Ribosomal Protein Phosphorylation in Rat Cerebral Cortex in Vitro

INFLUENCE OF CYCLIC ADENOSINE 3':5'-MONOPHOSPHATE*

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Earlier investigations of ribosomal protein phosphorylation in rat cerebral cortex in vitro have been extended to include an analysis of the effects of adenosine 3':5'-monophosphate (cyclic AMP) on this process. Cerebral cortical slices from 14-day-old rats were incubated for 2 h with $^{32}$P-orthophosphate, washed free of extracellular radioactivity, then incubated again in the presence of N$^{6}$O$^{2}$-dibutyryl adenosine 3':5'-monophosphate (dibutyryl cyclic AMP) or related substances. Addition of 1 mM dibutyryl cyclic AMP consistently stimulated phosphorylation of ribosomal proteins in the cerebral 40 S subunit, whereas overall phosphorylation of proteins in the 60 S subunit was not increased by the cyclic nucleotide. The minimum dose of dibutyryl cyclic AMP which was effective in stimulating phosphorylation of 40 S ribosomal proteins was about 0.1 mM. Stimulation was also produced with monobutyryl and other dibutyryl derivatives of cyclic AMP, as well as the phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine. N$^{6}$O$^{2}$-Dibutyryl guanosine 3':5'-monophosphate and sodium butyrate (1 mM) were without effect on ribosomal protein phosphorylation. Following electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate, the protein or proteins of the 40 S subunit which exhibited enhanced phosphorylation in the presence of dibutyryl cyclic AMP appeared to be located in a single radioactive band with a mobility which corresponded to a molecular weight of approximately 32,000. Two-dimensional electrophoresis of the ribosomal proteins on polyacrylamide gels containing urea revealed that the small subunit contained three ribosomal proteins which were phosphorylated in cerebral cortical tissue in vitro. The most highly labeled protein (S6) had a molecular weight of approximately 32,000 and consisted of at least five distinct species in different states of phosphorylation. Under basal conditions, the bulk of the S6 protein existed in the nonphosphorylated state.

Incubation of cerebral cortical tissue with 1 mM dibutyryl cyclic AMP increased the proportion of phosphorylated congeners of the S6 protein as indicated by their relative absorbances at 600 nm compared to the nonphosphorylated species. The radioactivity associated with each of these congeners was also increased. The other two phosphorylated proteins of the 40 S subunit had electrophoretic properties which were similar to those of ribosomal proteins S2 and S3. Phosphorylation of these proteins was considerably less than that of the S6 protein. The cerebral 60 S subunit contained several proteins which were phosphorylated in vitro. Three of these were basic proteins with electrophoretic mobilities similar to those of ribosomal proteins L6, L13, and L14. At least three other proteins associated with the 60 S subunit which were less basic were also phosphorylated. The latter proteins have not been identified as corresponding electrophoretically to constitutive ribosomal proteins. These investigations indicate that although ribosomal protein phosphorylation in cellular preparations of rat cerebral cortex in vitro involves proteins of both subunits, stimulation of this process by cyclic AMP is largely attributable to a single ribosomal protein of the cerebral 40 S subunit. The possible relationship of these findings to alterations in brain protein synthesis accompanying the transmission of neural impulses is discussed.

Considerable evidence supports the concept that adenosine 3':5'-monophosphate (cyclic AMP) plays an important role in neurotransmission (1-7). Several neurotransmitter amines increase the accumulation of this cyclic nucleotide in nervous tissue (8) by activating adenylyl cyclase systems (9-11). Catecholamine-sensitive adenylyl cyclase activity is partly localized in synaptic membrane fractions of rat cerebral cortex (12) and corpus striatum (13). Moreover, topical application of cyclic AMP or dibutyryl cyclic AMP to postsynaptic neurons normally responsive to catecholamines can mimic the inhibitory effects of these amines on neuronal firing (14-16). Certain of the synaptic actions of the catecholamines may be mediated by cyclic AMP-regulated protein kinases which catalyze the phosphorylation of synaptic membrane proteins (17-19). However, the cyclic nucleotides have additional actions in neural tissue which are related to neurotransmission.
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Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis (20), appears to be both activated (21) and induced (22) by cyclic AMP. Activation may involve phosphorylation of the enzyme by a cyclic AMP-regulated protein kinase (23, 24). Although the mechanisms underlying cyclic AMP induction of the synthesis of tyrosine hydroxylase and other neural proteins (25) are not known, there is evidence that protein synthesis in the brain is unusually sensitive to environmental influences and that this sensitivity is partly associated with alterations in ribosomal properties (26–28).

In the course of investigations of the nature of these ribosomal alterations, we have recently observed that several proteins which are present in purified ribosomes and ribosomal subunits from rat cerebral cortex are readily phosphorylated during incubation of cellular preparations in vitro (29). As part of continuing investigations of the possible relationship of regulatory mechanisms in protein synthesis to specialized function in the central nervous system, an analysis of the effects of cyclic nucleotides on protein phosphorylations in this organ has been undertaken. The present report indicates that ribosomal protein phosphorylation in rat cerebral cortex is markedly enhanced by cyclic AMP in vitro. This action appears to be highly selective for one ribosomal phosphoprotein.

**EXPERIMENTAL PROCEDURES**

**Incubation of Cerebral Cortical Slices**—Young male rats of an inbred Sprague-Dawley strain, 13 to 15 days old and weighing about 30 g, were used in these studies. At this stage of development, adenylylate cyclase systems in the cerebral cortex respond maximally to catecholamines with increased production of cyclic AMP (30). The animals were decapitated, and the brains were rapidly removed and placed on filter paper moistened with cold incubation medium in a Petri dish kept on ice. Cerebral cortical slices (0.3 mm thick) were prepared with a McIlwain tissue chopper (31) and washed briefly by suspension in cold modified Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 mM glucose. The buffer was prepared as described by Cohen (25), except that KH2PO4 was replaced by KCl. Following a brief centrifugation period (15 s), the medium was decanted and the slices (about 600 mg) were transferred to a plastic container containing 10 ml of the same medium, pH 7.4, containing 100 mM KCl, 6 mM β-mercaptoethanol, 50 mM Tris/HCl, pH 7.6 (4°), and ribonuclease inhibitor protein (2.5 units/ml) from rat liver (33). The slices were stirred at 0° while 7 volumes of cold acetone were added dropwise to precipitate the ribosomal proteins. This suspension was centrifuged at 105,000 × g and 0° in a Spinco 50 Ti rotor. Although the 40 S subunit obtained by the single dissociation and fractionation procedure described above appeared to be free of 60 S subunits, the 60 S subunits were significantly contaminated with 80 S ribosomes or 40 S subunits, as judged by the appearance of radioactive components of the 60 S ribosomal protein of the small subunit on autoradiographs of two-dimensional electrophoregrams. Redistribution of these components after refractionation under nonradioactive conditions of electrophoresis, the pooled fractions of each subunit were diluted to a final concentration of 3 mM MgCl2, and pooled by centrifugation for 20 h at 1,000 × g and 0° in a Spincio 50 Ti rotor. Although this procedure appeared to be free of 60 S subunits, the 60 S subunits were significantly contaminated with 80 S ribosomes or 40 S subunits, as judged by the appearance of radioactive components of the 60 S ribosomal protein of the small subunit on autoradiographs of two-dimensional electrophoreograms. Redistribution of these components after refractionation under nonradioactive conditions of electrophoresis, the pooled fractions of each subunit were diluted to a final concentration of 3 mM MgCl2, and pooled by centrifugation for 20 h at 1,000 × g and 0° in a Spincio 50 Ti rotor. Although the 40 S subunit obtained by the single dissociation and fractionation procedure described above appeared to be free of 60 S subunits, the 60 S subunits were significantly contaminated with 80 S ribosomes or 40 S subunits, as judged by the appearance of radioactive components of the 60 S ribosomal protein of the small subunit on autoradiographs of two-dimensional electrophoregrams. Redistribution of these components after refractionation under nonradioactive conditions of electrophoresis, the pooled fractions of each subunit were diluted to a final concentration of 3 mM MgCl2, and pooled by centrifugation for 20 h at 1,000 × g and 0° in a Spincio 50 Ti rotor.
Phosphorylation in Rat Cerebral Cortex in Vitro

Cerebral cortical slices were incubated for 2 h in medium containing 300 μCi of \[^{32}P\]orthophosphate/ml, washed to remove \[^{32}P\]orthophosphate that had not been taken up by the tissue, and then incubated 1 additional hour in fresh medium in the presence or absence of 1 mM dibutyryl cyclic AMP. Free ribosomes were isolated and dissociated in the presence of high concentrations of KCl. The 60 S subunits were radiolabeled at the same conditions. Purified ribosomal proteins were subjected to one-dimensional electrophoresis on polyacrylamide gels containing 0.1% sodium dodecyl sulfate.

**Table I**

<table>
<thead>
<tr>
<th>Specific activity</th>
<th>40 S</th>
<th>60 S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42.1</td>
<td>57.3</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP</td>
<td>73.6</td>
<td>99.1</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>71.8</td>
<td>95.1</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP</td>
<td>123.7</td>
<td>87.3</td>
</tr>
</tbody>
</table>

* Total radioactivity in gel determined on 1-mm slices following solubilization in 30% hydrogen peroxide.

Radioactivity determined directly on ribosomal protein preparations.

Influence of dibutyryl cyclic AMP on phosphorylation of proteins in cerebral cortex in vitro

Cerebral cortical slices were incubated for 2 h in medium containing 300 μCi of \[^{32}P\]orthophosphate/ml, washed to remove \[^{32}P\]orthophosphate that had not been taken up by the tissue, and then incubated 1 additional hour in fresh medium in the presence or absence of 1 mM dibutyryl cyclic AMP. The radioactivity was then determined directly on ribosomal protein preparations.

**RESULTS**

Influence of Dibutyryl Cyclic AMP on Ribosomal Protein Phosphorylation in Rat Cerebral Cortex in Vitro

Cerebral cortical slices from immature rats have been shown to incorporate radioactivity from \[^{32}P\]orthophosphate into proteins of both ribosomal subunits (29). In the present experiments, addition of dibutyryl cyclic AMP to cerebral cortical slices which had previously been incubated for 2 h with \[^{32}P\]orthophosphate consistently resulted in stimulation of protein phosphorylation in the 40 S subunit (Table I). In 10 experiments, this stimulation averaged 69% 1 h after the addition of 1 mM dibutyryl cyclic AMP. In contrast, \[^{32}P\] labeling of proteins present in the cerebral large subunit was not increased under the same conditions. Specific radioactivity of the large subunit proteins was uniformly greater than that of the small subunit proteins when the ribosomes and subunits were prepared by the high salt procedure from cerebral cortical slices incubated in the absence of dibutyryl cyclic AMP. In contrast, when the ribosomes and subunits were prepared by the low salt procedure, proteins associated with the small subunit incorporated considerably more radioactivity than proteins of the large subunit during incubation periods which varied from 1 to 3 h (29). Since exposure of ribosomes and ribosomal subunits to high concentrations of KCl tends to remove initiation factors and proteins adventitiously associated with the ribosomes (41, 42), the present results indicate that this treatment was more effective in removing proteins associated with the cerebral 40 S subunit than those associated to the 60 S subunit.

Increased phosphorylation of protein of the cerebral 40 S subunit in the presence of dibutyryl cyclic AMP was associated with a single protein band separated by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (Fig. 1). This protein band had the electrophoretic mobility of the S₈₀ protein species with a molecular weight of about 32,000 which has been described earlier (29). The selectivity of dibutyryl cyclic AMP for the S₈₀ protein species was observed with 40 S ribosomal subunits isolated in media of low ionic strength (Fig. 1a), as well as with comparable preparations obtained after exposure to high concentrations of KCl (Fig. 1b). A lag period of about 15 min was noted after addition of 1 mM dibutyryl cyclic AMP. Thereafter, phosphorylation of the S₈₀ ribosomal protein species was markedly enhanced for at least 3 h.

In addition to the \(S₈₀\) ribosomal protein species, two other radioactive bands consistently appeared on sodium dodecyl sulfate gels of ribosomal proteins from 40 S subunits prepared in high salt media (Fig. 1b). These bands contained materials with molecular weights of approximately 10,000 and 62,000. The component with the higher molecular weight (\(S₉₀\)) is protein in nature (29) and was also observed when the ribosomal subunits were isolated in low salt media (Fig. 1a). The band with a molecular weight of about 10,000 is nonproteinaceous (29) and was removed by the low salt procedure. Phosphorylation of proteins in the \(S₉₀\) band was not significantly
enhanced by dibutyryl cyclic AMP.

**Specificity and Sensitivity of Stimulatory Action of Dibutyryl Cyclic AMP on Ribosomal Protein Phosphorylation in Rat Cerebral Slices** — The minimum effective concentration of dibutyryl cyclic AMP in stimulating phosphorylation of the S6 protein band of the 40 S ribosomal subunit of rat cerebral cortex was about 0.1 mM (Table II). This stimulatory action could be attributed to cyclic AMP per se. Thus, the monobutyryl-8-methylthio derivative of cyclic AMP, as well as 1-methyl-3-isobutylxanthine, a potent phosphodiesterase inhibitor which favors the endogenous accumulation of cyclic AMP (43), also exhibited considerable stimulatory activity (Table II). Monobutyryl derivatives of cyclic AMP were less effective than dibutyryl cyclic AMP in stimulating phosphorylation of the S6 protein species. The 8-hydroxy derivative of dibutyryl cyclic AMP was even more effective than dibutyryl cyclic AMP in this regard (not shown). Sodium butyrate, formed from butyryl derivatives of cyclic AMP in brain tissue (44), and dibutyryl cyclic GMP, each in 1 mM concentration, were devoid of activity (Table II).

**Identity of Phosphorylated Ribosomal Protein of Cerebral 40 S Subunit Responsive to Dibutyryl Cyclic AMP** — Two-dimensional electrophoresis of the ribosomal proteins from the cerebral small subunit revealed the presence of at least four distinct 32P-labeled species associated with an elongated protein-stained spot (Fig. 2). This major phosphorylated protein of the 40 S subunit from rat cerebral cortex appeared to be analogous to the S6 protein of rat liver ribosomes (45).

Superposition of the autoradiographs over the corresponding electrophoretograms revealed that the radioactive spots associated with the cerebral S6 protein were located closer to the anode than the most intensely stained portion of this protein. This phenomenon was more clearly demonstrated when the region corresponding to the elongated S6 protein on the two-dimensional gel was excised, scanned for absorbance at 600 nm, then cut into 1-mm wide sections for determination of radioactivity. The radioactivity in the S6 region was associated with five protein species which stained relatively lightly on the gels (Fig. 3). The major stained portion of the cerebral S6 ribosomal protein was not in itself radioactive. Incubation of cerebral cortical slices in the presence of 1 mM dibutyryl cyclic AMP for 3 h not only enhanced the radioactivity incorporated into the phosphorylated congeners of the S6 protein, but also increased the relative absorbances of these species compared to that of the unlabeled, nonphosphorylated S6 protein, which contained an increasing number of phosphoserine or phosphothreonine residues, or both. This conclusion has also been drawn from similar studies of the S6 protein of rat...
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First dimension 3 1 . . . , ,,

Control

Dibutyryl cyclic AMP

FIG. 2. Two-dimensional electrophoresis of radioactive proteins present in the 40 S ribosomal subunit prepared from rat cerebral cortical slices following incubation with $^{32}$P orthophosphate in the presence or in the absence of 1 mM dibutyryl cyclic AMP. Conditions of incubation and preparation of ribosomal subunits were as described in the legend to Table I. Proteins extracted from the small subunit were subjected to two-dimensional electrophoresis on polyacrylamide gels containing urea. The amounts of protein applied to the first dimensional gels were: control, 196 μg; dibutyryl cyclic AMP, 208 μg. a and b, electrophoretograms stained with Coomassie brilliant blue; the arrows point to the cerebral S6 ribosomal protein. c and d, autoradiographs against Kodak No-Screen medical x-ray film. Magnifications of the electrophorograms and the autoradiographs are the same.

Liver ribosomes (45, 46) and the analogous ribosomal protein (S13) of rabbit reticulocytes (35).

Two additional radioactive phosphoproteins appeared on two-dimensional electrophoretograms of ribosomal proteins isolated from the cerebral small subunit in media of high ionic strength (Fig. 2, c and d). The more prominently labeled of these ribosomal phosphoproteins traveled slightly slower in both dimensions than the slowest moving radioactive component of the S6 protein and corresponded closely in position with the S2 protein of Sherton and Wool (47, 48) from rat liver and muscle ribosomes. The location of this protein is shown in the schematic of the cerebral 40 S ribosomal proteins in Fig. 4a. The more weakly labeled of these two additional phosphoproteins of the small subunit appeared to correspond with the upper component of the ribosomal protein species labeled S3. When the S2 and S3 protein spots were eluted from the two-dimensional gel slabs and subjected to third dimensional electrophoresis on sodium dodecyl sulfate gels, all of the detectable radioactivity was concentrated in regions of these gels which corresponded to molecular weights of approximately 35,000 and 33,000, respectively. These molecular weights are similar to those obtained for the S2 and S3 ribosomal proteins from other eukaryotic tissues under comparable conditions of analysis (see Ref. 50). Because of the relatively low level of radioactivity in the S2 and S3 proteins, we have not yet been able to determine whether dibutyryl cyclic AMP is capable of stimulating the phosphorylation of these proteins in vitro.

When the 40 S ribosomal proteins were subjected to reverse electrophoresis in the first direction from cathode to anode, followed by the usual second dimensional procedure, no additional phosphorylated proteins were detected. This finding substantiated the conclusion that isolation of cerebral 40 S subunits in high salt media effectively removed phosphorylated proteins which were not integral constituents of the ribosome. However, the results of several studies have suggested that proteins which are part of the ribosomal structure may also be removed by exposure of the ribosomes to media of high ionic strength (49, 51, 52). This possibility appeared to be supported by the present studies. Thus, protein S1 which was clearly visible on two-dimensional electrophoretograms of cerebral monomeric ribosomes and 40 S subunits isolated without repeated exposure to high concentrations of KCl, was not detected when the cerebral 40 S subunits were prepared by the high salt procedure described above, unless the gels were overloaded with ribosomal proteins. Although a protein with the electrophoretic properties of S1 was not detected in 40 S subunits prepared from rabbit reticulocyte ribosomes in the presence of 500 mM KCl (53), the analogous protein of rat liver or muscle 40 S subunits seemed to be less readily removed by exposure of the ribosomes to high concentrations of KCl (48).

S. Roberts and B. S. Morelos, unpublished observations.
Phosphorylated Ribosomal Proteins of Cerebral 60 S Subunit—Earlier investigations from this laboratory had revealed the presence of several radioactive proteins in 60 S subunits obtained from cerebral cortical slices following incubation with \[^{32}P\]orthophosphate (29). In the present experiments, redissociation and refractionation of the cerebral 60 S subunits in the presence of high concentrations of KCl removed certain of these proteins, leaving two major radioactive bands which could be detected on sodium dodecyl sulfate gels (Fig. 5). These radioactive bands corresponded to the L11 and L12 ribosomal protein species previously described, with molecular weights which approximated 37,000 and 15,000, respectively (29). Addition of 1 mM dibutyryl cyclic AMP to the incubation medium did not increase phosphorylation of either of these bands.

When ribosomal proteins of the 60 S subunit from cerebral cortical slices were subjected to electrophoresis in the first direction toward the cathode at pH 8.6 on polyacrylamide gels containing urea, most of the radioactivity remained at the anode. Autoradiographs of conventional two-dimensional electrophoretograms revealed that a considerable fraction of this anodal radioactivity was actually localized in several proteins which were immobile in the first dimension, but migrated toward the cathode at pH 5.2 in the second dimension (Fig. 6, a and c). These findings suggested that these phosphorylated proteins were relatively less basic than the bulk of 60 S ribosomal proteins. This conclusion was supported by subjecting the 60 S ribosomal proteins to one-dimensional electrophoresis in the opposite direction at pH 8.6, followed by the usual second dimensional procedure. Under these circumstances, three lightly stained protein spots were observed which moved toward the anode (Fig. 6b, arrows) and were radioactive (Fig. 6d). They appeared to correspond to phosphorylated proteins of the 60 S subunit which remained at the anodic origin at pH 8.6 (Fig. 6c). The most highly labeled protein spot, which traveled most rapidly in the second dimension, may have been composed of two or more proteins, possibly phosphorylated forms of the same protein (Fig. 6d). Electrophoresis of the eluted, radioactive material on sodium dodecyl sulfate gels revealed that its mobility corresponded to that of the cerebral L1 protein species with a molecular weight of approximately 15,000. Molecular weights of the other two radioactive proteins observed on the reversed polarity two-dimensional gels ranged from 36,000 to 38,000. Thus, the proteins were components of the radioactive L11 peak observed on sodium dodecyl sulfate gels of cerebral 60 S proteins (see Fig. 5).

The present studies also revealed that at least three basic proteins of the 60 S ribosomal subunit were phosphorylated after incubation of cerebral cortical slices with \[^{32}P\]orthophosphate (Fig. 6c). These three radioactive proteins appeared to correspond closely in electrophoretic mobility to ribosomal proteins L12, L13, and L14 on stained two-dimensional gels of the cerebral 60 S subunit (Fig. 4b). If the gels were heavily loaded with protein, an additional phosphorylated basic protein was seen, corresponding in position to protein L29 (not shown). The cerebral 60 S subunit was completely free of contamination with the 40 S subunit as judged by the absence of radioactive congeners of the S6 protein on the autoradiographs (Fig. 6c). When the radioactive

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FIG. 4. Schematics of the two-dimensional electrophoretograms of the proteins of cerebral ribosomal subunits. a, proteins from the 40 S subunit; b, proteins from the 60 S subunit. Certain proteins were absent or difficult to see on the stained electrophoretograms of the separated subunits unless the gels were overloaded with protein, but were clearly visible on electrophoretograms of cerebral 40 S ribosomes prepared in media of high ionic strength. These proteins are indicated by cross-hatched spots. Ribosomal proteins are labeled according to the nomenclature of Sherton and Wool (47, 48). Unnumbered spots could not be related to ribosomal proteins identified by numbers in this classification. Separation of the protein complexes designated S3 and L14, each into two or more protein-stained spots, has also been observed by Traut et al. (49).

FIG. 5. Polyacrylamide gel electrophoresis of radioactive proteins present in the 60 S ribosomal subunit prepared from rat cerebral cortical slices following incubation with \[^{32}P\]orthophosphate. Electrophoresis in the first dimension was from anode to cathode in a medium composed of two or more proteins, possibly phosphorylated forms of the same protein (Fig. 6d). Electrophoresis of the eluted, radioactive material on sodium dodecyl sulfate gels revealed that its mobility corresponded to that of the cerebral L1 protein species with a molecular weight of approximately 15,000. Molecular weights of the other two radioactive proteins observed on the reversed polarity two-dimensional gels ranged from 36,000 to 38,000. Thus, the proteins were components of the radioactive L11 peak observed on sodium dodecyl sulfate gels of cerebral 60 S proteins (see Fig. 5). The present studies also revealed that at least three basic proteins of the 60 S ribosomal subunit were phosphorylated after incubation of cerebral cortical slices with \[^{32}P\]orthophosphate (Fig. 6c). These three radioactive proteins appeared to correspond closely in electrophoretic mobility to ribosomal proteins L12, L13, and L14 on stained two-dimensional gels of the cerebral 60 S subunit (Fig. 4b). If the gels were heavily loaded with protein, an additional phosphorylated basic protein was seen, corresponding in position to protein L29 (not shown). The cerebral 60 S subunit was completely free of contamination with the 40 S subunit as judged by the absence of radioactive congeners of the S6 protein on the autoradiographs (Fig. 6c). When the radioactive

FIG. 6. Two-dimensional electrophoresis of radioactive proteins present in the 60 S ribosomal subunit prepared from rat cerebral cortical slices following incubation with \[^{32}P\]orthophosphate. Electrophoresis in the first dimension was from anode to cathode in a medium containing high concentrations of KCl. Redissociation and refractionation in media containing high concentrations of KCl, or in the absence (O—O) of 1 mM dibutyryl cyclic AMP, after a preliminary incubation of 2 h with 170 &muCi of \[^{32}P\]orthophosphate/ml of medium. The 60 S subunit was subjected to redissociation and refractionation in media containing high concentrations of KCl. Ribosomal proteins were analyzed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate. See legend to Table I for further explanations.
material which was associated with the L14 spot on two-dimensional slabs was eluted and subjected to third-dimensional electrophoresis on sodium dodecyl sulfate gels, this material was found to possess a molecular weight of about 26,000. Because of the low level of radioactivity in the basic 60 S ribosomal proteins which were phosphorylated in cerebral cortical tissue in vitro, they were not readily detected in slices prepared from sodium dodecyl sulfate gels (Fig. 5). Moreover, it was not possible to determine whether their phosphorylation was individually enhanced by exposure of the tissue to dibutyryl cyclic AMP. However, as noted earlier, overall phosphorylation of cerebral 60 S subunit proteins was not increased under these conditions (Table I).

**Discussion**

Several reports have appeared which dealt with the effects of cyclic AMP on ribosomal protein phosphorylation in intact eukaryotic cells. Roos (54) noted that addition of cyclic AMP (or adrenocorticotropic hormone) to functional tumor cell cultures of mouse adrenal origin enhanced ribosomal protein phosphorylation. Barden and Labrie (55) demonstrated that addition of dibutyryl cyclic AMP to slices of bovine anterior pituitary gland slices stimulated phosphorylation of one major ribosomal phosphoprotein component. Subunit localization or molecular weights of the phosphorylated components were not determined in either study. Cawthorn et al. (56) found that cyclic AMP and dibutyryl cyclic AMP increased ribosomal protein phosphorylation in intact rabbit reticulocytes. The increased phosphorylation was associated with a protein component of the 40 S subunit with a molecular weight of approximately 27,500. This protein was probably identical to the S13 ribosomal protein of rabbit reticulocyte 40 S subunits described by Traugh and Porter (35), which undergoes enhanced phosphorylation in the presence of cyclic AMP-regulated protein kinases. Greller and Wool (46) showed that phosphorylation of a single protein derived from the small subunit of rat liver ribosomes was stimulated by administration in vivo of cyclic AMP, dibutyryl cyclic AMP, or glucagon. Subsequent isolation of this ribosomal protein (designated S6) revealed that it possessed a molecular weight of about 31,000 (see Ref. 50). Recently, Schubart et al. (57) demonstrated that phosphorylation of a ribosomal protein of the 40 S subunit from hamster islet tumor cells was stimulated in vivo by the addition of glucagon, 8-bromoadenosine 3':5'-monophosphate, or 1-methyl-3-isobutylxanthine. This protein had a molecular weight of approximately 28,000.

In the present investigations with cerebral cortical tissue from immature rats, addition of derivatives of cyclic AMP or the phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine, markedly enhanced phosphorylation of a single ribosomal protein of the small subunit, but did not increase phosphorylation of proteins of the large subunit. This phosphorylated protein of the cerebral 40 S subunit appeared to be analogous to the S6 protein of rat liver ribosomes (46, 48, 50) and the S13 protein of rabbit reticulocyte ribosomes (35, 53). In common with the major phosphorylated protein of 40 S ribosomal subunits from other eukaryotic cells, the cerebral S6 ribosomal protein had a molecular weight of about 32,000 and contained several corine or threonine residues, or both, which were capable of enhanced phosphorylation in the presence of cyclic AMP. It may be noted that the more acidic (more highly phosphorylated) derivatives of the cerebral S6 protein, which are normally present only in trace amounts on stained electrophorograms, were actively labeled even in the absence of added cyclic AMP. This observation may reflect the fact that decapitation causes marked accumulation of cyclic AMP in the brain (58).

The cerebral S6 protein was one of the four radioactive phosphoprotein components of the small subunit detected on sodium dodecyl sulfate gels when free ribosomes from cerebral slices, incubated in the presence of [32P]orthophosphate, were isolated and dissociated into subunits in media of low ionic strength (29). Exposure of the ribosomes and subunits to media containing high concentrations of KCl did not remove the cerebral S6 protein or eliminate the response to cyclic AMP, but did result in total disappearance of all but one of the other phosphorylated protein components present on sodium dodecyl sulfate gels following electrophoresis of the 40 S ribosomal proteins. This additional component had a molecular weight of approximately 62,000, was markedly reduced in concentration when the subunits were isolated in high salt media, and was not affected by dibutyryl cyclic AMP. In view of the relatively high molecular weight of this component compared to the size of ribosomal proteins from the small subunit of other eukaryotic cells (50, 53, 59), and the fact that it was largely removed by exposure to high concentrations of KCl, its partial persistence under the latter conditions may reflect residual contamination of the small cerebral subunit with nonribosomal proteins.

In addition to the S6 protein, two other phosphorylated components appeared on conventional two-dimensional gels of cerebral 40 S proteins. These components corresponded electrophoretically to the S2 and S3 proteins of rat liver ribosomes with molecular weights in the 30,000 to 35,000 range (50). 32P labeling of S2 and S3 ribosomal proteins has not been observed in rat liver in vivo (45, 46, 60, 61) even after exposure to cyclic AMP. However, phosphorylation of a ribosomal protein with the electrophoretic properties of S2 has been noted in Krebs II ascites cells in vitro (62) and in HeLa cells infected with vaccinia virus (63). In addition, two phosphorylated proteins which trail the S3 protein complex in the first dimension have been noted in two-dimensional autoradiographs of 40 S ribosomal proteins from rabbit reticulocytes (35). An additional phosphorylated protein of the small ribosomal subunit, with a molecular weight of about 25,000, has been described in cultured insulinoma cells of the hamster (57). This ribosomal protein did not exhibit alterations in phosphorylation in the presence of cyclic AMP.

The status of phosphorylated proteins in eukaryotic 60 S ribosomal subunits is less certain than that of proteins of the 40 S subunit. Several investigators have found that overall phosphorylation of proteins associated with the large subunit in vivo or in cellular preparations in vitro proceeded at a rate that varied from approximately 65 to 120% of the rate for 40 S ribosomal proteins (29, 35, 46, 63). In the present investigations with cerebral cortical slices incubated in the absence of dibutyryl cyclic AMP or other additives, overall phosphorylation of ribosomal proteina isolated from redissociated and refractionated 60 S subunits was 20 to 40% greater than that of the 40 S proteins. It has been suggested that phosphorylated proteins associated with the 60 S ribosomal subunit in cellular preparations from eukaryotic cells represent mainly contamination with nonribosomal proteins (29, 46). In the present studies, three or more phosphorylated proteins were isolated from 60 S ribosomal subunits of incubated cerebral cortical slices which exhibited relatively acidic properties on two-dimensional polyacrylamide gels. These cerebral proteins, with molecular weights of approximately 15,000 and 37,000,
may be analogous to phosphorylated proteins associated with 60 S ribosomes in cellular preparations of rabbit reticulocytes (35, 50) and Krebs II ascites cells (62), which have not been identified as constitutive ribosomal proteins. However, several proteins of the 60 S subunit which were more basic were also phosphorylated in cerebral cortical tissue in vitro. Three of these proteins migrated from anode to cathode at pH 8.6 with mobilities that were similar to the mobilities of the ribosomal proteins L6, L13, and L14 of Shorten and Wool (47). Phosphorylation of these proteins has not been described for cellular preparations of other eukaryotic tissues in vivo or in vitro (35, 57, 61, 62, 64).

The present investigations support earlier findings from this laboratory which indicated that several proteins of both ribosomal subunits are capable of being phosphorylated by endogenous protein kinases in cellular preparations of rat cerebral cortex in vitro (29). However, which of these ribosomal proteins undergo phosphorylation in vivo remains to be determined. Although current investigations suggest that phosphorylation of initiation factors alters their capacity in protein synthesis (65–68), the physiological functions which may be served by phosphorylation of structural proteins of the ribosome are essentially unknown. It is not unlikely that the latter phenomenon is involved in several diverse functions of the ribosome, including processes which are not directly related to protein synthesis (69). Several investigators have suggested that ribosomal protein phosphorylation may be involved in attachment of the ribosome to messenger RNA, initiation factors or other components of the protein-synthesizing system, and that these processes are affected when phosphorylation is enhanced by intracellular accumulation of cyclic AMP resulting from altered cellular activity (29, 35, 46, 55, 70–72). The present results are consistent with the possibility that the accumulation of cyclic AMP in neural tissue that accompanies the release of certain neurotransmitter amines (9) may modify translational processes in protein synthesis by increasing the phosphorylation of a specific protein of the small ribosomal subunit. Moreover, in view of the strong likelihood that each 40 S cerebral ribosome contains only one congener of this protein (see Ref. 46), it is possible that neurotransmitter-induced production of cyclic AMP is capable of increasing the heterogeneity of the cerebral ribosomal pool and, thereby, perhaps selectively altering the synthesis of specific proteins which may function in neurotransmission.

Acknowledgments — We wish to thank Rita Kern and Beatrice S. Morelos for valuable technical assistance.

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