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Key words: corneal wound healing, corneal burns, alkali (corneal performation), vitamin C

REFERENCES

Sensitizing activity of 9,13-dics retinal in bleached photoreceptors of the skate.

ROSALIE CROUCH and DAVID R. PEPPERBERG.

9,13-dics Retinal was externally applied to photoreceptors of isolated skate retina that previously had been desensitized by bleaching irradiation. This treatment led to a significant lowering of photoreceptor threshold and to the intracellular formation of isorhodopsin II, an artificial visual pigment containing 9,13-dics retinal as its chromophore. These results suggest that isorhodopsin II can function in situ to promote an increase in the visual sensitivity of skate photoreceptors.

Although all naturally occurring visual pigments contain as their chromophore the 11-cis form of retinal (or 3-dehydroretinal), the 11-cis isomer is not unique in its ability to bind at the chromophoric site of opsin. Recently, it has been shown that a photosensitive pigment, "isorhodopsin II," is formed on the incubation of extracted opsin with 9,13-dics retinal, a stereoisomer of the native chromophore. In certain respects, the photochemistry of isorhodopsin II is similar to that of rhodopsin and "classic" isorhodopsin (isorhodopsin I, containing 9-cis retinal); for example, the bleaching of all three pigments leads to the formation of opsin and all-trans retinal. In other ways, isorhodopsins I and II differ significantly from rhodopsin; the isopigments maximally absorb light at relatively shorter wavelengths, and their quantum efficiencies of bleaching are less than that of the naturally occurring pigment. Furthermore, the formation of isorhodopsin II in vitro occurs much more slowly than does the formation of rhodopsin or isorhodopsin I. The distinctive properties of these pigments clearly depend on the configuration of their retinal chromophore.

The fact that opsin can combine with synthetic isomers of retinal raises an interesting question: Do the resulting analogs of rhodopsin exhibit physiological activity if they are induced to form within the photoreceptors? Recent evidence has suggested that isorhodopsin I can indeed act in the visual process. For example, treatment of the partially bleached, isolated skate retina with 9-cis retinal leads to both the formation of isorhodopsin I in the receptors and a substantial lowering of photoreceptor threshold. Here we present evidence that 9,13-dics retinal, when applied to the skate retina under similar conditions, also promotes the formation of pigment (isorhodopsin II) and a sensitization of the receptors. Our findings suggest that isorhodopsin II can at least partially mimic the visual activity of rhodopsin.

Methods. The isolated skate retina, utilized in the present experiments, is a preparation in which the photoreceptors do not ordinarily regenerate visual pigment. Sections of retina (approximately 3 by 5 mm) were isolated from the tapetal region of the dark-adapted eye; the techniques of dissection and electrophysiological recording, as well as the composition of the Ringer's solution, were as previously described. Photoreceptor po-
Fig. 1. Sensitization of skate photoreceptors induced by treatment with 9,13-dicis retinal. In each experiment, the bleaching irradiation was terminated at time zero; prior to bleaching, relative photoreceptor thresholds were approximately -5.1 and -6.1 log units, respectively, for the retinas described by the upper and lower curves. A suspension containing 9,13-dicis retinal (2,800 nmol/ml in ethanol-Ringer's solution, 2:100 by volume) was applied dropwise to each retina at the times indicated by arrows; each application was performed within a period of 1 min. Approximate quantities of 9,13-dicis retinal delivered during these applications were, respectively: for the preparation described by the upper curve, 130, 70, 130, and 80 nmol; for the preparation described by the lower curve, 90, 160, and 210 nmol.

Pigment chromophores contained within treated retinas were extracted and analyzed chromatographically. The procedure used involved the isolation of retinal from (denatured) opsin by extraction with methylene chloride; previous work with pigments formed in vitro has indicated that the removal of retinal chromophores by this method promotes <2% isomerization of the retinal. Analyses were performed on isolated retinas that had been mounted on Ringer-moistened filter paper (Whatman, qualitative), incubated at 15° to 19° C in the presence of retinal, and then stored in the dark at -20° C. All centrifugations were for 10 min, 500 × g, at 4° C. Pigment chromophores were extracted as follows. First, the filter paper surrounding the retina was trimmed away, and the retina (together with the underlying filter paper) was gently homogenized with a Teflon pestle in 2 ml of petroleum ether (b.p. 30° to 60° C). The homogenate was centrifuged, and the pellet was washed with 2 ml of petroleum ether and then stored in the dark at -20° C. All centrifugations were for 10 min, 500 × g, at 4° C. Pigment chromophores were extracted as follows. First, the filter paper surrounding the retina was trimmed away, and the retina (together with the underlying filter paper) was gently homogenized with a Teflon pestle in 2 ml of petroleum ether (b.p. 30° to 60° C). The homogenate was centrifuged, and the pellet was washed with 2 ml of petroleum ether. The washing with petroleum ether, which extracted retinal not bound as chromophore (see Results), was repeated 10 times; after the sixth wash, no retinal was detectable in the supernatant by hplc. To the washed pellet, 2 ml of petroleum ether was then added; the resuspended pellet was ground in a glass tissue grinder, and the resulting suspension was centrifuged. The pellet was washed once more with
Fig. 2. Voltage-intensity data obtained during the experiment shown by the lower curve of Fig. 1 (AC amplification; bandpass of 0.1 to 1,000 Hz). The time intervals during which the data were obtained (as referred to time zero in Fig. 1) were: (Δ), −24 to −15 min (fully dark-adapted); (o), 60 to 64 min (following bleaching); and (●) 202 to 208 min (following treatment with 9,13-dicis retinal). Shown in the inset are the responses which yielded the data points labeled A, B, and C on the voltage-intensity curves.

methylene chloride, and the supernatants obtained in the first and second washes with methylene chloride were combined and dried over sodium sulfate. The anhydrous extract was filtered (Millipore, 0.5 μm pore), and the methylene chloride was removed under vacuum. The dried sample was then redissolved in 50 μl of methylene chloride.

Extracted isomers of retinal were analyzed by hplc; the system used for the separation of isomers was identical to that described above, except that a μ-Porasil column (solvent consisting of ether-hexane, 2:98) was substituted for the preparative column. Isomers of retinal were identified by their pattern of elution from the chromatographic column. With the use of the appropriate extinction coefficient at 365 nm, the amount of each isomer in the fractionated sample was calculated from the absorbance of each compound in the elution profile. At 365 nm, the extinction coefficients of 9,13-dicis, 9-cis, 11-cis, and all-trans retinal are >2 × 10^4 M⁻¹ cm⁻¹; the resolution of the absorbance monitor (about 5 × 10⁻⁴ optical density units) established a limit of detection of about 0.03 nmol for each isomer in the eluate.

Results. Fig. 1 shows the effect of 9,13-dicis retinal on photoreceptor threshold of the previously bleached, isolated skate retina. In each of the two experiments illustrated, a dark-adapted retina was first irradiated to an extent that bleached at least 90% of the rhodopsin initially present in the receptors. Following the stabilization of receptor threshold at a new, elevated value, each retina was treated (at the times indicated by arrows) with a suspension containing 9,13-dicis retinal (2.8 μmol/ml); on a molar basis, the amount of retinal applied at each time (≥70 nmol) greatly exceeded the total amount of opsin (<1 nmol) contained in the section of excised retina. This treatment with 9,13-dicis retinal promoted a significant lowering
of receptor threshold from the stable value attained after the bleaching irradiation; in the two experiments shown, the induced decreases ultimately amounted to about 1.3 and 1.5 log units, respectively. Interestingly, these induced sensitizations proceeded more slowly than those previously observed on similar treatment of the skate retina with 11-cis and 9-cis retinal. Following the initial application of the 11-cis or 9-cis isomer, the lowering of threshold is largely complete within about 25 min. Over a comparable period in the present experiments, the sensitizing effect of 9,13-dicis retinal (record C) is illustrated. A comparison of these records indicates that the treatment with retinal reverses the effect of bleaching, in that it induces a lengthening of the time-to-peak of the PIII response near threshold.

Table I. Extraction of isolated retinas previously treated with 9-cis retinal

<table>
<thead>
<tr>
<th>Treatment of isolated retina*</th>
<th>Extract</th>
<th>Relative amounts of isomers of retinal†</th>
<th>Total amount of retinal contained in pooled extract (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleached plus 9-cis retinal</td>
<td>Petroleum ether</td>
<td>1.00 &lt;0.05 0.10</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Methylene chloride</td>
<td>1.00 &lt;0.05 0.10</td>
<td>1</td>
</tr>
<tr>
<td>Unbleached plus 9-cis retinal</td>
<td>Petroleum ether</td>
<td>1.00 &lt;0.05 0.10</td>
<td>104</td>
</tr>
<tr>
<td></td>
<td>Methylene chloride</td>
<td>0.05 1.00 0.10</td>
<td>1</td>
</tr>
</tbody>
</table>

*The total quantity of 9-cis retinal applied to each retina was about 300 nmol.
†Normalized to the amount of the predominant isomer. Accuracy of values, ±0.05.
‡Previously irradiated to an extent that bleached at least 90% of the rhodopsin initially present.

Table II. Extraction of isolated retinas previously treated with 9,13-dicis retinal

<table>
<thead>
<tr>
<th>Treatment of isolated retina*</th>
<th>Relative amounts of isomers of retinal in methylene chloride extract†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9,13-dicis</td>
</tr>
<tr>
<td>Bleached plus 9,13-dicis retina†</td>
<td>1.00</td>
</tr>
<tr>
<td>Bleached plus 9,13-dicis retinal</td>
<td>1.00</td>
</tr>
<tr>
<td>Unbleached plus 9,13-dicis retinal</td>
<td>0.05</td>
</tr>
<tr>
<td>Unbleached plus 9,13-dicis retinal</td>
<td>0.05</td>
</tr>
</tbody>
</table>

*Each experiment utilized a single retina. The total quantity of 9,13-dicis retinal applied to each retina was between about 250 and 630 nmol. Separate experiments with 9-cis retinal indicated that about 0.5 nmol of retinal was contained in each of the methylene chloride extracts under the present conditions.
†Normalized to the amount of the predominant isomer. Accuracy of values, ±0.05.
‡Previously irradiated to an extent that bleached at least 90% of the rhodopsin initially present.

of increased sensitivity. Shown in the inset of this figure are representative PIII responses recorded before and after the bleaching of the retina (records A and B, respectively), and following treatment with 9,13-dicis retinal (record C). A comparison of these records indicates that the treatment with retinal reverses the effect of bleaching, in that it induces a lengthening of the time-to-peak of the PIII response near threshold.

Viewed in the context of previous findings linking the degree of visual sensitivity with the content of visual pigment in the photoreceptors, our electrophysiological results suggest that exogenous 9,13-dicis retinal promotes the formation of isorhodopsin II in bleached skate photoreceptors. There existed, however, the alternate possibility that little or no isorhodopsin II was actually formed in the treated receptors but rather that the applied 9,13-dicis retinal was converted to another isomer (e.g., 9-cis retinal) that possessed sensitizing activity. It has been reported, for example, that the isomerization of retinal can be catalyzed by contaminating bacteria present in physiological preparations. Because the quantity of 9,13-dicis retinal available for the present study was relatively limited, it was not feasible to measure the formation of pigment within treated retinas by
spectrophotometry. As an alternative means of identifying pigment(s) formed, we chromatographically analyzed pigment chromophores contained within the photoreceptors of treated retinas. The method employed, which involves the extraction of chromophores with methylene chloride, has previously been used for isolating retinal bound to rat and bovine opsin. 

The workability of this method in our studies depends on the efficiency with which excess applied retinal (i.e., retinal that is not bound at the chromophoric site of opsin) can be extracted from treated retinas. For this initial extraction, we used petroleum ether. To obtain information on the specificity of the extraction with petroleum ether, we investigated the effects of treatment with 9-cis retinal, which is known to combine with the opsin contained in bleached skate photoreceptors. Two unbleached (i.e., dark-adapted) and two previously bleached sections of isolated retina were incubated with 9-cis retinal for a period of 145 min; the petroleum ether and methylene chloride extracts obtained from identically treated retinas were each pooled and then subjected to hplc. From the chromatographic profile of each extract, we determined the relative abundance of 9-cis, 11-cis, and all-trans retinal, as well as the total amount of retinal present. The results of these experiments with 9-cis retinal, shown in Table I, indicate that the initial, petroleum ether extract contains by far the major portion of the total retinal recovered from each pair of retinas. (A large portion of the retinal applied to each retina settles into the supporting filter paper.) Much of this retinal is removed prior to the biochemical analysis by the trimming-away of the filter paper surrounding the retina. Moreover, the retinal isolated in the petroleum ether extract of both the bleached and the unbleached retinas is predominantly of the 9-cis form. The data further show that the composition of the methylene chloride extract is, by contrast, highly dependent on whether the retinas were bleached or unbleached; 9-cis retinal predominates in the extract of the bleached retinas, and 11-cis retinal predominates in the extract of the unbleached retinas. The data of Table I therefore indicate that the extraction with petroleum ether efficiently removes excess applied retinal; they further indicate that the subsequent extraction with methylene chloride specifically isolates pigment chromophores present within the treated retinas.

Table II shows the composition of the methylene chloride extracts obtained from isolated retinas that were treated with 9,13-dicis retinal. In the four experiments described, the total periods of incubation with the 9,13-dicis isomer were varied (between 77 and 150 min); however, each period of incubation roughly matched or exceeded the length of time required for the sensitization of bleached retinas (Fig. 1). The data of Table II indicate that previously bleached retinas treated with 9,13-dicis retinal contain the 9,13-dicis isomer as the predominant pigment chromophore; that is, isorhodopsin II is the predominant pigment contained in the photoreceptors. Table II further shows that in unbleached retinas treated with 9,13-dicis retinal, the 11-cis retinal of the native rhodopsin remains the predominant chromophore. Thus it is unlikely that isorhodopsin II forms in the bleached receptors merely by the displacement of another chromophore by the applied 9,13-dicis retinal. The all-trans retinal contained in all of the methylene chloride extracts (10% to 25% of the level of the predominant isomer) probably arises by the isomerization of pigment chromophores during the isolation procedure.

Discussion. Our results show that under physiological conditions, isorhodopsin II can be generated within bleached photoreceptors of the isolated skate retina. Incubation of the retina with 9,13-dicis retinal, which promotes the formation of this pigment, also leads to a significant lowering of photoreceptor threshold. These findings indicate that the reactivity of opsin toward the 9,13-dicis isomer does not arise merely during the isolation of receptor outer segment membranes, nor does it depend on the presence of an extracting detergent. Rather, 9,13-dicis retinal binds at the chromophoric site of opsin in the native environment of the photoreceptor.

It is well established that in the intact eye or eyecup preparation, the slow recovery of visual sensitivity that follows bleaching irradiation occurs in concert with the regeneration of rhodopsin in the photoreceptors. A link between receptor sensitivity and rhodopsin content is also evident in the bleached isolated retina that has been treated with 11-cis retinal. In the present study it has not been possible to measure the kinetics of formation of isorhodopsin II in the photoreceptors. However, in view of the previous findings, we interpret our results to suggest that the sensitization observed here directly reflects the activity of isorhodopsin II formed intracellularly. Under this interpretation, the comparatively slow lowering of threshold promoted by 9,13-dicis retinal is quite consistent with the relatively slow rate at which isorhodopsin II forms in vitro.
Interestingly, receptor thresholds in our preparations, though significantly lowered by the treatment with 9,13-dicis retinal, remain several log units above the level exhibited by the dark-adapted retina (which contains a full complement of rhodopsin). As yet, we do not know what accounts for this apparent limit on the restoration of sensitivity induced by applied 9,13-dicis retinal; however, the following three possibilities have occurred to us. First, such a result could be due to a severe limitation on the extent of isorhodopsin II formation in our preparations. Although we cannot rule out this possibility, it seems unlikely to us in view of (1) the large excess of 9,13-dicis retinal applied to the retinas and (2) previous findings indicating the favorable generation (70% to 80% completion) of isorhodopsin II from extracted rat and bovine opsin.1,10 A second possibility is that the experimental conditions required in the present study (long incubations following 90% bleaching, and exposure of the retina to ethanol and excess retinal) lead to a partial desensitization of the photoreceptors. Previous experiments with 11-cis retinal have suggested that such conditions may limit the increases in sensitivity that can be induced by external application of the chromophore; a desensitizing effect of the treatment with 9,13-dicis retinal could furthermore contribute to the apparent delay in the net lowering of threshold (Fig. 1). A third, particularly intriguing, possibility is that the inherent ability of isorhodopsin II to influence receptor sensitivity may be relatively weak. For example, as a consequence of its configuration in functional photoreceptor membranes, isorhodopsin II may exhibit a severely diminished photosensitivity or may be deficient in some catalytic activity exhibited by rhodopsin during the course of dark adaptation.

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Key words: visual pigment, visual adaptation, photo-receptor sensitivity, isorhodopsin II, retinal, 9,13-dicis retinal

REFERENCES


The photopathology of retinal lesions produced by extended exposure (1000 sec) to low corneal power levels

*In conducting the research described in this report, the investigators adhered to the Guide for Laboratory Animals Facilities and Care of the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences—National Research Council.

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