CYCLIC-NUCLEOTIDE PHOSPHODIESTERASE

An Early Defect in Inherited Retinal Degeneration of C3H Mice

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ABSTRACT

Cyclic nucleotides have been implicated in the differentiation and function of the vertebrate retina. In the normal retina of DBA mice, the specific activity of cyclic-nucleotide phosphodiesterase (PDE), with cyclic-AMP as the substrate (cAMP-PDE), increases eightfold between the 6th and 20th postnatal day. Kinetic analysis of retinae from newborn mice reveals a PDE with a single Michaelis constant (K_m) value for cyclic-AMP (low K_m-PDE). After the 6th postnatal day, a second PDE with a high K_m for cyclic-AMP (high K_m-PDE) can be demonstrated. The appearance and increasing activity of the high K_m-PDE coincides with the differentiation and growth of photoreceptor outer segments. Additionally, the high K_m-PDE is shown by microchemical techniques to be concentrated in the photoreceptor cell layer and the low K_m-PDE within the inner layers of the normal retina. In C3H mice afflicted with an inherited degeneration of the photoreceptor layer, the postnatal increase in the specific activity of cAMP-PDE is substantially lower than in the normal retina. The postnatal increase in the specific activity of cAMP-PDE in two regions of the brain of C3H mice is the same as in the normal strain. A deficiency in high K_m-PDE activity in the C3H retina is evident on the 7th postnatal day, when the activity of low K_m-PDE, photoreceptor morphology, and rhodopsin content of these retina are essentially normal. In the adult C3H retina, the PDE activity with cyclic-GMP and cyclic-UMP as substrates is significantly below that of the normal retina. These data indicate that an alteration in cyclic-AMP metabolism occurs before photoreceptor cell degeneration in the retinae of C3H mice.

INTRODUCTION

The C3H/HeJ mice are carriers of an autosomal recessive mutation (*rd*) which causes a selective degeneration of the photoreceptor layer of the retina (27, 29). The retina of C3H mice and those of a normal strain (DBA/1J) develop similarly until the 8th postnatal day when degenerative changes arise in the ultrastructure of the inner and outer segments of the C3H photoreceptor cells (4, 8, 9, 21, 22, 23, 30). A depletion of DNA in the developing retinae of C3H mice suggests that cell death may begin even earlier (16). Degenerative cell death begins within the central retina and spreads to the periphery, suggesting that some aspect of photoreceptor differentiation may be adversely affected by the mutant gene. In view of the increasing evidence that cyclic nucleotides play a

role in the differentiation (6) and function (1, 3, 20, 24) of the retina, the activity of cyclic-nucleotide phosphodiesterase (PDE) and its kinetic properties were determined in developing and mature retinae of normal and C3H mice using cyclic-AMP as the substrate (cAMP-PDE). The activity of PDE with other cyclic nucleotides was determined in adult retinae of both strains. Postnatal changes in cAMP-PDE were also determined in two brain regions of these mice as indices of central nervous system (CNS) maturation.

MATERIALS AND METHODS

DBA/1J and C3H/HeJ mice were obtained from the Jackson Laboratories, Bar Harbor, Maine, and breeding couples supplied the young animals. Mice of both strains were sacrificed at various time intervals after birth (±8 h). After decapitation, the retina, anterior hypothalamus, and somatosensory cortex were dissected in ice-cold saline. The neural retina was dissected as previously described (15), and homogenized in 0.2–0.5 ml of cold water. Samples of hypothalamus and cortex weighing 2–3 mg were homogenized in 1–2 ml of water. The anterior hypothalamus was dissected rostral to the optic tract as indicated by stereotaxic coordinates prepared in our laboratory. These agree favorably with those published for rat brain (7, 11). The somatosensory cortex was defined according to the cortical maps of Woolsey (34). The protein content of each homogenate was determined by the method of Lowry et al. (18).

In some experiments, mice were quick frozen, and freeze-dried samples of retina were prepared as previously described (14, 16). Additionally, the retina was subdivided into a receptor and a bipolar-plus-ganglion-cell layer. The retinal samples were weighed and homogenized, as described above.

The activity of PDE was determined by the method of Breckenridge and Johnston (2) with only minor modifications. For cAMP-PDE, the complete reaction mixture of 75 μl contained 0.05 M imidazole-HCl (pH 7.6), 2 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin, 20 μg/ml alkaline phosphatase (calf intestine, Boehringer Mannheim Corp., San Francisco, Calif.), 2 or 4 mM cyclic-AMP, and homogenate equivalent to 20–40 μg protein.[1] After 5 min of equilibration at 37°C without substrate, the reaction was started by the addition of cyclic-AMP. The reaction was stopped after various time intervals by the addition of 8 μl of ice-cold 55% TCA. The complete reaction volume was assayed for inorganic phosphate by a microadaptation of the method of Martin and Doty (19), as described by Lewin and Hess (13). The absorption of the phosphomolybdate complex was measured at 730 nm in a Gilford spectrophotometer. The quantity of phosphate liberated during the reaction was determined after subtraction of appropriate blanks by reference to an inorganic phosphate standard curve. The hydrolysis of cyclic-AMP by the homogenates was linear with time for 20–30 min of incubation. The values of specific activity (nanomole of cyclic-AMP hydrolyzed per milligram of protein per minute) were calculated from the initial rates of hydrolysis of cyclic-AMP.

For the kinetic analysis of PDE, the 3′,5′-nucleotides, cyclic-AMP, cyclic-GMP, cyclic-UMP, and cyclic-CMP, obtained from Sigma Chemical Co., St. Louis., Mo., were used in the concentration range from 6.25×10^{-6} M to 4×10^{-3} M and the reaction interval was 20 min.[2] The kinetic data was plotted as $1/$[cyclic nucleotide] vs. $1/v$ observed, and the linear portions of these plots were extrapolated to the oridnate. Michaelis constant (K_m) and maximum enzyme activity (V_{max}) values were calculated from the X and Y intercepts, respectively.

RESULTS

Postnatal Changes of cAMP-PDE Specific Activity in Homogenates of DBA and C3H Retinae

During postnatal life, the specific activity of cAMP-PDE in the retinae of normal mice increases eightfold between the 6th and 20th postnatal day (DBA, Fig. 1) and reaches its maximal activity in adult retinae. In sharp contrast to the normal, the specific activity of cAMP-PDE in C3H retinae shows only a small, gradual increase during postnatal life and fails to show the rapid increase after the 6th postnatal day (C3H, Fig. 1). The deficiency in cAMP-PDE specific activity in developing retinae of C3H mice precedes the onset of ultrastructural damage in the C3H photoreceptor layer.

[1] Concentrations of 4 mM cyclic-AMP were used routinely for the determination of PDE activity in the DBA retina and young C3H retina; however, kinetic studies (Fig. 2B) showed this level of substrate to be inhibitory in the 10 day old C3H and an optimal concentration of 2 mM cyclic-AMP was used for PDE determination in the retinae of older C3H mice.

[2] The commercial alkaline phosphatase included in the incubation medium was capable of completely hydrolyzing a wide concentration range of all 4-nucleoside-monophosphates during the 20 min incubation.

Postnatal Changes in cAMP-PDE Specific Activity in Brain Regions of DBA and C3H Mice

Both the changes in activity of cAMP-PDE during development and the specific enzyme activity are identical in the hypothalamus and somatosensory cortex of developing DBA and C3H mice (Table I). The postnatal pattern of change in cAMP-PDE activity in these brain regions of the two species of mice is comparable to that reported for the rat cerebrum (10, 26, 33). The kinetic properties of cAMP-PDE are also identical in the two brain regions of normal and C3H mice, with an apparent single K_m of 0.80 mM for cyclic-AMP.

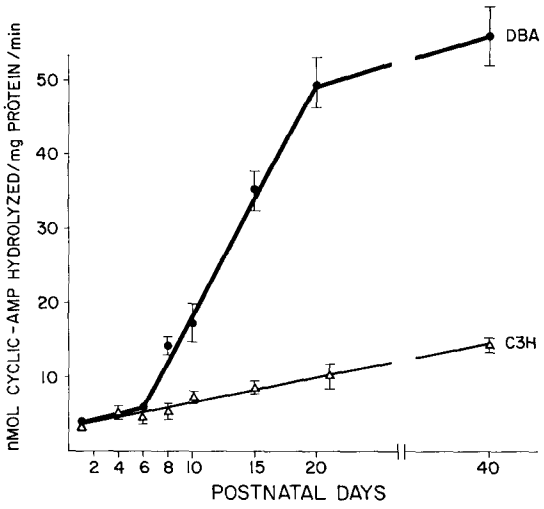

FIGURE 1 cAMP-PDE specific activity in DBA (●) and C3H (△) mouse retinae as a function of postnatal age. Each point represents the mean ± standard error for five or more individual determinations.

The similarity in K_m values and specific activity of cAMP-PDE in the brain regions is in contrast to the differences in these characteristics in the retinae of DBA and C3H mice. These observations are suggestive of there being no strain differences other than the *rd* mutation.

Kinetic Characteristics of Retinal PDE

The kinetic characteristics of PDE in retinal homogenates were determined at various stages of postnatal development. Shortly after birth, an apparent K_m for cyclic-AMP of 0.13 mM (low K_m-PDE) is observed in the retinae of normal and C3H mice (Fig. 2 A). A similar single low K_m value was found in the 8-, 12- (Fig. 2 B), and 21-day C3H retina as well as that of the adult (Table II).

In DBA, the kinetic analysis of cAMP-PDE in retinal homogenates becomes more complex during postnatal development. After 8 postnatal days, two K_m values for cyclic-AMP can be demonstrated in the normal retina. Fig. 2 B illustrates a Lineweaver-Burk plot of data from 12-day DBA retinae which shows K_m values for cyclic-AMP of 0.13 mM and 2.5 mM (high K_m-PDE).

The kinetic characteristics of PDE in homogenates of adult DBA and C3H retina were determined with cyclic-AMP, cyclic-GMP, and cyclic-UMP as substrates (Table II). In these experiments, cyclic-CMP is ineffective as a substrate. Of the cyclic nucleotides, only cyclic-AMP shows complex kinetics for PDE in homogenates of DBA retinae. The single K_m for cyclic-GMP in homogenates of DBA retinae is intermediate between the two K_m values observed with the cyclic-AMP. The single K_m for cyclic-UMP is greater than that of cyclic-AMP or cyclic-GMP. The V_{max} values for the hydrolysis of all three nucleotides appear to be of the same order of magnitude.

TABLE I

cAMP-PDE Activity in Two Regions of the Central Nervous System of DBA and C3H Mice during Postnatal Development

The cAMP-PDE specific activity is expressed as nanomoles of cyclic-AMP hydrolyzed per milligram of protein per minute. Each value represents the mean ± the standard error for five to six determinations.

Postnatal age (days)	Hypothalamus		Cortex	
	DBA	C3H	DBA	C3H
1–6	16.0 ± 1.3	16.0 ± 1.0	22.7 ± 4.0	22.2 ± 4.0
15	30.7 ± 2.5	32.8 ± 4.0	53.7 ± 7.7	62.7 ± 6.3
20	37.3 ± 3.8	43.7 ± 4.2	63.8 ± 7.5	74.0 ± 3.8

In homogenates of mature C3H retinae, only a single K_m can be demonstrated for any of the cyclic nucleotides with essentially identical V_{max} values (Table II). In these homogenates, the K_m for cyclic-GMP is similar to that of the normal retinae, while the specific activity of PDE is far below that of the normal retina. The K_m for cyclic-UMP and the V_{max} with this substrate are substantially lower than in the normal retinae. It should be noted that the K_m and V_{max} values of the low-K_m cAMP-PDE are essentially identical in the DBA retinae and the surviving cellular layers of the C3H retinae.

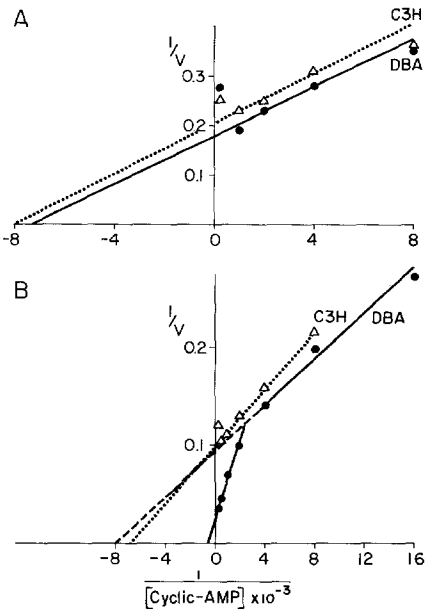

FIGURE 2 Lineweaver-Burk plots of cAMP-PDE activity in homogenates of DBA (●——●) and C3H (△...△) retinae: (A) 2 postnatal days and (B) 12 postnatal days. Each point represents the mean from two experiments. The enzyme activity, V, is expressed in nanomoles of cyclic-AMP hydrolyzed per milligram of protein per minute. For the 12 day old DBA retina (B), the broken Lineweaver-Burk plot was extrapolated (- - -) to show a second apparent Michaelis constant (low K_mPDE) for cyclic-AMP.

cAMP-PDE Activity in the Photoreceptor and Bipolar-Plus-Ganglion-Cell Layer

The specific activity and the kinetic characteristics of PDE with cyclic-AMP as substrate were measured in microdissected samples of receptor and bipolar-plus-ganglion-cell layers of retinae from adult DBA mice. On an average, the activity of cAMP-PDE in samples of freeze-dried retinae is reduced by about 65% from that observed in fresh retinal homogenates. Nevertheless, the kinetic characteristics are similar in the two preparations (Fig. 3 B). In three experiments with cyclic-AMP as substrate, the average specific activity of the receptor layer is 25.17 and that of the bipolar-plus-ganglion-cell layer is 8.50 nmol cyclic-AMP hydrolyzed/mg protein per min. Kinetic studies demonstrate that a cAMP-PDE with a K_m of 2.5 mM for cyclic-AMP is concentrated in the photoreceptor layer and a cAMP-PDE with a K_m of 0.14 mM is concentrated in the bipolar-plus-ganglion-cell layer (Fig. 3 A).

DISCUSSION

It has been suggested that the inherited retinal degeneration of C3H mice is caused by an error in

TABLE II

Kinetic Properties of PDE in Retinae of Adult Mice

The K_m and V_{max} values were calculated from the X and Y intercepts of the respective Lineweaver-Burk plots. Each value represents the mean ± the standard error for three to five determinations. The enzyme incubations were carried out for 20 min, as described in the Methods. V_{max} is expressed as nanomoles of cyclic nucleotide hydrolyzed per milligram of protein per minute.

	DBA		C3H	
Cyclic nucleotide	K_m (mM)	V_{max}	K_m (mM)	V_{max}
Cyclic-AMP	0.16 ± 0.03 2.82 ± 0.33	20.67 ± 2.17 93.67 ± 11.77	0.18 ± 0.02	17.77 ± 1.83
Cyclic-GMP	0.66 ± 0.04	88.50 ± 13.83	0.55 ± 0.06	16.50 ± 2.67
Cyclic-UMP	5.51 ± 0.79	61.83 ± 4.83	1.14 ± 0.10	14.67 ± 0.33

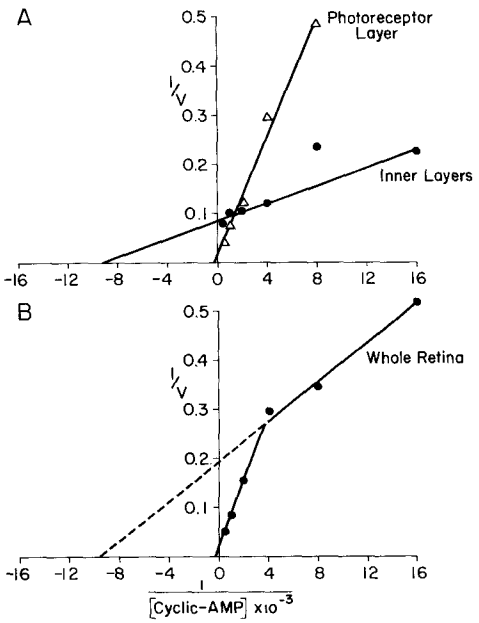

FIGURE 3 Lineweaver-Burk plots of cAMP-PDE activity in: (A) freeze-dried microdissected photoreceptor layer (△) and bipolar-plus-ganglion-cell layer (●) and (B) freeze-dried samples of whole retina (●) from adult DBA mice. The enzyme activity, V (nanomoles of cyclic-AMP hydrolyzed per milligram of protein per minute), was determined in duplicate samples at each level of substrate. An extrapolation of the broken Lineweaver-Burk plot (– – –) shows a second apparent Michaelis constant (low K_m-PDE) for cyclic-AMP in the whole DBA retina (B).

some aspect of photoreceptor cell differentiation (5, 16, 21). The particular biochemical system which is aberrant due to the mutation has remained unknown even though a number of secondary biochemical abnormalities have been observed (5, 12, 14, 16, 17, 22, 25).

This report presents data in support of the concept that cyclic-AMP metabolism is normal within the brain of C3H mice, but is abnormal in the C3H retina by the 7th day of postnatal life.

The differentiation of photoreceptor cells in the mouse retina follows closely upon the formation of these cells by mitotic division of neuroblast cells (23, 28). The process of differentiation begins within the central retina and proceeds peripherally to the ora serrata (21). Electron microscope studies show that some primitive photoreceptor cells are recognizable within the central retina on the 2nd and 3rd postnatal days, but it is not until the 6th to the 7th postnatal day that receptor outer segment formation begins (4, 23).

Starting in the central retina on about the 8th postnatal day, a wave of degeneration of C3H photoreceptor cells proceeds peripherally to the ora serrata (21). Photoreceptor cells appear to mature until a rudimentary outer segment has formed and then degeneration commences (4, 9, 27, 30). The first signs of ultrastructural deterioration occur as disruptions of the mitochondria of the inner segments and disorientation of rod outer segment lamellae (30). DNA measurements of the complete C3H retinae suggest that some cellular death may occur before the first formation of rod outer segment lamellae (16).

During the period of photoreceptor cell differentiation and rod outer segment growth, the specific activity of cAMP-PDE increases abruptly in the retinae of normal DBA mice. The cAMP-PDE in the developing DBA retina shows complex kinetics, from which two apparent K_m values can be determined. Such complex kinetics have been reported for cAMP-PDE of cattle retina (24) and rat brain (31, 32). The postnatal increase in cAMP-PDE specific activity in the complete retinae of DBA mice reflects the increase in activity of the high K_m-PDE (Figs. 2 B, 3 B, and Table II). The activity of this enzyme is concentrated in the photoreceptor cell layer (Fig. 3 A), and the postnatal increase in high K_m-PDE corresponds with the postnatal increase in the length of photoreceptor outer segments and with the increase in rhodopsin content (5). These observations are consistent with the finding that cattle rod outer segments contain a PDE with a relatively high K_m for cyclic-AMP (25).

In contrast to the normal retina, only a low K_m-PDE for cyclic-AMP can be identified in the retinae of the C3H mice. The high K_m-PDE is not observed at any stage of maturation of C3H retinae. Throughout postnatal development, the activity of a low K_m-PDE is concentrated in the inner layers of the normal retina (Fig. 3 A), and it seems likely that this kinetically distinct class of PDE is associated with the synaptic complexes of the inner retina, where both cyclic-AMP and cyclic-GMP may be functionally associated with neuronal transmission (3).

In summary, our observations indicate that the developing C3H retina is missing the activity of a PDE which shows a high K_m for cyclic-AMP. The earliest indication of enzyme deficiency in C3H retinae occurs by 7 postnatal days. This suggests that the metabolism of cyclic-AMP is abnormal in the photoreceptor layer before this age.

Three alternative possibilities could explain the observed deficiency of cAMP-PDE activity in developing C3H retina: (*a*) The high K_m-PDE enzyme may be produced during development of C3H retinae but, due to some defect, it is enzymatically inactive, (*b*) the developing C3H retina may fail to synthesize this enzyme, or (*c*) some endogenous substance might inhibit or interfere with the assay of the high K_m-PDE. Further work will be necessary to distinguish between these alternatives and to elucidate the mechanism underlying the deficiency of cAMP-PDE activity in developing retinae of C3H mice.

The absence of a high K_m-PDE enzyme in developing C3H retinae should have profound effects upon the metabolism of cyclic-AMP in the photoreceptor cells. The degree to which the levels of cyclic-AMP are abnormal during this period will depend upon the relative activities of adenyl cyclase and PDE. The resultant balance of these enzymes throughout the developmental period will establish the steady-state level of cyclic-AMP which is probably crucial to the synchronized changes during differentiation of the photoreceptor cell. From the present data, it is evident that a deficiency in PDE activity exists in the C3H retina before the onset of photoreceptor cell degeneration. In order to develop a working hypothesis for the mechanism causing this disease, additional studies are in progress to determine the activity of adenyl cyclase and the steady-state level of cyclic-AMP in the developing normal and C3H retinae.

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