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Involvement of Cyclic GMP Phosphodiesterase Activator in an Hereditary Retinal Degeneration

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Abstract

CYCLIC NUCLEOTIDES mediate many aspects of normal cellular metabolism; thus, degradation as well as synthesis of these intracellular mediators must be strictly regulated. Phosphodiesterase (PDE), the enzyme of cyclic nucleotide catabolism, is present in mammalian tissues in multiple forms, which differ in substrate specificity, kinetic characteristics and sub-cellular localisation. Moreover, a calcium-dependent protein activator (now called calmodulin) has been characterised that specifically activates at least one of the PDE types although other types of PDE are known to be activator independent. Thus, several mechanisms are present *in vivo* which allow strict control of PDE. A unique cyclic GMP-PDE is compartmentalised in the outer segments of retinal photoreceptor cells; its activity is low in the dark-adapted state but increases dramatically on light adaptation. The resulting drop in cyclic GMP content could serve as a chemical 'signal' in the normal visual process. However, despite much investigation of various cyclic nucleotide systems, no definitive information has been obtained which clearly links a disorder of cyclic nucleotide metabolism with a disease process elsewhere than in retina. We have recently presented preliminary evidence that an abnormality in cyclic GMP metabolism could be present in the retinas of Irish setter dogs with inherited rod–cone dysplasia that could lead to greatly increased cyclic GMP content, as had been reported in mice with inherited retinal degeneration. We now report that the basic defect in the disease seems to be a failure to switch PDE type and a concomitant decrease in protein activator concentration early in postnatal development, at the time of photoreceptor differentiation.

Disciplines

Eye Diseases | Ophthalmology | Veterinary Medicine

the mean distance between gap junctions linking neighbouring L_1 units should be greater in the direction parallel to the streak than perpendicular to it. Assuming that cytoplasmic resistance is much lower than junctional resistance, the signal attenuation will occur mainly at these junctions. We further assume a uniform distribution of gap junctions along the cell surface. The signal attenuation per unit length normal to the slit stimulus will then be greater in the direction perpendicular to the streak resulting in receptive fields elongated along the streak.

The mechanism described above is based upon a network of L_1 units each of which has a common orientation. A random distribution of orientations of elongated cells would result in more equal mean distances between junctions along any two orthogonal directions. This type of anatomical arrangement is in fact seen in Golgi-impregnated L_1-type horizontal cells in the peripheral retina. Here, our preliminary physiological findings indicate that L_1-type horizontal cells have response eccentricities closer to 1.0 with no consistently observed preferred orientation along any particular axis.

In this paper, we have shown that the streak region of the turtle retina contains at least one population of cells with specialised properties; the axonal arborisations and the receptive fields of L_1 horizontal cells in this region are elongated in the direction of the streak. The functional significance of the specialisations in the streak region may be better understood from ganglion cell recordings in this region. However, such measurements have only been made in the visual streak of another species, the rabbit[1]. Amongst rabbit ganglion cells in this region, orientation selective units were found which responded only to slits of light which were oriented either parallel or perpendicular to the streak. Although a full understanding of receptive fields at the ganglion cell level requires a greater knowledge of signal processing in the inner and outer plexiform layers, the oriented horizontal cell receptive fields described here may have significance for the development of orientation specificity in the visual streaks of species whose retinas contain this specialisation.

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Involvement of cyclic GMP phosphodiesterase activator in an hereditary retinal degeneration

CYCLIC NUCLEOTIDES mediate many aspects of normal cellular metabolism[1]; thus, degradation as well as synthesis of these intracellular mediators must be strictly regulated. Phosphodiesterase (PDE), the enzyme of cyclic nucleotide catabolism, is present in mammalian tissues in multiple forms, which differ in substrate specificity, kinetic characteristics and subcellular localisation[2]. Moreover, a calcium-dependent protein activator (now called calmodulin) has been characterised that specifically activates at least one of the PDE types[3,4] although other types of PDE are known to be activator independent[5,6]. Thus, several mechanisms are present *in vivo* which allow strict control of PDE. A unique cyclic GMP-PDE is compartmentalised in the outer segments of retinal photoreceptor cells[7–10]; its activity is low in the dark-adapted state but increases dramatically on light adaptation[10–12]. The resulting drop in cyclic GMP content could serve as a chemical 'signal' in the normal visual process[13,14]. However, despite much investigation of various cyclic nucleotide systems, no definitive information has been obtained which clearly links a disorder of cyclic nucleotide metabolism with a disease process elsewhere than in retina[15,16]. We have recently presented preliminary evidence that an abnormality in cyclic GMP metabolism could be present in the

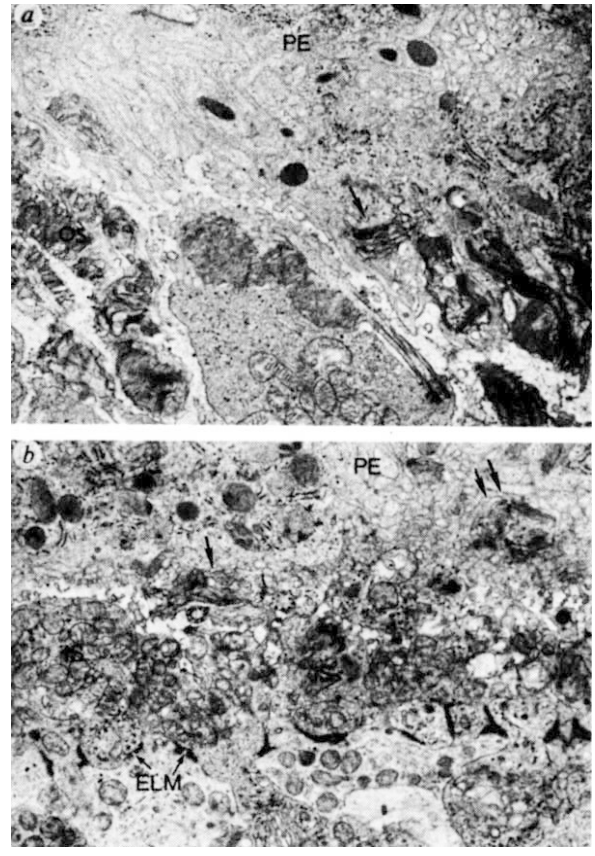

Fig. 1 *a*, Photoreceptor layer from a 13-d-old normal Irish setter. Photoreceptor outer segments have begun to elongate and contain well organised stacks of outer segment (OS) lamellar disks. Some of these seem to have been engulfed (arrow) by the pigment epithelium (PE). *b*, Photoreceptor layer from a 13-d old affected Irish setter. Small photoreceptor inner segments (IS) project through the external limiting membrane (ELM). There is minimal outer segment material; some is apposed to the PE apex (arrow) and some seems to be located within the PE cytoplasm (double arrow) ×10,400.

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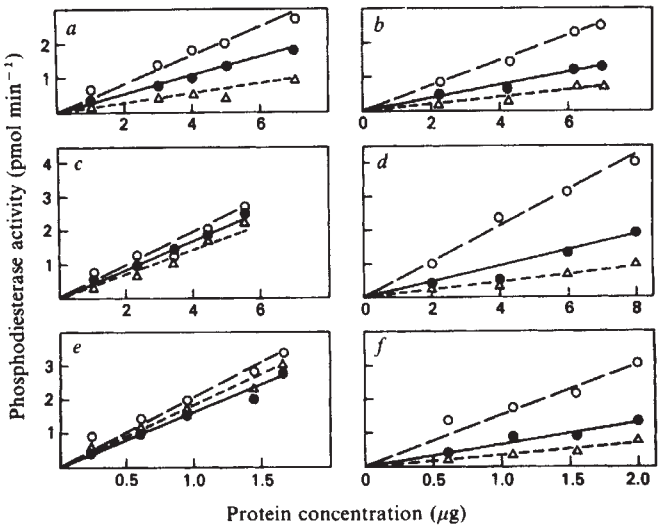

Fig. 2 Cyclic GMP phosphodiesterase activity in retinas of different ages. *a*, Control 9-d-old; *b*, affected 9-d-old; *c*, control 31-d-old; *d*, affected 34-d-old; *e*, control 48-d-old; *f*, affected 48-d-old. ●, No additions; ○, 10 μg purified brain activator added; △, 250 μM EGTA added. Experimental points are averages of triplicates which differed by no more than 15%.

retinas of Irish setter dogs with inherited rod–cone dysplasia[15] that could lead to greatly increased cyclic GMP content, as had been reported in mice with inherited retinal degeneration[16]. We now report that the basic defect in the disease seems to be a failure to switch PDE type and a concomitant decrease in protein activator concentration early in postnatal development, at the time of photoreceptor differentiation.

The Irish setters used were bred to develop rod–cone dysplasia, and controls were heterozygous Irish setters unaffected by the disease. Tissues were obtained as previously described[15]; briefly, animals were anaesthetised with pentobarbital, their eyes enucleated and the retinas quickly dissected on ice and frozen in liquid nitrogen. Protein was determined by the method of Lowry *et al.*[17] with bovine serum albumin as standard. Bovine brain activator and activator-deficient PDE were prepared as previously described[18]. The activator-deficient enzyme had a basal activity of 930 pmol cyclic AMP hydrolysed per mg protein per min; this was increased fourfold in the presence of optimal concentrations of Ca^{2+} and activator protein. PDE activity was measured using an assay mixture containing 40 mM Tris-HCl (*p*H 8.0), 3 mM $MgSO_4$, 50 μM $CaCl_2$, 1 μM ^{3}H-cyclic AMP (36.6 Ci mmol^{-1}) or ^{3}H-cyclic GMP (8.28 Ci mmol^{-1}) (NEN) and an appropriate amount of retinal homogenate in a total volume of 100 μl. When appropriate, 250 μM EGTA or 10 μg of purified brain activator was added before PDE assay. Samples were incubated for 12 or 15 min at 30°C, the reaction was terminated by boiling for 45 s, and 50 μg snake venom (*Crotalus atrox*, Sigma) was added. Samples were incubated for a second 10-min period at 30°C and 1.0 ml of IRP-58 resin (200–400 mesh, Rohm and Haas) added. After centrifugation, ^{3}H-adenosine or ^{3}H-guanosine in the supernatant was determined by liquid scintillation spectrometry. Recovery of ^{3}H-nucleoside was >90%. Retinal protein activator was assayed by testing its ability to stimulate the activity of activator-deficient, purified cyclic nucleotide PDE enzyme (0.68 μg per 0.1 ml assay mixture) using boiled retinal aliquots as the source of activator. One unit of activator is defined as the amount required to give half-maximal stimulation of the activator-deficient PDE enzyme.

The second week after birth is critical in retinal development in dogs, as the photoreceptors begin to differentiate during this period (Fig. 1). At day 9 (Table 1 and Fig. 2*a*, *b*), cyclic GMP-PDE activity was already lower in the dystrophic dog (244 pmol per mg protein per min) than in heterozygous controls (309 pmol per mg per min). However, cyclic AMP-PDE activity was the same in affected and control retinas at 9 days. Cyclic GMP-PDE activity in control retinas rose sevenfold (Table 1) by the time of photoreceptor maturation at 7 weeks[19]. Cyclic GMP-PDE activity observed in affected retinas during development was lower than that of control retinas, whereas no difference was observed in cyclic AMP-PDE activity (Table 1). Measurement of endogenous protein activator for cyclic GMP-PDE using activator-deficient brain PDE enzyme indicated lower amounts in affected than in control retinas in each age group studied. No differences were observed with the protein activator using ^{3}H-cyclic AMP as substrate (not shown).

Activity of cyclic GMP-PDE was then investigated in the presence of exogenous brain activator protein or EGTA (Fig. 2). The latter was used because Ca^{2+} is necessary for activator-induced increases in PDE activity. The addition of brain activator increased PDE activity in the 9-d control retina but added EGTA decreased activity (Fig. 2*a*). In the 9-d affected retina (Fig. 2*b*), the inhibitory effect of EGTA was also apparent, as was the stimulatory effect of brain activator. At about 5 and 7 weeks, neither brain activator nor EGTA had any marked effect on PDE activity in the control retina (Fig. 2*c*, *e*). In contrast, EGTA continued to exert an inhibitory effect in the dystrophic retina, and the addition of brain activator restored PDE activity to the levels seen in the control (Fig. 2*d*, *f*).

The expression of PDE activity in cells seems complex in that it can be regulated by several factors including activator protein and Ca^{2+} (refs 20, 21) in addition to the basic activity of the enzyme itself. It has been suggested[22] that the concentration of cyclic GMP in particular is controlled by a PDE protein activator. It was therefore not unexpected to find effects of activator and Ca^{2+} on cyclic GMP-PDE activity in 9-d control retinas. Morphologically, photoreceptor outer segments begin to develop at this time. However, it was surprising to find that after outer segment elongation and maturation added brain activator and Ca^{2+} had negligible effects on PDE activity in these retinas. This indicated that the activator-dependent PDE activity had switched to an activator-independent ('adult') type (Fig. 2). In contrast, in affected retinas, no such switch was found to occur as the outer segments began to develop. Exogenous activator markedly stimulated PDE activity throughout the 7-week experimental period, whereas endogenous activator levels fell to about half of that observed in control retinas. It is not known if endogenous activator levels are actually limiting in affected retinas. The finding that exogenous brain activator restores PDE activity to close to that seen in the control *in vitro*, however, could be important in future attemps to halt or at least slow the progress of the disease.

Table 1 Phosphodiesterase activity and protein activator activity in control and affected retinas

Animal age (day)	Animal type	Phosphodiesterase activity (pmol per mg protein per min)		Cyclic GMP-PDE activator concentration (units per mg protein)
		Cyclic AMP	Cyclic GMP	
9	Control	102	309	125
9	Affected	102	244	101
31	Control	104	887	53
34	Affected	104	326	23
48	Control	143	2,275	95
48	Affected	151	539	56

Phosphodiesterase activity was measured by determination of ^{3}H-nucleoside with an ion-exchange method (see text). Protein activator was determined in boiled retinal aliquots using purified bovine brain PDE which was deficient in activator. One unit of protein activator activity is that amount which is required to give half-maximal stimulation of the activator-deficient brain PDE enzyme. Values given are averages of triplicate determinations in two experiments which agreed to within 15%.

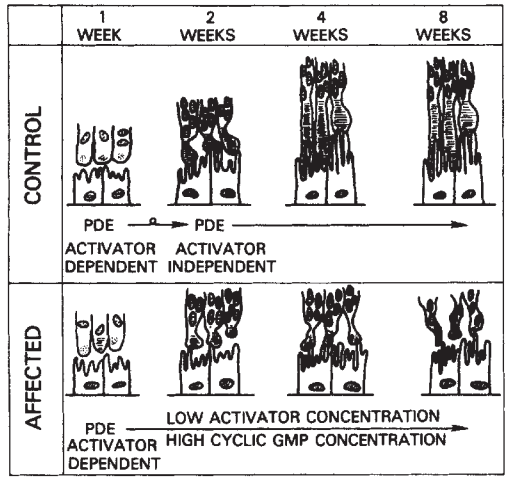

Fig. 3 Schematic outline of morphological and biochemical events in the development of outer segments (OS) in control and affected retinas. In both control and affected retinas, no OS are observed in the first postnatal week. OS appear in the second week, develop normally (4–5 weeks) in the control retinas but quickly degenerate in affected retinas. Biochemically, the cyclic GMP-PDE enzyme switches from an activator-dependent to an activator-independent type in control retinas but not in affected retinas. Protein activator concentration is substantially decreased in affected retinas.

It is well known that enzyme patterns change during development[23,24]. Recent evidence suggests that, at least in some tissues, PDE activity changes during differentiation with regard to its sensitivity to regulation by the Ca^{2+}-dependent protein activator[25,26]. Our data strongly indicate that a normal switch in PDE type fails to occur in affected retinas during photoreceptor development and that the low level of activator present in these retinas may not be sufficient to maintain adequate cyclic GMP-PDE activity (Fig. 3). It seems, therefore, that for the first time calmodulin can be directly linked to a disease process. It will be interesting to see if a similar situation exists in other degenerative diseases or if this biochemical defect is unique to inherited degeneration of the neural retina.

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Evidence that cyclic GMP regulates membrane potential in rod photoreceptors

CYCLIC NUCLEOTIDES have been proposed as mediators of the decreased Na^+ permeability caused by illumination of vertebrate photoreceptors[1,2]. We show here that cyclic GMP depolarises the rod outer segment (ROS) approximately to the Na^+ equilibrium potential within milliseconds after being injected intracellularly, that previous light adaptation antagonises this depolarisation, and that the injection of cyclic GMP without illumination initiates a repolarisation after a time lag which is longer than that following a light flash and proportional to the injection time. The results are interpreted in terms of a model in which cyclic GMP levels are controlled by the resultant of cyclase and phosphodiesterase (PDE) velocities. The PDE velocity of hydrolysis in the dark is increased by prior light adaptation in the presence of increased substrate (cyclic GMP). The data suggest that cyclic GMP is an important factor in the regulation of the membrane potential of the ROS.

Biochemical experiments on the cyclic nucleotide system of isolated homogenised ROS suggest that illumination decreases the concentration of cyclic GMP by activating a GTP-dependent PDE[3,4]. The rate of light-initiated hydrolysis of cyclic GMP by PDE is apparently sufficiently fast to mediate the rapid initial decrease in Na^+ permeabilty[5,6]. The cyclic GMP that is not hydrolysed presumably increases Na^+ permeability. The mechanism of increased Na^+ permeability of the ROS plasma membrane would be by phosphorylating a membrane protein[6,7] as proposed for other tissues. Superfusion with cyclic GMP or a PDE inhibitor has been shown to depolarise rods[8]. We have injected cyclic GMP intracellularly through the recording pipette into ROS of the isolated retina of the toad, *Bufo marinus*, to determine the effects of excess cyclic GMP on membrane potential, latency and amplitude of the light response. We reported[9] that this excess cyclic GMP increases both the latency and amplitude of the response. Here, using the same preparation and methods, we found that excess injected cyclic GMP depolarised the rod membrane. This depolarisation in dark-adapted preparations frequently exceeded the zero level (Figs 1, 2) and attained a maximum value of +11 mV (Fig. 1*a*, *d*). A reversal potential of 0 to +10 mV was obtained for the isolated *Necturus* rod[10]. A value of +11 mV implies an intracellular Na^+ concentration ($[Na^+]_i$) of 66 mM, based on our extracellular concentration of 103 mM. This compares with 50 mM $[Na^+]_i$ estimated using another technique[11]. Therefore, +11 mV seems to approximate to the equilibrium potential for Na^+. The maximum depolarisation we observe is consistent with the interpretation that the injected excess cyclic GMP renders the ROS plasma membrane permeable to Na^+.

Intracellular injection of cyclic GMP in the absence of illumination should initiate a recovery from depolarisation after a time proportional to the amount injected, provided the dark concentration is less than the K_m of PDE which is 70 μM cyclic