellular fractions is that one cannot know whether all elements of the preparation contribute equally to the measured parameters. Thus, the observation of neuronal markers in the GCP fraction per se does not prove the neuronal origin of all components of the GCP fraction; such evidence will have to be furnished by immunocytochemical analyses. Nevertheless, together with the ultrastructural characteristics of GCPs, the high degree of morphologic homogeneity of the GCP fraction, and the absence of synaptic structures from it (Pfenninger et al., 1983), the biochemical results suggest that most—if not all—GCPs are indeed nerve growth cone fragments.

The observed high degree of similarity (qualitatively) between GCP and SYN phosphoprotein patterns is entirely consistent with a precursor-product relationship between growth cones and presynaptic terminals. Several reports have documented the presence of neurotransmitters in, and their release from, nerve growth cones (e.g., Tennyson et al., 1972; Hume et al., 1983; Young and Poo, 1983). The presence in GCPs of synapsin I, a protein bound to synaptic vesicles in the mature nervous system (De Camilli et al., 1983; Huttner et al., 1983) may be related to these findings. It should be pointed out, however, that synaptic vesicles are rare organelles in GCPs (constituting ~2% of all GCP membranes; Pfenninger et al., 1983). Likewise, synapsin I phosphorylation is a minor kinase activity of the GCP fraction.

Although GCP and SYN phosphoprotein patterns are qualitatively similar, they are quantitatively very different. The quantitative comparison between the two fractions has to be done with caution because (i) SYNs include postsynaptic elements, which are absent from the GCP fraction, (ii) SYN fractions are not as homogeneous as the GCP preparation, and (iii) SYNs and GCPs are likely to be derived from somewhat different populations of neurons. With these qualifications in mind, one can nevertheless make the following observations: whereas synapsin I (a presynaptic component in the adult) is a predominant phosphoprotein of SYNs, it is a relatively minor substrate of GCPs.⁵ On the other hand, the Ca²⁺/CM-dependent pp46 is a very prominent kinase substrate in GCPs but not in SYNs. It is of particular interest that this latter phosphoprotein is a major membrane-associated protein of the GCP (P. Simkowitz, L. Ellis and K. H. Pfenninger, manuscript in preparation). In addition, the 40,000-dalton and, especially, the 80,000-dalton (acidic) substrates of Ca²⁺/phospholipid-dependent kinase are major phosphoproteins of GCPs, but they occur in SYNs as minor substrates based on total protein content or relative to synapsin I phosphorylation.

The pre-eminence of Ca²⁺/phospholipid-dependent kinase substrates in growth cones is of significance. This kinase is believed to

⁵ The subunits of synapsin I kinase also are major phosphoproteins in SYNs, but not in GCPs. However, recent reports suggest that some of this kinase is associated with the postsynaptic density (Kennedy et al., 1983a), which has no equivalent in the GCP.

be the phorbol ester receptor (Castagna et al., 1982; Kikkawa et al., 1983; Nidel et al., 1983), and phorbol esters are potent growth and tumor promoters. Phorbol ester receptors are more concentrated in brain than in any other tissue (Kuo et al., 1980; Nagle et al., 1981; Kikkawa et al., 1982). In the fetal rat, the highest concentrations of phorbol ester receptors have been correlated with regions of particularly active neuronal sprouting in the central nervous system (e.g., the marginal zone of the developing spinal cord at 15 days of gestation; Murphy et al., 1983). Although the role of protein kinase C and its substrates is not understood, it is tempting to speculate that they may be involved in regulatory mechanisms controlling neurite growth.

Our results demonstrate that several specific components of the mature synaptic terminal are already present in the fetal brain and GCP fraction, presumably in the sprouting axon, but in apparently quite different relative quantities. These observations raise a number of new questions: (i) Are these quantitative differences a reflection of developmental regulation of these kinases and/or their substrates? (ii) Are the protein kinase activities we find in GCP homogenates latent *in vivo* (i.e., present but not operational prior to target cell contact), or are they actually functional in the growth cone? (iii) If they are functional, do they subserve unique or common roles in growth cones and mature nerve terminals?

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