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Rod/cone dysplasia in Irish setters

Presence of an altered rhodopsin

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On the basis of the amino acid sequence of bovine rhodopsin, a series of peptides from the *C*-terminus (Rhod-4 and Rhod-1) and external loops (Rhod-10) were synthesized. Rabbit antisera to these peptides recognize the rhodopsin molecule in whole retina from 8-week-old normal and affected rcdl (rod/cone-dysplasic) Irish setters (8- and 4-weeks-old). When the rhodopsin content was equalized by using a solid-phase radioimmunoassay, the reaction with anti-peptide antisera to the *C*-terminal octapeptide (residues 341–348) is severely decreased in the rcdl-dog retinas. The results of mixing experiments suggest that this is not due to proteolytic clipping of the rhodopsin *C*-terminus from the affected dogs. Treatment of retinas with 1.0 mM-NaF, a phosphatase inhibitor, or pretreatment with alkaline and acid phosphatases does alter the reaction of the rhodopsin with anti-rhodopsin antisera. This suggests that the decreased reaction of the affected rhodopsin with the anti-peptide antisera may partially result from differences in intrinsic rhodopsin phosphorylation. However, since the reaction of rcdl retinas cannot be restored to that of the normals, these results suggest that the rhodopsin molecule from the rcdl dogs may be structurally altered in other ways.

INTRODUCTION

In the photoreceptors of retinal-dystrophic Irish setter dogs, abnormal cyclic GMP metabolism is associated with a selective degeneration of rods by about 8 weeks of age [1,2]. An abnormality in cyclic GMP hydrolysis has been observed in both *rd* mice and dogs before the affected visual cells show signs of morphological pathology [3,4]. In both cases, the phosphodiesterase (PDE) activity is depressed and cyclic GMP accumulates in the affected photoreceptors [2,5].

Although kinetic studies of PDE enzyme activity have proven useful to characterize the normal retinal enzymes, they have been of limited value in assessing the nature of the biochemical defect in animals. The PDE complex is composed of three subunits which adhere to rod-outer-segment membranes in the presence of Mg^{2+} [6]. When in soluble form, this PDE is activated by histone, a characteristic which is unique to outer-segment PDE. Recently Lee *et al.* [7] have used the properties of histone activation and reaction with monoclonal anti-PDE antibodies to prove that the PDE protein is present in dystrophic Irish setters at 34 days of age. The PDE exhibited histone activation, immunocross-reactivity with monoclonal anti-PDE antibodies and had the same M_r on SDS/PAGE as the PDE from the adult unaffected Irish-setter retinas [7]. However, the degree of histone activation was decreased in the affected state. We have used polyclonal anti-PDE antiserum to quantify the retinal PDE from affected and normal Irish Setter dogs. Our results suggest that the PDE protein is not reduced in amount at early ages (J. Cunnick, M. Rider, L. J. Takemoto & D. J. Takemoto, unpublished work). Rhodopsin, transducin and cyclic GMP PDE are all part of the light-activated cascade response that eventually results in the hydrolysis of cyclic GMP [8]. A defect in any of these three membrane-bound polypeptides is therefore capable of disrupting the visual cascade response. Fairly recently we reported that rhodopsin is altered in affected Irish setters [9]. By using site-specific antisera directed against synthetic peptides of bovine rhodopsin, we have demonstrated altered reactivity in the affected dogs. One of these altered regions is the *C*-terminus of rhodopsin, which is near known phosphorylation sites [10]. These regions may serve as recognition regions for retinal transducin [11,12].

We now report that the *C*-terminal alteration is apparent in affected dogs at early stages (4 weeks old) and is partially due to changes in rhodopsin phosphorylation. These findings suggest that the defect in the affected dog may be, either directly or indirectly, at the level of the rhodopsin molecule itself.

EXPERIMENTAL PROCEDURES

Materials

Eyes from 8-week-old and 4-week-old affected rcdl and from 8-week-old normal Irish setter dogs were obtained from Dr. Greg Acland, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, U.S.A., through grant EYO 6855 from the National Institutes of Health. The eyes were removed under anaesthesia and shipped frozen to the laboratory. All eyes were stored at −70 °C.

Methods

Retina preparation. The eyes were thawed and retinas removed immediately, at 4 °C, under room light. Retinas were suspended in 30% (w/v) sucrose/65 mM-NaCl/

Abbreviations used: rcdl, rod/cone dysplasic; PDE, phosphodiesterase; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; r.i.a., radioimmunoassay; KLH, keyhole-limpet haemocyanin; Rhod-1, rhodopsin-(332–339)-peptide; Rhod-4, rhodopsin-(341–348)-peptide; Rhod-9, rhodopsin-(309–316)-peptide (Met-Asn-Lys-Gln-Phe-Arg-Asn-Lys); Rhod-10, rhodopsin-(232–239)-peptide.

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2 mM-$MgCl_2$/5 mM-Tris/HCl (pH 7.4)/1 mM-dithiothreitol/0.5 mM-PMSF. After centrifugation at 10 min, the retinal pellets were resuspended and homogenized in 50 mM-Tris/HCl (pH 7.4)/1 mM-$MgCl_2$/0.5 mM-PMSF. Total protein content was measured [13], and the rhodopsin content was equalized by using a solid-phase r.i.a. as described by Suter [14].

Sample incubations. In some cases, retina samples were treated with 1 mM-NaF to enhance the phosphorylation of rhodopsin [15]. Retinal samples (1–10 μg of total protein) were incubated for 12–14 h at 4 °C in 70 mM-potassium phosphate (pH 7.4)/3 mM-$MgCl_2$/1 mM-ATP/1 mM-NaF/0.5 mM-PMSF. For analyses with carboxypeptidase, samples were incubated as described above, with or without added ATP or NaF, with 5 μg of carboxypeptidase Y (Pierce Chemical Co.; baker's yeast enzyme; 30 units/0.5 mg of protein). Pretreatment of samples with phosphatase was as described above, at room temperature, with 10 μg of alkaline phosphatase (Sigma; human; 3.5 units/mg) and 10 μg of acid phosphatase (Sigma; potato type IV-S; 4.7 units/mg).

Antisera to synthetic peptides. Peptides were synthesized manually by using the solid-state method of Hodges & Merrifield [16] as modified by Gorman [17], with the exception that cleavage of peptides and protecting groups was performed by use of HBr and anhydrous trifluoroacetic acid [18]. Peptides were purified by cellulose thin-layer electrophoresis using a buffer consisting of acetic acid/formic acid/water (3:1:16, by vol.). Peptide spots were revealed with ninhydrin, and corresponding unsprayed lanes were eluted with aq. 0.5% NH_3.

To determine amino acid composition and to quantify the peptides, 0.5% of each total sample was hydrolysed *in vacuo* with 5 M-HCl. Amino acids were determined by using a reverse-phase C_{18} h.p.l.c. column (Vydak) and *o*-phthalaldehyde as a detecting agent [19].

All peptides had the expected amino acid composition when determined after HCl hydrolysis. Quantification was accomplished by peak integration and comparison with known amino acid standards.

Peptides are listed in Table 1. These peptides were each cross-linked to keyhole-limpet haemocyanin (KLH) as follows. The KLH (3 mg of total protein; Sigma) was added in 0.2 ml of distilled water to 1.0 mg of peptide in 0.2 ml of distilled water. To this was added 0.2 ml of 30 mM-glutaraldehyde. The reaction proceeded, with shaking, at room temperature for 12–14 h. After this time the reaction was quenched by the addition of 1 ml of 2 M-$NaBH_4$. Samples were then dialysed against 1 mM-NH_4HCO_3, pH 7.0, and stored at −20 °C until further use.

Rabbits were injected at least five times, subcutaneously, every 2 weeks with 20 μg of KLH peptide in a suspension of either KLH peptide/Freund's complete adjuvant (first injection) or KLH peptide/Freund's incomplete adjuvant (subsequent injections). Sera were tested by using a solid-phase r.i.a.

R.i.a. Since peptides do not bind to nitrocellulose, we have developed a solid-phase r.i.a. which is a modification of that described by Suter [14]. Polystyrene tubes were coated with 0.2% glutaraldehyde for 3 h, followed by washing three times with 0.1 M-sodium phosphate, pH 5.0. The desired peptide or retinal tissue was added (1–2 μg of protein) and incubated for 2 h at 37 °C. This reaction resulted in the covalent coupling of the sample. Tubes were then washed three times with 0.15 M-NaCl and 0.5% Tween-20 to block non-specific binding. Antisera were added, generally at 1:250 dilution, in 0.1 M-sodium phosphate(pH 7.4)/0.9% NaCl/0.05% Tween-20/0.02% NaN_3 (antisera buffer), and incubated overnight at room temperature. Tubes were then washed with 10 mM-Tris/HCl(pH 8.0)/0.05% Tween-20, three times, then twice with water. Finally, samples were allowed to react for 1 h at room temperature with ^{125}I-protein A (approx. 200000 c.p.m./tube) diluted in antisera buffer, washed three times with the same buffer, and counted for radioactivity in a γ-radiation counter.

Table 1. Rhodopsin peptides

Peptides were synthesized, purified, and coupled to KLH as described in the Materials and methods section by using the known amino acid sequence of bovine opsin. Numbering is from the *N*- to the *C*-terminus.

Code name	Amino acid residue numbers	Sequence
Rhod-4	341–348	Glu-Thr-Ser-Gln-Val-Ala-Pro-Ala
Rhod-1	332–339	Glu-Ala-Ser-Thr-Thr-Val-Ser-Lys
Rhod-10	232–239	Glu-Ala-Ala-Ala-Gln-Gln-Gln-Glu

Western blots and SDS/PAGE. After protein determination [13], retinal samples were solubilized in sample buffer containing 2% (v/v) 2-mercaptoethanol, 3% SDS, and 50 mM-Tris/HCl, pH 6.8. SDS/(7.5 or 10%, w/v) PAGE was performed as described by Laemmli [20]. Samples were electroblotted on to nitrocellulose by using a Transphor (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.) transfer system at 0.2 A for 3 h. Nitrocellulose was blocked by incubation for 16 h in buffer A [10 mM-Tris/HCl (pH 7.5)/50 mM-NaCl/2 mM-EDTA/0.1% NaN_3] containing 2% (w/v) bovine serum albumin. Blots were incubated 6–8 h in antisera, washed in buffer A and, finally, allowed to react with approx. 1×10^6 c.p.m. of ^{125}I-protein A. The nitrocellulose blots were washed, dried, and exposed to Kodak XR-1 film, using du Pont Cronex intensifying screens, at −70 °C. Autoradiograms were scanned in a densitometer (Bio-Rad) and quantified on a Shimadzu integrator. Analyses of phosphorylated rhodopsin on SDS/PAGE were as previously described [15].

RESULTS

Rhodopsin content

We have previously reported that the reaction of rcdl-affected Irish-setter rhodopsin with *C*-terminal-specific anti-peptide antisera (anti-Rhod-4) was reduced when compared with that of the normal, unaffected, animal [9]. Since the 8-week-old dogs used in that study already had considerable outer-segment degeneration, it was difficult to ascertain the cause–effect relationships. In the present

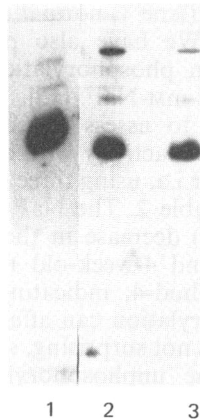

Fig. 1. Western blots of normal and affected rcdl dog retinal homogenates equalized for rhodopsin content and allowed to react with anti-Rhod-10 antisera

Gels (10% polyacrylamide) were electroblotted and allowed to react as described in the Materials and methods section. Lane 1, bovine depleted rod-outer-segment discs (25 μg of protein); lane 2, normal dog retina (8 weeks old); lane 3, rcdl retina (4 weeks old). Densitometric scans and peak area integrations were: lane 2: top band, 94578; middle band, 31275; bottom band (rhodopsin monomer) 216430; lane 3: top band, 84345; middle band, 34851; bottom band, 174831.

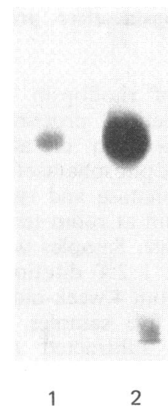

Fig. 2. SDS/PAGE of normal dog retinal homogenates with and without NaF in the presence of [γ-^{32}P]ATP

Phosphorylation reactions were as described in the Materials and methods section, using 8-week normal dog retinal homogenates (10 μg of protein), [γ-^{32}P]ATP (10 μCi/sample) and 1.0 mM-NaF. The Figure shows an autoradiogram. Lane 1, normal phosphorylated sample without NaF; lane 2, normal phosphorylated sample + 1.0 mM-NaF.

study we analysed retinas from younger Irish setters (4 weeks old).

Each retinal homogenate was assayed for rhodopsin by using anti-Rhod-10 antisera to standardize the rhodopsin content in each case. Samples were then diluted so that the amounts of rhodopsin protein/μl of sample were identical. The amount of rhodopsin/μg of protein was lower in rcdl samples (usually about one-half that in normal samples) so that, generally, normal samples were diluted to equalize for rhodopsin. However, since this varied with each dog eye, standardization was necessary in each case.

When rhodopsin content was equalized by RIA with anti-Rhod-1 and anti-Rhod-10, samples were analysed by Western blots using anti-Rhod-10. Fig. 1 depicts the results of such a blot of retinal homogenates from 8-week-old normal and 4-week-old affected dogs. The results indicate no difference in reaction of normal (lane 2) and affected (lane 3) samples when equal amounts of rhodopsin are added to each lane. Lane 1 is an immunoblot of bovine discs with anti-Rhod-10. These results demonstrate the specificity of the anti-peptide (Rhod-10) antisera for rhodopsin. We have previously reported on the specificity of the anti-Rhod-4 antisera [9]. Anti-Rhod-1 antisera fails to react on a Western blot.

Table 2. R.i.a. of rhodopsin after phosphorylation

After equalization with anti-Rhod-1 and anti-Rhod-10 (see the Materials and methods section), 1.15 μl of retinal homogenates were incubated with 1 mM-NaF + 1 mM-ATP, or with buffer alone, for 19 h at 4 °C. Samples were assayed by r.i.a using antisera at 1:250 dilution. Values are c.p.m./tube and are means ± S.D. for triplicate experiments. Background radioactivities have been subtracted and were: anti-Rhod-4, 119 ± 12 c.p.m.; anti-Rhod-1, 145 ± 22 c.p.m.; anti-Rhod-10, 80 ± 9 c.p.m. Statistical significance: $^*P \leqslant 0.05$ compared with normal −NaF.

	Antisera reaction (c.p.m./assay tube)		
Sample	Anti-Rhod-4	Anti-Rhod-1	Anti-Rhod-10
8-Week-old normal			
−NaF	4712 ± 173	512 ± 123	966 ± 48
+NaF	4210 ± 205*	621 ± 46	1096 ± 163
8-Week-old rcdl			
−NaF	776 ± 76	605 ± 63	927 ± 143
+NaF	878 ± 5	553 ± 83	889 ± 32
4-Week-old rcdl			
−NaF	806 ± 40	638 ± 128	1078 ± 73
+NaF	682 ± 22	457 ± 69	1004 ± 123

Table 3. R.i.a. of rhodopsin after pretreatment with phosphatase

*After equalization of rhodopsin contents with anti-Rhod-9, 1–4 μg of retinal protein homogenates was incubated with 10 μg each of alkaline phosphatase (3.5 units/mg), then acid phosphatase (4.7 units/mg) (Sigma human alkaline phosphatase and type IV-S potato acid phosphatase) for 15 min at room temperature. The ATP concentration was 1 mM. Samples were then assayed by r.i.a. using antisera at 1:250 dilution. Normal and rcdl retinal samples were from 4-week-old Irish setters. Values are means ± S.D. for five samples. Background radioactivities have been subtracted and were: normal, 135 ± 16 c.p.m.; rcdl, 182 ± 12 c.p.m. Statistical significance: *$P \leqslant 0.001$ when compared with the −ATP sample.

Sample treatment	Antisera reaction to Rhod-4 (c.p.m./assay tube)
Normal	
No additions	4380 ± 740
+ Phosphatase − ATP	7360 ± 290
+ Phosphatase + ATP	6680 ± 60*
Rcdl	
No additions	1490 ± 50
+ Phosphatase − ATP	2800 ± 150
+ Phosphatase + ATP	2210 ± 130*

Rhodopsin r.i.a.

We have previously reported that the level of phosphorylation of rhodopsin is altered in affected as against normal dog retinas [15]. In order to determine if this could cause the decreased reaction with anti-Rhod-4 antisera, retinas were incubated with NaF before r.i.a. NaF is a non-specific phosphatase inhibitor which dramatically increases rhodopsin phosphorylation in normal and affected dog retinas [15]. Enhanced phosphorylation of normal dog rhodopsin with NaF is illustrated in Fig. 2 (lane 1, normal dog; lane 2, normal dog + 1 mM-NaF). We have also previously observed enhanced rhodopsin phosphorylation of rcdl-affected-dog retinas with 1 mM-NaF [15]. This suggests that NaF can be used to assess the effects of increased phosphorylation on reactivity with anti-Rhod-4.

The results of an r.i.a. using three antipeptide antisera are illustrated in Table 2. The NaF caused a slight but significant ($\simeq$ 15%) decrease in the reaction of the 8-week-old normal and 4-week-old rcdl retinal samples with antisera to Rhod-4, indicating that the level of rhodopsin phosphorylation can affect the reaction with anti-Rhod-4. This is not surprising, since the antisera are directed against the unphosphorylated peptide, and, therefore, phosphorylation would alter the epitope recognized by the antisera. However, the decrease was small ($\simeq$ 15%), and in no case were the rcdl levels comparable with normal levels of reaction to the antisera. One reason for the decreased reaction of anti-Rhod-4 with the rcdl retinas could be stable over phosphorylation of the rhodopsin. To determine if this occurs, samples were dephosphorylated with phosphatases before reaction with antisera. Table 3 illustrates the results of such an experiment. It is apparent that dephosphorylation increases reactivity of both normal and rcdl samples at 4 weeks of age (68% increase for normal and 88% increase for rcdl). Re-addition of ATP caused an increase in phosphorylation (results not shown) and a slight, but significant ($P \leqslant 0.001$), decrease in reactivity. It is thus apparent that the degree of phosphorylation can alter the reaction with the anti-Rhod-4 antisera. Furthermore, dephosphorylation significantly increases the reaction of the rcdl samples.

The decreased reaction of the affected retinas was apparent at 4 weeks of age, suggesting that this decrease is an early event in the disease process. However, although this decreased reaction is also apparent at 8 weeks of age, we were unable to manipulate the level of reaction by altering rhodopsin phosphorylation (Table 2). This suggests that, by 8 weeks of age, the disease has progressed to the point where irreversible alterations in rhodopsin have occurred. Later alterations could include

Table 4. R.i.a. of rhodopsin after treatment with carboxypeptidase Y

Incubations and the r.i.a. were as described in Table 2, except that the incubation time was 15 h. [NaF] was 1 mM, [ATP] was 1 mM and carboxypeptidase Y was added at 5 μg/tube. Background radioactivities have been subtracted and were: anti-Rhod-4, 119 ± 22 c.p.m.; anti-Rhod-1, 145 ± 22 c.p.m.; anti-Rhod-10, 80 ± 6 c.p.m. Results are means ± S.D. for four samples.

Sample	Antisera reaction (c.p.m./assay tube)		
	Anti-Rhod-4	Anti-Rhod-1	Anti-Rhod-10
8-Week normal			
Control	4093 ± 160	671 ± 133	955 ± 115
+ Carboxypeptidase + NaF + ATP	2303 ± 105	575 ± 77	883 ± 28
+ Carboxypeptidase − NaF − ATP	1197 ± 107	624 ± 181	816 ± 42
8-Week rcdl			
Control	805 ± 98	988 ± 107	784 ± 89
+ Carboxypeptidase + NaF + ATP	559 ± 58	1017 ± 203	782 ± 89
+ Carboxypeptidase − NaF − ATP	555 ± 76	2239 ± 408	828 ± 79
4-Week rcdl			
Control	802 ± 55	862 ± 14	886 ± 104
+ Carboxypeptidase + NaF + ATP	538 ± 22	2140 ± 145	906 ± 88
+ Carboxypeptidase − NaF − ATP	408 ± 34	2016 ± 140	813 ± 104

Table 5. R.i.a. of rhodopsin after mixing of affected and normal samples

Samples were incubated as described in Table 2, except that incubation of mixtures was of 1.15 μl of each and was for 28 h at 4 °C. Background radioactivities have been subtracted and were 156 ± 27 c.p.m. Values are means ± S.D. for five samples. Values in parentheses are percentage decreases compared with the normal reaction.

Sample	Antisera reaction to anti-Rhod-4 (c.p.m./assay tube)
8-Week normal	
Expt. 1	4773 ± 44
Expt. 2	4470 ± 105
8-Week rcdl	
Expt. 1	990 ± 131
Expt. 2	850 ± 62
8-Week normal + 8-week rcdl	
Expt. 1	4456 ± 520 (22 %)
Expt. 2	3869 ± 299 (28 %)

post-translational modifications, stable structural changes or proteolysis.

Table 4 illustrates that the decreased reaction with anti-Rhod-4 can be mimicked by clipping samples with carboxypeptidase Y. This enzyme clips sequentially at the *C*-terminus of rhodopsin. Initially, time-course studies were conducted to determine the course of proteolysis by monitoring the loss of reactive Rhod-1 on a r.i.a. (i.e. the next sequence in from the *C*-terminus). Optimum incubation time was from 12 to 17 h at 4 °C. Incubation of samples for 15 h (Table 4) with carboxypeptidase Y resulted in the loss of anti-Rhod-4 reactivity in all samples. Loss was greater in normal (71 %) than in the 4-week rcdl (49 %) or 8-week rcdl (31 %) samples. Significantly, when rhodopsin was phosphorylated by including NaF and ATP in the reaction mixture, the effect was that of a protection from clipping by carboxypeptidase Y. Phosphorylation may cause a conformational change in rhodopsin, such as to render it less susceptible to proteolysis. Such an effect was not observed in the 8-week-old rcdl samples (Table 4).

The effect of such treatment on the Rhod-1 site was unusual. This region of rhodopsin is a probable site of phosphorylation by rhodopsin kinase (residues 332–339) and is a recognition region for the transducin [11]. After proteolysis the normal sample showed no change in anti-Rhod-1 reaction. However, the affected samples showed a dramatic increase in anti-Rhod-1 reactivity (998 c.p.m. as against 2239 c.p.m. for 8-week-rcdl; 862 c.p.m. as against 2016 c.p.m. for 4-week rcdl). This may reflect an increased accessibility of this region in affected dogs after proteolytic clipping of the *C*-terminus. Since such a change was not observed in the normal samples at 15 h, this further suggests that rhodopsin is altered in the Rhod-1 region in the disease state.

If proteolytic clipping of the *C*-terminus of rhodopsin occurs in affected samples, then mixing of affected and normal retinal homogenates should result in a loss of normal-sample anti-Rhod-4 reactivity. Table 5 demonstrates that long-term incubation of affected samples with normal samples (28 h) lowers reactivity with anti-Rhod-4. This suggests that the low reaction of the affected samples with anti-Rhod-4 antisera may be partially due to proteolytic clipping of this region. However, 4-week-old rcdl samples did not 'clip' normal retinal preparations, indicating that this may simply be a result of a general proteolysis due to the advanced disease state as 8 weeks of age (results not shown).

Table 6. Peptides reacting with anti-Rhod-4

Peptides were synthesized as described in the Materials and methods section. Each was assayed by using excess peptide in the r.i.a. (> 10 μg). Rhod-4 − 1 corresponds to residues 341–347 and Rhod-4 − 2 to 341–346. Antisera were used at 1:250 dilution. Background radioactivities have been subtracted and were: Rhod-4, 228 ± 81 c.p.m.; Rhod-4 − 1, 113 ± 8 c.p.m.; Rhod-4 − 2, 151 ± 27 c.p.m. Results are means ± S.D. for four samples. Rhod-4 − 1 comprises residues 341–347 (Glu-Thr-Ser-Gln-Val-Ala-Pro) and Rhod-4 − 2 comprises residues 341–346 (Glu-Thr-Ser-Gln-Val-Ala).

Antigen	Reaction (c.p.m./assay tube)
Rhod-4	25868 ± 2163
Rhod-4 − 1	1338 ± 253
Rhod-4 − 2	706 ± 94

Although we cannot rule out a very slow 'clipping' of the *C*-terminus, one might expect that, if that occurred, the reaction of the 4-week-old affected sample would be slightly higher than that of the 8-week-old affected sample. The effects of NaF and mixing experiments suggest that an alteration has occurred in the affected rcdl rhodopsin molecule. Results of proteolysis experiments further indicate specific changes in the Rhod-1 region as well as in the Rhod-4 region.

In summary, we have found a loss of reactivity of rhodopsin with an anti-peptide antisera directed against the *C*-terminus. This is expressed as high reaction in normal retinas and low in both 8- and 4-week-old affected rcdl retinas. The altered reaction can be partially reversed by phosphorylation; however, in no instance can full reactivity be restored to the rcdl rhodopsin. These data suggest that: (1) rcdl rhodopsin is altered in phosphorylation and/or (2) rcdl rhodopsin is altered in conformation due to some stable genetic alteration.

In order to assess the epitope recognized by the anti-Rhod-4 antisera, we have synthesized peptides that each have deleted a single amino acid and have allowed these to react in the r.i.a. Table 6 shows that deletion of a single amino acid (Rhod-4 − 1) from the *C*-terminal peptide diminishes the ability of the antisera to react. Thus the antisera appear to have major components that react with the entire Rhod-4 peptide. Likewise, substitution of a single amino acid (serine or threonine by valine; i.e. phosphorylation sites) gives the same results (not shown). This suggests that the anti-Rhod-4 antiserum is exquisitely sensitive to changes in the rhodopsin *C*-terminus.

DISCUSSION

Irish setter dogs recessively inherit a rod/cone dysplasia. Retinal degeneration occurs in the homozygous affected animals until all rods have degenerated by 18–20

weeks of age, leaving only a small number of cone cells left in the photoreceptor layer. Before photoreceptor degeneration, an abnormality in cyclic GMP metabolism is apparent and is expressed as a failure to light-activate the rod-specific cyclic GMP PDE [2,4]. The PDE protein is present and is histone-activatable; however, the activity is severely diminished [7]. This probably causes the elevated cyclic GMP levels (10-fold) and subsequent retinal degeneration [2].

The cause of the lowered cyclic GMP PDE activity remains a mystery. The PDE protein in affected dogs was measurable both by its reaction (and M_r) on a Western blot that was allowed to react with polyclonal anti-PDE antisera (J. Cunnick, M. Rider, L. J. Takemoto & D. J. Takemoto, unpublished work), and by silver staining of an affected-dog retinal preparation which was immunoprecipitated with ROS-1, a monoclonal anti-PDE antibody [7].

Several possibilities could explain the lowered cyclic GMP PDE activity in the affected Irish setter. Since the PDE protein is present and active (although its activity is very low), the protein could be altered at the gene level, or post-translationally, resulting in a defective PDE enzyme. We have previously reported alterations in phosphatase activity in affected dogs [15]. A change in the kinase/phosphatase system could cause dramatic post-translational alterations in this protein.

Alternatively, earlier events in the light-cascade mechanism could result in the failure to light-activate the PDE. Rhodopsin, transducin and cyclic GMP PDE are all part of this light-mediated cascade. Therefore defects at any point in this cascade cycle would disrupt the final events of light activation of the PDE. We have not found defects in the retinal guanosine-5′-triphosphatase activity in normal, heterozygous or affected dogs. This suggests that rhodopsin is capable of interacting with the Tα subunit of transducin. The ability of the Tα subunit to activate rcdl dog retinal PDE has not been measured. Likewise, we are still uncertain as to whether the PDE interacts only with Tα or also with rhodopsin. Recently we have reported effects of PDE-γ on Tα [24].

The sites on rhodopsin which interact with transducin have been identified by competition with synthetic peptides [11,12] and by binding with monoclonal antibodies [21]. These include a region which is 12 amino acids upstream from the *C*-terminus (residues 317–339; [12]) and a region within the 5–6 external loop of rhodopsin (J. Cunnick, M. Rider, L. J. Takemoto & D. J. Takemoto, unpublished work [21]). It has been previously reported that proteolytic clipping of the *C*-terminal 12 amino acids of rhodopsin with thermolysin does not abolish rhodopsin interaction with transducin or subsequent PDE activation [22,23].

Clearly the *C*-terminal 12 amino acids are not required for activation *in vitro*. However, alterations in any regions within the *C*-terminus could block, or alter the accessibility of, the binding regions, resulting in decreased PDE activation in the rcdl state *in vivo*.

In the present study we have used anti-peptide antibodies to probe for changes in rhodopsin in the normal and rcdl affected Irish setter. We probed as our epitopes the *C*-terminus (Rhod-4), a region which is within the *C*-terminal transducin-binding site (Rhod-1), and a region in the 5–6 loop (Rhod-10). These regions specifically contain six phosphorylation sites that are phosphorylated by rhodopsin kinase. Once rhodopsin content had been equalized in all samples, the only difference in the reaction to antisera was a very pronounced difference in the Rhod-4 region. This was expressed as a high reaction in normal and much reduced in both 8- and 4-week-old affected rcdl samples. The reaction of the rcdl samples to the anti-Rhod-4 antisera was partially restored only by altering the phosphorylation state of rhodopsin with phosphatases. Since increased reactivity was also noted in normal samples, this may suggest that the reaction with anti-Rhod-4 is partly dependent on the rhodopsin phosphorylation state. Since the antisera is directed against an unphosphorylated peptide, dephosphorylation of rhodopsin should increase reactivity. However, the fact that the rcdl reactivity never approaches that of the normal suggests that other alterations are also present in the rhodopsin molecule. At the present time it is not possible to determine which phosphorylation sites are altered in rcdl rhodopsin. Production of antisera to phosphopeptides will be necessary.

Some surprising clues as to the altered sites on rhodopsin were provided by proteolysis experiments. The reaction of all samples was decreased by clipping with carboxypeptidase Y. However, after proteolysis there was a significant increase in reactivity with anti-Rhod-1 in rcdl affected samples (Table 4). This suggests that both the Rhod-1 and Rhod-4 regions may be altered in the affected animals.

However, since we have not seen differences in GTPase activity in normal, carrier or affected (rcdl) animals, this suggests that the altered rhodopsin does not affect interactions with Tα. Subsequent steps such as T$\beta\gamma$-rhodopsin interactions, Tα–PDE interactions or PDE–rhodopsin interactions (if present) have not yet been assessed. Clearly, these must be done in order to determine if the changes in rhodopsin ultimately effect changes in PDE activity in the rcdl Irish setter.

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