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Metabolism in the Cytosol of Intact Isolated Cattle Rod Outer Segments as Indicator for Cytosolic Calcium and Magnesium Ions†

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ABSTRACT: The metabolism of the chromophore of rhodopsin in the cytosol compartment of isolated intact cattle rod outer segments was used as an indicator for changes of the cytosolic Mg^{2+} and Ca^{2+} concentration upon changes of the external Mg^{2+} and Ca^{2+} concentration. The reduction of retinal to retinol upon photolysis of rhodopsin in situ in intact rod outer segments was critically dependent on the availability of cytosolic Mg^{2+}. The latter is necessary as chelator of endogenous adenosine 5′-triphosphate (ATP). Lowering the cytosolic Ca^{2+} concentration beneath 10^{-7} M resulted in an inhibition of the rate of retinol formation. This is presumably due to a light-activated process, which competes with retinol formation for the supply of high-energy phosphate from a common pool. These results led to the following conclusions. Changes of the external Mg^{2+} concentration are only followed by substantial changes of the cytosolic Mg^{2+} concentration when the ionophore A23187 is present. Changes of the external Ca^{2+} concentration are followed by parallel changes of the cytosolic Ca^{2+} concentration either when external Na^+ is present or in the presence of A23187. Li^+ and K^+ could not substitute for Na^+ in the former case, but K^+ diminished the effectivity of Na^+ at low Na^+ concentrations and enhanced it at high Na^+ concentrations. It is concluded that the control of the cytosolic Ca^{2+} concentration in isolated intact rod outer segments is predominantly provided for by Na–Ca exchange, i.e., by coupled fluxes.

Changes of the extracellular Ca^{2+} concentration in retinal rod photoreceptor cells result in changes of the membrane voltage and the membrane current in the dark but not in bright light (Yoshikami & Hagins, 1973; Hagins & Yoshikami, 1974; Brown & Pinto, 1974; Lipton et al., 1977; Bastian & Fain, 1979). These results were explained by the assumption that changes of the extracellular Ca^{2+} concentration are followed in these cells by concomitant changes of the cytosolic Ca^{2+} concentration in the same direction and that raising the cytosolic Ca^{2+} concentration subsequently results in a progressive blocking of ionic channels in the plasma membrane, which are open in the dark and are closed by bright light. This suggestion was strengthened by experiments in which the cytosolic Ca^{2+} concentration was manipulated more directly either by the introduction of Ca^{2+}-chelating substances into the cytosol (Brown et al., 1977; Hagins & Yoshikami, 1977) or by the application of Ca^{2+} ionophores (Hagins & Yoshikami, 1974; Bastian & Fain, 1979). In one of these studies (Hagins & Yoshikami, 1977) it was suggested that changes of the extracellular Mg^{2+} concentration likewise are followed by concomitant changes of the cytosolic Mg^{2+} concentration. In a study using isolated intact cattle rod outer segments, it was demonstrated that efflux of internal Ca^{2+} from these rod outer segments is selectively stimulated by Na^+ ions, most likely by a Na–Ca exchange mechanism (Schnetkamp, 1980). However, most of the internal Ca^{2+} is stored within disks, and it was difficult to assess the contribution of cytosolic Ca^{2+} (Schnetkamp, 1979). Therefore, in this study an attempt has been made to use metabolism confined to the aqueous cytosol compartment as an indicator for the cytosolic Ca^{2+} and Mg^{2+} concentrations.

In the vertebrate retina (Baumann, 1972; Baumann & Bender, 1973; Brin & Ripps, 1977) as well as in isolated intact rod outer segments (Bridges, 1962; Paulsen et al., 1975; Kaplan & Liebman, 1977; Schnetkamp et al., 1979) the final photoproduct, formed upon photolysis of rhodopsin, is *all-trans*-retinol (λ_{max} = 330 nm). From the various slow photoproducts, *all-trans*-retinal, metarhodopsin II (both λ_{max} = 380 nm), and metarhodopsin III (λ_{max} = 455–470 nm), the chromophore is reduced to *all-trans*-retinol by an intrinsic retinol dehydrogenase and by NADPH[1] (Futterman, 1963). In the rod outer segment cytosol this reaction is rate limited by the recycling of NADPH (Schnetkamp et al., 1979). The latter occurs in a series of reactions, which are most likely identical with the pentose phosphate pathway and fueled by MgATP (Futterman et al., 1970; Schnetkamp & Daemen, 1981). Thus, the reduction of the chromophore appears to be dependent on the availability of cytosolic Mg^{2+} and may serve as an indicator for the latter.

Exposure to Ca^{2+}-deficient media has been reported to increase the cGMP content of mouse retinas by 10-fold (Cohen et al., 1978) and to reduce the ATP and GTP content of

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[1] Abbreviations used: ATP, adenosine 5′-triphosphate; GTP, guanosine 5′-triphosphate; cGMP, guanosine cyclic 3′,5′-monophosphate; OAc, acetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; NADP, β-nicotinamide adenine dinucleotide phosphate; NADPH, β-nicotinamide adenine dinucleotide phosphate, reduced form; Tris, tris(hydroxymethyl)aminomethane; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone.

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isolated frog rod outer segments (Biernbaum & Bownds, 1979). These changes of the nucleotide metabolism may affect the availability of substrates necessary for the metabolism of the chromophore. If, furthermore, these effects of Ca^{2+}-deficient media were due to changes of the cytosolic Ca^{2+} concentration, they could serve as an indicator for the mechanism underlying the latter.

Experimental Procedures

All procedures with rod outer segments were carried out in darkness or in dim red light unless illumination procedures are explicitly specified.

Preparations. Intact cattle rod outer segments were stabilized and purified as described previously (Schnetkamp et al., 1979) except for two minor modifications. Firstly, the Ficoll percentage in the light medium of the continuous gradient was reduced from 5% to 3.5%. This reduced the amount of material, which did not enter the gradient. Secondly, the washing step after the gradient centrifugation was performed at increased centrifugation speed (20 min, 6000*g*, 10 °C). Routinely, a yield of ~15 nmol of rhodopsin/retina was obtained with the above procedure. All the experiments described in this paper were performed on the same day the preparation was made. Storage of the preparation as a concentration suspension (150 μM rhodopsin) at 4 °C for periods up to 6 h did not noticeably change the results.

Spectral Recordings. Spectral recordings of suspensions of rod outer segments were performed on a Beckman UV5260 spectrophotometer with the cuvette placed directly in front of the photomultiplier. All experiments were performed at room temperature (21–23 °C). A concentrated suspension of intact cattle rod outer segments was diluted to a final rhodopsin concentration of 15–25 μM (volume 1 mL) in the standard medium containing 600 mM sucrose, 0.5% Ficoll 400, and 20 mM Tris-HCl (pH 7.4). The indicated additions were made to this medium. The EDTA and EGTA solutions were buffered with 20 mM Tris-HCl to a final pH of 7.4. Other additions never resulted in changes of the medium pH exceeding 0.1 pH unit. In view of the relative insensitivity of the intact rod outer segments to osmotic manipulation, additions of electrolytes were not osmotically compensated for by leaving out the corresponding amount of sucrose. Spectra were scanned with a recording speed of 4 nm/s starting at 650 nm. The absorbance at 650 nm (apparent absorbance due to light scattering) was set to 0. Photolysis of rhodopsin was carried out for 10 s with white light bleaching ~85% of the rhodopsin present as determined by subsequent addition of detergent (1% Triton X-100) and hydroxylamine (50 mM).

Analysis of Retinol Formation. The time course of quantitative retinol formation (see Figure 5) has been analyzed as described before (Schnetkamp et al., 1979). In other cases, where only the relative rate of retinol formation was needed, it was preferred to plot the observed increase of absorbance at 330 nm without correction for changes of the other photoproducts (Figures 3, 4, and 6). The rate of retinol formation (Table I) is defined as the difference in absorbance observed at 330 nm 15 min after bleaching between the sample involved and a sample treated with the ionophore A23187 (5 μM) and EDTA (0.5 mM). In the latter case no retinol formation occurred (see Figure 1 and its discussion), and the changes observed at 330 nm were due to changes of other photoproducts [metarhodopsin II and metarhodopsin III; see Schnetkamp et al. (1979)]. Thus, in the presence of both A23187 and EDTA the rate of retinol formation is set at 0%. When only EDTA was added to the standard medium (no A23187) retinol formation readily occurred (see Figure 1), and the amount of retinol formed under these conditions is set at 100% in Table I. In the data used for Table I, 100% retinol formation reflected absorbance changes at 330 nm of 0.1–0.2 absorbance unit.

Determination of NADPH Synthesis. NADPH synthesis was determined by a continuous registration of the absorbance at 340 nm of intact rod outer segments solubilized in 0.5% Triton X-100. NADP (200 μM) was present during the assay, and the recordings were started by the addition of the indicated substrates. A molar absorbance of 6400 for NADPH was used. The experiment was carried out with unbleached material.

ATP Determination. ATP determinations were performed on a Lumac luminometer (Lumac B. V., The Netherlands) with purified luciferase (that did not respond to GTP). An aliquot of a rod outer segment suspension was solubilized in the Lumac detergent in the presence of the Lumac nucleotide releasing agent. ATP calibration curves showed the same slope with or without solubilized rod outer segments present.

Results

Retinol Formation as Indicator for Cytosolic Mg^{2+} Ions. The development of the slow photoproducts in intact isolated cattle rod outer segments upon photolysis of rhodopsin in the presence of a strong chelator for divalent cations in the external medium is shown in Figure 1a. In the first spectrum recorded after bleaching, metarhodopsin II (λ_{max} = 380 nm) was observed, which subsequently decayed into metarhodopsin III (λ_{max} = 455 nm) and *all-trans*-retinol (λ_{max} = 330 nm). Metarhodopsin III arose only as a transient product, which likewise decayed to retinol (spectrum 5 to spectrum 6). Thus, the presence of a chelator for Mg^{2+} in the external medium did not seriously affect the formation of retinol even for a time period of 1 H. If, in contrast, the permeability of the rod plasma membrane was increased by the addition of the divalent cation ionophore A23187, retinol formation was completely abolished when external EDTA was present (Figure 1b).[2] Under these conditions a simple metarhodopsin II → metarhodopsin III transition with an isosbestic point at 415 nm was observed in agreement with results obtained with water-washed rod outer segment membranes (van Breugel et al., 1979). Subsequently, Mg^{2+} (in excess of the EDTA present) was added to the suspension after the recording of spectrum 4, shown in Figure 1b, and further spectra were recorded at different times after the addition of Mg^{2+} (Figure 1c). The increase of the absorbance at 330 nm at the expense of the absorption at 380 and 455 nm (Figure 1c) indicated the recovery of retinol formation under these conditions at about the same rate as observed without the presence of A23187 and external Mg^{2+} (compare parts a and c of Figure 1). In the latter experiment Ca^{2+} could only poorly replace Mg^{2+}, resulting in a greatly reduced rate of retinol formation (not shown). The above observations demonstrate that the reduction of the chromophore upon bleaching of rhodopsin is critically dependent on the presence of Mg^{2+} in the cytoplasm of rod outer segments.

The pentose phosphate pathway, which supplies the reducing agent NADPH for the reduction of the chromophore, can be

[2] The difference observed between the first spectra recorded in parts a and b, respectively, of Figure 1 did not arise from different bleaching levels, but from different proton concentrations at the membrane surface seen by the rhodopsin molecules. It should be noted that on the time scale of the experiments described in this study no actual proton gradients existed in intact rod outer segments. This is described in detail somewhere else (Schnetkamp et al., 1981).

Table I: Effect of Different Medium Conditions on Rate of Retinol Formation in Intact Isolated Cattle Rod Outer Segments[a]

additions to the standard medium	% with respect to control in standard medium	SD	no. of observations
A23187 (5 μM), Mg^{2+} (2 mM), and Ca^{2+} (50–100 μM)[b]	87	10	4
A23187 (5 μM), Mg^{2+} (2 mM), and EGTA (0.5 mM)	19	9	4
NaOAc (50 mM), KOAc (5 mM), Ca^{2+} (1 mM), and EGTA (0.5 mM)	110	14	8
NaOAc (50 mM), KOAc (5 mM), and Ca^{2+} (50–100 μM)[b]	77	3 (range)	2
NaOAc (50 mM), KOAc (5 mM), and EGTA (0.5 mM)	30	11	8
LiOAc (50 mM), KOAc (5 mM), and EGTA (0.5 mM)	88	12	3
KOAc (55 mM) and EGTA (0.5 mM)	85	9	3

[a] The rate of retinol formation is determined as described under Experimental Procedures. The experiments are carried out at room temperature (21–23 °C) in the standard medium, 600 mM sucrose, 0.5% Ficoll 400, and 20 mM Tris-HCl (pH 7.4), with the indicated additions. [b] Endogenous Ca^{2+} present in the preparation [see Schnetkamp (1979)].

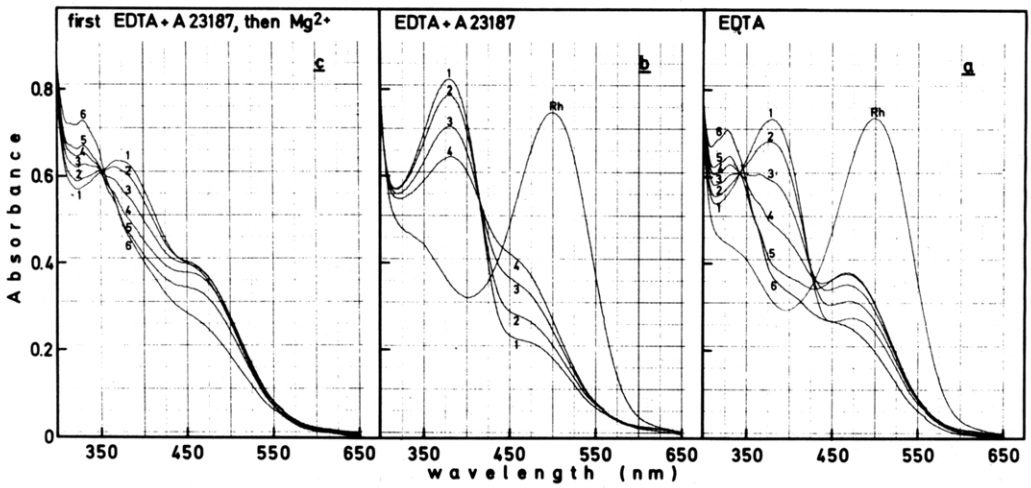

FIGURE 1: Dependence of retinol formation on cytosolic Mg^{2+}. Photolysis spectra of intact rod outer segment suspensions in the standard medium are shown following an exposure to light bleaching ~85% of the rhodopsin present. The following additions were made to the standard medium (600 mM sucrose, 0.5% Ficoll 400, and 20 mM Tris-HCl, pH 7.4): (a) 0.5 mM EDTA; (b) 0.5 mM EDTA plus 5 μM A23187; (c) 0.5 mM EDTA plus 5 μM A23187 plus 2 mM $MgCl_2$. Spectra marked by Rh are the suspension spectra before the bleach. Spectra 1–6 are started 20 s and 3, 8, 15, 25, and 90 min, respectively, after the exposure to light (a and b) or after the addition of Mg^{2+} (c). In the latter case Mg^{2+} was added to the suspension after the recording of spectrum 4 in Figure 1b.

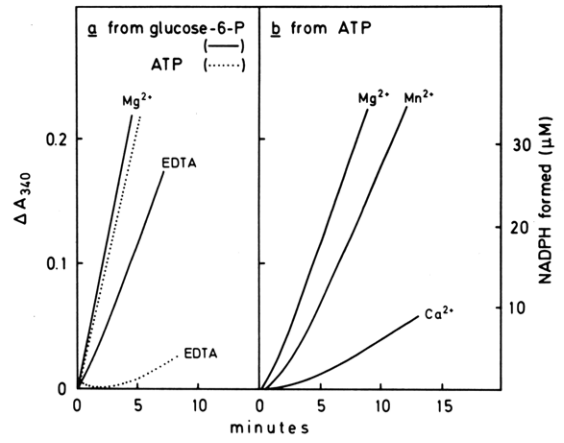

FIGURE 2: Metal ion requirement of NADPH synthesis by rod outer segment cytosol enzymes. Continuous registrations are shown of the absorbance at 340 nm of solubilized intact rod outer segments. Rod outer segments are solubilized in order to ensure a full accessibility of enzymes and added substrates. The experiment is carried out in the dark in order to avoid complications of the photolysis products absorbing at 340 nm. Final incubation medium: 200 mM sucrose, 0.25% Ficoll 400, 20 mM Tris-HCl, 10 mM glucose, 200 μM NADP, and 0.5% Triton X-100 (pH 7.4). The recordings are started by the following additions: (a) (solid lines) 150 μM glucose 6-phosphate and (broken lines) 150 μM ATP [divalent cation concentrations are indicated at the traces by Mg^{2+} (2 mM $MgCl_2$) or EDTA (1 mM EDTA)]; rhodopsin concentration, 8.5 μM; (b) 200 μM ATP and the indicated divalent cations (2 mM of the chloride salts); rhodopsin concentration, 4.2 μM.

fueled either by ATP and glucose or by glucose 6-phosphate (which is formed as the first intermediate from the former) but not by GTP (Schnetkamp & Daemen, 1981). The experiment shown in Figure 2a revealed that only the first step in the pentose phosphate pathway, the phosphorylation of glucose, was critically dependent on Mg^{2+} but for the further steps Mg^{2+} was not essential. From this and the above experiment shown in Figure 1 the conclusion seems justified that in situ in the rod outer segment cytosol MgATP is the true substrate for the recycling of NADPH necessary for the reduction of the chromophore. Similarly, as noted before, Ca^{2+} could only poorly replace Mg^{2+} as chelator of ATP in the synthesis of NADPH (Figure 2b).

Effect of Cytosolic Ca^{2+} on Retinol Formation. In the experiment shown in Figure 1 EDTA in the external medium did not affect the formation of retinol. Since EDTA chelates Ca^{2+} as well as Mg^{2+}, this experiment also shows that reduction of the external Ca^{2+} concentration [Ca^{2+} present in the preparation at 30–60 μM; see Schnetkamp (1979)] did not affect the rate of retinol formation. However, it is not self-evident that this treatment resulted in a concomitant lowering of the cytosolic Ca^{2+} concentration. The most direct way to control the latter in isolated intact rod outer segments is by application of the ionophore A23187, which, at least in the micromolar concentration range, appears to equilibrate the free Ca^{2+} concentration in all intracellular compartments (Schnetkamp, 1979; Kaupp et al., 1979). The data represented

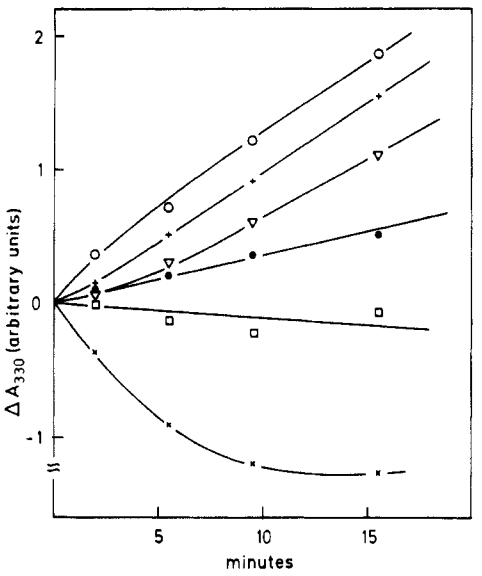

FIGURE 3: The effect of external Na^+ on the rate of retinol formation. To a suspension of intact rod outer segments in the standard medium containing 0.5 mM EDTA is further added the following: no further additions (○); 4 mM NaCl (▽); 4 mM NaCl plus 5 mM KCl (+); 50 mM NaCl (●); 50 mM NaCl plus 5 mM KCl (□); 5 μM A23187 (×). A spectrum is recorded in the dark, the samples are exposed to light bleaching 85% of the rhodopsin present and, spectra are recorded 20 s and 2.5, 6, 10, and 16 min after the illumination. The data are plotted as the difference in absorbance at 330 nm between the first spectrum recorded after the illumination and the subsequent spectra as a function of the time elapsed between the recording of the spectra. Rhodopsin concentration, 16 μM.

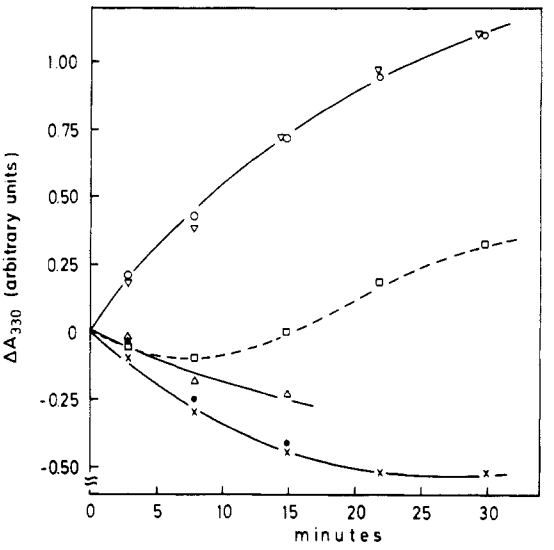

FIGURE 4: The effect of changes of the external Na^+/Ca^{2+} ratio in the dark and in the light on the rate of retinol formation. To a suspension of intact rod outer segments in the standard medium is added 50 mM NaOAc, 5 mM KOAc, and 0.5 mM EGTA. Aliquots are subsequently given the following treatments: omission of NaOAc and KOAc (○); no further treatment (×); incubation for 5 min in the dark, followed by addition of 1 mM $CaCl_2$ (▽); incubation for 40 min in the dark, followed by addition of 1 mM $CaCl_2$ (□); addition of 1 mM $MgCl_2$ (△); addition of 0.1 mM ouabain (●). A spectrum is recorded in the dark, the samples are exposed to light bleaching 85% of the rhodopsin present, and spectra are recorded 20 s and 3, 8, 15, 22.5, and 30 min after the illumination. The data are plotted as the difference in absorbance at 330 nm between the first spectrum recorded after illumination and the subsequent spectra (see Figure 3). Rhodopsin concentration, 25 μM.

in the first two lines of Table I show that in the presence of A23187 and Mg^{2+} the rate of retinol formation was strongly reduced when the Ca^{2+} concentration was lowered beneath 10^{-7} M (by addition of EGTA, which at physiological pH selectively chelates Ca^{2+} as compared to Mg^{2+}). In a recent study Schnetkamp (1980) showed that in the presence of external EDTA Na^+ induced a significant reduction of the total Ca^{2+} content of isolated intact rod outer segments. Therefore, in the experiment shown in Figure 3 the effect of Na^+ (EDTA present) on the rate of retinol formation was investigated. The data are presented as the change in the absorbance of the rod outer segment suspension at 330 nm. The open symbols indicate the rate of retinol formation in the absence of Na^+ by an increase of the absorbance at 330 nm. No retinol formation was observed in the presence of both EDTA and A23187 (crosses), and the absorbance at 330 nm decreased due to the decay of metarhodosin II (see Figure 1 and its discussion). The addition of 4 mM Na^+ to the external medium resulted in a significantly reduced increase of the absorption at 330 nm (Figure 3, triangles) indicating a decreased rate of retinol formation, whereas addition of 50 mM Na^+ had an even stronger inhibitory effect on the rate of retinol formation. External K^+ reduced the effect of Na^+ on the Ca^{2+} content of isolated intact rod outer segments at external Na^+ concentrations below 20 mM and enhanced the effect of Na^+ at concentrations greater than 20 mM (Schnetkamp, 1980). A similar reversal of the action of K^+ is noted in Figure 3. At low Na^+ (4 mM) the combined addition of Na^+ and K^+ had a weaker inhibitory effect on the rate of retinol formation as compared to the effect of Na^+ alone, whereas K^+ alone is ineffective (see Table I). In contrast, at high Na^+ (50 mM) the combined addition of Na^+ and K^+ is more effective than the addition of Na^+ alone (Figure 3). The most consistent and strong inhibitory effects on the rate of retinol formation could be obtained by the use of acetate salts rather than chloride salts, and, therefore, in the experiments described below the combination of 50 mM sodium acetate plus 5 mM potassium acetate was used.

The data presented in lines 3–5 of Table I indicate that in the presence of external Na^+ and K^+ (both acetate salts) changes of the external Ca^{2+} concentration led to significant changes in the rate of retinol formation. The final two lines of Table I indicate that these changes could not be obtained when Na^+ was replaced by Li^+ or when only K^+ (55 mM) was present. In Figure 4 the rate of retinol formation was analyzed by plotting the absorbance changes of rod outer segment suspensions under different conditions at 330 nm (see also description of Figure 3). Again, the inhibitory effect of external Na^+ in the presence of the Ca^{2+} chelator EGTA on the rate of retinol formation is clear (compare with Table I). The action of EGTA could be completely counteracted by the addition of Ca^{2+} (in excess of the chelator), but not by Mg^{2+} (Figure 4).

The effectivity of the combined addition of Na^+ and K^+ could possibly be explained by the activation of a Na,K-ATPase, which might account for a rapid hydrolysis of endogenous ATP. However, the lack of any effect of ouabain (Figure 4) does not support this idea.

Light Is a Necessary Requirement for Action of Low Ca^{2+} on Retinol Formation. In the above experiments bleaching of rhodopsin was required to provide the substrate for the reduction of the chromophore. The next experiment, therefore, was to look for the effect of a preincubation in the dark with low Ca^{2+} (EGTA present), Na^+, and K^+ present, followed by the addition of external Ca^{2+} (in excess of the EGTA present). Subsequently, the photolysis of rhodopsin was observed. The result of such an experiment is shown in Figure 4 and demonstrates that a 5-min preincubation in this low Ca^{2+} medium was without noticeable effect on the rate of retinol formation, and even a 40-min preincubation was clearly not as effective

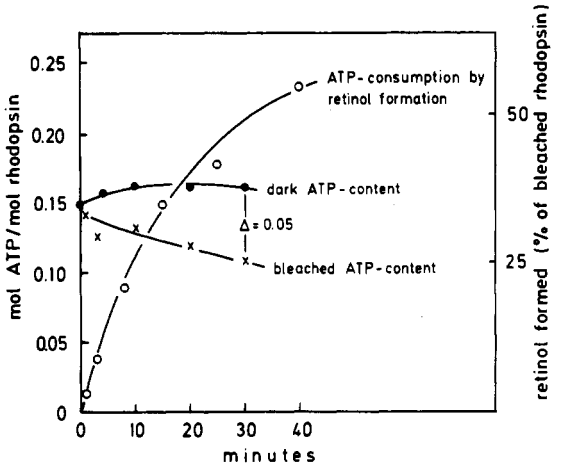

FIGURE 5: Observed ATP content and calculated ATP consumption because of retinol formation. A suspension of intact rod outer segments in the standard medium (plus additional 100 μM EDTA) is exposed to light bleaching 85% of the rhodopsin present. (●) ATP content of aliquots, which were kept in the dark; (×) ATP content at different times after the bleach: (○) ATP consumption calculated from the reduction of the chromophore (1 ATP → 2 NADPH → 2 retinol), which is quantitatively determined as described under Experimental Procedures. Rhodopsin concentration, 19.7 μM.

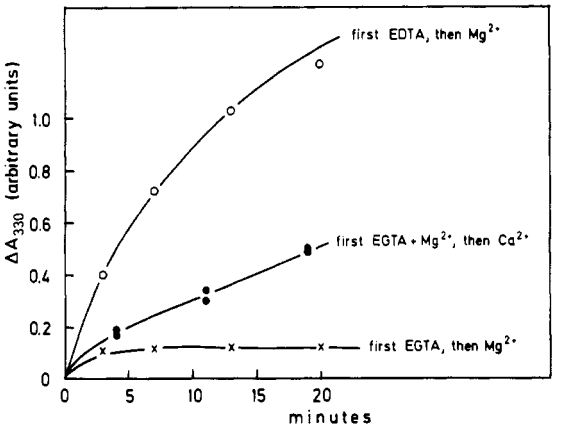

FIGURE 6: Reversibility of effect of low Ca^{2+} concentrations on the rate of retinol formation. Suspensions of intact rod outer segments in the standard medium are pretreated for 15 min in a manner similar to that shown in Figure 1b and retinol formation is started in a manner similar to that shown in Figure 1c. The data are plotted as the increase at 330 nm (see Figure 4 and Figure 1c). (○) Pretreatment with 0.5 mM EDTA plus 5 μM A23187, start by addition of 2 mM $MgCl_2$; (×) pretreatment with 0.5 mM EGTA plus 5 μM A23187, start by addition of 2 mM $MgCl_2$; (●) pretreatment with 0.5 mM EGTA plus 5 μM A23187 plus 2 mM $MgCl_2$, start by addition of 1 mM $CaCl_2$. Rhodopsin concentration, 19.1 μM.

as a short exposure to this low Ca^{2+} medium in the light. This means that in the above low Ca^{2+} medium exposure of intact rod outer segments to light had two effects. First, light merely provides the substrate "*all-trans*-retinal". Secondly, light decreased the availability of reducing power for the retinol formation.

Correlation between Retinol Formation and ATP Consumption. A reduction of the rate of retinol formation arises from the decreased rate of NADPH recycling, which in its turn is provided for by the ATP-fueled pentose phosphate pathway (Futterman et al., 1970; Schnetkamp & Daemen, 1981; this study, see Figures 1 and 2). Curiously, no clear correlation could be detected between the rod outer segment ATP content and the rate of retinol formation. When, for example, the rod outer segments were exposed to the standard medium, containing 50 mM Na^+, 5 mM K^+ (both acetate salts), and EGTA, retinol formation was strongly and rapidly inhibited (see above), but the relatively low ATP content (0.15 mol of ATP/mol of rhodopsin) decreased only slowly ($t_{1/2}$ > 10 min, data not shown). Furthermore, the decrease of the ATP content was about the same in the dark as in the light similar as has been reported for the ATP content of isolated frog rod outer segments (Biernbaum & Bownds, 1979). The calculated ATP consumption by retinol formation (1 ATP → 2 NADPH → 2 retinol) under the standard medium conditions was compared with the observed drop of the in situ ATP content of isolated intact cattle rod outer segments (Figure 5) to illustrate this apparent contradiction. The measured ATP content did not decrease by much more than 30% (0.05 mol of ATP/mol of rhodopsin) during the first 0.5 h following an 85% bleach of the rhodopsin present, despite the fact that the amount of retinol formed would have required at least 0.2 mol of ATP/mol of rhodopsin. The additional observation that the mitochondrial uncoupler FCCP (5 μM) did not affect the rate of retinol formation (data not shown) suggests that the constant ATP level of isolated rod outer segments was not due to mitochondrial ATP synthesis.

Reversibility of Action of Light and Low Ca^{2+} on Retinol Formation. It was suggested above that light is a necessary requirement for the inhibitory effect of low Ca^{2+} on the rate of retinol formation to become expressed. The nature of this action of light was further investigated in the experiments shown in Figure 6. Intact rod outer segments were pretreated with the ionophore A23187 and either EGTA or EDTA. Photolysis of rhodopsin was followed under these conditions for 15 min and resulted in both cases in a simple metarhodospin II → metarhodopsin III transition (similar to that observed in Figure 1b). This can be understood by the fact that cytosolic Mg^{2+} was either chelated by EDTA or in the case of EGTA sufficiently diluted in the external medium. Subsequently, Mg^{2+} was added in excess over the chelators present and the increase of absorbance at 330 nm was followed (similar to the procedure shown in Figure 1c). Normal retinol formation (indicated by the increase of absorbance at 330 nm) was observed, when EDTA was present (see also Figure 1c). In contrast, when EGTA was present, retinol formation was severely inhibited. These observations can be understood on account of the differential affinity of EDTA and EGTA for Mg^{2+}. The excess of Mg^{2+} added will liberate endogenous Ca^{2+} ions present in the preparation from their EDTA chelate, but not from their EGTA chelate. Therefore, addition of Mg^{2+} to a rod outer segment suspension containing EDTA and A23187 resulted in a free external Ca^{2+} concentration comparable to that without EDTA present. Under these conditions retinol formation was not inhibited (Table I, line 1).

In the third case, shown in Figure 6, the recovery of retinol formation was investigated after previous bleaching under conditions of inhibited retinol formation but with Mg^{2+} present (i.e., under conditions which allow the consumption of ATP–EGTA with A23187 and Mg^{2+} present). During the first 15 min after the bleach little retinol developed under these conditions (Table I). Subsequently, Ca^{2+} was added (in excess over the EGTA present) and a partial recovery of retinol formation was observed as indicated by the increase of the absorbance at 330 nm (Figure 6). In similar experiments partial to little recovery was commonly observed, but it never exceeded 50% of the rate of retinol formation under control conditions (as in Figure 1a). The same result was obtained after previous inhibition of retinol formation in the presence of Na^+ and K^+ (both acetate salts) and EDTA (see Figure 4 and Table I) and subsequent addition of Ca^{2+} 15 min after the bleach. In preliminary experiments, rod outer segments

were incubated under these conditions for 5–15 min in the dark following a small bleach (<5% of the rhodopsin bleached). Subsequently, Ca^{2+} was added, a rhodopsin spectrum was recorded, and the photolysis of rhodopsin was measured after a full bleach. The application of small bleaches (0.1–1% of the rhodospin bleached) resulted in a markedly reduced rate of retinol formation as compared to a sample kept in the dark. These experiments will be the subject of another paper.

Discussion

In the present study an attempt is made to analyze the metabolism of the aqueous cytosol compartment of isolated intact cattle rod outer segments with the option to find indicators which respond to changes of the cytosolic Ca^{2+} and Mg^{2+} concentration. Until now, the cytosol compartment of such small cells or cellular organelles like cattle rod outer segments has proven to be not easily accessible to external measuring methods. Thus, it is necessary to use metabolic routes, which involve water-soluble and not membrane-bound enzymes and substrates. In this study it is found that the availability of endogenous ATP for the recycling of the reducing agent NADPH is suited for this purpose, since it involves the water-soluble pentose phosphate pathway (Futterman et al., 1970, Schnetkamp & Daemen, 1981). Furthermore, NADPH is necessary (Futterman, 1963) and rate limiting (Schnetkamp et al., 1979) for the reduction of the chromophore to retinol. The latter can be conveniently followed spectrophotometrically (Figure 1).

The advantage of the above analysis as compared to a nucleotide analysis (ATP or GTP and their hydrolysis products) is illustrated by the observations discussed in relation to Figures 5 and 6. There appeared to be no clear correlation between the availability of ATP to fuel the pentose phosphate pathway and the actual ATP concentration. Thus, the cytosolic ATP concentration remained at a low, but fairly constant, level despite a major expenditure by retinol formation (Figure 5). In addition, under these conditions phosphorylation of rhodopsin should have occurred and consumed at least 0.5 mol of ATP/mol of rhodopsin (Kühn et al., 1973; Weller et al., 1975; Paulsen et al., 1975; Kühn & Bader, 1976; McDowell & Kühn, 1977). These considerations strongly suggest that ATP is steadily replenished from a high-energy phosphate pool, which exceeds the equivalent of 1 mol of ATP/mol of rhodopsin. The low and light-stable ATP content of isolated cattle rod outer segments is in good agreement with values obtained with isolated frog rod outer segments (Biernbaum & Bownds, 1979). If the ATP/GTP ratio in cattle rod outer segments would be similar as observed by these authors for frog rod outer segments, the conclusion would be justified that the major store of high-energy phosphate in rod outer segments is neither ATP nor GTP. In accordance with this picture, in the presence of rod outer segment enzymes, phosphate groups can be readily transferred between the various guanine and adenine nucleotides, and also another phosphate donor like phosphocreatine readily phosphorylates AMP to yield ATP (Schnetkamp & Daemen, 1981). This means that nucleotides may be strongly and mutually buffered. On the other hand, mitochondrial synthesis of ATP does not seem to play an important role in view of the ineffectivity of the mitochondrial uncoupler FCCP to inhibit retinol formation (data not shown).

Do Changes of External Mg^{2+} Concentration Affect That of the Cytosol? Mg^{2+} serves in the rod outer segment cytosol as the primary chelator of ATP (Figures 1 and 2) and is essential for its functioning (Figures 1 and 6). The observations shown in Figure 1 clearly demonstrate that removal of external Mg^{2+} by EDTA had no noticeable consequences for the availability of Mg^{2+} in the cytosol. When, however, the plasma membrane was made permeable to Mg^{2+} by the addition of the ionophore A23187, the cytosolic Mg^{2+} concentration adjusted to the external Mg^{2+} concentration. From this the conclusion seems justified that the plasma membrane of rod outer segments does not contain a mechanism which transports Mg^{2+} and is virtually leakproof to Mg^{2+}. This is in contrast to the findings of Hagins & Yoshikami (1977) on intact rod cells in the rat retina treated with phospholipid vesicles and agrees with the finding that the specific exchange system present in these intact cattle rod outer segments does not transport Mg^{2+} (Schnetkamp, 1980). Furthermore, these preparations also appear to be remarkably leakproof to net transport of Ca^{2+} other than by the above indicated specific exchange transport (Schnetkamp, 1979).

Low Cytosol Ca^{2+} Inhibits Retinol Formation. In the presence of the ionophore A23187 free Ca^{2+} concentrations in all compartments of isolated intact rod outer segments appear to be equilibrated (Schnetkamp, 1979; Kaupp et al., 1979). When in the presence of this ionophore the external Ca^{2+} concentration was reduced to below 10^{-7} M, the rate of retinol formation was strongly inhibited (Table I, Figure 6). This result suggests that a low cytosolic Ca^{2+} concentration modulates some part of the aqueous metabolism in the rod outer segment cytosol, which is involved in the supply of ATP for the pentose phosphate pathway (the retinol dehydrogenase and the pentose phosphate pathway itself are not dependent on Ca^{2+}; see also Figure 2a). Further experiments revealed that this effect was only manifested when two further conditions were satisfied. First, the ready reversibility upon a dark preincubation in low Ca^{2+} (Figure 4) as opposed to a light preincubation (Figure 6) implies that bleached rhodopsin must be present. Secondly, Mg^{2+} is required (Figure 6). Considering the preceding discussion, these results suggest that light (or better bleached rhodopsin) activates a process which strongly competes with the pentose phosphate pathway for replenishment from a common pool of high-energy phosphate. In agreement with this, readdition of Ca^{2+} after a preincubation in the light in a Ca^{2+}-deficient medium apparently switched off this process and restored retinol formation, but only partially, as if the available pool of high-energy phosphate was decreased substantially. One possible candidate for the above process could be an increased turnover of cGMP at low cytoplasmic Ca^{2+} levels as indicated by the large drop of the cGMP content of mouse retinas upon illumination under these conditions (Cohen et al., 1978).

Cytosolic Ca^{2+} Concentration Is Controlled by External Na^+/Ca^{2+} Ratio. The observations, shown in Table I and Figures 3 and 4, demonstrate that in the presence of external Na^+ changes of the external Ca^{2+} concentration affected the rate of retinol formation in a manner similar to that observed for changes of the external Ca^{2+} concentration in the presence of A23187. This strongly suggests that in the presence of external Na^+ changes of the external Ca^{2+} concentration are followed by changes of the cytosolic Ca^{2+} concentration in the same direction by the operation of a Na–Ca exchange mechanism. Changed nucleotide levels upon changes of the external Ca^{2+} concentration have been reported by Cohen et al. (1978) and by Biernbaum & Bownds (1979). For the present discussion it is relevant to note that in both studies the external medium always contained Na^+. A Na–Ca exchange mechanism, which could account for the ionic requirements noted in this study for the inhibition of retinol formation, has recently been found in rod outer segments (Schnetkamp, 1980). Thus, Li^+ and K^+ cannot substitute for Na^+ both in

Na–Ca exchange and in the inhibitory effect on retinol formation (Table I). Furthermore, K^+ diminishes the effectivity of low Na^+ concentrations ($\leq$10 mM) in its effect on both the rate of retinol formation and the stimulation of Ca^{2+} efflux from rod outer segments, whereas at high Na^+ concentrations (50 mM) K^+ enhanced both actions of Na^+ (Figure 3; Schnetkamp, 1980).

Na–Ca exchange in isolated intact cattle rod outer segments occurs for the greater part directly between the external medium and the intradiskal space bypassing the cytosol (Schnetkamp, 1980). Thus, it might be possible that in some way the intradiskal Ca^{2+} is responsible for the observed effects on the rate of retinol formation. However, there appears to exist no clear correlation between the total Ca^{2+} content of rod outer segments (predominantly localized within disks) and the rate of retinol formation. For example, in the presence of A23187 Mg^{2+} substantially reduced the total Ca^{2+} content of rod outer segments (Schnetkamp, 1980) but has no clear inhibitory effect on the rate of retinol formation (Table I, Figure 1c). Alternatively, a low Na^+ concentration (4 mM) in the presence of EGTA has a substantial effect on the rate of retinol formation (Table I) but only minor effects on the total Ca^{2+} content of rod outer segments. This enforces the notion that the cytosolic Ca^{2+} concentration modulates the availability of ATP and, therewith, modulates the rate of retinol formation. Furthermore, the cytosolic Ca^{2+} concentration seems to be controlled by a Na–Ca exchange system with properties very similar to that responsible for Na^+-stimulated Ca^{2+} efflux from rod outer segments. From this the tentative conclusion could be drawn that a single transport system, which communicates between disk and plasma membrane, is capable of performing transport between the external medium and both the cytosol and the intradiskal space.

Does Na–Ca Exchange in Rod Outer Segments Require High-Energy Phosphate? Biernbaum and Bownds have suggested that changes of the GTP content of isolated frog rod outer segments upon changes of the external Ca^{2+} concentration might indicate the presence of a GTP-driven Ca^{2+} pump (Biernabum & Bownds, 1979). In squid axons Ca^{2+} extrusion can be performed by a Na–Ca exchange mechanism and by an uncoupled Ca^{2+} efflux, of which only the latter is driven by ATP (DiPolo & Beaugé, 1979; DiPolo et al., 1979). In isolated cattle rod outer segments, changes of the external Na^+/Ca^{2+} ration appear to have a reversible effect on the cytosolic Ca^{2+} concentration and appear to change the latter over a broader range without Na^+ present (Table I, Figures 3 and 4). Therefore, in this preparation Na–Ca exchange seems to be a major mechanism for the control of the cytosolic Ca^{2+} concentration. An indirect involvement of nucleotides in this mechanism similar to that which has been described for squid axons (Baker & McNaughton, 1976) remains possible, but a uniport Ca^{2+} efflux by an ATP-driven Ca^{2+} pump seems less likely. In the presence of A23187 and Ca^{2+} in excess over the normal value such a pump would be short circuited and forced to run continuously. However, a supposed Ca^{2+} pump does not seem to dissipate sufficient high-energy phosphate under uncoupling conditions so that it interferes with the energy supply for the reduction of the chromophore over a time period of up to 1 h (see Figure 1c). An uncoupled Ca^{2+} efflux of magnitude similar to that reported for squid axons [0.04 pmol of Ca^{2+} cm^{-2} s^{-1} (DiPolo et al., 1979); compare with the unidirectional flux of Ca–Ca and Na–Ca exchange in cattle rod outer segments, which amounts $\sim$5 pmol of Ca^{2+} cm^{-2} s^{-1} (Schnetkamp, 1979, 1980)] would consume $\sim$1 mol of ATP/mol of rhodopsin h^{-1} on the assumption of 2 Ca^{2+} transported/ATP hydrolyzed.

Physiological Implications of Na–Ca Exchange. The experiments, reported in this study, support the interpretation that in rod cells changes of the extracellular Ca^{2+} concentration in the presence of external Na^+ may affect the membrane voltage and membrane current due to concomitant changes of the cytosolic Ca^{2+} concentration. A Ca^{2+} extrusion mechanism in rod outer segments, which is dependent on the electrochemical gradient of Na^+ across the plasma membrane, implies that abolition of this gradient (in isolated outer segments) or reversal of this gradient (substitution of Na^+ by other cations in the perfusate of retinas or retina slices) in the presence of Ca^{2+} will result in a rise of the cytosolic Ca^{2+} concentration. This in its turn might lead to a closure of the light-regulated channels, and, therefore, ion substitution experiments may lead to erroneous conclusions about the ion selectivity of the light-regulated channels [see Fain & Lisman (1981)]. As Fain and collaborators have shown, in toad rods photoreceptor potentials can be recorded, when, for example, Na^+ is substituted by Li^+ in Ringer solution in which Ca^{2+} is buffered to 10^{-8} M, whereas in normal Ca^{2+} Ringer solution this substitution abolishes the photoreceptor potential (Brown & Pinto, 1974) and the dark current (Hagins & Yoshikami, 1975) in rod cells.

Control of the cytosolic Ca^{2+} concentration in rod cells by a Na–Ca exchange mechanism in the rod plasma membrane is also suggested by the light-induced Ca^{2+} efflux from rod cells in the toad retina reported recently (Gold & Korenbrot, 1980). The light-induced Ca^{2+} efflux observed by these authors is clearly dependent on the external Na^+/Ca^{2+} ratio, and a maximal rate of Ca^{2+} efflux of 1.2×10^6 Ca^{2+} ions rod^{-1} s^{-1} is observed. The maximal rate of Na^+–Ca^{2+} and Ca^{2+}–Ca^{2+} exchange observed for isolated bovine rod outer segments ranges between 10^6 and 10^7 Ca^{2+} ions rod^{-1} s^{-1} (Schnetkamp, 1979, 1980).

On the other hand, the results of the present and a previous study with intact isolated cattle rod outer segments (Schnetkamp, 1980) reveal a new interpretation problem. In these preparations changes of the external Na^+/Ca^{2+} ratio result in a rapid and massive change of both the cytosolic and intradiskal Ca^{2+} concentrations by coupled fluxes, at which intradiskal Ca^{2+} can be directly transported to and from the external medium bypassing the cytosol. Similarly, application of Ca^{2+} ionophores results in an adjustment of both the cytosolic and intradiskal Ca^{2+} concentrations to the external Ca^{2+} concentration and a concomitant uncoupling of Ca^{2+} pumps. The close apposition of disk and plasma membrane in rod outer segments ensures a distribution of ionophore molecules over both membranes as has been demonstrated before for the case of A23187 (Schnetkamp, 1979; Kaupp et al., 1979). In conclusion, several options are possible to interpret experiments on rod cells, in which the external Ca^{2+} concentration is manipulated or monitored, and it remains to be established that the effects of Ca^{2+} on the physiology of rod cells are due to variations of the cytosolic Ca^{2+} concentration.

Acknowledgments

I thank Drs. W. Junge, W. J. de Grip, U. B. Kaupp, H.-W. Trissl, and L. H. Pinto for their interest in the work and for reading the manuscript. I am also grateful to M. Offerman for preparing the drawings. Dr. F. J. M. Daemen generously provided the facilities for the experiment shown in Figure 5.

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