Nerve Growth Cones Isolated from Fetal Rat Brain

II. Cyclic Adenosine 3':5'-Monophosphate (cAMP)-binding Proteins and cAMP-dependent Protein Phosphorylation¹

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Abstract

Cyclic adenosine 3':5'-monophosphate (cAMP)-binding proteins and cAMP-dependent protein phosphorylation were examined in growth cone particles (GCPs) prepared from fetal rat brain. Several major proteins which specifically bind a photoactivatable analogue of cAMP are observed in GCPs and correspond to isoelectric variants of the regulatory subunits of the cAMP-dependent protein kinase described in adult brain. We found no evidence for differential compartmentalization of specific cAMP-binding proteins in subcellular fractions of fetal brain or within GCPs. cAMP-stimulated phosphoproteins of GCPs are similar to cAMP-dependent protein kinase substrates characterized in nerve terminals (synaptosomes) of adult brain and include the nerve terminal-specific protein, synapsin I. However, as shown in the companion paper (Katz, F., L. Ellis, and K. H. Pfenninger (1985) *J. Neurosci.* 5: 1402-1411), this synaptic phosphoprotein is not the major kinase substrate in the GCP fraction. The finding of synapsin I in a subcellular fraction prepared from fetal brain suggests that components of the mature nerve terminal are already present in fetal brain during neuronal sprouting and prior to synaptogenesis.

The biochemical analysis of the nerve growth cone is a prerequisite for the elucidation of the molecular mechanisms underlying neuronal differentiation. The growth cone plays a pivotal role in neuronal sprouting, neuron-target cell interaction, and synaptogenesis.

In a previous paper (Pfenninger et al., 1983) we have described a subcellular fraction from fetal rat brain that is highly enriched in pinched-off cell fragments that are morphologically indistinguishable from axonal nerve growth cones, so-called "growth cone particles" (GCPs). Additional evidence for the identity of GCPs includes: (i) GCPs co-purify in mixing experiments with identified growth cones from neurons sprouting *in vitro* (Pfenninger et al., 1983), and (ii) a monoclonal antibody (5B4) prepared against GCPs recognizes a neuronal antigen which is associated with growing axons in developing brain and is enriched in GCP membranes (Wallis et al., 1983). Thus, there are now both cytological and biochemical data consistent with the nerve growth cone origin of GCPs.

We are interested in analyzing the biochemical composition of the growth cone and in studying the developmental transition from nerve growth cone to mature nerve terminal. A substantial body of information concerning the biochemistry of the nerve terminal and, especially, of nerve terminal-specific proteins has been derived from studies of synaptic phosphoproteins (for review, see Nestler and Greengard, 1983). However, little is yet known on the presence or distribution of synapse-specific phosphoproteins in fetal brain. By the use of the GCP fraction, we attempt to answer the questions as to (i) whether there are any of the well characterized synaptic phosphoproteins present in fetal brain and possibly enriched in the GCP, and (ii) whether there are phosphoproteins that are unique to fetal brain and possibly the GCP.

In the present study we describe cAMP-dependent kinase activity and cAMP-binding proteins in GCPs. The cAMP-dependent protein kinase, which is believed to mediate all cAMP-dependent processes in mammalian cells (Kuo and Greengard, 1969), consists of two regulatory subunits which bind cAMP, associated with two identical catalytic subunits. Although the catalytic subunit appears to be of a single type (Beavo and Mumby, 1982), the regulatory subunits fall into two main classes, RI and RII, each of which contains several isoelectric variants. Thus, the characterization of cAMP-dependent kinases is facilitated by the analysis of their cAMP-binding, regulatory subunits. In the present paper, we describe first the analysis of cAMP-binding proteins of the GCP. Second is a study on cAMP-dependent kinase substrates and a comparison of these GCP substrates with those of the nerve terminal, i.e., the synaptosome. The companion paper (Katz et al., 1985) describes calcium-dependent protein phosphorylation in the GCP and extends the comparison with the synaptosome to include this class of kinases. A preliminary report of our findings has been presented (Ellis et al., 1983).

Materials and Methods

Materials. Ultrapure sucrose was from Schwarz-Mann. The following chemicals were from Sigma: Tris(hydroxymethyl)aminomethane (Tris as TRIZMA base), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), ethyleneglycol-bis(β -aminoethyl ether) *N,N'*-tetra-acetic acid

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(EGTA), adenosine 3':5'-cyclic monophosphate (cAMP), 8-bromoadenosine 3':5'-cyclic monophosphate (8-Br-cAMP), and guanosine 3':5'-cyclic monophosphate (cGMP). γ - ^{32}P -ATP was from Amersham. 8-Azido- ^{32}P -cAMP was from ICN Biochemicals. Protein standards for SDS-PAGE were from Pharmacia (14,400 to 94,000) and Bio-Rad (14,400 to 200,000). All other chemicals were reagent grade or better. Purified catalytic subunit of the cAMP-dependent protein kinase prepared from bovine muscle was the kind gift of Dr. Mark Eppler.

Preparation of GCPs. GCPs were prepared from 17-day-gestation rat brain as described in detail in a previous paper (Pfenninger et al., 1983). A low-speed supernatant was layered onto a discontinuous sucrose density gradient and spun to equilibrium ($248,000 \times g_{\text{max}}$ for 40 min, 4°C) in a VTi50 rotor (Beckman L8-55). Bands were removed from each step of the gradient: A, 0.32 M (load)/0.75 M; B, 0.75 M/1.0 M; and C, 1.0/2.7 M. The A-band was diluted in 0.32 M sucrose and pelleted by centrifugation ($39,000 \times g_{\text{max}}$ for 30 min, 4°C). This pellet (consisting of partially broken GCPs) was resuspended in 6 mM Tris-HCl (pH 8.1) by homogenization, first in a Teflon-glass, then in a glass-glass homogenizer, and was used directly in these experiments. Unless otherwise indicated, GCPs were used fresh, immediately after preparation.

Preparation of synaptosomes. Synaptosomes were prepared from adult rat cerebral cortex according to the method described by Cohen et al. (1977). The synaptosome fraction collected from the 1.0/1.2 M interface of the sucrose density gradient was diluted in 4 vol of 0.32 M sucrose (containing 1 mM NaHCO_3 , 1 mM MgCl_2 , and 0.5 mM CaCl_2) and pelleted at $27,200 \times g_{\text{av}}$ for 30 min. This pellet was resuspended and either homogenized as described for GCPs or subfractionated as explained below.

Analysis of cAMP-binding proteins. cAMP-binding proteins of the GCP were characterized using a photoaffinity analogue of cAMP, 8-azido- ^{32}P -cAMP, and a procedure modified after that described by Walter et al. (1978). The reaction mixture (50 μl final volume) contained 50 mM HEPES (pH 6.8), 10 mM MgCl_2 , 0.2% Triton X-100, 20 to 50 μg of GCP protein, and 31.25 to 500 nM 8-azido- ^{32}P -cAMP. The addition of Triton X-100 was necessary to make the binding proteins accessible to the reagents.

The reaction was preincubated for 30 min at 4°C . Controls included a 10-fold or greater molar excess of cAMP in the reaction at concentrations indicated in the figure legends. Photolysis was performed for 10 min. Reactions were terminated by the addition of: (i) 25 μl of twice concentrated sample buffer (Laemmli, 1970), followed by heating at 100°C for 3 min for SDS-PAGE, or (ii) 50 μl of 4% Nonidet P-40 (NP-40) and 10% β -mercaptoethanol, for two-dimensional gel analysis. Samples were stored at -20°C until used for electrophoresis.

For analysis of soluble versus membrane-bound cAMP-binding proteins, five reaction volumes were pooled, the reaction was stopped by rapid freezing in liquid nitrogen (without the addition of sample buffer), and membranes and lysate were prepared as follows. The thawed GCP homogenate was diluted to 4 ml with 6 mM Tris-HCl, pH 8.1, containing 0.5 mM EDTA, and stirred at 4°C for 45 min (cf. Cohen et al., 1977). The lysed material was loaded onto a 1.0-ml cushion of 1 M sucrose containing 0.5 mM EDTA (Castle and Palade, 1978) and spun at $200,000 \times g_{\text{max}}$ for 60 min at 4°C in an SW55Ti rotor (Beckman L8-55). Soluble proteins were precipitated from the supernatant by the addition of 50% ice-cold trichloroacetic acid (TCA) to a final concentration of 8.3% and collected by centrifugation ($11,200 \times g_{\text{max}}$ for 20 min, at 4°C). The resulting pellet was washed three times with ice-cold diethylether to remove TCA (Fujiki et al., 1982) and solubilized in the appropriate sample buffer for electrophoresis. The crude membrane fraction was collected at the Tris/1 M sucrose interface, and treated with 5 ml of 0.3 M Na_2SO_4 plus 20 $\mu\text{g}/\text{ml}$ of saponin for 30 min at 4°C , with stirring (Castle and Palade, 1978). The salt-washed membranes were collected as a pellet ($200,000 \times g_{\text{max}}$ for 60 min, at 4°C), washed twice with water, and solubilized for electrophoresis as described above.

cAMP-dependent phosphorylation. cAMP-dependent phosphorylation of GCP proteins was performed essentially as described by Lohmann et al. (1978), but with the following modifications. The reaction mixture, in a final volume of 50 μl , contained 50 mM HEPES (pH 7.4), 10 mM MgCl_2 , 1 mM EGTA, 5 μM γ - ^{32}P -ATP, with or without 5 μM cAMP. Samples were preincubated in the presence or absence of cAMP for 1 min at 30°C . This was followed by the addition of γ - ^{32}P -ATP and further incubation for 15 sec at 30°C . The reactions were terminated for one- or two-dimensional gel analysis as described above.

For the comparison of kinase activities associated with soluble versus particulate fractions, GCPs or synaptosomes (which had been 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 $200,000 \times g_{\text{max}}$ for 60 min at 4°C (SW55Ti rotor in Beckman L8-55). The

supernatant was used as lysate for the assays. The pellet was resuspended by homogenization (glass-glass) in double-distilled water and used as the particulate fraction (without wash at high ionic strength).

Electrophoresis of GCP proteins. SDS-PAGE was performed as described by Laemmli (1970). The resolving gels consisted of 5 to 15% acrylamide gradients ($140 \times 240 \times 0.75$ mm) with a 20-mm stacking gel (3%), prepared as described previously (Piccioni et al., 1982). Samples were run for a length of 200 mm in the resolving gel, as monitored by the bromophenol blue tracking dye. Two-thirds of the total reaction volume (50- μl reaction plus 25 μl of stop solution) was loaded for each sample. Molecular weights were calibrated using the following standard proteins: lysozyme (14,400), soybean trypsin inhibitor (20,000), carbonic anhydrase (30,000), ovalbumin (43,000), bovine serum albumin (67,000), phosphorase B (94,000), β -galactosidase (116,000), and myosin (200,000). Peptide maps were prepared as described by Cleveland et al. (1977).

Two-dimensional gels with isoelectric focusing (IEF) in the first dimension were run as described previously (O'Farrell, 1975), with the following modifications: (i) samples contained 20 to 50 μg of protein in a final volume of 100 μl ; (ii) gels were poured to a height of 130 mm in tubes of 160×2 mm; and (iii) for the analysis of acidic proteins, Ampholines of pH 5 to 7 (0.8%), pH 3.5 to 5 (0.8%), and pH 3.5 to 10 (0.4%) were used in the sample buffer and IEF gels. Non-equilibrium pH gradient gel electrophoresis (NEPHGE) was performed as described previously (O'Farrell et al., 1977), with modifications *i* and *ii* above. In addition, the sample buffer and first-dimension gels contained Ampholines of pH 3.5 to 10 (1.6%) and 3.5 to 5 (0.4%). Electrophoresis was done at constant voltage for 1600 volt-hours. All gels for the second dimension (SDS-PAGE) were 5 to 15% gradient gels prepared as described above.

Gels were stained with Coomassie blue (Fairbanks et al., 1971) and destained (Weber and Osborn, 1969). Autoradiography of the dried gels was performed using Kodak X-Omat film with a single intensifying screen (Dupont Quanta II or Quanta III) for the times and at the temperatures indicated in the legend of each figure.

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 directly by liquid scintillation spectrometry (Huttner and Greengard, 1979) using Omnifluor-toluene scintillation fluid (New England Nuclear).

Results

GCPs contain cAMP-binding proteins. The regulatory subunits of cAMP-dependent kinases can be labeled covalently with a photoaffinity analogue of cAMP, 8-azido- ^{32}P -cAMP (Walter et al., 1977a, 1978). When GCP proteins are labeled in this manner and analyzed by one-dimensional SDS-PAGE and autoradiography, prominent radioactive bands of 53,000, 52,000, and 48,000 daltons are observed (Fig. 1, a to c). The same set of labeled bands is also observed in the homogenate and low-speed supernatant of 17-day-gestation rat brain, from which the GCP fraction is prepared by density gradient centrifugation (Fig. 1d). Within the range of GCP protein concentrations used in these experiments (25 to 50 $\mu\text{g}/50$ - μl reaction), the incorporation of 8-azido- ^{32}P -cAMP is dose dependent at reagent concentrations of 31.25 to 500 nM (Fig. 1b). Apparent half-maximal labeling of GCP proteins is observed between 62.5 and 125 nM. At a concentration of 62.5 nM reagent, binding is linear over a protein concentration range of 10 to 50 $\mu\text{g}/50$ μl (Fig. 1c; determined by liquid scintillation spectrometry of excised bands).

To assess the specificity of labeling by the azido reagent, competing concentrations of cold cAMP were included in the reaction. At a 10- to 20-fold molar excess of cold cAMP, competition is usually complete (Fig. 1a). However, even a large molar excess of cold cAMP sometimes fails to compete with the labeling of the 52,000-dalton band of GCPs (Fig. 1, d and e).

To answer the question of whether cAMP-binding proteins are associated selectively with a membrane compartment, we reacted pelleted and resuspended GCPs with the azido reagent, lysed them completely in hypotonic buffer at high pH, and prepared a salt-washed membrane pellet. As illustrated in Figure 1e (lane 3), this membrane preparation contains two labeled bands of 53,000 and 52,000 daltons and, just barely visible, a band of 48,000. Their

Figure 1. One-dimensional SDS-PAGE of cAMP-binding proteins. Autoradiograms were exposed at -80°C for the times indicated. *a*, cAMP-binding proteins of the GCP. Each sample contained $30\ \mu\text{g}$ of GCP protein plus the amount of 8-azido- ^{32}P -cAMP and unlabeled cAMP indicated for each lane. Exposure, 17 hr. Lane 1, 500 nM 8-azido- ^{32}P -cAMP plus $10\ \mu\text{M}$ cAMP; lane 2, 500 nM 8-azido- ^{32}P -cAMP plus $5\ \mu\text{M}$ cAMP; lane 3, 500 nM 8-azido- ^{32}P -cAMP (no cAMP); lane 4, 62.5 nM 8-azido- ^{32}P -cAMP (no cAMP); lane 5, 62.5 nM 8-azido- ^{32}P -cAMP plus $1\ \mu\text{M}$ cAMP; lane 6, 62.5 nM 8-azido- ^{32}P -cAMP plus $10\ \mu\text{M}$ cAMP. *b*, Incorporation of radioactivity as a function of 8-azido- ^{32}P -cAMP concentration. Each sample contained $30\ \mu\text{g}$ of GCP protein. Exposure, 7.5 hr. Lanes 1 to 5 contained, respectively, the following concentrations of 8-azido- ^{32}P -cAMP (in nanomolar concentration): 31.25, 62.5, 125, 250, and 500. *c*, Incorporation of 8-azido- ^{32}P -cAMP as a function of GCP protein concentration. Each sample contained 62.5 nM 8-azido- ^{32}P -cAMP. Exposure, 3.5 days. Lanes 1 to 5 contained 10, 20, 30, 40, and $50\ \mu\text{g}$ of GCP protein, respectively. *d*, Incorporation of 8-azido- ^{32}P -cAMP into subcellular fractions of 17-day-gestation rat brain. Each sample contained $30\ \mu\text{g}$ of protein and 62.5 nM 8-azido- ^{32}P -cAMP. Exposure, 7.5 hr. Lane 1, homogenate; lane 2, homogenate plus $40\ \mu\text{M}$ unlabeled cAMP; lane 3, low-speed supernatant; lane 4, low-speed supernatant plus $40\ \mu\text{M}$ unlabeled cAMP; lane 5, GCP; lane 6, GCP plus $40\ \mu\text{M}$ unlabeled cAMP. *e*, Incorporation of 8-azido- ^{32}P -cAMP into GCP or synaptosomal membrane proteins. Exposure, 17 hr. Lane 1, $30\ \mu\text{g}$ of GCP protein plus 62.5 nM 8-azido- ^{32}P -cAMP and $40\ \mu\text{M}$ cAMP; lane 2, $30\ \mu\text{g}$ of GCP protein plus 62.5 nM cAMP 8-azido- ^{32}P -cAMP; lane 3, membranes prepared from $50\ \mu\text{g}$ of lysed GCPs, plus 400 nM 8-azido- ^{32}P -cAMP; lane 4, membranes prepared from $50\ \mu\text{g}$ of lysed synaptosomes plus 400 nM 8-azido- ^{32}P -cAMP.

Figure 2. Two-dimensional gels (IEF, SDS-PAGE) of cAMP-binding proteins of the subcellular fractions of fetal rat brain. Samples *a* to *c* contained approximately $25\ \mu\text{g}$ of protein. The lysate sample (*d*) contained 25% of the total sample protein, whereas the membrane preparation (*e*) contained 100%. The spots shown are the only labeled proteins visible on the autoradiographs. Films were exposed at -80°C for the times indicated. *a*, Homogenate, 17-day-gestation rat brain (exposure, 3 days); *b*, low-speed supernatant (exposure, 3 days); *c*, GCP (exposure 8.5 hr); *d*, lysate of GCP (exposure 16 hr); *e*, membranes of GCP (exposure 16 hr).

Figure 3. cAMP-dependent phosphorylation of GCP proteins. Each lane contained 18 μ g of GCP protein, 50 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 0.2 mM EGTA, and 5 μ M γ - ^{32}P -ATP, plus the additions indicated for each lane. *Lane 1:* Coomassie blue-stained lane from the gel of lanes 2 to 7. The major bands visible at $M_r = 52,000$ and 42,000 are tubulin and actin, respectively. *Lane 2:* No additions. *Lane 3:* cGMP (5 μ M). *Lane 4:* 8-Br-cAMP (5 μ M). *Lane 5:* cAMP (5 μ M). *Lane 6:* cAMP (5 μ M) plus approximately 1 μ g of purified catalytic subunit of cAMP-dependent protein kinase. *Lane 7:* EGTA (1 mM) plus 1.3 mM $CaCl_2$. *b, Lane 1:* No additions. *Lane 2:* cAMP (5 μ M) plus approximately 1 μ g of purified catalytic subunit of cAMP-dependent protein kinase (as lanes 2 and 6 of a). *Lane 3:* 8-azido- ^{32}P -cAMP. Exposures: a, lanes 2 to 7, and b, lanes 1 and 2, 2 hr at room temperature; b, lane 3, 16 hr at $-80^\circ C$ with intensifying screen. a and b are from separate gels.

relative quantities are altered compared to intact GCPs; the 52,000-dalton band is now predominant (cf. below).

In comparison to the GCP labeling pattern, crude membranes of synaptosomes (Gray and Whittaker, 1962), prepared from adult rat brain, also contain bands of 53,000, 52,000, and 48,000 daltons (Fig. 1e, lane 4). An additional band of 40,000 daltons is visible in the synaptosomal membrane preparation only. This additional band is observed in the GCP preparation only after much longer exposures of the autoradiograms. It has been described in other studies of mammalian cAMP-binding proteins as a proteolysis product of the other, higher molecular weight species (e.g., Rangel-Aldao et al., 1979).

Analysis of labeled cAMP-binding proteins by two-dimensional gel electrophoresis (IEF and SDS-PAGE) reveals a qualitatively identical set of isoelectric variants of the cAMP-binding proteins in the homogenate (Fig. 2a), the low-speed supernatant (Fig. 2b), and the GCP (Fig. 2c). The upper 53,000- and 52,000-dalton bands visible on one-dimensional gels have isoelectric points on these two-dimensional gels of approximately 5.4 and 5.3, respectively. The lower 48,000-dalton band is resolved into three species with isoelectric points of 5.7 to 5.6. Based on spot size and density, the quantity of the more basic 48,000-dalton species, relative to that of the 52,000-dalton species, is reduced in GCPs compared to low-speed supernatant or homogenate.

Upon hypotonic lysis of the GCP and salt wash, the majority of the cAMP-binding proteins are released into the lysate. (Because of the inevitable use of 0.2% Triton X-100 during the binding reaction with the azido reagent, we cannot exclude the possibility that some membrane-associated proteins appear in the lysate under these conditions.) On two-dimensional gels, a set of cAMP-binding proteins identical to those of the unlysed GCP is present in the soluble compartment (Fig. 2d; cf. Fig. 2c). The GCP membrane preparation,

after permeabilization and washing with high salt, retains traces of the same set of cAMP-binding proteins (Fig. 2e). The most prominent species remaining is the 52,000-dalton protein (assessed by superimposition of autoradiograms), as was observed on the one-dimensional gels.

GCPs contain substrates for a cAMP-dependent protein kinase. GCPs were incubated with 5 μ M γ - ^{32}P -ATP and various co-factors to assess cAMP-dependent protein kinase activity. The phosphoproteins were analyzed by SDS-PAGE and autoradiography (Fig. 3a). The reagents 8-Br-cAMP (Fig. 3a, lane 4) and cAMP (Fig. 3a, lane 5) stimulate the phosphorylation of major bands of approximately 52,000, 53,000, 56,000, 77,000, 80,000, and 300,000 daltons. Several substrates in the molecular weight range of 100,000 to 250,000 are also phosphorylated. Experiments which include the phosphodiesterase inhibitor isomethylbutylxanthine give identical results (data not shown). The observed phosphorylation is enhanced by the inclusion of the exogenous catalytic subunit of the cAMP-dependent protein kinase (Fig. 3a, cf. lanes 5 and 6). Under these conditions, the enhancement may not be equal for all polypeptide bands, and we observe additional bands of 42,000 and 43,000 daltons. The prominent 53,000-dalton phosphoprotein co-migrates with the 53,000-dalton cAMP-binding protein labeled with 8-azido- ^{32}P -cAMP (Fig. 3b, cf. lanes 2 and 3). In contrast to cAMP, cGMP does not stimulate protein kinase activity in GCPs.

The cAMP-stimulated phosphorylation of proteins (Fig. 3a, lanes 5 and 6) is distinct from that dependent upon calcium (Fig. 3a, lane 7). However, one or more proteins of 80,000 daltons are substrates for kinases of both classes. The analysis of substrates of calcium-dependent protein kinases is the subject of the following paper (Katz et al., 1985).

When cAMP-dependent phosphoproteins are analyzed in two dimensions using NEPHGE in the first dimension, phosphoproteins

Figure 4. Two-dimensional gel analysis of GCP phosphoproteins. *a*, NEPHGE of cAMP-dependent phosphoproteins. A very basic protein with $M_r = 80,000$ is indicated by the arrow. Exposure, 6 hr at -80°C . *b*, IEF of cAMP-dependent phosphoproteins. Exposure, 20 hr at 80°C .

of approximately 45,000, 53,000, and 80,000 daltons are seen (Fig. 4*a*). One of the major 80,000-dalton proteins appears as a streak under these conditions. A second major protein at 80,000 daltons is very basic and appears as a doublet (Fig. 4*a*, arrow). When these two basic spots are excised and analyzed by peptide mapping using *Staphylococcus aureus* V8 protease, both are found to contain a phosphopeptide of 10,000 daltons (Fig. 5). This pair of basic 80,000-dalton proteins is also phosphorylated by a calcium-dependent protein kinase (see Katz et al., 1985).

When cAMP-stimulated phosphoproteins of the GCP are analyzed on two-dimensional gels using acidic IEF in the first dimension, a pair of proteins with an isoelectric point of approximately 4.3 is evident at about 80,000 daltons (Fig. 4*b*). However, the major phosphoprotein observed in this size range is a very acidic protein, with an isoelectric point of approximately 4.0. One-dimensional peptide map analysis (*S. aureus* V8 protease) of this 80,000-dalton species yields phosphopeptides of 13,000 and 9,000 daltons (Fig. 5). Other cAMP-dependent substrates visible on these IEF gels include proteins at 18,000, 45,000, and 53,000 daltons, with isoelectric points of approximately 4.6, 5.1, and 5.3, respectively.

cAMP-dependent phosphoproteins are enriched in subcellular fractions of fetal brain. Important aspects of this study are the relative distribution and the possible enrichment of cAMP-dependent phosphoproteins and their kinases during subcellular fractionation of fetal rat brain and, in particular, the question as to whether any of these substrates or kinases are unique to GCPs. Therefore, we analyzed aliquots from each fraction generated during the preparation of GCPs for cAMP-dependent protein phosphorylation (Fig. 6; cf. "Materials and Methods"). At the protein concentration employed (50 $\mu\text{g}/50\text{-}\mu\text{l}$ reaction), the homogenate (Fig. 6, *H*), low-speed pellet (Fig. 6, *P*), and high-speed supernatant (Fig. 6, *O*; soluble protein of the load of the discontinuous gradient) contain very little radioactivity. The low-speed supernatant (Fig. 6, *S*) exhibits bands at 52,000, 53,000, and 80,000 daltons. All subfractions of the low-speed

supernatant, except for the soluble component of GCPs (Fig. 6, *L*; proteins released during GCP lysis), show a further enrichment of substrates, especially around 53,000 and 80,000 daltons, and at considerably higher molecular sizes up to 300,000 daltons. As one goes from the A-fraction (which contains the GCPs and a large amount of soluble proteins from the load; Pfenninger et al., 1983) to the pelleted GCPs (Fig. 6, *G*) and to a crude GCP membrane subfraction (Fig. 6, *M*), one observes, in general, an increase in intensity of the various polypeptide bands (except for the 80,000-dalton and some other, less prominent species). Also noteworthy are the enrichment of the upper band of the 300,000-dalton doublet and of the 56,000-dalton band in the particulate subfraction of GCPs (Fig. 6, *M*), and the apparent absence of phosphoproteins in the GCP's soluble component (Fig. 6, *L*). However, the evaluation of these results has to take into account the possibility that subfractionation may lead to the separation of kinases and/or co-factors from substrates. Therefore, the absence of phosphoproteins from a particular fraction may have several explanations. The heavier, very heterogeneous subfractions of the low-speed supernatant (Fig. 6, *B* and *C*; see "Materials and Methods" and Pfenninger et al., 1983) contain small glial profiles, subcellular organelles, and (especially the C-fraction) lysed GCPs. They have several bands in common with GCPs. This similarity is especially striking for the B-fraction, whereas the C-fraction exhibits a more distinct overall pattern.

Discussion

Regulatory subunits of cAMP-dependent protein kinase

The photoactivatable analogue of cAMP, 8-azido- ^{32}P -cAMP, has been used to label covalently cAMP-binding proteins in a variety of tissues including brain (for review, see Walter and Greengard, 1982). Several lines of evidence, including behavior in electrophoresis and ion exchange chromatography, have demonstrated that the major bands labeled with this reagent, at M_r of approximately 48,000 and

Figure 5. One-dimensional peptide maps of the 80,000-dalton acidic and basic proteins. Radioactive spots were located on the autoradiograms in Figure 4, excised from the dried gels, and digested with *S. aureus* V8 protease according to the procedure of Cleveland et al. (1977). Lane 1, The 80,000-dalton acidic protein (cf. Fig. 4b, pl 4.0); lane 2, the lower member of the 80,000-dalton basic doublet (arrow in Fig. 4a); lane 3, the upper member of the 80,000-dalton basic doublet (cf. Fig. 4a, arrow).

55,000, correspond to the regulatory subunits of type I and type II cAMP-dependent protein kinase, respectively (Uno et al., 1977b; Walter et al., 1977a, 1978; Rangel-Aldao et al., 1979).

In the present study, we have found a number of GCP proteins that can be labeled with 8-azido- ^{32}P -cAMP. On the basis of their molecular weights ($M_r = 48,000$ and $53,000$) and isoelectric points ($\text{pI} = 5.6/5.7$ and $5.3/5.4$), these proteins behave identically to the regulatory subunits of type I (RI) and type II (RII) cAMP-dependent protein kinases, respectively. A protein which co-migrates with the 53,000-dalton cAMP-binding protein is phosphorylated in a cAMP-dependent manner. This is also consistent with the identification of this protein as RII because autophosphorylation in the presence of cAMP is characteristic of RII (Erllichman et al., 1974; Hoffman et al., 1975; Walter et al., 1977b; Rangel-Aldao et al., 1979).

A third cAMP-binding protein of the GCP migrates at 52,000 daltons and has an isoelectric point of 5.3. Rosen and collaborators (Rangel-Aldao et al., 1979) have demonstrated that the phospho- and dephospho-forms of RII can be resolved into bands of slightly different molecular weights by SDS-PAGE in Tris-glycine buffer systems, as we employed them in the present study. It is likely, therefore, that the 52,000-dalton GCP protein is the nonphosphorylated form of RII. We have often observed that the labeling of this protein with 8-azido- ^{32}P -cAMP cannot be inhibited completely by an excess of cold cAMP. Interestingly, the affinity for cAMP is greater for the phosphorylated than for the dephosphorylated form of RII prepared from bovine heart (see Hoffmann et al., 1975). A difference in cAMP-binding properties may explain the incomplete competition observed for the 52,000-dalton GCP protein.

Diversity of isoelectric variants of RI and RII suggests the possibility that kinase function could be controlled by the differential subcellular compartmentalization of distinct regulatory subunits. Previous studies have demonstrated that membrane-bound and cytosolic cAMP-dependent protein kinases in brain (Walter et al., 1978; Rubin et al.,

Figure 6. cAMP-dependent protein phosphorylation in various subcellular fractions of fetal rat brain (cf. "Materials and Methods" and Pfenninger et al., 1983). Each lane contained $17 \mu\text{g}$ of protein (samples were once frozen and thawed prior to assay). Phosphorylation was carried out according to the standard protocol, with the following additions: $5 \mu\text{M}$ cAMP, approximately $1 \mu\text{g}$ of purified catalytic subunit, and 0.1% Triton X-100. H, homogenate; P, low-speed pellet; S, low-speed supernatant; O, high-speed supernatant (soluble fraction of the low-speed supernatant, i.e., of the load of the discontinuous gradient); G, pelleted GCPs; A, A-fraction (GCPs plus soluble proteins of the load); M and L, crude membranes and lysate of GCPs, respectively; B and C, B- and C-fractions (very heterogeneous, dense subfractions of S, collected from the discontinuous gradient).

1979) and heart (Corbin et al., 1977) are apparently identical with respect to several criteria (however, see Uno et al., 1976, 1977a). In contrast, in the nervous system of the marine mollusc *Aplysia californica*, membrane and cytosolic compartments have distinct sets of cAMP-binding proteins (Eppler et al., 1982; however, the relationship of these proteins to the regulatory subunits of the cAMP-dependent protein kinase is unclear in this case). In fetal brain, we do not observe such compartmentalization. When GCPs are labeled with the azido reagent and subsequently lysed, the majority of the labeled proteins are soluble, but a small amount of the same subset of proteins remains membrane associated. Similarly, during subcellular fractionation of fetal brain, the pattern of isoelectric variants of the cAMP-binding proteins remains qualitatively constant, despite the fact that the GCP fraction contains a highly specific subset of particles compared to homogenate or low-speed supernatant. This observation is comparable to the results obtained for various subcellular fractions from adult mouse brain (Panter et al., 1981). Quantitatively, on the other hand, there appears to be a marked enrichment in GCPs of RII relative to RI, and of the 52,000-dalton species relative to the 53,000-dalton protein (see Fig. 2). Whether this has functional significance is unknown.

The pattern of cAMP-binding proteins of GCPs is similar (by SDS-PAGE) to that of synaptosomal membranes (cf. Walter et al., 1979) and to the two-dimensional IEF pattern of whole adult rat brain described by Panter et al. (1981). In the adult rat there is a set of brain-specific, basic variants of RII in addition to those shown in Figure 2. We have observed similar variants in our preparation, but they are minor species present in variable amounts and visible only after longer exposure of the autoradiograms (data not shown). Although quantitative differences may exist,⁵ we find no evidence of a qualitative developmental regulation of cAMP-binding proteins in rat brain.

The interpretation that the cAMP-binding proteins of GCPs are regulatory subunits of the cAMP-dependent protein kinase is further supported by the demonstration of endogenous cAMP-stimulated kinase activity in the GCP fraction. Only one catalytic subunit of cAMP-dependent protein kinase has been observed in adult brain, and the same kinase appears to be highly conserved in diverse tissues and phyla (Kuo and Greengard, 1969; Weber et al., 1981). The kinase activity we observe in fetal brain is likely to be identical to the adult kinase activity because (i) it has the same regulatory subunits, and (ii) the addition of exogenous catalytic subunit (from adult bovine muscle) results in the quantitative enhancement of ³²P incorporation, without changing the pattern of phosphoproteins. However, we cannot rule out the possibility of a unique fetal catalytic subunit that shares regulatory subunits with the adult variant, or of a fetal kinase whose cAMP-binding site is not recognized by the azido reagent used in our assay.

Substrates of cAMP-dependent protein kinase in GCPs

The addition of exogenous cAMP to the growth cone preparation stimulates the phosphorylation of a series of major proteins of 300,000, 80,000, 77,000, 56,000, 53,000, and 52,000 daltons. At least some of them have synaptic counterparts, and they can be characterized as follows.

Phosphoproteins of 77,000, 56,000, 53,000, and 52,000 daltons.

The electrophoretic behavior of these GCP substrates is reminiscent of four phosphoproteins described by Nestler and Greengard (1983) in the adult nervous system. By SDS-PAGE we observe a prominent phosphorylated band of approximately 77,000 daltons (Fig. 3). In the same molecular weight range there are two prominent spots on IEF gels that are more basic than the acidic 80,000-dalton protein and two streaks on NEPHGE gels that are more acidic than a very basic 80,000-dalton phosphoprotein (synapsin I, see below). One of these streaks or spots may thus correspond to the synaptic protein IIIa, a cAMP-dependent kinase substrate which forms multiple spots of pI 6.8 to 7.1 in the same molecular weight range (Browning et al., 1982). Our 56 kd phosphoprotein may also correspond to a synaptic protein. This protein, IIIb, forms a charge train of pI = 6.6 to 7.3, at 55,000 daltons, according to Huang et al. (1982); on NEPHGE gels, our 56,000-dalton species migrates in a corresponding region (Fig. 4a). The similarities between the GCP 77,000- and 56,000-dalton substrates and phosphoproteins IIIa and IIIb, respectively, are of interest because the latter proteins are believed to be neuron specific; their phosphorylation is stimulated not only by cAMP but also by membrane depolarization and by certain biogenic amines (Forn and Greengard, 1978; Huang et al., 1982; Tsou and Greengard, 1982). Like our 56,000-dalton substrate, protein IIIb is preferentially found in particulate fractions (Walaas et al., 1983).

The 53,000-dalton substrate has already been described and identified as RII. The 52,000-dalton phosphoprotein is one of the

major substrates of the cAMP-dependent protein kinase in fetal brain, as it is one of the few phosphoproteins detectable in low-speed supernatant and homogenate. The 52,000-dalton species is comparable to a substrate observed in synaptic membranes of developing brain (2 days postnatal; Lohmann et al., 1978) and referred to as IIb by Greengard and collaborators (Sieghart et al., 1978; Walter et al., 1979). Interestingly, the level of protein IIb seems to decline during postnatal development of rat brain from day 2 to day 20 (Lohmann et al., 1978).

Phosphoproteins of 300,000 daltons. Prominent substrates of the cAMP-dependent protein kinase in GCPs include a pair of proteins of approximately 300,000 daltons. As described in the following paper (Katz et al., 1985), the 300,000-dalton proteins of the GCP are also substrates for calcium-dependent protein kinases. Several authors have described the cAMP- and calcium-dependent phosphorylation of the high-molecular-weight microtubule-associated protein, MAP 2, in both soluble preparations from adult brain microtubules (Sloboda et al., 1975; Vallee, 1980) and particulate preparations from adult brain (Lohmann et al., 1980; Walaas et al., 1983). In adult rat brain, MAP 2 is localized exclusively in neurons and, in particular, in dendrites (Matus et al., 1981). However, recent studies using monoclonal antibodies to MAP 2 have suggested that MAP 2 also occurs in areas of axonal sprouting in developing brain (Frankfurter et al., 1983). Thus, the 300,000-dalton substrate in GCPs is likely to be identical to MAP 2. Interestingly, a major fraction of soluble RII in brain (here shown to be present in GCPs) is associated with MAP 2 (Vallee et al., 1981; Miller et al., 1982; Theurkauf and Vallee, 1982).

Phosphoproteins of 80,000 daltons. As seen in two-dimensional (NEPHGE) gels, there are several major substrates of cAMP-dependent protein kinase near 80,000 daltons. (a) Some of these phosphoproteins have already been discussed (77,000 daltons, above) and compared with protein IIIa. (b) A pair of very basic proteins of about 80,000 daltons can be cleaved by V8 protease to generate a phosphopeptide of 10,000 daltons. As described in the following paper (Katz et al., 1985), this basic protein doublet is also phosphorylated in a calcium/calmodulin-dependent manner. These molecular properties—electrophoretic behavior, kinase dependence, and phosphopeptide content—indicate that our basic 80,000-dalton substrate of GCPs is closely related to or identical with synapsin I. Furthermore, this doublet is recognized by an anti-synapsin I antibody kindly provided by Dr. Greengard's laboratory (K. H. Pfenninger, C. Ouimet and P. Greengard, work in progress). Synapsin I is a highly specific neuronal marker protein concentrated in the adult brain in nerve terminals and associated with synaptic vesicles (Ueda and Greengard, 1977; Huttner and Greengard, 1979; Huttner et al., 1981; De Camilli et al., 1983). (c) An additional 80,000-dalton phosphoprotein found in our studies has a very acidic isoelectric point (4.0) and, upon V8 protease cleavage, resolves into phosphopeptides of 13,000 and 9,000 daltons. Based on these characteristics, this substrate is identical with a substrate of calcium/phospholipid-dependent kinase described in GCPs by Katz et al. (1985) and present in synaptosomes of adult brain (Wu et al., 1982). However, Wu et al. (1982) did not observe cAMP-dependent phosphorylation of the synaptic acidic 80,000-dalton protein. It may be significant that the component phosphopeptides of the GCP substrate observed in our cAMP studies are identical to those seen after calcium/phospholipid stimulation (Katz et al., 1985). Therefore, we have to consider the possibility that the apparent cAMP stimulation observed in this study could be the result of an indirect effect of cAMP or of GCP properties not shared by synaptosomes.

Similarities of GCPs and synaptosomes

Various phosphoproteins and/or their kinase(s) are highly enriched in particulate subfractions of the low-speed supernatant of fetal brain, including GCPs. The comparison of fetal cAMP-dependent protein kinase substrates with well characterized phosphoproteins of adult brain suggests that a number of such adult brain proteins

⁵ Such quantitative comparisons are particularly difficult given (i) the differences in the degree of homogeneity of the fractions and (ii) the report by Malkinson and Butley (1981) that 8-azido-³²P-cAMP-binding sites on RII are developmentally regulated in certain tissues, and that reagent binding does not necessarily reflect the actual amounts of RII present. Phosphorylation sites also appear to be regulated during development.

have counterparts in the GCP fraction. Most prominent among them is synapsin I, a nerve terminal marker. The nature of calcium-dependent kinase substrates in GCPs described by Katz et al. (1985) further supports the conclusion that, qualitatively, the phosphoprotein patterns of GCP and synaptosome fractions are very closely related. With any biochemical analysis of a subcellular fraction, one can never be entirely certain that all components of the fraction contribute equally to the measured parameter. Nevertheless, the significance of our conclusion is at least 2-fold. (i) The enrichment of nerve terminal markers in the GCP fraction, a morphologically highly homogeneous preparation free of synaptosomes (Pfenninger et al., 1983), supports our contention that GCPs are indeed of neuronal origin. Given their ultrastructural characteristics, GCPs are thus most likely to be derived from nerve growth cones. If our contention is correct, then (ii) nerve growth cones contain some of the known regulatory machinery present in nerve terminals. The possible functional role of this machinery in growth cones is discussed in the following paper (Katz et al., 1985).

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