RECIPROCAL CHANGES IN Ca^{2+}/PROTEIN ACTIVATOR-DEPENDENT AND-INDEPENDENT CYCLIC AMP PHOSPHODIESTERASE DURING THE DEVELOPMENT OF CHICK EMBRYOS

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SUMMARY

The changes in hepatic Ca^{2+}/protein activator-dependent and independent cAMP phosphodiesterase activity during the development of chick embryo were investigated. Nearly all of the cAMP phosphodiesterase found in the liver during the early embryonic stage was the Ca^{2+}/protein activator-dependent enzyme; however, this enzyme progressively diminished and the independent enzyme increased proportionately with embryonic development. In the adult liver, little or no Ca^{2+}/protein activator-dependent enzyme activity could be observed. These results indicate that the concentration of intracellular cAMP may be regulated by these two enzymes in compliance with the development of the chick.

INTRODUCTION

Since 3',5'-cyclic AMP (cAMP) plays important regulatory roles in many biological functions(1,2), it is reasonable to assume that the intracellular concentrations of cAMP are delicately controlled. This consideration, in the main, has led a numbers of researchers(3-8) to investigate the regulatory properties of cyclic nucleotide phosphodiesterase, the enzyme responsible for the hydrolytic destruction of nucleotide. Regarding the mechanism of regulation for this enzyme, Teo et al.(9) has demonstrated that the partially purified enzyme can be reactivated by a specific protein activator found in crude bovine heart extract. Other workers have also demonstrated the existence of such a protein activator in mammalian tissues(4-5). Recently, Teo and Wang(9), and Kakiuchi et al.(10) have shown that the activation of cyclic nucleotide phosphodiesterase is completely dependent on the protein activator and Ca^{2+}.

While investigating the regulation of cAMP levels, we observed that the

ratio of the activity of the Ca^{2+}/protein activator-dependent enzyme to that of the activator-independent enzyme in the liver differs according to the developmental stage of the chick embryo. The present study reveals the degree to which the Ca^{2+}/protein activator-independent cAMP phosphodiesterase fraction progressively increases, while the dependent enzyme fraction progressively decreases during development. These results suggest the participation of Ca^{2+}/protein activator in the regulation of cAMP phosphodiesterase activity in the liver at the early stage of chick embryonic development.

MATERIALS AND METHODS

Fertilized eggs from Rhode Island Red hens were obtained from the Sato Farms, Kyoto. The [^{3}H]-cAMP was obtained from New England Nuclear. The snake venom (Naja naja) was obtained from the Sigma Chemical Company. All the other reagents were purchased from Nakarai Chemicals, Kyoto.

Enzyme assay

The assay used for phosphodiesterase activity was that described by Thompson and Appleman(11). The first stage reaction mixture of 0.5 ml contained 50 mM Tris-Cl^- buffer(pH 8.0), 10 mM $MgCl_2$, 0.5 % bovine serum albumin, 1 μM cAMP, and an appropriate concentration of enzyme. After incubation for 30 min at 37°C, the tubes containing the reaction mixture were transferred to a boiling water bath for 1 min to terminate the reaction. The reaction mixture was then further incubated with sufficient snake venom, for 30 min at 37°C. The reaction was stopped by the additions of 0.8 ml 1:1 slurry Bio-Rad resin, AG 1X2, 200-400 mesh. The amount of radioactivity of the ^{3}H-adenosine left in the supernatant after centrifugation in a clinical centrifuge was measured by means of a liquid scintillation spectrometer. All assays were carried out at 20 % or less total reaction to be in the linear portion of the enzyme assay. One unit of enzyme activity was defined as the amount cleaving nmole of cAMP per hour. Protein was determined by the method of Lowry et al.(12).

Enzyme preparation

The livers isolated from the chick embryos were homogenized in 9 volumes of 50 mM Tris-Cl^- buffer, containing 10 mM $MgCl_2$(pH 7.5). The homogenate was centrifuged at 105,000 X g for 2 hrs. The supernatants were then applied to a DEAE-cellulose column(2.0 X 5.0 cm), previously equilibrated with 50 mM Tris-Cl^-, pH 7.5, containing 10 mM $MgCl_2$. The column was washed with 250 ml of the same buffer and developed with a linear 400 ml KCl gradient from 0 to 0.5 M KCl in the same buffer.

Enzyme activator preparation

Isolated brains from 21st day chick embryos were homogenized in 9 volumes of 50 mM Tris-Cl^- buffer(pH 7.5), and centrifuged at 105,000 X g for 1 h. The supernatant was boiled for 10 min, and centrifuged at 10,000 X g for 20 min. The supernatant was dialyzed against 4 liters of 50 mM Tris-Cl^-(pH 7.5) overnight, and used for the experiments.

RESULTS AND DISCUSSION

To confirm the Ca^{2+}-dependence of the hepatic cAMP phosphodiesterase of chick embryo, EGTA was added to the reaction mixture. As seen in Fig. 1, 1 mM

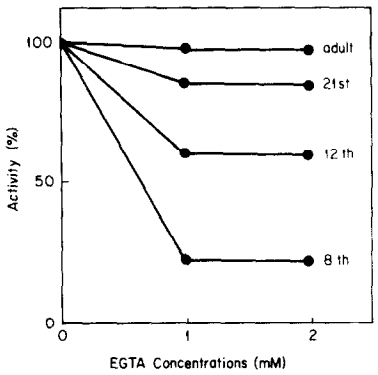

Fig. 1.
Effect of EGTA concentrations on hepatic cAMP phosphodiesterase activity in developing chick embryos and adult hens. The assay was carried out with 0.09-0.16 unit enzyme. Activity in the absence of EGTA was settled as 100 %. The values are the mean of two experiments.

EGTA brought an approximately 80 % inhibition in the 105,000 X g supernatant prepared from the liver of an 8th day embryo as enzyme. Futhermore, if Ca^{2+} was added in the assay, a significantly high enzyme activity was also observed (data not shown). These results indicate that the chick liver also contains Ca^{2+}/protein activator-dependent cAMP phosphodiesterase as seen in rat brain (5) and in bovine heart(12).

The inhibitory capacity of EGTA appears to be inversely proportionate to chick embryo development, i.e. the rate of inhibition decreases with embryo growth until little or no inhibition can be observed in the adult liver. Futhermore, the EGTA inhibitory rate found in a mixture of equal volumes of the 8th day and adult enzyme preparations showed the mean value as that calculated from the respective values (data not shown). These results suggest that the Ca^{2+}/protein activator-dependent enzyme would gradually decrease and the independent one increase as the embryo develops.

To clarify this point further, DEAE-cellulose column chromatography was carried out with a 105,000 X g supernatant prepared from the livers of several embryonic stages, and the activities were assayed in the presence of Ca^{2+}/ protein activator and EGTA, respectively. As depicted in Fig. 2-B, two dis-

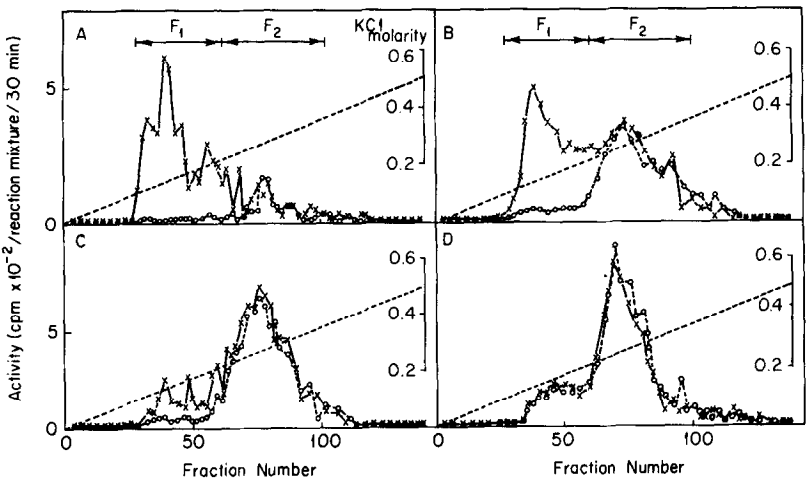

Fig. 2.
Fractionation of the hepatic cAMP phosphodiesterase activity in chick embryos and adults. The DEAE-cellulose chromatography was performed as described in the text. The flow rate was 3 ml per 5 min and the fraction size was 3 ml. Aliquots of the column fraction were assayed for phosphodiesterase activity in the presence of 1mM EGTA (o----o), or 1 mM $CaCl_2$ plus 40 μg protein activator (x——x). The fractions comprising tubes #28-60 and #61-100 were designated as F_1 and F_2, respectively.

tinct fractions containing cAMP phosphodiesterase were detected with the 12th day embryo, when it was assayed in the presence of Ca^{2+} and protein activator. These fractions were designated as F_1 and F_2, respectively. Note that if the assays were carried out in the presence of EGTA, a very low, but not zero, enzyme activity was detected in F_1 while no decrease in enzyme activity was observed in F_2. These results indicated that in the 12th day embryo liver, F_1 contained mainly Ca^{2+}/protein activator-dependent, and F_2 contained mainly independent cAMP phosphodiesterase. In addition, the elution patterns of the cAMP phosphodiesterase of the 8th day, 21st day, and adult livers are depicted in Figs. 2-A, C, and D, respectively. The data of Fig. 2 clearly show the degrees to which the Ca^{2+}/protein activator-independent cAMP phosphodiesterase progressively increased while the dependent enzyme conversly diminished during embryo development. The highest value of F_1/F_2 calculated from the data of

TABLE 1

Comparison of Enzyme Activites between F_1 and F_2

Stages	Fractions	Total Activity units (a)	F_1/F_2 (b)	Dependent / Independent (c)
8th day	F_1	28.1	3.5	44.5
	F_2	8.1		1.5
12th day	F_1	17.6	1.0	11.3
	F_2	17.7		1.1
21st day	F_1	9.0	0.3	4.9
	F_2	30.8		1.1
Adult	F_1	13.1	0.4	1.1
	F_2	30.6		0.9

a. The assay was carried out in the presence of both 1 mM $CaCl_2$ and 40 μg protein activator. The values are the mean of the two experiments. The recovery of enzyme activity after DEAE-cellulose column chromatography (Fig. 2) was found to be 40-45 % of the applied activity in all experiments, when assays were carred out in the presence of Ca^{2+} plus protein activator.
b. The ratio was calculated from the values of column (a).
c. The ratio of Ca^{2+}/protein activator-dependent to independent enzyme activity was calculated from the values obtained from the two assay methods described in the legend to Fig. 2.

Fig. 2 was found in the 8th day embryo, the earliest embryonic stage studied here (Table 1). The liver of the 12th day embryo contains approximately equal amounts of the two forms of cAMP phosphodiesterase, and 21st day and adult livers show large amount of the Ca^{2+}/protein activator-independent enzyme, i.e., the F_1/F_2 is low (Table 1). Furthermore, the ratio of the dependent to independent enzyme activity was also calculated from the data depicted in Fig. 2 (Table 1, column c). The value found in F_1 decreased in the order of development and reached to 1.0 in the adult liver. The independent enzyme activity found in the F_1 of the 8th day embryo seems to increase during development, and it was found to be predominantly independent in adult liver

(Fig. 2-D and Table 1, column c). However, the significant change in ratio seen in F_2 was not detected during development.

The physiological significance of these reciprocal changes occurring in the two phosphodiesterases during embryo development is unknown. However, protein activator was found in the liver from rat embryo(14). We also observed this point with chick embryo (data not shown). Therefore, it is interesting to speculate that in chick liver, the Ca^{2+}/protein activator-dependent cAMP phosphodiesterase may participate in the regulation of the intracellular cAMP levels during the early embryonic stage and that the independent enzyme may do so after hatching. Attempts to identify the factor(s) promoting these reciprocal enzymic change are now in progress in our laboratory.

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