Phosphorylation of Multiple Proteins of both Ribosomal Subunits in Rat Cerebral Cortex *in vivo*

EFFECT OF ADENOSINE 3':5'-CYCLIC MONOPHOSPHATE

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Investigations were carried out on the phosphorylation of ribosomal proteins *in vivo* in cerebral cortices of immature rats. Two-dimensional electrophoresis revealed that the cerebral 40S subunit contained at least four ribosomal proteins which were phosphorylated in animals given [32 P]orthophosphate intracisternally. These proteins exhibited electrophoretic properties similar to those of the constitutive basic proteins S2, S3a, S5 and S6. The cerebral 60S subunit contained several proteins that were phosphorylated *in vivo*, including three basic proteins with electrophoretic mobilities similar to those of ribosomal proteins L6, L14 and L19. Four other proteins associated with the 60S subunit that were more acidic were also phosphorylated. Phosphorylated congeners of 40S and 60S ribosomal proteins could often be detected in distinct protein-stained spots on two-dimensional electrophoretograms. The cerebral S6 protein consisted of at least five distinct species in different states of phosphorylation. Administration of N^6O^2 -dibutyryl cyclic AMP increased the proportion of the more phosphorylated congeners of the S6 protein, but appeared to have little or no effect on phosphorylation of other cerebral ribosomal proteins. The phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine also stimulated S6-protein phosphorylation; N^2O^2 -dibutyryl cyclic GMP had no effect on this process. These observations indicate that several ribosomal proteins of both subunits are normally phosphorylated in rat cerebral cortex *in situ*. The results also suggest that selective and specific alterations in the phosphorylation state of the S6 ribosomal protein of the cerebral 40S subunit may accompany the production of cyclic AMP during neural activation.

Earlier investigations from this laboratory revealed the presence of several proteins in both ribosomal subunits that were phosphorylated in cellular preparations of rat cerebral cortex *in vitro* (Ashby & Roberts, 1975; Roberts & Ashby, 1978). Elevated concentrations of cyclic AMP consistently stimulated phosphorylation of the S6 protein of the 40S subunit *in vitro*, but appeared to have little or no effect on phosphorylation of other cerebral ribosomal proteins (Roberts & Ashby, 1977a,b, 1978). Since the spectrum of ribosomal proteins phosphorylated in mammalian tissues *in vivo* may differ qualitatively and/or quantitatively from that observed in cellular preparations and reconstituted cell-free systems *in vitro* (Krystosek *et al.*, 1974; Wool & Stöffler, 1974; Francis & Roberts, 1978), the present investigations were undertaken to delineate the nature of ribosomal proteins that normally undergo phosphorylation in rat cerebral cortex *in situ* and the effect of cyclic nucleotides on this process.

Experimental

Treatment of animals

Young male rats of an inbred Sprague–Dawley strain, 8 days old and weighing about 20 g, were used in these investigations. At this age in the rat, brain-barrier systems are not well developed and administered substances are more readily taken up by the brain than in older animals (Dobbing, 1968). The animals were kept warm under an incandescent lamp throughout the experiment.

Phosphorylated ribosomal proteins in cerebral cortices of these animals were labelled by intracisternal administration of 2 mCi of [32 P]orthophosphate in 20 μ l of 0.9% (w/v) NaCl at pH 7. The intracisternal route was selected because only very small amounts of radioactivity were incorporated into brain ribosomal proteins when [32 P]orthophosphate was given intraperitoneally or intravenously. The animals were routinely killed by decapitation at intervals of

30–120 min after the injection of the radioactive isotope.

Dibutylryl cyclic AMP, dibutylryl cyclic GMP and 3-isobutyl-1-methylxanthine were administered both intraperitoneally and intracisternally. Solutions of these substances were adjusted to pH 7 before administration. The precise dosages and conditions employed in each experiment are described in the Results section. At the termination of each experiment, brains were rapidly removed, placed on filter paper, wet with 0.9% (w/v) NaCl, in a Petri dish kept on ice, and immediately transferred to a cold-room. Cerebral cortices were then dissected free of underlying tissue, weighed on a torsion balance, washed with buffer to remove extracellular radioactivity, and homogenized as described below. Brain homogenates from 15–20 animals were pooled to provide sufficient material for isolation and characterization of ribosomal proteins.

Preparation of cerebral ribosomes and ribosomal subunits

Since cerebral polyribosomes are unusually sensitive to ribonuclease action (Zomzely *et al.*, 1968; Simpkins *et al.*, 1973; Roberts & Morelos, 1976), special precautions were taken to minimize polyribosome degradation by endogenous and exogenously introduced ribonucleases. These measures routinely included sterilization of media, glassware and plastic ware, addition of a purified ribonuclease-inhibitor preparation from rat liver (Shortman, 1961) to all media used for ribosome isolation, and gentle homogenization in media of relatively high Mg^{2+} concentration (Zomzely *et al.*, 1966). Cerebral tissue was homogenized in medium (1:3, w/v) containing 0.25 M-sucrose, 12 mM- $MgCl_2$, 100 mM-KCl, 6 mM- β -mercaptoethanol, 50 mM-Tris/HCl, pH 7.6 (4°C) and ribonuclease-inhibitor protein (50 μ g/ml). This amount of inhibitor contained 3–5 units of activity in the assay described by Shortman (1961). Only three passes of a loosely fitting Teflon pestle in a glass homogenizing tube were used, since more forceful homogenization resulted in polyribosome breakdown and did not improve recovery. The resulting homogenates were centrifuged at 8000g for 10 min at 0°C to obtain the post-mitochondrial supernatant fraction. Samples of this preparation were treated with 10% (w/v) trichloroacetic acid to precipitate the proteins. After centrifugation, radioactivity in the supernatant fraction was determined as a measure of ^{32}P uptake into the cerebral cytosol.

Purified polyribosomes were prepared by two procedures. When the influence of cyclic nucleotides on the state of aggregation of cerebral polyribosomes was to be assessed by sucrose-density-gradient analysis, the postmitochondrial supernatant fractions were centrifuged in a Spinco 50Ti rotor for

4 or 20 h at 226400g and 0°C through 2 M-sucrose which contained the same additives as the homogenization medium, including ribonuclease inhibitor. The resulting pellets were resuspended in this medium and layered on to linear 10–40% (w/v) sucrose gradients containing the same medium in a Spinco SW 50.1 swinging-bucket rotor. After centrifugation at 185000g and 0°C for 60 min, the gradients were analysed for material that absorbed u.v. light at 254 nm by the use of an Isco model 640 density-gradient fractionator.

A second procedure was used to obtain salt-washed polyribosomes that were to be dissociated into subunits for isolation and gel-electrophoretic analysis of the ribosomal proteins. This procedure was based on the finding that repeated exposure of ribosomes during isolation to media containing high concentrations of KCl removes proteins that presumably are not part of the ribosomal structure (Warner & Pène, 1966; Sherton & Wool, 1974a). Cerebral free polyribosomes were prepared as described above, with the exception that the 2 M-sucrose medium contained 500 mM-KCl. The polyribosomes were then resuspended in the same high-salt medium containing 0.25 M-sucrose, and centrifuged at 226400g and 0°C for 3.5 h. These purified salt-washed ribosomes were dissociated as described earlier (Roberts & Ashby, 1978). Briefly, the ribosomes were incubated at 37°C for 20 min in the presence of 0.2 mM-puromycin/800 mM-KCl/12.5 mM- $MgCl_2$ /20 mM- β -mercaptoethanol/50 mM-Tris/HCl, pH 7.5 (22°C). The subunits were then separated by centrifugation of this suspension in a Spinco SW27 swinging-bucket rotor for 17.5 h at 131000g and 20°C on a linear 10–30% (w/v) sucrose gradient containing 5 mM- $MgCl_2$, 580 mM-KCl, 10 mM- β -mercaptoethanol and 10 mM-Tris/HCl, pH 7.5 (22°C). Centrifugation of these gradients containing cerebral ribosomal subunits at 28°C, as described by Leader & Wool (1972) for hepatic preparations, resulted in considerable breakdown of the cerebral 40S subunits. The cerebral subunits were individually isolated by collecting those fractions of the 40S and 60S bands that exhibited absorbances above the half-heights of the peaks on the density-gradient recordings. The pooled fractions of each subunit were diluted to give a final concentration of 3 mM- $MgCl_2$ and pelleted by centrifugation for 20 h at 226400g and 0°C in a Spinco 50Ti rotor. The 60S subunits were redissociated and refractionated as described above to remove contaminating 80S ribosomes and 40S subunits. Cerebral 40S and 60S subunits isolated by this procedure were virtually free of cross-contamination (Roberts & Ashby, 1978).

Electrophoresis of ribosomal proteins

Ribosomal proteins were isolated from the purified cerebral 40S and 60S subunits as described earlier

(Barritault *et al.*, 1976; Roberts & Ashby, 1978). The proteins were dissolved in 125–250 μ l of medium composed of 8 M-urea and 10 mM- β -mercaptoethanol; samples (10 μ l) were taken for measurements of protein (Lowry *et al.*, 1951) and radioactivity. A measured volume of the ribosomal protein preparation (usually 100 μ l) was subjected to two-dimensional electrophoresis by a modification of the procedure of Howard & Traut (1974), as outlined by Roberts & Ashby (1978). The first-dimensional cylindrical gel contained 8% (w/v) acrylamide and 6 M-urea buffered at pH 8.6, and was maintained at 23°C during electrophoresis. The proteins were stacked for 1 h with a current of 3 mA/gel. Electrophoresis of ribosomal proteins placed at the anode was allowed to proceed towards the cathode at 3–4 mA/gel for 14–16 h; electrophoresis from cathode to anode was conducted at 1 mA for 12–14 h. Electrophoresis in the second dimension was carried out in slab gels containing 18% (w/v) acrylamide and 6 M-urea buffered at pH 4.5. The ribosomal proteins were stacked at the anode for 1 h at 40 V and then subjected to electrophoresis toward the cathode for 5–6 h at approx. 300 V. The slab-gel apparatus was cooled by circulating refrigerant kept at 0°C. The slabs were stained with Coomassie Brilliant Blue for 3 h and then destained by diffusion overnight. In some instances, the gel slabs were subsequently dried and subjected to radioautography against Kodak No-Screen X-ray film for periods varying from 5 h to 21 days. In other cases, the region of the gel slabs corresponding to the cerebral S6 protein of the 40S subunit was excised, scanned at 600 nm, and then cut into 1 mm sections for scintillation counting after solubilization in 30% (v/v) H_2O_2 (Ashby & Roberts, 1975). In yet another procedure, phosphorylated ribosomal proteins were individually cut out of the two-dimensional gel slabs and eluted as described earlier (Roberts & Ashby, 1978). These samples were subjected to electrophoresis alongside standard proteins of known molecular weight on polyacrylamide slab gels containing sodium dodecyl sulphate (Laemmli, 1970). The third-dimensional gels were subsequently stained, dried and subjected to radioautography. Molecular weights of the radioactive proteins were estimated by comparison with the mobilities of the protein markers.

Analysis of phosphorylated amino acids present in ^{32}P -labelled ribosomal proteins from rat cerebral cortex was carried out by high-voltage paper electrophoresis after hydrolysis of the purified proteins in 6 M-HCl (Ashby & Roberts, 1975).

Radioactivity measurements

Radioactivity in solubilized ribosomal proteins and gel slices, as well as in the cerebral cytosol after removal of material precipitated by trichloroacetic

acid, was measured in a Packard Tri-Carb scintillation spectrometer. Samples of these preparations were mixed with 10 ml of scintillation fluid and counted for radioactivity alongside an appropriate dilution of the [^{32}P]orthophosphate solution that was given intracisternally. Where indicated, the counts were corrected to the value of this radioactive standard on the first day of the experiment. Efficiency of detection of ^{32}P radioactivity exceeded 90%.

Materials

Sucrose (ribonuclease-free), urea and Tris (all ultra-pure) were obtained from Schwarz/Mann, Orangeburg, NY, U.S.A. Urea solutions were further purified by passage through a column of Bio-Rad AG 501-X8(D) Dowex resin before use. [^{32}P]Orthophosphate (carrier-free) in 0.02 M-HCl (285 Ci/mg), obtained from ICN Pharmaceuticals, Irvine, CA, U.S.A., was neutralized with 0.2 M-NaOH and supplemented with NaCl to a final concentration of 0.9% (w/v) NaCl just before administration. Reagents and apparatus for electrophoresis of ribosomal proteins in polyacrylamide gels were all obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.), except sodium dodecyl sulphate which was from Sigma Chemical Co., St. Louis, MO, U.S.A. Sigma was also the source of the standard proteins used for molecular-weight estimations by electrophoresis in sodium dodecyl sulphate-containing gels (Ashby & Roberts, 1975), puromycin dihydrochloride, β -mercaptoethanol and the sodium salts of N^6O^2' -dibutyryl cyclic AMP and N^2,O^2' -dibutyryl cyclic GMP. Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), supplied 3-isobutyl-1-methylxanthine. Inorganic reagents were analytical reagent grade from Mallinckrodt, St. Louis, MO, U.S.A. or J. T. Baker, Phillipsburg, NJ, U.S.A. Scintillation-counting solution (3a70B) was obtained from Research Products International, Elk Grove Village, IL, U.S.A.

Results

Phosphorylation of ribosomal proteins in rat cerebral cortex in vivo

Administration of [^{32}P]orthophosphate intracisternally resulted in substantial labelling of 40S ribosomal proteins of rat cerebral cortex. Incorporation of radioactivity continued actively for at least 90 min (Fig. 1). The apparent phosphorylation rate of proteins in highly purified preparations of the cerebral 60S subunit was relatively low *in vivo*, i.e. less than 10% of the phosphorylation rate of the 40S ribosomal proteins. This finding was in contrast with the results of earlier experiments, where the specific radioactivity of 60S proteins was uniformly greater than that of the small subunit proteins when

cellular preparations of rat cerebral cortex were incubated *in vitro* with [32 P]orthophosphate in the absence of exogenous cyclic AMP (Roberts & Ashby, 1978). In common with earlier results from experiments *in vitro* (Ashby & Roberts, 1975), incorpora-

tion of 32 P into cerebral ribosomal proteins *in vivo* involved principally esterification of serine and threonine hydroxy groups (results not shown).

Two-dimensional electrophoresis was carried out on the 40S ribosomal proteins isolated from cerebral cortices of untreated rats that were not given an intracisternal injection of [32 P]orthophosphate. In these analyses, electrophoresis in the first dimension was prolonged to permit better detection of the phosphorylated derivatives of 40S ribosomal proteins. The pattern obtained (Fig. 2a) was qualitatively similar to that observed earlier with the corresponding protein preparation from cerebral-cortical slices that were incubated *in vitro* for 1–3 h (Roberts & Ashby, 1978). However, the more highly phosphorylated congeners of the S6 protein, which exhibited reduced mobility in the first dimension at pH 8.6, were more prominent in 40S proteins isolated directly from cerebral cortices than in comparable samples derived from incubated tissue. Intracisternal administration of [32 P]orthophosphate had no discernible effect on patterns of cerebral 40S ribosomal proteins obtained by two-dimensional electrophoresis (see below). When radioautographs of the latter electrophoretograms were exposed for relatively short periods of time (6 h to 2 days), almost all of the radioactivity was localized in the S6 protein (Fig. 2b). However, more prolonged exposure of the electrophoretograms to radioautography (10–15 days) revealed that cerebral ribosomal proteins with the electrophoretic properties of S2, S3 and S5 proteins were also phosphorylated *in vivo* (Fig. 2c). In addition, a radioactive spot that tailed S10 protein was observed. No additional 32 P-labelled proteins were detected when the 40S ribosomal proteins were subjected to electrophoresis in the first dimension from cathode to anode, followed by the usual second-dimensional procedure.

Fig. 1. Kinetics of incorporation of radioactivity from [32 P]orthophosphate into ribosomal proteins of rat cerebral cortex *in vivo*.

Immature rats were each given 2 mCi of [32 P]orthophosphate intracisternally in 20 μ l of 0.9% NaCl. The animals were killed by decapitation 30–120 min later. Proteins of the purified 40S and 60S subunits, pooled from 12 animals at each time interval, were dissolved in 8 M-urea containing 10 mM- β -mercaptoethanol, and sampled for measurements of total protein and radioactivity. Specific radioactivities, corrected for decay over a period of 9 days, are shown for 40S (●) and 60S (○) subunits. The results shown are representative of three separate experiments.

Fig. 2. Two-dimensional electrophoresis of proteins isolated from 40S ribosomal subunits of rat cerebral cortex.

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 (a) 259 μ g, (b) 312 μ g and (c) 352 μ g. (a) Electrophoretogram of 40S proteins from untreated rats, stained with Coomassie Brilliant Blue; electrophoresis in the first dimension was allowed to proceed for 16 h at 4 mA/gel. (b) Radioautograph, exposed for 1 day, of 40S proteins from rats given 2 mCi of [32 P]orthophosphate intracisternally 1 h before decapitation; electrophoresis in the first dimension was allowed to proceed for 14 h at 3 mA/gel. (c) Radioautograph, exposed for 2 weeks, of a two-dimensional gel similar to that in (b). Magnifications of the electrophoretogram and the radioautographs are similar.

Proteins S2 and S3 were previously shown to be phosphorylated *in vitro* (Roberts & Ashby, 1978). In these earlier studies, protein S3 was observed to consist of two or more components with slightly different electrophoretic mobilities in the second dimension on polyacrylamide gels containing urea (see also Fig. 2a). Two of these components, protein S3 and the faster-moving protein S3a, have now been identified in several eukaryotic cell types (McConkey *et al.*, 1979). A third component (protein S3b), with slightly greater mobility in both dimensions, may also be present in the S3 complex. When the radioactive tail of the S3 complex was eluted, along with the portion of the complex that exhibited the same or lower mobility in the second dimension, and then subjected to electrophoresis on third-dimensional gels containing sodium dodecyl sulphate, two protein bands were observed (Fig. 3a). Only the species with the lower mobility on these gels (S3a) was radioactive (Fig. 3b). Presumably, the non-radioactive band was principally protein S3, but protein S3b, with similar or identical molecular weight, may also have been present (Collatz *et al.*, 1977). Selective phosphoryla-

tion of protein S3a has not previously been described. The molecular weights of proteins S2, S3a, S5 and S6 were approx. 32000, 33000, 21000 and 32000 respectively. These molecular weights are similar to those reported earlier for the analogous ribosomal proteins from rat liver (Collatz *et al.*, 1976, 1977).

Phosphorylated satellites of S2, S3 and S5 proteins were often present as components of distinct spots or tails closely following the non-phosphorylated forms on the stained electrophoretograms, especially when electrophoresis in the first dimension was prolonged (Fig. 2a). Since the only proteins that could be detected in material eluted from these satellite spots had the same molecular weights as their companion proteins (Fig. 3a), this finding suggested that substantial proportions of the S2, S3 and S5 proteins were normally present in phosphorylated states *in situ*, even though they incorporated radioactive phosphate rather slowly. However, it is possible that the satellite spots contained other proteins as well. This possibility is suggested by the observation that protein S7, which did not incorporate detectable radioactivity from [32 P]orthophosphate

Fig. 3. Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate of phosphorylated 40S ribosomal proteins excised from two-dimensional gels

Immature rats were each given 2mCi of [32 P]orthophosphate intracisternally in 20 μ l of 0.9% NaCl. The animals were killed by decapitation 1 h later. Proteins extracted from the small subunit were subjected to two-dimensional electrophoresis on polyacrylamide gels containing urea. After the two-dimensional slab gels were stained with Coomassie Brilliant Blue, the protein spots corresponding to S2, S5, S6 and the upper portion of the S3 complex were cut out, along with their radioactive tails. The gel sections, pooled from two to four electrophoretograms, were chopped into small pieces and extracted overnight with stirring in 1 ml of a solution containing 1% sodium dodecyl sulphate, 6M-urea and 20mM- β -mercaptoethanol. These samples were then concentrated by dialysis against solutions containing 0.1% sodium dodecyl sulphate, 20mM- β -mercaptoethanol, 5mM-sodium phosphate, pH 7.2, and increasing concentrations of sucrose (10–40%, w/v). The resulting samples, each containing about 10 μ g of protein, were heated at 65°C for 15 min before application to slab gels containing 10% acrylamide and 0.1% sodium dodecyl sulphate. Standard protein mixtures were similarly treated. These mixtures contained the following proteins with molecular weights as listed by Weber & Osborn (1969): rabbit muscle phosphorylase α , 94000; bovine serum albumin, 68000; yeast alcohol dehydrogenase, 37000; bovine pancreas α -chymotrypsinogen A, 25700; horse heart cytochrome *c*, 11700. Electrophoresis was conducted at 20mA/gel for 4.5 h at room temperature. (a) Electrophoretogram stained with Coomassie Brilliant Blue. (b) Radioautograph of the same electrophoretogram exposed for 3 weeks.

in vivo, also split into two components when electrophoresis in the first dimension was extended (Fig. 2a). These two components exhibited identical mobilities on polyacrylamide gels containing sodium dodecyl sulphate (results not shown). Subdivision of protein S7 into two species has not previously been noted on two-dimensional electrophoretograms of eukaryotic 40S ribosomal proteins.

Phosphorylation of 60S ribosomal proteins isolated directly from rat cerebral cortex (Fig. 4a) was qualitatively similar to comparable preparations obtained from cerebral slices after incubation *in vitro* (Roberts & Ashby, 1978; S. Roberts & B. S. Morelos, unpublished observations). Administration of [32 P]orthophosphate intracisternally resulted in labelling of at least three basic proteins of the 60S subunit *in situ* with electrophoretic mobilities corresponding to the mobilities of proteins L6, L14 and L19 (Fig. 4b). The most prominent radioactive component (L14) exhibited a mol.wt. of approx. 26000. Proteins L6 and L19 had mol.wts. of approx. 33000 and 24500 respectively. These molecular weights are comparable with those reported earlier for the analogous ribosomal proteins of the 60S subunit from rat liver (Tsurugi *et al.*, 1977, 1978). In the present investigations, the phosphorylated components of proteins L6, L14 and L19 could sometimes be detected in spots or tails closely following the non-phosphorylated forms of these proteins on stained electrophoretograms (Fig. 4a).

Several relatively acidic proteins of the cerebral 60S subunit were also phosphorylated *in vivo*. Certain of these proteins, which remained at the anodic origin during electrophoresis in the first dimension at pH 8.6, were more substantially labelled than the basic ribosomal proteins (Fig. 4b). At least four of these radioactive proteins exhibited

appreciable mobility when the 60S ribosomal proteins were subjected to electrophoresis toward the anode at pH 8.6 in the first dimension (Fig. 4c). The most highly labelled protein spot, which travelled most rapidly in the second dimension, was composed of two or more phosphorylated derivatives. The radioactive derivatives of this protein all exhibited mol.wts. of approx. 13500 on third-dimensional polyacrylamide gels containing sodium dodecyl sulphate. Molecular weights of the other radioactive proteins observed on the reversed-polarity two-dimensional gels varied from 33000 to 35000.

Influence of cyclic nucleotides on ribosomal protein phosphorylation in rat cerebral cortex in vivo

Administration of dibutyryl cyclic AMP intraperitoneally, 30min after intracisternal injection of [32 P]orthophosphate, significantly stimulated incorporation of radioactivity into ribosomal proteins of the cerebral 40S subunit in immature rats (Table 1, Expts. 1 and 2). Dosages varying from 1 to 5 mg/100 g body wt. were effective. Higher doses (e.g. 10 mg/100 g) tended to inhibit ribosomal protein phosphorylation and resulted in evidence of neural excitation, including hyperirritability and twitching. The potent inhibitor of cyclic nucleotide phosphodiesterase, 3-isobutyl-1-methylxanthine, also stimulated phosphorylation of cerebral 40S ribosomal proteins after intraperitoneal administration (Expt. 2). The effective dose of 3-isobutyl-1-methylxanthine was considerably lower than that of dibutyryl cyclic AMP (i.e. 0.25–0.5 mg/100 g), and doses in excess of 2 mg/100 g were toxic and sometimes lethal. Phosphorylation of cerebral 40S ribosomal proteins *in vivo* was also stimulated by very low doses of dibutyryl cyclic AMP and 3-isobutyl-1-methyl-

Fig. 4. Two-dimensional electrophoresis of proteins isolated from 60S ribosomal subunits of rat cerebral cortex. Immature rats were each given 2 mCi of [32 P]orthophosphate intracisternally in 20 μ l of 0.9% NaCl. The animals were killed by decapitation 1 h later. Proteins extracted from the large subunit were subjected to two-dimensional electrophoresis on polyacrylamide gels containing urea. The amounts of protein applied to the first-dimensional gels were (a) 275 μ g, (b) 368 μ g and (c) 346 μ g. (a) Two-dimensional electrophoretogram stained with Coomassie Brilliant Blue; the 60S proteins were applied to the anode in the first dimension. (b) Radioautograph of a similar two-dimensional gel of 60S proteins exposed for 3 weeks. (c) Radioautograph of a two-dimensional gel obtained when the 60S proteins were applied to the cathode in the first dimension; exposure time was 3 weeks. The 60S acidic proteins corresponding to the radioactive spots on reversed, polarity gels stained too weakly to provide a useful photograph.

Table 1. *Influence of cyclic nucleotides on phosphorylation of proteins in ribosomal subunits of rat cerebral cortex in vivo*
 Immature rats, weighing about 20g, were each given 2mCi of [32 P]orthophosphate intracisternally in 20 μ l of 0.9% NaCl. The substances listed were administered intracisternally in conjunction with the radioactive isotope, or intraperitoneally 30min later in approx. 0.25ml of 0.9% NaCl. Control animals in the latter groups received an equivalent volume of 0.9% NaCl. The rats were killed by decapitation 60min after the first injection. Free ribosomes were isolated and dissociated in the presence of high concentrations of KCl. The 60S subunits were redissociated under similar conditions. Purified ribosomal proteins were dissolved in 8M-urea containing 10mM- β -mercaptoethanol. Samples (10 μ l) were taken for determination of protein and radioactivity. Specific radioactivities have been corrected for radioactive decay over the 10-day period between administration of isotope and measurement of radioactivity.

Treatment	Dosage ($\mu\text{g/rat}$)	Specific radioactivity (c.p.m./ μg of protein)	
		40S	60S
Intraperitoneal administration			
Expt. 1			
Control (0.9% NaCl)	—	117.4	14.0
Dibutyryl cyclic AMP	500	172.7	10.3
Dibutyryl cyclic GMP	500	122.4	9.5
Expt. 2			
Control (0.9% NaCl)	—	145.6	13.7
Dibutyryl cyclic AMP	250	179.0	16.0
3-Isobutyl-1-methylxanthine	100	185.2	15.6
Intracisternal administration			
Expt. 3			
Control	—	107.8	9.4
Dibutyryl cyclic AMP	25	145.8	8.6
3-Isobutyl-1-methylxanthine	10	180.1	11.8
Expt. 4			
Control	—	135.3	13.3
Dibutyryl cyclic AMP	25	176.1	12.7
Dibutyryl cyclic GMP	25	140.2	10.5

xanthine administered by the intracisternal route (Expts. 3 and 4). The phosphodiesterase inhibitor produced a greater stimulation than the cyclic nucleotide. Dibutyryl cyclic GMP, administered by either route, did not stimulate phosphorylation of cerebral 40S ribosomal proteins (Expts. 1 and 4). Incorporation of radioactivity from [32 P]orthophosphate into proteins of the cerebral 60S subunit appeared to be unaffected by administration of these various substances. However, the accuracy of the latter measurements was limited by the relatively low rate of phosphorylation of the 60S ribosomal proteins. Uptake of 32 P into the cerebral cytosol was not enhanced at intervals of 30–120min after intracisternal administration of either dibutyryl cyclic AMP or 3-isobutyl-1-methylxanthine (Table 2).

Two-dimensional electrophoresis of ribosomal proteins isolated from the cerebral small subunit indicated that administration of dibutyryl cyclic AMP intraperitoneally or intracisternally selectively increased phosphorylation of the more highly phosphorylated congeners of the S6 protein, which travelled more slowly in the first dimension than did the non-phosphorylated form. The stimulatory effect of dibutyryl cyclic AMP was apparent in the stained electrophoretograms (Fig. 5b) and especially

Table 2. *Influence of dibutyryl cyclic AMP on 32 P uptake into cytosol of rat cerebral cortex in vivo*

Immature rats, weighing about 20g, were each given 2mCi of [32 P]orthophosphate and, where indicated, 25 μ g of dibutyryl cyclic AMP or 3-isobutyl-1-methylxanthine in a total of 20 μ l of 0.9% NaCl by the intracisternal route. The rats were killed by decapitation at stated intervals thereafter. 32 P uptake was estimated from the amount of radioactivity in the trichloroacetic acid-soluble material of the post-mitochondrial supernatant fraction pooled from six animals in each group. The results shown are representative of three separate experiments.

Time after administration (min)	$10^{-5} \times ^{32}\text{P uptake (c.p.m./g of cortex)}$		
	Control	Dibutyryl cyclic AMP	3-Isobutyl-1-Methylxanthine
30	1995	2083	1646
60	2652	2486	2535
120	4127	3825	3918

in the radioautographs (Fig. 5e), as increases in the relative proportions of stained and radioactive material in the more acidic components of the S6

protein. Dibutyl cyclic GMP was without effect on this phenomenon (Figs. 5c and 5f). The stimulatory action of dibutyl cyclic AMP on S6 protein phosphorylation was even more clearly evidenced in other experiments, 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. In control animals, the radioactivity in the S6 protein region was associated with four or five protein species on the gel (Fig. 6). An additional congener of the S6 protein, which moved most rapidly in the first dimension at pH 8.6, was non-radioactive. Administration of dibutyl cyclic AMP increased the relative amounts of the phosphorylated congeners of the S6 protein, as judged by changes in the absorbance profile. This phenomenon was reflected in the striking shift in incorporation of ^{32}P into the two most highly phosphorylated congeners of the S6 protein. Since the total incorporation of radioactivity into the cerebral 40S ribosomal proteins was significantly increased by the cyclic nucleotide under these circumstances (Table 1), it is unlikely that the differences between the profiles of control animals and animals given dibutyl cyclic AMP can be ascribed solely to variations in the rate of dephosphorylation. Dibutyl cyclic GMP had little or no effect on either the absorbance or radioactivity profile of S6 protein (Fig. 6). The effects of

intraperitoneal or intracisternal administration of 3-isobutyl-1-methylxanthine on the S6 protein profiles were comparable with those of dibutyl cyclic AMP (results not shown).

Influence of cyclic nucleotides on polyribosomal aggregation in rat cerebral cortex in vivo

Polyribosomal disaggregation has been described in cell-free preparations of brain obtained from young animals subjected to a wide variety of challenges *in vivo* (Roberts, 1977). Since phosphorylation of the S6 ribosomal protein may normally be a more active process in polyribosomes than in monoribosomes (Bitte & Kabat, 1972; Leader & Coia, 1978a), experiments were carried out to determine whether polyribosomal profiles in cerebral cortex of the immature rat were altered by administration of the dibutyl derivatives of cyclic AMP and cyclic GMP. Leader & Coia (1978a) reported that treatment of baby-hamster kidney fibroblasts in culture with cyclic AMP and theophylline resulted in polyribosomal disaggregation.

Parenteral administration of dibutyl cyclic AMP, in concentrations sufficient to stimulate phosphorylation of the cerebral S6 ribosomal protein, did not alter the state of aggregation of cerebral polyribosomes (Fig. 7). The proportion of polyribosomes (trimer or larger) in preparations

Fig. 5. Influence of cyclic nucleotides on two-dimensional electrophoretograms of proteins isolated from 40S ribosomal subunits of rat cerebral cortex

Immature rats were each given 2 mCi of [^{32}P]orthophosphate intracisternally in 20 μl of 0.9% NaCl. In two groups of animals, the intracisternal solution also contained either 25 μg of dibutyl cyclic AMP or 25 μg of dibutyl cyclic GMP. All rats were killed by decapitation 60 min later. 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, 423 μg ; dibutyl cyclic AMP, 397 μg ; dibutyl cyclic GMP, 452 μg . (a), (b) and (c) Electrophoretograms stained with Coomassie Brilliant Blue; (d), (e) and (f) radioautographs exposed for 1 day.

Fig. 6. Influence of cyclic nucleotides on phosphorylation of the S6 ribosomal protein of rat cerebral cortex *in vivo*

Immature rats were each given 2mCi of [32 P]orthophosphate intracisternally in 20 μ l of 0.9% NaCl. After 30min, each animal was injected intraperitoneally with 0.5mg of dibutyryl cyclic AMP in 0.25 ml of 0.9% NaCl (b), the same dose of dibutyryl cyclic GMP (c) or an equal volume of 0.9% NaCl (a). All rats were killed by decapitation 1 h after the first injection. The amounts of protein applied to the first-dimensional gels were: control, 519 μ g; dibutyryl cyclic AMP, 402 μ g; dibutyryl cyclic GMP, 385 μ g. After the two-dimensional slab gels were stained with Coomassie Brilliant Blue, a horizontal rectangular section corresponding to the S6 protein and its more acidic derivatives was cut out and scanned for A_{600} (—). This section of the gel was then divided into 1 mm slices for determination of radioactivity (●). Radioactivity measurements were not corrected for decay.

pelleted through heavy sucrose for 20h equalled about 80%, and was unaffected, over intervals that varied from 30min to 2h, by administration of cyclic AMP intraperitoneally or intracisternally in concentrations up to twice as high as those shown in Table 1. Dibutyryl cyclic GMP, which did not stimu-

Fig. 7. Influence of cyclic nucleotides on sedimentation properties of cerebral polyribosomes of immature rats

The cyclic nucleotides (b and c) were administered intraperitoneally in amounts equivalent to 2.5mg/100g body wt. dissolved in 0.9% NaCl. (a) Control animals received an equal volume of 0.9% NaCl. The animals were killed 30min later and postmitochondrial supernatant fractions were prepared from cerebral cortices pooled from three animals in each group. Polyribosomes were isolated by centrifugation of these fractions for 20h at 226400g. Sucrose-density-gradient analyses of the purified polyribosomes were carried out as described in the text. The monoribosome peaks are designated '80S'. The proportion of polyribosomes (trimer or larger) in each group, as determined by planimetry, was as follows: control, 82.1%; dibutyryl cyclic AMP, 80.7%; dibutyryl cyclic GMP, 82.7%.

late S6 protein phosphorylation, was also without effect on the state of aggregation of cerebral ribosomes under these conditions. Comparable results were obtained when the polyribosomes were isolated by centrifugation of postmitochondrial supernatant fractions for only 4h instead of 20h, except that the proportions of polyribosomes were somewhat greater (results not shown). These experiments clearly demonstrate that the diverse effects of parenterally administered cyclic nucleotides on ribosomal protein phosphorylation in rat cerebral cortex cannot be attributed to variations in relative concentrations of monoribosomes and polyribosomes.

Discussion

The present investigations demonstrate that several structural proteins of both ribosomal subunits undergo phosphorylation in mammalian cells *in situ*. The roster of constitutive ribosomal proteins of rat cerebral cortex that incorporated radioactivity from [32 P]orthophosphate *in vivo* included proteins with the electrophoretic mobilities and molecular weights of S2, S3a, S5 and S6 of the 40S subunit, as well as L6, L14 and L19 of the large subunit. In addition, at least four proteins of the 60S subunit, which exhibited relatively acidic properties on two-dimensional polyacrylamide gels, were labelled in cerebral cortex *in vivo*. Phosphorylation of proteins S2, S3, S6, L6 and L14 had earlier been described in experiments with rat cerebral-cortical slices *in vitro* (Roberts & Ashby, 1977b, 1978). The component of the S3 protein complex which is phosphorylated *in vivo* (S3a) was not resolved in the latter investigations. Phosphorylation of a basic ribosomal protein with the electrophoretic properties of S2 has also been described in HeLa cells infected with vaccinia virus (Kaerlein & Horak, 1976) and in Krebs II ascites-tumour cells *in vitro* (Rankine *et al.*, 1977). Moreover, phosphorylation of proteins with the mobilities of S3, L6 and L14 has been noted in ascites-tumour cells *in vitro* (Leader & Coia, 1978c). The relatively acidic phosphorylated proteins of the cerebral 60S subunit probably correspond to acidic proteins of the large subunit that are phosphorylated in yeast cells (Zinker & Warner, 1976), Krebs II ascites-tumour cells (Rankine *et al.*, 1977; Leader & Coia, 1978b), baby-hamster kidney fibroblasts (Leader & Coia, 1978b), HeLa cells in culture (Horak & Schiffmann, 1977; Schiffmann & Horak, 1978) and rat liver (Arpin *et al.*, 1978). Several additional ribosomal proteins of both subunits are phosphorylated in diverse ribosomal preparations exposed to protein kinases *in vitro* (Eil & Wool, 1971, 1973; Traugh & Porter, 1976; Horak & Schiffman, 1977; Schiffmann & Horak, 1978), but it is not known whether these phosphorylations reflect cellular processes.

Quantitative, as well as qualitative, differences were noted in the phosphorylation of cerebral ribosomal proteins *in vivo* and *in vitro*. Thus earlier experiments had shown that the specific radioactivity of 60S proteins was uniformly greater than that of the small subunit proteins when cellular preparations of rat cerebral cortex were incubated *in vitro* with [32 P]orthophosphate in the absence of exogenous cyclic AMP (Roberts & Ashby, 1978). In contrast, phosphorylation of cerebral 60S ribosomal proteins *in situ* was much slower than phosphorylation of 40S proteins in the present studies. These results may indicate that phosphate group turnover in the cerebral S6 protein normally proceeds quite rapidly

in vivo, whereas dephosphorylation of this protein exceeds rephosphorylation *in vitro*. This process could result in loss of the more highly phosphorylated congeners of the S6 protein and a reduction in the relative rate of labelling of the 40S proteins. An additional explanation for the observed differences in phosphorylation state of cerebral ribosomal proteins *in vivo* and *in vitro* is suggested by the finding that incubation of cerebral-cortical slices results in partial disaggregation of polyribosomes (Ashby & Roberts, 1975). Phosphorylation of the S6 ribosomal protein in eukaryotic cells appears to be more active in polyribosomes than in monoribosomes (Bitte & Kabat, 1972; Leader & Coia, 1978a).

Phosphorylation of S6 and other ribosomal proteins in rat cerebral cortex *in situ* appears to occur at a more rapid rate than in other mammalian tissues that have been studied. Incorporation of radioactivity from [32 P]orthophosphate into a wide spectrum of ribosomal proteins of both cerebral subunits was readily observed on radioautographs of two-dimensional electrophoretograms. Moreover, the phosphorylated derivatives of the basic ribosomal proteins could frequently be detected in spots or tails closely following the non-phosphorylated species in the first dimension on stained two-dimensional electrophoretograms. In contrast, phosphorylated derivatives of basic ribosomal proteins other than S6 have not been described on stained electrophoretograms of ribosomal preparations from rat liver or muscle (Sherton & Wool, 1974b; Treloar *et al.*, 1977). Several lines of evidence support the view that the phosphorylated state of ribosomal proteins in rat cerebral cortex reflects continuing neural activity and the normally high activity of cyclic nucleotide systems in the mammalian brain (Butcher & Sutherland, 1962; Sutherland *et al.*, 1962; von Hungen & Roberts, 1974; Daly, 1977), rather than stress-induced alterations in cerebral production of cyclic nucleotides associated with handling and killing of the animals. Thus exposure of rats to a wide variety of psychological and physical stresses, including handling, may actually decrease brain concentrations of cyclic AMP (Skinner *et al.*, 1978). Moreover, although cyclic AMP and cyclic GMP accumulate in the central nervous system after decapitation (Breckenridge, 1964; Kakiuchi & Rall, 1968; Dinnendahl, 1975), a similar spectrum of phosphorylated proteins was observed in cerebral cortices of rats killed under anaesthesia with pentobarbital sodium (S. Roberts & B. S. Morelos, unpublished observations). This procedure not only does not produce an elevation of cyclic nucleotide concentrations in the brain, but also prevents the increase in these concentrations resulting from decapitation (Daly, 1977).

The selectivity and specificity of cyclic AMP and its derivatives in stimulating phosphorylation

of S6 protein in rat cerebral cortex *in vivo* and *in vitro* has been noted with other cellular systems, including rat liver *in vivo* (Gressner & Wool, 1976) and hamster islet tumour cells *in vitro* (Schubart *et al.*, 1977). The present experiments suggest that phosphorylation of cerebral ribosomal proteins other than S6 may be directed by cyclic nucleotide-independent protein kinases. However, cyclic nucleotide-induced alterations in the phosphorylation of ribosomal proteins other than S6 may be obscured by slow turnover of phosphate groups in these proteins. This possibility is consistent with the observation that the catalytic subunit of cyclic AMP-regulated protein kinase from rat cerebral cortex was capable of phosphorylating the S2 and S3 ribosomal proteins, as well as S6, in isolated cerebral ribosomes incubated *in vitro* with [γ - 32 P]ATP (T. A. Francis & S. Roberts, unpublished observations).

Available data are inadequate to determine whether many or all of the ribosomal protein phosphorylations detected in rat cerebral cortex *in vivo* are normally of wide occurrence in eukaryotic cells *in situ*. In any event, the relative capacities of different ribosomal proteins to be phosphorylated, as well as regulation of these processes, appear to vary greatly in different cell types. Since each ribosome presumably possesses only one copy of each of the various species of phosphorylated proteins, and many different combinations are possible, the heterogeneity of the ribosome pool could be very great. Although the functional roles of ribosomal protein phosphorylations remain to be elucidated, evidence has been cited from cross-linking experiments that ribosomal protein S6 may be involved in the binding of mRNA to the 40S subunit in eukaryotic cells (Anderson *et al.*, 1977). In the mammalian nervous system, differential regulation of ribosomal protein phosphorylation by cyclic nucleotides and other consequences of neural activation may provide a mechanism whereby the translational activity of diverse classes of ribosomes in specific neuronal pathways can be modulated selectively during the transmission of neural impulses.

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