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ANTISERA TO SYNTHETIC PEPTIDES OF BOVINE RHODOPSIN: USE AS SITE-SPECIFIC PROBES OF DISC MEMBRANE CHANGES IN RETINAL DYSTROPHIC DOGS

D. J. Takemoto*, B. Spooner** and L. J. Takemoto**

*Department of Biochemistry and **Division of Biology
Kansas State University, Manhattan, KS 66506

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Based on the amino acid sequence of bovine rhodopsin, five peptides corresponding to the carboxyl terminus and one loop region have been synthesized. Rabbit antisera to these peptides recognize rhodopsin in whole bovine and dog retinas. Antisera were used to detect differences in specific regions of rhodopsin in dystrophic vs normal dog retinas. As detected on both "dot blots" and Western blots, rhodopsin from retinas of dystrophic dogs has a reduced reaction with antisera to peptides, Rhod-4 and Rhod-10 (# 341-348 and 232-239, respectively). Since these sites on rhodopsin are possible binding sites for transducin and rhodopsin kinase, an alteration in these regions would have profound effects in the dystrophic state.

Visual transduction in retinal rods involves an internal transmitter substance that couples the signal of photoisomerization of rhodopsin at the disk membrane to the decrease in Na^{2+} conductance at the plasma membrane. Light-induced activation of cyclic GMP phosphodiesterase (PDE) in the rod outer segment of photoreceptors has been suggested as a key step in this transduction process (1).

In the photoreceptors of retinal degenerative Irish Setter dogs, abnormal cGMP metabolism is associated with a genetic mutation that causes early, selective degeneration of photoreceptor cells (2). The dogs develop a biochemical abnormality in cGMP metabolism at around 8 weeks of age (3). The retinal ROS discs from the affected dogs are deficient in the light-activated PDE activity resulting in an accumulation of cGMP (4).

ABBREVIATIONS

ROS, rod outer segments; PDE, 3',5'-cyclic nucleotide phosphodiesterase; rd, retinal dystrophic; cGMP, cyclic guanosine 3',5'-monophosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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Activation of this PDE and concomitant hydrolysis of cGMP is mediated by a "GTP-binding protein complex". This complex, referred to as transducin, serves as the link between the PDE and photolyzed rhodopsin. A defect in any of these three proteins, rhodopsin, PDE, or transducin, might abrogate the visual transduction response. In this report, we have examined specific structural changes which occur in rhodopsin from retinal degenerative Irish Setter dogs. Alterations have been found in two regions which have been previously hypothesized to be possible recognition sites for rhodopsin kinase and for transducin.

MATERIALS AND METHODS

Animals

Eight-week-old dystrophic Irish Setter dog eyes were obtained through the National Eye Institute. Normal dog eyes were purchased from Pel-Freeze. The frozen eyes were thawed and retinas were dissected immediately under room light.

Antisera to Rhodopsin Carboxyl Terminus

Peptides were synthesized manually using the solid-state method of Hodges and Merrifield (5) as modified by Gorman (6) with the exception that cleavage of peptides and protecting groups was performed by HBr and anhydrous trifluoroacetic acid (7). Peptides were purified by cellulose thin-layer electrophoresis using a buffer of acetic acid:formic acid:H_2O (3:1:16), then visualized with ninhydrin and the corresponding unsprayed lanes were scraped from the plates and extracted with 0.5% NH_4OH. These peptides were then lyophilized and redissolved in distilled water and stored at -20°C. To determine amino acid composition and to quantitate the peptides, 0.5% of each total sample was hydrolyzed in 6 N HCl and amino acid content was determined using an HPLC reverse phase C_{18} column (Vydak) and o-phthalaldehyde as a detecting agent (8). Peptides had expected amino acid compositions as determined after HCl hydrolysis. Quantitation was accomplished by peak integration and comparison with known amino acid standards.

The peptides synthesized are listed in Table 1. These peptides were crosslinked to keyhole limpet hemocyanin (KLH) as follows. The KLH (3 mg total protein; Sigma) was added in 0.2 ml distilled water to 1.0 mg of peptide in 0.2 ml distilled water. To this was added 0.2 ml of 30 mM glutaraldehyde. The reaction took place on a shaker at room temperature for 12 h. Following this, the reaction was quenched by addition of 1 ml of 2 M sodium borohydride. The sample was then dialyzed against 1 mM ammonium bicarbonate (pH 7.0). Rabbits were injected 5 times, every two weeks, with 200 µg of KLH-peptide in a 50:50 suspension of KLH-peptide: Freund's complete adjuvant (1st injection) or: Freund's incomplete adjuvant (next 4 injections). Sera were tested after 4 weeks on a nitrocellulose dot blot, using purified bovine rhodopsin.

Polyclonal Antisera Preparation

Retina from 1000 fresh bovine eyes were dissected and ROS isolated as previously described (9,10). Rhodopsin was purified from other ROS membrane-bound proteins by separation on 10% SDS-PAGE. The identity of each protein was verified by peptide mapping (11-15). Approximately 30 µg of purified

protein was dissolved in 200 µl of water plus 100 µl of Freund's complete adjuvant. Adult female rabbits were injected, subcutaneously, five times at 20 day intervals. Sera were obtained and used at a 1:1000 dilution.

Western Blots

After protein determination according to Bradford (16) using bovine serum albumin as a standard, suspensions of dog retinas were solubilized in sample buffer containing SDS and electrophoresed on 7.5% or 10% SDS-PAGE according to Laemmli (17). The proteins were then electroblotted onto nitrocellulose using an EC electrophoretic transfer system at 5 V (100 mA) for 3 h. Unreacted nitrocellulose was blocked by incubation for 16 h in buffer A (0.01 M Tris, [pH 7.5], 0.01% sodium azide) containing 2% w/v bovine serum albumin (BSA). Blots were then incubated for 8 h in antisera or in control, pre-immune sera and washed in buffer A. After extensive washing, the nitrocellulose sheets were incubated for 3 h in buffer A with 2% BSA and 1 x 10^6 cpm/ml of radioiodinated protein A (1-2 µg/ml) at room temperature. The blots were then washed, dried, and exposed overnight at -70°C to Kodak XR-1 film using Dupont Cronex intensifying screens.

Solid Phase Radioimmunoassay

Radioimmunoassay using "dot blots" was essentially as described by Jahn et al (18). Protein content of whole retina was determined using BSA as a standard (16). Each retinal suspension was diluted to approximately 1 mg/ml with buffer A and spotted onto squares (1 cm x 1 cm) drawn onto nitrocellulose paper (Schleicher and Schuell, 0.45 microns pore size). The nitrocellulose was dried for 15 min, then incubated overnight in buffer A containing 2% (w/v) BSA. Blots were then incubated in antisera and then protein A, as described above for Western blots. Following autoradiography each spot was cut out and counted in a gamma counter. Initially dose curves with differing amounts of retinal protein were used to verify that the reaction was within the linear range. In all experiments binding of antisera to tropomyosin, was used to equalize the amount of protein used from rd and normal dog retina. The linear portion of the curve was from 1.0 to 20.0 µg total retinal protein for the "dot blots".

RESULTS AND DISCUSSION

Peptides synthesized are listed in Table 1. All correspond to regions of the carboxyl terminus of bovine rhodopsin except Rhod-10, a loop region. Antisera were prepared and tested for specificity. Figure 1 illustrates a dot blot using each antisera preparation reacted with rhodopsin, ROS, or 2 unrelated proteins. Antisera did not crossreact with bovine α crystallin of lens or with BSA. All antisera were tested on quantitative dot blots using whole retina from normal or dystrophic dogs. These results are summarized in Table 2. From these results, it is evident that decreased reactivity was observed only in regions corresponding to Rhod-4 (N/rd ratio = 2.06-3.22) and slightly in Rhod-10 (N/rd ratio = 1.45-1.86). A polyclonal anti-rhodopsin antisera, included as a control, also had less reaction with rd vs normal dog retina. Since no change was observed in the other regions of rhodopsin, this

TABLE 1

Rhodopsin Peptides[a]

Code Name	Amino Acid Residue Number	Sequence
Rhod-3	317-321	H_2N-MET-VAL-THR-THR-LEU-COOH
Rhod-4	341-348	H_2N-GLU-THR-SER-GLN-VAL-ALA-PRO-ALA-COOH
Rhod-5	245-253	H_2N-LYS-ALA-GLU-LYS-GLU-VAL-THR-ARG-MET-COOH
Rhod-7	66-73	H_2N-LYS-LYS-LEU-ARG-THR-PRO-LEU-COOH
Rhod-10	232-239	H_2N-GLU-ALA-ALA-ALA-GLN-GLN-GLN-GLU-COOH

[a] Peptides were synthesized and purified as described in the Methods using the known amino acid sequence of bovine opsin as a model. Numbering of the sequence starts with the amino terminus as Residue # 1 and the carboxyl terminus as Residue # 348. All peptides were dissolved in distilled water. Concentrations were determined by amino acid analysis as described in the methods.

suggests that the total amount of rhodopsin is not different in the dystrophic vs normal dog retinas. Rather, this specific decrease in antisera binding to Rhod-4 and Rhod-10 may be due to either a change in accessibility of these regions, or to a covalent modification of the rhodopsin molecule itself.

To differentiate between these possibilities, Western blots were performed on each sample in which a difference on dot blots was observed. In these experiments, presumably changes in native rhodopsin accessibility would be masked since the Western blot separation step is conducted on SDS-PAGE.

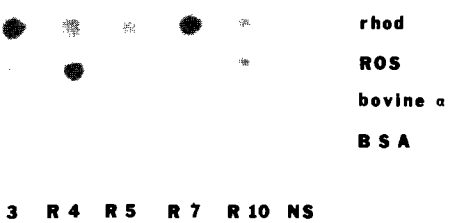

Figure 1: Dot blots of antisera raised against specific peptides which correspond to regions of bovine opsin (see Table 1 for sequence of peptides). Antisera was developed in rabbits as described in the Methods Section of this proposal. R3, R4, R5, R7 and R10 are antisera against peptides Rhod-3, Rhod-4, Rhod-5, Rhod-7 and Rhod-10 respectively. Antigens are labeled in the right column. Rhod = purified bovine rhodopsin; ROS = bovine rod outer segments; bovine α = alpha crystallin from bovine lens; BSA = bovine serum albumin. NS = normal pre-immune sera.

Table 2

Binding of Anti-Rhodopsin Antisera to Retinas

Sample[a]	cpm Bound[b]		N/RD Ratio[c]	
	Exp.1	Exp. 2	Exp. 1	Exp. 2
		Anti-Rhod-3		
RD	859±28(4)	1250±114(3)	1.11	1.49
Normal	958±66(4)	1864±34(3)		
		Anti-Rhod-4		
RD	2374±162(4)	2347±77(3)	2.06	3.22
Normal	4904±194(4)	7566±303(3)		
		Anti-Rhod-5		
RD	760±47(4)	1092±123(3)	1.10	1.33
Normal	836±84(4)	1456±33(3)		
		Anti-Rhod-7		
RD	785±57(4)	1403±102(3)	0.99	0.83
Normal	782±50(4)	1171±84(3)		
		Anti-Rhod-10		
RD	1880±103(4)	1504±174(3)	1.45	1.86
Normal	2730±162(4)	2798±111(3)		
		Polyclonal Anti-Rhodopsin		
RD	3061±476(5)	---	2.69	---
Normal	8238±209(6)	---		---

[a] Retinas were dissected and shaken for 1 min. in 30% sucrose w/v, 65 mM NaCl, 2 mM MgCl, 5 mM tris HCl (pH 7.4), 1 mM dithiothreitol, and 0.5 mM PMSF. Retinas were then pelleted at 10,000 rpm (Sorvall, SS-34) for 10 min. and spotted directly onto nitrocellulose as a suspension in 50 mM tris. HCl (pH 7.4). Each spot contained 7.6 µg total retinal protein as determined by the dye procedure of Bradford (16) using BSA as a standard. Linearity was assessed as described in the text.

[b] Each sample was reacted with anti-peptide antisera as described in the text. Numbers are the mean of cpm of ^{125}I-protein A bound per spot ± standard deviation. Numbers in parentheses are number of samples tested. Each individual spot was cut out and counted in a gamma counter. Antisera was used at 1:500 dilution. Protein-A was added at approximately 1-2 µg/ml; 1x10^6 cpm/blot. Dashed line indicates samples not tested.

[c] N/rd = ratio of the mean of cpm of ^{125}I-protein A in normal samples divided by the mean of cpm of ^{125}I-protein A in rd samples.

TABLE 3

Analyses of Retinal Rhodopsin from Normal and Dystrophic Dogs by Western Blots

Sample[a]	cpm Bound	N/RD Ratio
	Anti-Rhod-4	
RD	7226±165(4)	1.86
Normal	13504±1646(3)	
	Anti-Rhod-10	2.39
RD	2008±93(3)	
Normal	4802±61(3)	
	Polyclonal-Anti-Rhodopsin	
RD	3163±87(4)	3.46
Normal	10956±208(4)	

[a] Samples were prepared as described in Table 2 except that each retinal sample was dissolved in SDS - sample buffer and electrophoresed in a 10% SDS-PAGE gel (17). Following electroblotting and reaction with ^{125}I-protein A, the lanes from Figure 2 were cut out, both monomer and dimer, and counted in a gamma counter. Results are the mean ± standard deviation. Numbers in parentheses are total number of samples run.

This procedure would, thus, determine if differences in *in situ* accessibility of native rhodopsin occurs in the dystrophic state.

Table 3 illustrates the results of Western blots of *rd* and normal dog retinas. The *rd* samples had less reactivity than the normal samples. This was apparent using either anti-Rhod-10 or anti-Rhod-4 antisera. Once again, polyclonal antisera included as a control also had less reaction with *rd* samples. The results of Western blots using anti-Rhod-10 and anti-Rhod-4 are pictured in Figure 2.

In summary, our results using antisera against several regions on the carboxyl terminus and one loop region of rhodopsin suggest that of these regions, Rhod-4 and Rhod-10, are altered in dystrophic Irish Setter retina. Both of these regions have been hypothesized as sites of interaction for rhodopsin kinase and/or for transducin (1). An alteration in these regions would have profound effects on the interaction of transducin with photolyzed rhodopsin. This could, in turn, account for the failure to light-activate the

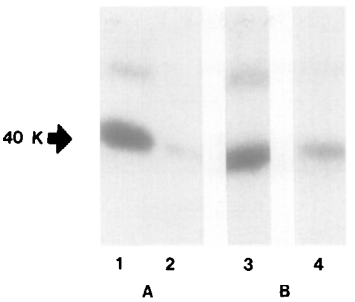

Figure 2: Western blot of whole retina from normal and dystrophic dogs. Each lane contains 22 µg total retinal protein. A.) Reaction with anti-rhod-10 antisera. B.) Reaction with anti-Rhod-4 antisera. Red blood cell membrane proteins were used as molecular weight markers (19). Lanes marked 1 are of normal dog retina; lanes marked 2 are of dystrophic dog retina.

PDE from the retina of dystrophic dogs, resulting in accumulation of cGMP and eventual retinal degeneration.

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REFERENCES

1) Shichi, H. (1983) in "Biochemistry of Vision" (H. Shichi, ed.) pp. 239-251, Academic Press, N. Y.
2) Bitensky, M.W., Gorman, R.E., Miller, W.H. (1971) Proc. Natl. Acad. Sci. U.S.A. 68,561-565.
3) Aguirre, G.D. and Rubin, L.F. (1975) J.A.V.M.A., 166, 157-164.
4) Aguirre, G., Farber, D., Lolley, R., Fletcher, R.T., and Chader, G. (1978) Science, 201, 1133-1134.
5) Hodges, R.S., and Merrifield, R.B. (1975) Anal. Biochem. 65, 241-272.
6) Gorman, J.J. (1984) Anal. Biochem. 136, 397-406.
7) Merrifield, R.B. (1964) Biochem. 3, 1385-1390.
8) Lookhart, G.L., Jones, B.L., Cooper, D.B., and Hall, S.B. (1982) J. Biochem. Biophys. Methods 7, 15-23.
9) Kuhn, H. (1980) Nature 283, 587-589.
10) Papermaster, D.S. and Dryer, W.J. (1974) Biochem. 13, 2438-244.
11) Takemoto, D.J., Haley, B., Hansen, J., Pinkett, O. and Takemoto, L.J. (1981) Biochem. Biophys. Res. Comm. 102, 341-347.
12) Takemoto, D. J., Hansen, J., Takemoto, L.J. and Houslay, M.D. (1982) J. Biol. Chem. 257, 14597-14599.
13) Takemoto, L.J., Hansen, J., Farber, D., Souza, D. and Takemoto, D.J. (1984) Biochem. J. 217, 129-133.
14) Takemoto, D.J., Hansen, J., Takemoto, L.J., Houslay, M., and Marchmont, R.J. (1984) Adv. in Cycl. Nucl. and Prot. Phos. Res. 16, 55-63.
15) Elder, J.H., Pickett, R.A., Hampton, J. and Lerner, R.A. (1977) J. Biol. Chem. 252, 6510-6515.
16) Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
17) Laemmli, U. (1970) Nature 227, 680-686.
18) Jahn, R., Schiebler, W., and Greengard, P. (1984) Proc. Natl. Acad. Sci. 81, 1684-1687.
19) Fairbanks, G., Steck, T.L., and Wallach, D. (1971) Biochem. 10, 2606-2617.