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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS

III. SPECIFICITY OF THE RETINALDEHYDE EFFECT ON CATION MOVEMENTS IN ROD OUTER SEGMENTS*

F. J. M. DAEMEN AND S. L. BONTING
Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)
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SUMMARY

1. Addition of all-*trans*- or 11-*cis*-retinaldehyde to suspensions of isolated dark-adapted bovine rod outer segments caused an efflux of K^+ and an influx of Na^+, as also occurs upon illumination of these structures.

2. All-*trans*-retinol and all-*trans*-retinoic acid did not have a significant effect on the cation levels.

3. These observations indicate that the aldehyde group of retinaldehyde is required for this cation exchange, suggesting that formation of a Schiff base plays an essential role in this process.

INTRODUCTION

BONTING AND BANGHAM[1] recently presented evidence supporting an ionic mechanism for the stimulation of the photoreceptor cell by light. Illumination of a suspension of dark-adapted bovine rod outer segments caused an influx of Na^+ and an equivalent efflux of K^+. The same effect, qualitatively and quantitatively, occurred upon addition of all-*trans*-retinaldehyde (retinal, retinene or vitamin-A aldehyde) to such a suspension. Increased cation leakage under the influence of retinaldehyde was also observed by these authors in model experiments with artificial membranes consisting of phosphatidylethanolamine, but not of phosphatidylcholine. This effect was shown to be due to the formation of a Schiff base link between the aldehyde group of retinaldehyde and the amino group of phosphatidylethanolamine, the amino group being absent in phosphatidylcholine. Blocking of the amino group made the surface charge of the phospholipid micelles more negative, which has been shown by BANGHAM, STANDISH AND WATKINS[2] to cause a very pronounced increase in the passive permeability for cations. A similar phenomenon was demonstrated for erythrocyte membranes by BERG, DIAMOND AND MARFEY[3], who treated erythrocytes with the amino group reagent 2,4-dinitrofluorobenzene. It was, therefore, suggested that release of retinaldehyde during photolysis of rhodopsin would lead to the observed

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cation leakage in outer segments through formation of a Schiff base with phospholipid amino groups (or other strategically located amino groups) in the rod-sac membrane[1].

This hypothesis would require that the aldehyde group of retinaldehyde is essential for the observed cation movements in rod outer segments. The purpose of the present study was, therefore, to determine the specificity of the retinaldehyde effect, by comparing the effects of all-*trans*-retinaldehyde, 11-*cis*-retinaldehyde, all-*trans*-retinol (vitamin-A alcohol) and all-*trans*-retinoic acid (vitamin-A acid) in incubation experiments with isolated bovine rod outer segments.

MATERIALS AND METHODS

The vitamin-A derivatives were purchased from Distillation Products Industries Inc., and were stored in the dark at —20°, sealed *in vacuo* in small amounts.

Retinas from 60 cattle eyes were homogenized in 60 ml 0.32 M sucrose. After differential centrifugation as previously described[1], 6 ml of a rod outer segment suspension in 0.32 M sucrose were obtained. From this suspension 300-μl aliquots were pipetted into a total of 15 35 mm $\times$ 7 mm centrifuge tubes. The vitamin-A derivatives (15 mM solutions in ethanol) were added to the experimental tubes in a final concentration of 0.075 mM, while an equal volume of ethanol was added to the control tubes, in such a way that two experimental tubes with different vitamin-A derivatives were followed by one control tube. The tubes were kept in the same sequence throughout. After mixing by vibration for 10 sec, the tubes stayed for 10 min at room temperature and were thereupon centrifuged for 30 sec at 10000 $\times$ *g*. From each centrifuged tube 200 μl supernatant were transferred to a clean tube and diluted with water to 4 ml. After removing the remaining supernatant by suction the sediments were dried for 1 h at 105°. All manipulations up to this point were carried out in dim red light. The dry sediments were dissolved by gentle heating in 100 μl concentrated HNO_3 and the resulting solutions quantitatively transferred to test tubes containing 4 ml water. The sodium and potassium contents of the supernatants and of the sediments were determined by flame photometry.

Upon standing in darkness without addition of any compound, a steady loss of both Na^+ and K^+ from the outer segments occurs, leading to average Na^+ and K^+ levels in the supernatants of approx. 1 and 0.5 mM respectively under the conditions used. Any effect of the tested compounds is superimposed on this steady loss of both cations. In order to eliminate this steady loss from the data, control tubes and experimental tubes were handled as much as possible in the same way, as described above. In calculating the cation movements the Na^+ and K^+ concentrations in supernatants and sediments of the experimental tubes were subtracted from the corresponding concentrations in the control tubes. These differences were then referred to dry weight of outer segment preparation present in the tubes. This dry weight was determined by weighing aliquots of the original suspension after drying for 2 h at 105°, correcting for sucrose present in the medium.

RESULTS

The absolute shifts in supernatants and sediments for each added substance were averaged for Na^+ and K^+ separately, and are listed in Table I, Columns 2 and 4.

TABLE I

CHANGES IN Na^+ AND K^+ CONTENTS OF ISOLATED ROD OUTER SEGMENTS UPON INCUBATION WITH VITAMIN-A DERIVATIVES

Gain of cation in the outer segments is indicated by +, loss by −. Final concn. of vitamin-A derivatives, 75 μM.

Compound added	*Change in* Na^+		*Change in* K^+	
	mmoles/kg dry wt.	*% of* Na^+ *in outer segments*	*mmoles/kg dry wt.*	*% of* K^+ *in outer segments*
All-*trans*-retinaldehyde	+0.26	+2.1	−0.21	−2.0
11-*Cis*-retinaldehyde	+0.36	+2.9	−0.10	−0.9
All-*trans*-retinol	+0.02	+0.2	−0.05	−0.5
All-*trans*-retinoic acid	+0.12	+1.0	+0.01	+0.1

Where the cation difference between experimental and control tubes indicated a cation gain of the outer segments under the influence of the added compounds, the difference was reckoned positive, while a cation loss was reckoned negative. The Na^+ and K^+ shifts, expressed as the percentage of the amounts present in the outer segments are shown in Columns 3 and 5 of Table I. The rod outer segments isolated from Dutch cattle in the present study retained considerably less K^+ (11 *vs.* 61 mmoles/kg dry weight) and had slightly higher Na^+/K^+ ratios (1.18 *vs.* 1.03) than those from British cattle in the previous study. This suggests that the first-mentioned outer segments were more fragile than the latter, and retained a much smaller part of their ionic content. In this light it is understandable that the absolute size of the ionic effects (averaged 0.23 mmole/kg dry weight) is much smaller than in the previous study (averaged 1.55 mmoles/kg dry weight), although the average percentage of cation changes upon addition of both retinaldehydes in the present study (2.0 %) was about the same as previously observed for all-*trans*-retinaldehyde (2.5 %).

The results compiled in Table I suggest that the two aldehydes caused a simultaneous gain in Na^+ and loss of K^+, but that retinol and retinoic acid did not cause

TABLE II

APPLICATION OF THE SIGN TEST TO THE CHANGES IN Na^+ AND K^+ CONTENTS OF ISOLATED ROD OUTER SEGMENTS UPON INCUBATION WITH VITAMIN-A DERIVATIVES

n, total number of pairs of control and experimental tubes, in which both sediment and supernatant were analyzed; +, number of cases of cation gain by the outer segments, from sediment and supernatant analysis, divided by 2; −, number of cases of cation loss, divided by 2; o, number of cases where experimental and control data did not differ, divided by 2 (division by 2 is necessary, because results from sediment and supernatant in the same tube are dependent). *P*: double-sided probability.

Compound added	*n*	*Change in* Na^+				*Change in* K^+			
		+	*o*	−	*P*	+	*o*	−	*P*
All-*trans*-retinaldehyde	85	54	9	22	<0.01	15	6	64	<0.01
11-*Cis*-retinaldehyde	23	17	4	2	<0.01	5	3	15	0.04
All-*trans*-retinol	31	14	3	14	1.00	13	2	16	0.71
All-*trans*-retinoic acid	25	15	2	8	0.21	9	3	13	0.52

such an effect. In order to verify this, particularly in view of the relative smallness of the differences in Na^+ and K^+ concentrations to be measured, statistical analysis was essential. This was carried out by expressing the results as an all-or-none effect and applying the sign test to the results listed in Table II. The effects on both ions were significant at the $P = 0.05$ level for the two retinaldehydes, while the effects of retinol and retinoic acid were not significant at this level.

DISCUSSION

An effect of light upon cation exchange in photoreceptor cells has now been reported by several investigators. SEKOGUTI[4] demonstrated a loss of K^+ from frog retina *in vitro* upon illumination. HAGINS and co-workers[5,6] showed that local illumination of squid rods *in vitro* caused an influx of Na^+ into the outer segment and an outward flux of K^+ from the rest of the cell. BUCKSER AND DIAMOND[7] noted an increased influx of ^{22}Na into the isolated, dark-adapted frog retina upon illumination. BONTING AND BANGHAM[1] observed in cattle rod outer segment suspensions upon illumination an efflux of K^+ and an equivalent influx of Na^+. These ionic effects were approximately equal over a range of illumination, causing from 100 % to less than 1 % bleaching of the rhodopsin present. Further evidence for an ionic effect of light can be deduced from the demonstration of a high activity of ouabain-sensitive (Na^+-K^+)-activated ATPase in rod outer segments by BONTING, CARAVAGGIO AND CANADY[8] and the observation by FRANK AND GOLDSMITH[9] that ouabain (0.1 mM) applied to the receptor side of frog retina *in vitro* rapidly abolished all light-induced electrical activity.

An effect of all-*trans*-retinaldehyde upon cation exchange in cattle rod outer segment suspensions, qualitatively and quantitatively equal to the effect of light, has been reported by BONTING AND BANGHAM[1]. The ionic effects remained approximately constant, when the final concentration of retinaldehyde was varied from 0.1 mM (equivalent to 1 mole retinaldehyde per mole rhodopsin) down to 1 μM (equivalent to 1 mole retinaldehyde per 100 moles rhodopsin). Besides confirming this effect of all-*trans*-retinaldehyde, the experiments reported here indicate that the action of added retinaldehyde is due to its aldehyde function and is not a general property of compounds of the vitamin-A type. The compounds tested were all added in a final concentration of 75 μM, approximately equivalent to a 1:1 molar ratio with respect to the rhodopsin present in the outer segments. This would compare to a condition of complete bleaching of all rhodopsin present.

Osmotic and membrane effects of vitamin-A derivatives have been found in a number of other systems. LUCY AND DINGLE[10] observed hemolysis of erythrocytes with retinol and retinaldehyde, but not with retinoic acid. Retinol caused rapid swelling of mitochondria isolated from rat liver, kidney and heart, while retinaldehyde and retinoic acid were hardly active in this respect[11]. Retinol and retinoic acid, but not retinaldehyde, caused release of lysosomal enzymes from lysosomes[12]. These various actions of retinol appear to be due to a general lytic effect, and hence they reinforce the specificity of the retinaldehyde effect on cation leakage in rod outer segments described in this paper. The lytic effects of retinol may also explain the observations by ETINGOF, SHUKOLYUKOV AND LEONT'EN[13] of a loss of both K^+ and Na^+ from rod outer segments upon addition of retinoyl acetate.

A number of questions are raised by the results reported here, which require further consideration. Since visual excitation occurs in msec after absorption of a light quantum by a rhodopsin molecule, does the effect of retinaldehyde on cation movements in rod outer segments take place in a similarly short time? Unfortunately the experiments reported here require a very much longer time, and it would be technically extremely difficult to devise an experiment allowing measurement of ion-exchange processes in rod outer segments within msec.

Another point is the location of the retinaldehyde effect on cation exchange. The visual pigment molecules are generally supposed to be located in the rod-sac membrane. Since release of retinaldehyde from opsin in mammalian rods *in vitro* is considered to be very slow and not to occur at all in squid rods, its action *in vivo* would have to be on the rod-sac membrane near the site of its original binding to opsin. Can the retinaldehyde added in our experiments have acted on this membrane? In our opinion this is quite likely, firstly because the isolated outer segments are broken cell fragments, secondly because retinaldehyde is able to penetrate into condensed monolayers of rhodopsin and phosphatidylethanolamine[1], assisted by the formation of a Schiff base[14].

The low Na^+ concentration (approx. 1 mM) in the suspending medium poses the question of how Na^+ influx can occur. First of all, it must be kept in mind that what is recorded here as a Na^+ influx was in effect observed as a reduction in the Na^+ efflux, which steadily occurs from isolated rod outer segments in darkness and without addition of retinaldehyde. A satisfactory reply to the problem would require a knowledge of the extra- and intra-saccular spaces and ion levels, which we do not have. Addition of Na^+ to the suspending medium leaves the K^+ effect intact, but the Na^+ effect relative to the Na^+ level in the medium becomes too small to be determined. The sacs in rod outer segments are isolated structures, which are osmotically active[15], whereas the plasma membrane appears relatively permeable to solutes. It therefore appears that the extra-saccular space is more or less continuous with the extra-cellular space. Presumably, the immediate effect of light or added retinaldehyde is an influx of Na^+ from the extra-saccular space to the intra-saccular space, followed by a leakage of K^+ from the latter compartment.

The demonstration of the specificity of the retinaldehyde effect upon cation exchange in rod outer segments is paralleled by the observation of similar specificities of retinaldehyde, as compared with retinol and retinoic acid, and of benzaldehyde, as compared with benzylalcohol and benzoic acid, in artificial phosphatidylethanolamine micelles[16]. The aldehyde specificity supports the hypothesis that the photolysis of rhodopsin upon illumination of photoreceptor cells leads to a Schiff-base formation between the aldehyde group of retinaldehyde and an amino group of a membrane constituent, possibly phosphatidylethanolamine or phosphatidylserine. The resulting more negative charge of the membrane would increase the passive permeability of the rod sac to cations, explaining the observed cation exchange. The quantitative feasibility of this proposed mechanism has previously[1] been shown by a number of theoretical calculations.

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