Developmental Changes in the Cyclic AMP-Dependent Phosphorylation and Dephosphorylation of a Protein Endogenous to Murine Brain and Liver

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Received July 18, 1977

Brain and liver cytosol extracts from mice of different ages were incubated with (γ-^{32}P)ATP. The phosphorylated substrates were separated by gel electrophoresis and examined by autoradiography. The amount of P^{32} that could be incorporated into a 49,000 M.W. protein (called protein 49) postnatally increased in brain but decreased in liver. Cyclic AMP stimulated both the phosphorylation and dephosphorylation of liver protein 49 to a greater extent in adults than in neonates. Brain protein 49 phosphorylation was more sensitive to cyclic AMP in neonates than in adults.

Since cyclic AMP-modulated protein phosphorylation is involved in several aspects of cell physiology (1), it would seem important to note any developmental changes in this reaction. Postnatal changes in the specific activities of cyclic AMP-dependent protein kinase enzymes (2-7) and in intracellular cyclic AMP concentrations (8-12) have been demonstrated in brain and liver. No reports of developmental changes in the effects of cyclic AMP on the phosphorylation of endogenous protein substrates have appeared. A 49,000 M.W. phosphoprotein (called protein 49) has been found in the particulate and soluble fractions of several rat and bovine tissues (13). The rate and extent of both its phosphorylation and dephosphorylation was regulated by cyclic AMP (13). Equivalent concentrations of cyclic GMP and cyclic AMP had opposite effects on murine brain protein 49 phosphorylation when the reaction was assayed in the presence of Ca^{++} (14). Steroid hormones induced the synthesis of a phosphoprotein phosphatase which dephosphorylated protein 49 in target tissues (15). Ontogenetic changes in the effects of cyclic AMP on both the phosphorylation and dephosphorylation of this endogenous protein substrate are herein described. Some of this work has appeared in preliminary form (16).

Methods

Tissues were removed from strain A/Umc mice, which had been fed ad libitum and ranged in age from 17 days gestation to 90 days after birth. Tissues from one litter of mice 11 days of age and younger were pooled; for older mice, tissues from

ISSN 0006-291X

2-3 mice were pooled. Tissues were homogenized at 4° and the following consecutive centrifugations were performed: 3000 x g, 5 min; 27,000 x g, 15 min; and 105,000 x g, 40 min. This last supernatant (cytosol) was used in all experiments. Protein concentrations were determined by the method of Lowry et al. (17).

The endogenous protein phosphorylation assay has been previously described (13). Cytosol fractions (15-50 µg of brain or 250-550 µg of liver) were incubated with 50 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH=6.5; either 10 mM $MgCl_2$, 5 mM $CaCl_2$ or 5 mM $ZnCl_2$; and 1.0-1.5 µM (γ-^{32}P)ATP (5-20 Ci/mmole) with or without cyclic nucleotides in a 100 µl volume. The reaction was terminated after 3 min at 30° with 50 µl of a sodium dodecyl sulfate (SDS)-dithiothreitol "stop" solution (18), and boiled for 2 min. Aliquots were subjected to SDS-polyacrylamide slab gel electrophoresis, and autoradiographs of the dried gels were made. The amount of phosphorylation, expressed as arbitrary units, was determined by measuring the band density on the autoradiograph with an Ortec 4310 Microdensitometer (19).

To study dephosphorylation, cytosols were first phosphorylated in the presence of Zn^{++} as described above. EDTA and unlabeled ATP were then added to give final concentrations of 15 mM and 0.7 mM, respectively, with or without 15 µM cyclic AMP. Incubation was continued for 10 min, the reaction terminated, and electrophoresis was done as described.

Results

Developmental Changes in the Phosphorylability of Protein 49 in the Absence of Cyclic AMP

The pattern of proteins phosphorylated by a tissue extract is determined in part by the divalent cation included in the assay as well as by the tissue source (Fig. 1). The mechanism by which cations modify the substrate-specificity of protein kinases is unknown. Protein 49 was the most heavily phosphorylated protein in brain cytosol with each cation used, and in liver cytosol when assayed in the presence of either Mg^{++} or Zn^{++}. In liver cytosol phosphorylated in the presence of Ca^{++}, however, a 53,000 M.W. protein incorporated more (^{32}P)-phosphate than protein 49.

Developmental changes in the phosphorylability of protein 49 were examined by phosphorylating equal amounts of cytosol from mice of different ages in the absence of cyclic AMP. Brain protein 49 phosphorylation began increasing before birth and continued to increase until a plateau was reached between 10-21 days (Fig. 2a). At this age phosphorylation had increased 8-fold above the fetal level. Liver protein 49 phosphorylation decreased with age and by the fifth postnatal week was less than 20% of the level at birth (Fig. 2b). These developmental time courses were not appreciably affected by the divalent cation present during assay.

Developmental Changes in the Effects of Cyclic AMP on Protein 49 Phosphorylation

The phosphorylation of protein 49 in rat and bovine tissues was affected by the presence of cyclic AMP, and the nature of this effect (whether stimulatory or

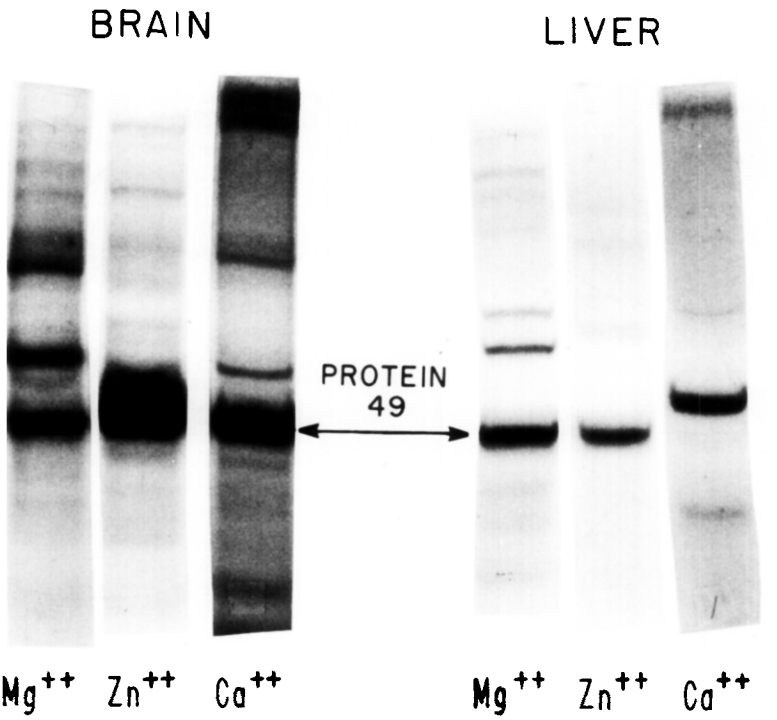

Figure 1: Endogenous protein phosphorylation in brain and liver cytosol, assayed in the presence of either Mg^{++}, Zn^{++}, or Ca^{++}.

inhibitory) depended on the divalent cation present during assay (13, 19). In both murine brain and liver the phosphorylation of protein 49 was stimulated by 10 μM cyclic AMP in the presence of Mg^{++}, and inhibited by 10 μM cyclic AMP in the presence of Zn^{++} or Ca^{++} (Table 1). These effects indicate that the 49,000 M.W. region in these mouse tissues corresponds to the protein 49 previously described in rat and bovine tissues. The phosphorylation of protein 49 was stimulated by 0.6 μM cyclic AMP in liver and brain when assayed in the presence of Ca^{++}. The biphasic response to cyclic AMP in the presence of Ca^{++} may involve alterations in the affinity of protein kinase for the CaATP substrate. No developmental differences were observed in the extent of inhibition by 10 μM cyclic AMP of protein 49 phosphorylation in the presence of Zn^{++} or Ca^{++}. The stimulatory effect of cyclic AMP in the presence of Mg^{++} increased with age in liver but decreased with age in brain. Stimulation of protein 49 phosphorylation by 0.6 μM cyclic AMP in the presence of Ca^{++} did

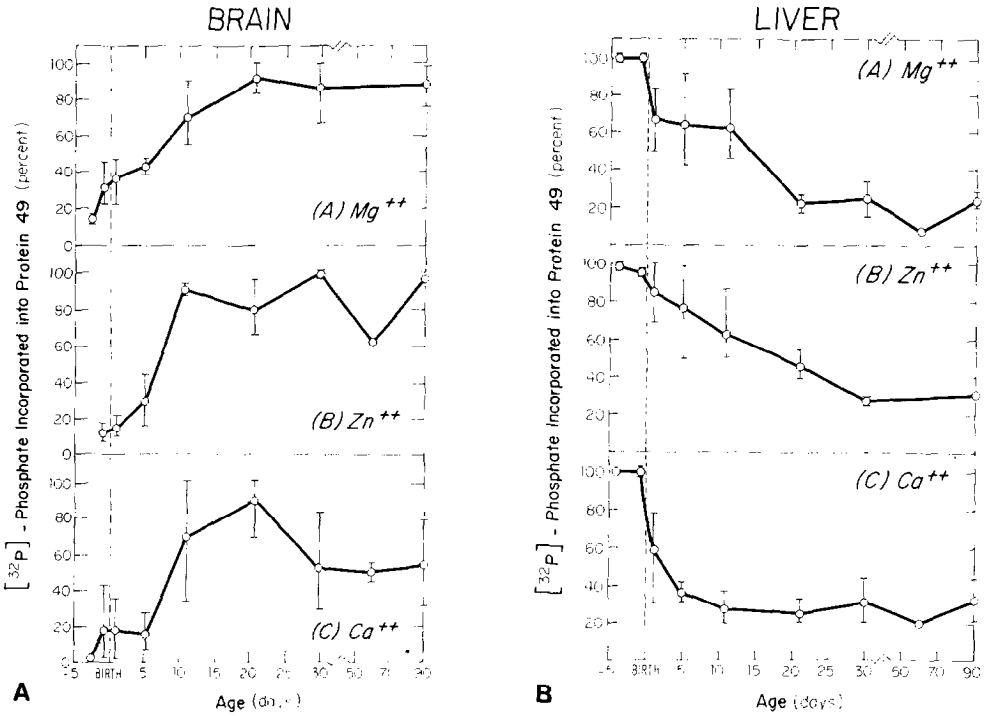

Figure 2: Protein 49 phosphorylation in brain (A) and liver (B) cytosols as a function of age. Phosphorylation was assayed in the presence of either Mg^{++}, Zn^{++} or Ca^{++}. The results of four experiments were compared by expressing the data as a percent of maximal protein 49 phosphorylation in a single experiment. The means and ranges of these percentages are shown.

not vary with age in liver, but was greater in neonatal brain extracts than in adult brain. The effect of 0.6 μM cyclic AMP on protein 49 phosphorylation could be mimicked by 10 μM cyclic GMP (14).

Developmental Changes in the Effect of Cyclic AMP on Protein 49 Dephosphorylation

Cyclic AMP dramatically stimulated the dephosphorylation of liver protein 49 but only slightly increased the amount of phosphate removed from brain protein 49 (Table 2). While there was no apparent age-related change in the dephosphorylation of brain protein 49, more phosphate was removed from liver protein 49 in the absence of cyclic AMP in neonatal extracts than in adults. The amount of phosphate removed from liver protein 49 in the absence of cyclic AMP decreased with age, until by ca. 50 days protein 49 dephosphorylation only occurred if cyclic AMP was present (Fig. 3).

TABLE 1: EFFECT OF CYCLIC AMP ON PROTEIN 49 PHOSPHORYLATION IN NEONATAL AND ADULT MURINE TISSUES

TISSUE	N	CATION	CYCLIC AMP (μM)	% EFFECT OF CYCLIC AMP NEONATAL	ADULT
LIVER	8	Mg^{++}	10	226 ±27	400 ±68
	8	Zn^{++}	10	49 ± 7	49 ± 6
	6	Ca^{++}	10	45 ± 7	55 ±24
	5	Ca^{++}	0.6	191 ±37	204 ±48
BRAIN	11	Mg^{++}	10	164 ±25	107 ± 6
	7	Zn^{++}	10	87 ± 6	85 ± 9
	8	Ca^{++}	10	61 ±18	53 ±10
	8	Ca^{++}	0.6	293 ±88	118 ±12

Phosphorylation was carried out in N separate experiments as described in Methods. The percent effect of an indicated concentration of cyclic AMP on the phosphorylation of protein 49 (+ cyclic AMP/-cyclic AMP) is expressed as Mean ± S.E.M.

TABLE 2: EFFECT OF CYCLIC AMP ON THE DEPHOSPHORYLATION OF PROTEIN 49 IN NEONATAL AND ADULT TISSUES

	PERCENTAGE ^{32}P REMOVED FROM PROTEIN 49			
	(- CYCLIC AMP)		(+ CYCLIC AMP)	
	NEONATAL	ADULT	NEONATAL	ADULT
LIVER	36 ± 6	9 ± 4	83 ± 3	77 ± 6
BRAIN	36 ± 5	29 ± 5	46 ± 8	44 ± 6

Dephosphorylation was carried out as described in Methods. Radioactive phosphate removed from protein 49 is expressed as percent of that present at the start of the dephosphorylation reaction. Each value represents Mean ± S.E.M. for 8 experiments.

Discussion

Changes in the in vitro phosphorylability of endogenous protein substrates of a given cell or tissue type under different biological states (e.g., different cell densities (20) or different states of malignancy (21)) have been reported. Several

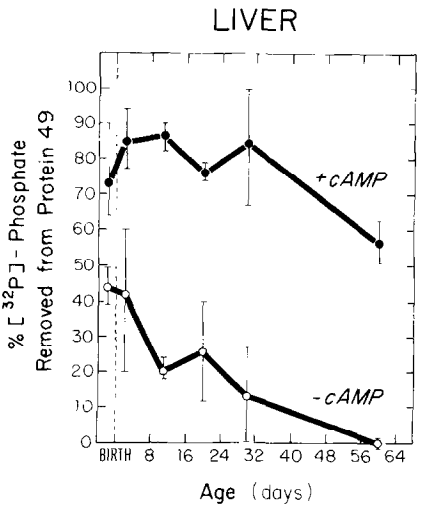

Figure 3: Developmental changes in the effect of cyclic AMP on the dephosphorylation of liver protein 49. Liver cytosol from mice of different ages was prepared, and the dephosphorylation reaction was done in the presence (+) or absence (-) of 15 μM cyclic AMP. The percentage of (^{32}P)-phosphate removed from protein 49 (mean and ranges) in 4 experiments is shown.

mechanisms have been postulated to account for such changes (20,21) including alterations in the concentration of the protein substrate, occupancy of phosphorylable sites by phosphate due to phosphorylation reactions which had occurred in vivo prior to assay, and changes in the types and amounts of protein kinase/phosphoprotein phosphatase enzymes or in the concentrations of substances which affect the activity of these enzymes. We have found no significant differences between neonatal and adult cyclic AMP-dependent protein kinase specific activities in murine brain or liver (22). The age-dependent changes in protein 49 phosphorylability were independent of which cation was present during assay (Fig. 2). Since cyclic AMP stimulates protein 49 phosphorylation in the presence of Mg^{++} but is inhibitory in the presence of Zn^{++}, the ontogenetic changes in phosphorylability measured in the absence of cyclic AMP were not a reflection of altered in vivo cyclic AMP levels. The mechanism for the developmental differences described here are unknown.

A decreased stimulation of brain protein kinase activity by cyclic AMP (2) and an increased stimulation of liver protein kinase activity by cyclic AMP (7) in

older rats have been described. Such changes could be accounted for by age-dependent alterations in the intracellular cyclic AMP concentrations of these tissues. The greater the amount of cyclic AMP present in vivo, the less effect cyclic AMP would have in in vitro assays. Rat brain cyclic AMP levels are higher in adults than in neonates (8,9), while liver cyclic AMP levels are maximal in neonates (10,11). The age-related alterations in the effects of cyclic AMP on the phosphorylation and dephosphorylation of protein 49 are consistent with the direction of these reported developmental changes in cyclic AMP levels.

There is evidence that protein 49 is the regulatory subunit of a cyclic AMP-dependent protein kinase and that its phosphorylation represents an autophosphorylation reaction. Purified autophosphorylated bovine brain protein kinases co-migrated with protein 49 on SDS gels (23). When purified protein kinase preparations (24) or crude tissue extracts (13) were labelled with 8-azido cyclic AMP, a photoaffinity analog of cyclic AMP specific for cyclic AMP binding sites, the major labelled protein co-migrated with protein 49 on SDS gels. Cyclic AMP reduced the extent of autophosphorylation of bovine brain kinase assayed in the presence of Zn^{++} or Ca^{++} (23). Protein 49, cyclic AMP-dependent protein kinase and cyclic AMP-dependent phosphatase activities were jointly solubilized from synaptic membranes with detergent or salt (25). It should be mentioned, however, that a similar solubilization treatment of erythrocyte membranes (26,27) resulted in extraction of cyclic AMP-binding activity while the major 48,000 M.W. phosphoprotein was retained in the detergent-insoluble fraction. Whatever the identity of protein 49, it is one of the few proteins phosphorylated in all tissue extracts studied, including extracts from tumors and cultured cells (21). Moreover, the sensitivity of its phosphorylation and dephosphorylation to cyclic AMP/divalent cation interactions make it unique among eukaryotic phosphoproteins.

The physiological significance of the decreased stimulation of brain protein 49 by cyclic AMP is unclear. The increased sensitivity of the turnover of phosphate from liver protein 49 by cyclic AMP in older mice may be functionally related to the complex ontogenetic changes that have been described in the sensitivity of liver adenylate cyclase to various hormones (28,29).

Acknowledgements

I would like to thank Miss Lora Hogy, Mrs. Carolyn McSwigan and Mr. Laurence Taaffe for their excellent technical assistance. This work was supported by a Basil O'Connor Starter Grant from the National Foundation - March of Dimes and USPHS grants CA 16228 and GM22492.

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