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THE ISOLATION OF STABLE CATTLE ROD OUTER SEGMENTS WITH AN INTACT PLASMA MEMBRANE

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Summary

A procedure is described to purify and stabilize cattle rod outer segments with an intact plasma membrane. Three criteria are applied to assess the integrity of the latter.
Upon photolysis in these rod outer segments: (1) exogenous ATP cannot phosphorylate rhodopsin located in the disk membrane. (2) Endogenous cofactors (NADPH, NADPH-regenerating system) are still available in the rod cytosol and consequently retinol is the final photoproduct of photolysis of rhodopsin. (3) The rod cytosol can maintain a pH different from that of the medium, since the later stages of rhodopsin photolysis are independent of the medium pH.

The stability and homogeneity of the preparation appear to be much better than those of freshly isolated frog rod outer segments, which have been used most frequently so far for experiments on the physiology of rod outer segments. In addition, these cattle rod outer segments remain intact during various manipulations and therefore considerably extend the experimental possibilities when intact rod outer segments are required.

Introduction

Vertebrate rod outer segments (rods) are connected by a narrow cilium to the rest of the photoreceptor cell. Upon mechanical agitation the outer segment easily breaks off and can be conveniently purified by means of density gradient centrifugation. It has generally been assumed that the enveloping plasma membrane reseals during this procedure. This assumption is crucial for

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experiments on the physiology of isolated rods and hence it is surprising, that so few attempts have been made to check this assumption. The criterion presently available to assay the intactness of the plasma membrane, the dye staining test [1], has only been used in a few recent studies [2—4] and exclusively for frog rods. The authors agree that even freshly isolated frog rods contain 30—70% leaky rods and the number of leaky rods increases upon manipulations (e.g. centrifugation and resuspension). Consequently purification of frog rods with an intact plasma membrane seems impossible when a 'physiological' isolation medium is used.

Cattle rods, isolated in Tris buffer and purified by sucrose density gradient centrifugation, appear completely leaky to small solutes like ATP [5]. Furthermore, the resulting preparation deteriorates fast (and may already be deteriorated to a considerable extent during isolation) upon manipulation and ageing with respect to two important parameters of calcium metabolism of rods, calcium storage capacity and calcium translocation capacity [5].

We have now devised isolation conditions which stabilize the rod structure and allow purification of rods, which appear stable and not deficient with respect to calcium metabolism. In addition, the integrity of the enveloping plasma membrane can be maintained. To assess the latter we have applied three criteria, based on the permeability properties of the plasma membrane to small solutes.

Materials and Methods

Isolation procedure for cattle rods with an intact plasma membrane. All procedures are carried out in darkness or in dim red light. Plastic labware is used throughout the procedures. Cattle eyes are collected at the local slaughterhouse as fresh as possible (frozen retinas are not suitable). The retinas are carefully dissected and collected in ice-cold isolation medium (0.5 ml/retina), containing 600 mM sucrose, 5% Ficoll 400, 10 mM D-glucose, 10 mM ascorbic acid, 1 mM $CaCl_2$, 20 mM Tris-HCl (pH 7.4). $CaCl_2$ acts as a stabilizing agent and ascorbate as an anti-oxidant. Before addition of the other components the sucrose/Ficoll solution is passed over a mixed-bed ion-exchange column. The outer segments are shaken off on a vortex mixer (30 s at maximal speed) and filtered through a teflon screen (125 mesh). The filtrate is applied on top of a gradient (7 ml/gradient) by means of a syringe fitted with plastic tubing (inner diameter 3 mm). The continuous gradient is made up by mixing equal volumes (13 ml/gradient) of isolation medium and 20% (w/w) sucrose, 16% (w/w) Ficoll 400.

The gradients are centrifuged for 1 h at 24 000 rev./min (40 000—100 000 × *g*) in an IEC B-60 ultracentrifuge (5—10° C). The rod-containing band is collected in a syringe fitted with plastic tubing, is diluted with two volumes of 600 mM sucrose, 20 mM Tris-HCl (pH 7.4) and centrifuged (20 min, 3000 × *g*, 5—10° C). An occasionally occurring upper band, containing rhodopsin, is discarded. The pellet is carefully resuspended in 600 mM sucrose, 5% Ficoll 400, 20 mM Tris-HCl (pH 7.4), to a final concentration of about 100 μM rhodopsin and stored at 4° C.

Spectral recordings. Spectral recordings of suspensions are performed on a

Rapid T3 spectrophotometer (Howaldtswerke-Deutsche Werft, Kiel, F.R.G.) or a Pye Unicam SP1750 UV spectrophotometer.

Spectral ratios are determined in 1% Ammonyx LO. Absorbance at 278 nm due to the presence of ascorbate can be reduced by the addition of hydrochloric acid (final concentration 0.3 M) after the 500 nm absorbance has been measured. Rhodopsin determinations are performed as described before [6].

The time course of photoproduct formation (see Fig. 4) is analyzed at three wavelengths: 330, 380 and 455 nm. In order to calculate the concentrations of the individual photoproducts retinol (λ_{max} = 330 nm), metarhodopsin II + all-*trans* retinal (λ_{max} = 380 nm) and metarhodopsin III (λ_{max} = 455 nm) with respect to the amount of rhodopsin photolyzed (separately determined) from the absorbance changes at these three wavelengths, the following procedure is used. The molar extinction coefficients of rhodopsin, metarhodopsin II + all-*trans* retinal, and metarhodopsin III are taken to be the same [7] and a value of 40 600 is used. The molar extinction coefficient of all-*trans* retinol (52 600 in ethanol) was found to be depressed by 25% when added to an aqueous suspension of rod membranes, concomitant with a shift of λ_{max} from 325 to 330 nm. To obtain changes of metarhodopsin II + all-*trans* retinal the absorbance changes at 380 nm are corrected for changes at 380 nm due to metarhodopsin III (25% with respect to 455 nm). To obtain changes of retinol the absorbance changes at 330 nm are corrected for changes of metarhodopsin II + all-*trans* retinal (38% with respect to 380 nm) and for changes of metarhodopsin III (20% with respect to 455 nm). The correction coefficients used (others are small and have been neglected) are estimated from nomograms of all-*trans* retinal and protonated retinylidene-phosphatidylethanolamine (van Breugel, P., unpublished results). These are similar to those used by Baumann [8], with the exception of the contribution of metarhodopsin III at 330 nm. These corrections may not be completely appropriate, but the final result appears to be subject to small errors only. This is illustrated by the fact that with water-washed rod membranes, in the absence of retinol formation, the absorbance changes observed at 330 nm agree within 10% with those calculated from the decay of metarhodopsin II and the formation of metarhodopsin III. Furthermore, during the first 15 min after photolysis the sum of the photoproducts amounts to 98—104% with respect to the rhodopsin photolyzed and then decays slowly to 86.5% after 60 min. This is most probably due to degradation of the retinol formed. For obvious reasons, the calculation is only fairly accurate if the scattering of the suspension (monitored at 650 nm) does not change during the measurement.

Other procedures. Rods with a leaky plasma membrane are prepared as described before [5,6], involving mild homogenization in a Tris-HCl buffer (0.16 M, pH 7.4), followed by sucrose density gradient centrifugation and washing with the same Tris-HCl buffer. A modified procedure to prepare stable leaky rods is discussed in the Results section.

Phosphorylation of rhodopsin with [γ-^{32}P]ATP is determined as described before [5].

Dodecyl sulfate disc gel electrophoresis is carried out according to Weber and Osborn [9] with 8% gels. Proteins are stained with Coomassie blue R250.

Results

General characterization of the preparation

The best preparations are invariably prepared from very fresh eyes. Under these conditions the density gradient shows, about half-way, one major band, which is used. An occasionally occurring upper band, often containing straight rods without shrunken appearance, and some material left on top of the gradient are carefully removed by suction prior to collection of the major band. The gradient does not contain further particulate material and ends in a small, tight pellet, reddish brown at the top and black at the bottom.

Examination of our rod outer segment preparations by phase contrast microscopy shows that the large majority of the particles can be identified as rods or rod fragments, but does not allow quantitative conclusions about the purity of the preparations or about the intactness of the plasma membrane. Only very few rods carry a part of the inner segment. By far the most rods give the impression to be shrunken and to be somewhat bended, undoubtedly by the hypertonic conditions. The amount of smaller rod fragments does not appear to affect the percent of leaky rods (see further).

The purity of the intact rod preparation is characterized by the spectral ratio A_{278}/A_{500} and by SDS gel electrophoresis. After a single lysis step to remove soluble cytosol proteins (and ascorbate, which gives considerable absorbance at

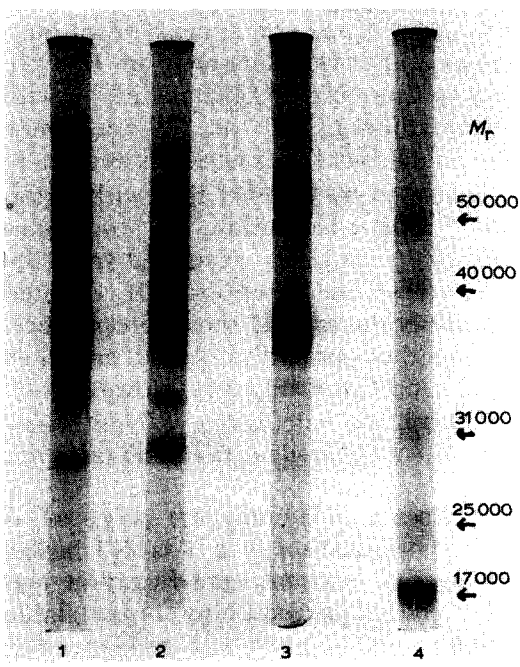

Fig. 1. SDS gels of different rod preparations. (1) Intact rods; (2) supernatant, obtained after lysis of intact rods; (3) pellet, obtained after lysis of intact rods, and (4) calibration gel. Intact rods, resuspended in the standard medium, are lysed by addition of 15 volumes of water. After addition of a sodium phosphate buffer (pH 7.0) to a final concentration of 40 mM the membranes are centrifuged for 16 h at 140 000 × *g*. The supernatant is dialyzed against water and subsequently concentrated. The concentrated supernatant does not contain a detectable amount of rhodopsin.

278 nm), the A_{278}/A_{500} ratio of these preparations lies routinely between 1.9 and 2.3, indicating a reasonable purity [6,10,11]. In intact rods 20—30% more protein may be present, as judged by the absorbance at 278 nm (after acid treatment), but this may still be partly due to ascorbate.

SDS gels (Fig. 1) of intact rods consistently show, as compared to lysed rods, the same number of additional bands, which appear in the 100 000 × *g* supernatant after lysis. This might indicate that they are soluble cytosol proteins. Leaky rods also contain these bands, though to a variable degree. The plasma membrane in these preparations apparently retains these large molecules in contrast to smaller molecules like ATP and NADPH (see next sections).

Criteria for the intactness of the plasma membrane

The dye staining test [1] unfortunately does not work with cattle rods. Isolated cattle rods do not stain with didansylcysteine irrespective of the state of the plasma membrane. A similar observation has been made by P. Hochstrate and H. Rüppel (personal communication). Therefore we have used three other criteria to assess the integrity of the plasma membrane: entry of exogenous ATP, retention of endogenous NADPH, and retention of protons. They are all based on the degree to which the plasma membrane forms a permeability barrier to small hydrophilic solutes.

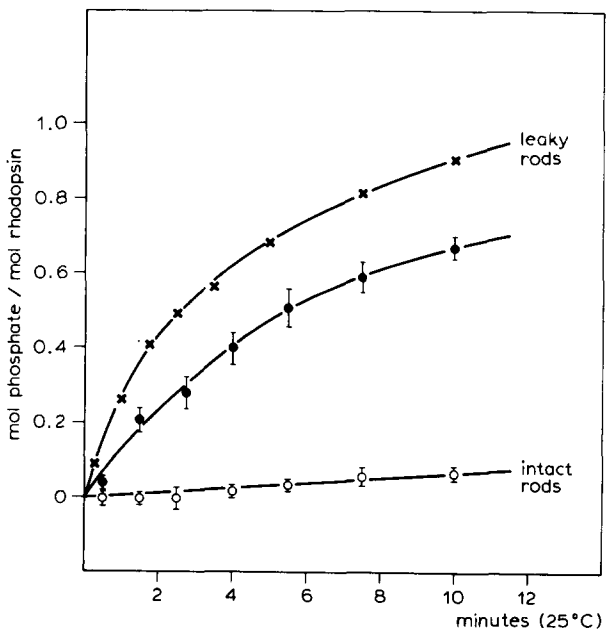

Fig. 2. Light-dependent phosphorylation of isolated cattle rods. In addition to the media: 2 mM $MgCl_2$, 1 mM Tris/ATP and [γ-^{32}P]ATP. Illumination is started 30 s before addition of ATP and sustained until complete bleaching. All data are corrected for phosphate incorporation in the dark (in leaky rods maximally 0.1 mol phosphate/mol rhodopsin in intact rods virtually absent). X, leaky rods, resuspended in 100 mM KCl, 20 mM Tris-HCl (pH 7.4), data from [5]; ○, intact rods in: 600 mM sucrose, 5%(w/v) Ficoll 400, 20 mM Tris-HCl (pH 7.4); ●, intact rods, resuspended in: 200 mM KCl, 20 mM Tris-HCl (pH 7.4). The latter two represent averages ± S.E. of 3—6 experiments with different preparations. Temperature: 25° C.

Entry of exogenous ATP

Upon photolysis rhodopsin can be phosphorylated with ATP by an intrinsic kinase, resulting in a phosphate ester, which is stable in vitro [12,13].

Hence, a suitable reaction to test the permeability to ATP is the phosphorylation of photolyzed rhodopsin, located in the disk membrane, with exogenous [γ-^{32}P]ATP. Fig. 2 shows that our intact rod preparations cannot be phosphorylated by exogenous ATP. Resuspension of these rods in an electrolyte medium makes the phosphorylation site accessible to [γ-^{32}P]ATP. The phosphorylation seems somewhat slower than in the leaky rod preparations used before [5]. Phosphorylation can also be observed when leaky rods are resuspended in the sucrose/Ficoll medium.

Retention of endogenous NADPH

If endogenous cofactors like ATP cannot enter the rod, conversely endogenous cofactors like NADPH should still be present in the rod cytosol. In the vertebrate retina, free and opsin-bound all-*trans* retinal, formed upon photolysis of rhodopsin, are reduced to all-*trans* retinol [8,14,15] by an intrinsic retinol dehydrogenase with NADPH as cofactor [16].

Fig. 3 demonstrates that after 80% bleaching of rhodopsin retinol (λ_{max} = 330 nm) is the final photoproduct in the intact rod preparation, while in leaky rods a mixture of all-*trans* retinal (λ_{max} = 380 nm) and metarhodopsin III (λ_{max} = 455 nm) has formed. Fig. 4 shows the time course of photoproduct formation after a 50% bleach. The difference between intact and leaky rods is

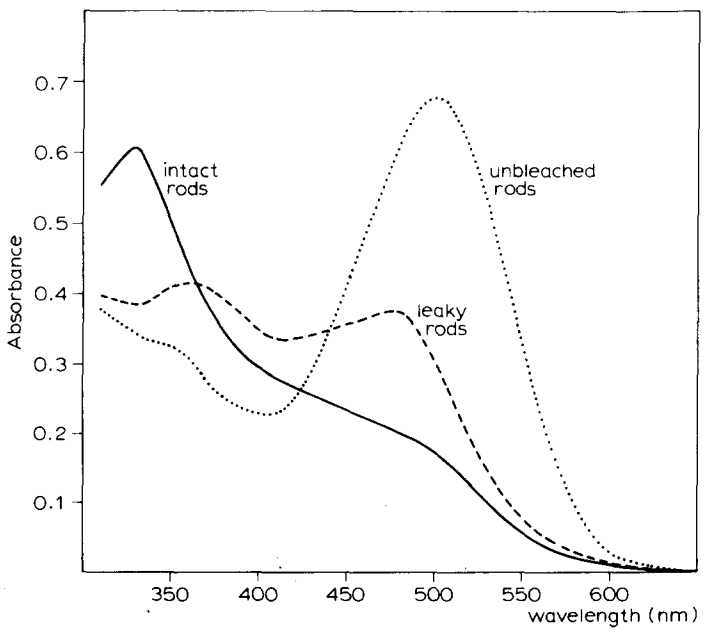

Fig. 3. Final photoproducts in intact and leaky rods. Suspension spectra are shown, which are normalized so that the rhodopsin spectra coincide. The absorbance at 650 nm is set to zero, · · · · · ·, unbleached rods; ———, intact rods following 80% photolysis; - - - - - -, leaky rods following 80% photolysis. Medium: 600 mM sucrose, 5% (w/v) Ficoll 400, 20 mM Tris-HCl (pH 7.4). Temperature: 25° C.

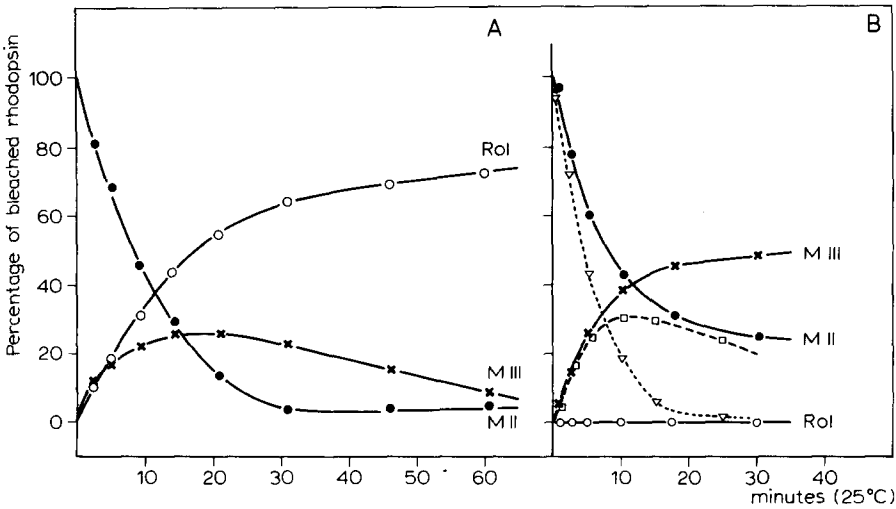

Fig. 4. Time course of photoproduct formation following 50% photolysis, in intact (A) and leaky rods (B). Retinol (○); metarhodopsin II + retinal (●); metarhodopsin III (X); leaky rods: metarhodopsin II + free retinal, with a 10-fold molar excess of added NADPH (△); leaky rods: metarhodopsin III with a 10-fold molar excess of added NADPH (□). Medium: 600 mM sucorse, 5% (w/v) Ficoll 400, 20 mM Tris-HCl (pH 7.4). Temperature: 25° C.

obvious. At lower bleaches relatively less metarhodopsin III is formed (not shown), similar to what has been described for the frog retina [17].

Fig. 4b shows that in leaky rods excess exogenous NADPH gives rise to a faster disappearance of 380 nm and 455 nm absorption (because of the overlapping absorption spectra of NADPH and retinol, retinol formation cannot be analyzed). This suggests, that in intact rods regeneration of NADPH is the rate-limiting step. In agreement with this, NADPH cannot be detected spectrophotometrically in the preparation, although nearly complete reduction of all the chromophore can be achieved. Hence, a considerable activity of a NADPH-regenerating system from glucose and ATP, which is specific for $NADP^+$ over NAD^+ can be demonstrated in our intact rods.

Retention of protons

Our interest in ion fluxes in rods has inspired us to look for a permeability test involving small ions. The pH dependence of the metarhodopsin I (λ_{max} = 480 nm) ⇌ metarhodopsin II (λ_{max} = 380 nm) equilibrium [7] can be used to establish whether the rod plasma membrane can act as a proton barrier and maintain a proton gradient.

Fig. 5 shows that in leaky rods exposure of metarhodopsin II to an external pH of 9 at 0°C results in a spectral shift to longer wavelengths, indicating a shift of metarhodopsin II to a pigment, isochromic with metarhodopsin I. Reduction of the temperature to 0°C without a change of external pH leaves metarhodopsin II unaffected. In intact rods metarhodopsin II persists when the external pH is adjusted to 9 at 0°C. From the subsequent disappearance of metarhodopsin II a time constant for the proton leakage through the rod plasma membrane in the order of minutes can be estimated. Upon addition of

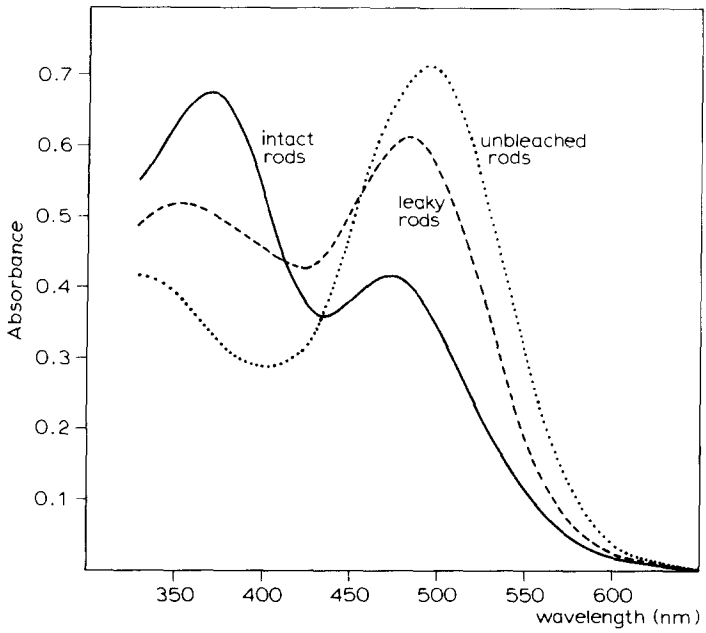

Fig. 5. pH dependence of the later stages of photolysis of rhodopsin in intact and leaky rods. A concentrated rod suspension (100 µl) in 600 mM sucrose, 5% (w/v) Ficoll 400, 20 mM Tris-HCl (pH 7.4), is bleached during 5 s (approx. 80% photolysis) at 25° C. Immediately 900 µl icecold 600 mM sucrose 5% (w/v) Ficoll 400, 50 mM Tris-HCl (pH 9.0) is added and a spectrum is recorded. The spectra of both preparations are normalized so that the rhodopsin spectra coincide. Absorbance at 650 nm is set to zero. · · · · · ·, unbleached rods; ———, intact rods following photolysis; - - - - - -, leaky rods following photolysis.

NH_4Cl (10 mM) to the external medium the existing proton gradient across the rod plasma membrane is equilibrated in seconds, resulting in a similar spectral shift to longer wavelengths as observed with leaky rods.

Stability of intact and leaky cattle rod outer segments

Intact cattle rods stored as a concentrated suspension in the sucrose/Ficoll medium remain intact for several days at 4° C. The structure still seems unaffected when viewed by phase contrast microscopy and electronmicroscopy and no phosphorylation by exogenous ATP can be observed. Formation of retinol is slowed down (about 50% after 24 h at 4° C), but still occurs to about the same extent after two days at 4° C. This indicates that the NADPH level has been lowered, but that metabolites required for NADPH regeneration are still sufficiently available. Intact rods remain inaccessible to exogenous NADPH. This can be demonstrated by following the time course of metarhodopsin III formation and decay with and without exogenous NADPH (similar as carried out in Fig. 4b).

The calcium storage and translocation capacities are also well preserved. The preparation can be subjected to various manipulations such as complete photolysis of rhodopsin, centrifugation and resuspension, sampling, osmotic manipulation, partial substitution of sucrose by electrolytes, without affecting the integrity of the plasma membrane, although the stability may be reduced (Schnetkamp, P.P.M., unpublished data).