Light-dependent Na^+-Ca^{2+} Exchange in Retinal Rod Discs

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Abstract. Experiments are described demonstrating that Na^+—Ca^{2+} exchange of retinal rod disc membrane is highly sensitive to light. The Na^+—Ca^{2+} exchanger was shown to possess two types of binding sites with different affinities for calcium. The low affinity binding sites (K_D^{Ca} = 5.8 μmol/l) are light-insensitive. After bleaching, K_D of the high affinity Ca^{2+}-binding sites an K_i for Na^+ changed from 0.2 to 0.3 μmol/l and from 3.2 to 0.7 mmol/l, respectively. Light inhibits the steady-state Ca^{2+} uptake by a factor of 1.5. Photocontrol of the Na^+—Ca^{2+} exchanger affinity is observed at the physiological level of rhodopsin bleaching.

Key words: Photoreceptor discs — Na^+—Ca^{2+} exchange — Light dependence

Introduction

When a photon is absorbed by the photosensitive pigment rhodopsin the retinal rod generates, using an intracellular transmitter, electrical response with a transient hyperpolarization of plasma membrane.

Yoshikami and Hagins (1971) were the first to propose that calcium functions as transmitter. The numerous attempts to prove the hypothesis have, however, failed. Now it is generally agreed that cGMP is the transmitter (Fesenko et al. 1985; Yau and Nakatani 1985) and Ca ions act as a modulator in the process of light adaptation of photoreceptor (Altman 1985).

There are some indications that light induces Ca^{2+} to move between membrane-separated compartments of the retinal rod outer segment. Actually, light induces Ca^{2+} release from the discs (Smith and Bayer 1979).

There is ample evidence that Ca^{2+} is presumably transported across the disc membrane using the Na^+—Ca^{2+} exchange mechanism (Schnetkamp et al. 1977; Caretta 1985; Fain and Schröder 1985; Volotovski and Khovratovich 1986; Schnetkamp 1986). It was shown in the present study that the disc Na^+—Ca^{2+} exchanger is highly light-sensitive and that it possesses binding sites with much

higher affinities for both sodium and calcium ions than those of the exchangers in other membrane systems.

Materials and Methods

Disc preparation: Cattle eyes were collected from a local slaughterhouse. Retinae from chilled eyes were placed in a 45% sucrose solution (1 ml/retina) containing 1 mmol/l $MgCl_2$, 0.1 mmol/l EDTA, 2 mmol/l dithiothreitol, 10^{-5} mol/l phenylmethylsulfonyl fluoride, 10^{-5} mol/l α-tocopherol, 70 mmol/l Na_2HPO_4/NaH_2PO_4, pH 7.0 (standard medium I) and shaken for 1 min. The suspension was spun at 6000x*g* for 5 min, the supernatant was filtered through 400 mesh nylon gauze and, after dilution with 3 volumes of the standard medium I without sucrose, it was centrifuged for 6 min at 2500x*g*. The pellet was resuspended with standard medium I containing 0.77 mol/l sucrose (3 ml/25 retinae) and layered on discontinuous density gradient in a 35 ml centrifuge tube containing three solutions of different densities: 5 ml of 1.14 g/ml, 12 ml of 1.13 g/ml and 12 ml of 1.11 g/ml prepared as 1.15 mol/l, 1.00 mol/l and 0.84 mol/l sucrose solutions in standard medium I. After centrifugation for 30 min at 100,000x*g* the band between 1.11 and 1.13 g/ml containing ROS was collected and diluted three times with the respective solution and centrifuged for 15 min at 20,000x*g* (Papermaster and Dreyer 1974).

Discs were prepared from ROS according to Smith et al. (1975). The ROS pellets were resuspended in 5% Ficoll-400 (1 ml/retina). The resulting suspension was kept at 4 °C for at least 2 h to allow all the ROS to burst. After 2 h centrifugation at 80,000x*g* intact discs were collected from the interface. The collected discs were washed by repeated centrifugation for 15 min at 20,000x*g* in the respective solution.

All the procedures were carried out under dim red light.

During the preparation both the solutions and the disc suspensions were kept at 0 °C and the measurements were performed immediately after the isolation.

The concentration of unbleached rhodopsin was determined from absorbance at 500 nm of the photoreceptor membranes suspension in 1% cetyltrimethylammonium bromide and 0.1 mol/l hydroxylamine assuming the molar extinction coefficient of 40,300 and M_r of 39,000 (Daemen et al. 1972).

The A_{280}/A_{500} absorption ratio in the solubilized disc membranes and ROS was routinely found to not exceed 1.9 and 2.1, respectively.

When needed, the samples were bleached at 0 °C by visible light of a tungsten lamp (100 W) placed at 30 cm from the sample.

All the measurements were carried out in a medium which contained (in millimoles per litre): imidazole-HCl 40, pH 7.0, $MgCl_2$ 2, EGTA 0.1 and different proportions of KCl and NaCl in a total concentration of 150 (standard medium II). The free calcium concentration in Ca^{2+}—Mg^{2+}—EGTA buffer was evaluated with the algorithm of Fabiato and Fabiato (1979) using the following binding constants: H.EGTA 1.70×10^9 $mol^{-1}.l$, Ca.EGTA 4.37×10^{10} $mol^{-1}.l$, Mg.EGTA 2.57×10^5 $mol^{-1}.l$ (Chijsen et al. 1984). Ca^{2+} contamination of distilled water was not detectable.

The protein content was determined according to Lowry's method as modified by Markwell et al. (1978).

Ca^{2+} uptake experiments: Ca^{2+} uptake was initiated by adding 25 μl of membrane suspension (up to 0.2 — 0.4 mg of protein per ml) to 1.25 ml of standard medium II containing various concentrations of free calcium (2 μCi $^{45}Ca^{2+}$). The mixture was incubated at 37 °C. Ca^{2+} uptake was stopped

by adding 5 ml of chilled calcium-free standard medium II. Then, the disc suspension was layered on glass-paper filters and washed two times within 30 s with 5 ml of ice-cold KCl-standard medium II containing 1 mmol/l EDTA. The filters were mixed with 5 ml Bray's cocktail for radioactivity measurements with a Mark- III scintillation counter.

For kinetic determinations aliquots were withdrawn from the suspension incubated for the indicated intervals at 37 °C. In some experiments the discs were overloaded with Na^+ by preincubation for 1 h at 4 °C in the standard medium containing 150 mmol/l NaCl. Then they were immediately used for experiments.

Ca^{2+} release experiments: Before the determination of Ca^{2+} release, the discs and ROS were overloaded with Ca^{2+} by preincubation for 1 h at 4 °C in standard medium II containing 150 mmol/l KCl and 200 μmol/l $CaCl_2$ (4 μCi $^{45}Ca^{2+}$). Ca^{2+} release was initiated by transferring 50 μl of the suspension of Ca^{2+} overloaded discs into 2.5 ml of standard medium II with different NaCl concentrations; the media were made calcium-free by the addition of 0.1 mmol/l EGTA. The amount of Ca^{2+} released was determined from sample radioactivity decrease after 10 min incubation at 37 °C. Ca^{2+} release was stopped by layering the membrane suspension on glass-paper filters and washing two times with ice-cold KCl-standard medium. Radioactivity of the filters after a 10 min incubation in the Na^+-free standard medium II was taken as 100%.

Na^+—Ca^{2+} exchange activity in both uptake and release experiments, i.e. residual ^{45}Ca radioactivity of the discs was measured under optimal conditions (disc overloading with Na^+ and absence of the ion in the medium in uptake experiments; disc overloading with Ca^{2+} and absence of the ion in the medium in release experiments). This means that the Na^+-dependent components of Ca^{2+} uptake and release were measured.

Determinations of K: The values of apparent K_D for calcium and K_i for sodium ions of the Na^+—Ca^{2+} exchanger were obtained graphically using the data of kinetic measurements. The Lineweaver-Burk plots were used for K_D^{Ca} determination according to the Michaelis-Menten equation. K_i^{Na} was determined with the use of the Dixon equation (Webb 1963).

Regeneration of bleached rhodopsin: Regeneration was initiated by adding exogenous 11-*cis*-retinal (10 mol per 1 mol of rhodopsin bleached) in ethanol to the membrane suspension. The final ethanol concentration did not exceed 1%. The samples were incubated at 37 °C for 30 min (Heuselman and Cusanovich 1976). Typically, 95-98% regeneration was obtained.

Reagents: Cetyltrimethylammonium bromide, phenylmethylsulfonyl fluoride, quinidine, ruthenium red, EDTA, EGTA, Tris, Imidazole (Sigma). Ionophores A23187 (Eli Lilly) and monensin (BDH). Dithiothreitol and α-tocopherol (Serva). Ficoll-400 (Pharmacia). $^{45}CA^{2+}$ (Amersham). 11-*cis*-retinal (Hoffman — La Roche). Glass-paper filters GF/C were from Whatman.

Results

Na^+-dependent Ca^{2+} accumulation and release. Addition of Ca^{2+} to the medium initiated calcium accumulation by the discs. Figure 1 shows the kinetics of this process in the retinal rod discs. The curves describing Ca^{2+} uptake reach a plateau approximately by the fifth minute of incubation. They reflect Ca^{2+}

Table 1. Effect of the cation composition of the pre- and incubation media on Ca^{2+} uptake in photoreceptor discs

Preincubation medium containing:	Ca^{2+}-accumulation capacity in the medium containing: KCl, 150 mmol/l		NaCl, 150 mmol/l	
	nmol / mg protein	%	nmol / mg protein	%
KCl, 150 mmol/l	1.51 ± 0.13	520	0.29 ± 0.04	100
NaCl, 150 mmol/l	3.98 ± 0.17	1373	0.68 ± 0.05	234

The value of Ca^{2+} uptake in 150 mmol/l KCl for the bleached discs preincubated in 150 mmol/l NaCl was taken as 100%. For the conditions of the experiment see legend to Fig. 1; the incubation time was 15 min. The data presented are mean ± S.E.M. values of 3 experiments.

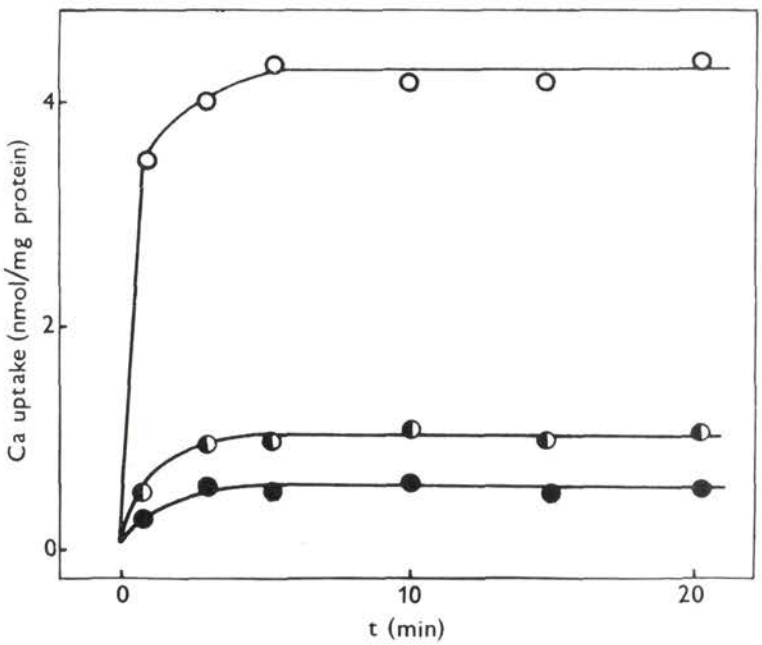

Fig. 1. Effect of A23187 and of replacement of K^+ by Na^+ on the time course of Ca^{2+} uptake by bleached retinal rod discs. The discs were Na^+-overloaded as described in Materials and Methods. The samples contained 0.2—0.4 mg/ml protein. Ca^{2+} uptake experiments were performed at the free Ca^{2+} concentration of 4.85 μmol/l with additions as indicated. The points are mean values of 3 experiments. ○ — 150 mmol/l KCl, ◐ — 150 mmol/l NaCl, ● — 150 mmol/l KCl + 5 μmol/l A23187.

Table 2. The influence of different additions on Ca^{2+} uptake in bleached photoreceptor discs

Additions	Ca accumulation activity, nmol/mg protein . 15 min
control	3.82 ± 0.19
+ monensin, 10 μmol/l	0.33 ± 0.03 ($P < 0.001$)
+ quinidine, 100 μmol/l	2.88 ± 0.18 ($P < 0.05$)
+ ruthenium red, 1 μmol/l	4.74 ± 0.13 ($P < 0.05$)
+ cGMP, 50 μmol/l	1.85 ± 0.14 ($P < 0.005$)

For the measurement conditions see legend to Fig. 1. Ca^{2+} uptake measurements were performed in the standard medium II containing 150 mmol KCl. The data presented are mean ± S.E.M. values of 3 experiments.

transport into the discs but not Ca^{2+} binding by external surface sites of the disc membrane because Ca^{2+} ionophore A23187 inhibits Ca^{2+} uptake. Ca^{2+} accumulation was suppressed by sodium ions as suggested by a comparison of the kinetics of Ca^{2+} accumulation in KCl and NaCl media. It is noteworthy that the Ca^{2+}-accumulating capacity of the discs was found to be 5-fold lower in the sodium medium, suggesting that Na^+-dependent Ca^{2+} transfer is the main contributor to Ca^{2+} uptake under the conditions used.

The most pronounced effects were observed after overloading of the discs with both Na^+ and K^+ ions (Table 1). Depending on the ionic composition of the Ca^{2+} uptake medium, the Na^+-enriched discs accumulated different amounts of Ca^{2+} (~ 0.7 nmol/mg protein in Na^+-containing and ~ 4.0 nmol/mg protein in K^+-containing media). The lowest value of this parameter (~ 0.3 nmol/mg protein) was measured in Na^+-depleted discs during the determination of Ca^{2+} uptake capacity in the Na^+-containing medium; this may suggest that Ca^{2+} uptake is determined by the ratios of inter- and intradisc Na^+ concentration. Considering the data presented in Table 1 it should be stressed that the most unfavourable condition for Na^+—Ca^{2+} exchange is at $K_i = Na_o$. On the contrary, at $K_o = Na_i$ Ca^{2+} uptake capacity is maximal. Steady-state level of Ca^{2+} accumulation at $K_i = Na_o$ was taken as 100% to trace the influence on it of different variations of inter- and intradisc ion concentrations.

Table 2 shows the effects of monensine, quinidine, ruthenium red and cGMP on Ca^{2+} uptake in dark-adapted discs. As is known, monensine eliminates the Na^+ gradient in membrane systems (Bayerdöffer et al. 1985), quinidine inhibits non-specifically Na^+—Ca^{2+} exchange (Ledvora and Heguvary 1983) and ruthenium red prevents the potential-driven Ca^{2+} transfer in mitochondria (Caroni et al. 1978). Both monensine and quinidine suppress Ca^{2+} uptake in the discs. On the contrary, ruthenium red did not inhibit Ca^{2+} uptake but even enhanced it. An appreciable effect was also observed under the action of cGMP

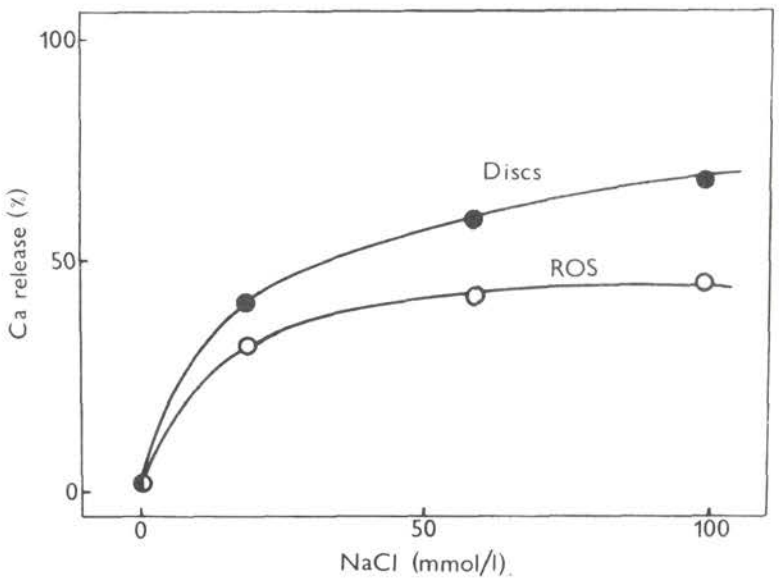

Fig. 2. Induction of Ca^{2+} release from bleached photoreceptor discs and ROS by sodium ions. Prior to Ca^{2+} release determination, the ROS and discs were overloaded with Ca^{2+} as described in Materials and Methods. The points are mean values of 3 experiments.

that binds specifically to the disc membrane (Volotovski et al. 1984; Caretta et al. 1985) and forms a non-selective ionic pore (Fatt 1982; Kaupp and Koch 1984); this results in an essential release of Ca^{2+} from the discs, a decrease in the cation gradient on the membrane and, consequently, in a lower efficiency of Ca uptake.

Figure 2 shows the effect of sodium ions on $^{45}Ca^{2+}$ release from Ca^{2+}-overloaded ROS and discs. The amount of Ca^{2+} released depends on the sodium concentration in the medium against the background of its constant osmolarity. Two points should be emphasized here: first, Na^+ induces Ca^{2+} release from the discs more effectively than from ROS and, second, unexchangeable pools of Ca^{2+} ions for ROS and discs are different, amounting to 60 and 40%, respectively.

Light effects on Na^+-dependent Ca^{2+} uptake. Direct measurements show that the Na^+-dependent Ca^{2+} uptake in the retinal rod discs is light-controlled (Fig. 3). The comparison of the Ca^{2+} accumulation kinetics in dark and bleached membranes reveals a decrease of the steady-state level of Ca^{2+} uptake after bleaching (from 6 to 4 nmol/mg protein). The light-dependent modulation of Ca^{2+} uptake

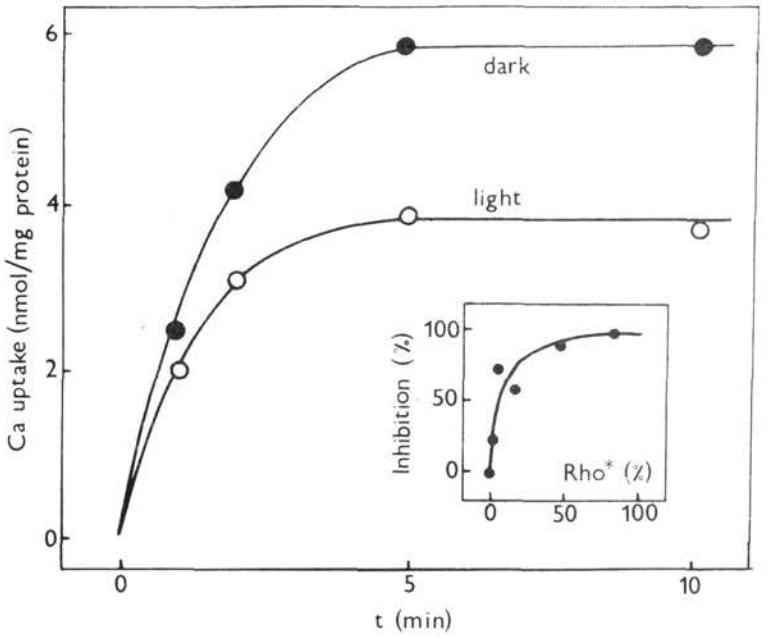

Fig. 3. Effect of light on the time course of Ca^{2+} uptake in retinal rod discs. Ca^{2+} uptake measurements were performed in the standard medium containing 150 mmol/l KCl. For other conditions see legend to Fig. 1. Insert: The dependence of light inhibition of Ca^{2+} uptake on the degree of rhodopsin bleaching. The points are mean values of 3 experiments.

in the discs occurs at very low percentages of rhodopsin bleaching, since the maximum light-dependent inhibiting effect is actually observed at values as low as 10% (Fig. 3, insert).

Additional experiments were performed to evaluate the inhibition and dissociation constants for Na^+ and Ca^{2+} ions, respectively, of the Na^+-dependent Ca^{2+}-transferring system. Figures 4*A* and 4*B* present the results of these experiments performed with dark and bleached disc preparations. As could be expected for Na^+—Ca^{2+}exchange, the efficiency of Ca^{2+} uptake fell both with decreasing Ca^{2+} concentration and with increasing Na^+ concentration in the medium. In double inverse coordinates (Fig. 4*A*) both curves for Ca^{2+} dependence show breaks suggesting the presence of two types of binding sites with low and high affinities for calcium. For the dark discs these sites have the following parameters: $K_D^1 = 5.8$ and $K_D^2 = 0.2$ μmol/l. No heterogeneity could be revealed in studying the affinity of the Ca^{2+} uptake system for the external Na^+ (Fig. 4*B*): K_i for the ion is about 3.2 mmol/l.

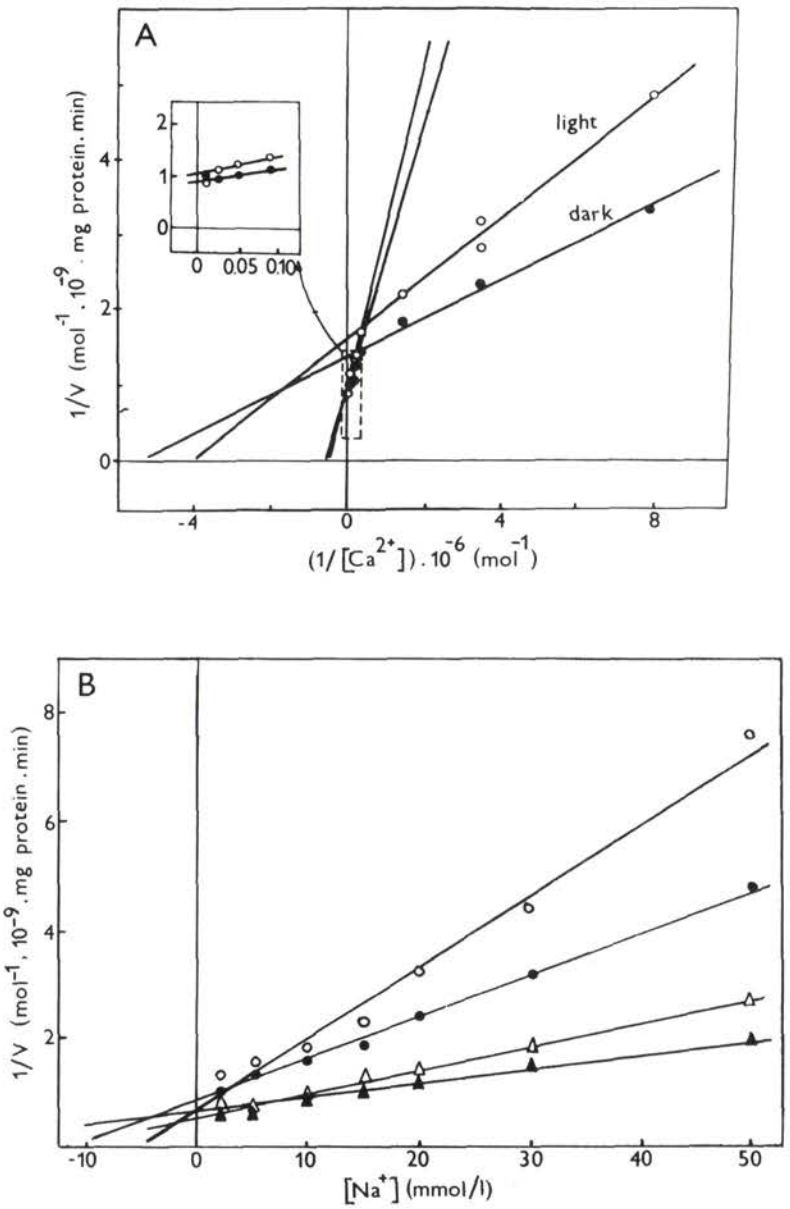

Fig. 4. Dependence of the Ca^{2+} uptake rate in the dark and bleached photoreceptor discs versus free Ca^{2+} (*A*) and Na^{+} (*B*) concentration in the medium. Lineweaver-Burk and Dixon plots. For the measurement conditions see legend to Fig. 1. Different Na^{+} concentrations were obtained by replacement of KCl in the incubation medium by NaCl at a total concentration of 150 mmol/l. Free Ca^{2+} concentration was varied by adding different $CaCl_2$ amounts to the Mg-EGTA-containing medium. The points are mean values of 3 experiments.

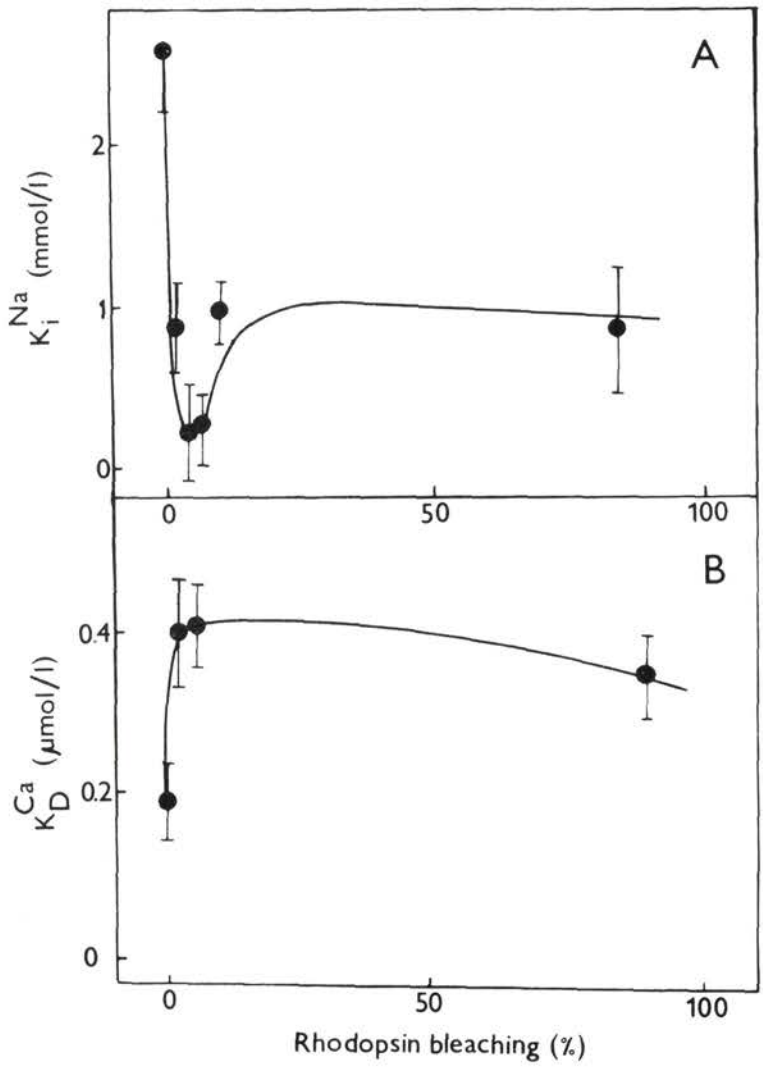

Fig. 5. The influence of rhodopsin bleaching on apparent K_i^{Na} (*A*) and K_D^{Ca} (*B*) of high affinity binding sites of the Na^+—Ca^{2+} exchanger in photoreceptor discs. For the measurement conditions see legend to Fig. 4. The points are means ± S.E.M. of 3 experiments.

It should be noted that the low affinity sites for Ca^{2+} are actually not regulated by light. The high affinity sites are, however, markedly light-sensitive. So, after rhodopsin bleaching the apparent dissociation constant for calcium

Table 3. The influence of rhodopsin bleaching on the inhibition constant for Na^+ of the Na^+—Ca^{2+} exchanger in the disc membrane

Rhodopsin state:	K_i^{Na} mmol/l	
	Control discs	Discs + ROS lysate + ATP
Dark	2.78 ± 0.37	1.91 ± 0.39
2% bleached	1.45 ± 0.28 ($P < 0.05$)	0.95 ± 0.05 ($P < 0.05$)
Regenerated	1.64 ± 0.31 ($P < 0.1$)	2.06 ± 0.10 (IS)

For conditions of K_i^{Na} determination see legend to Fig. 4. ROS lysate contained 0.9 mg/ml protein, and 0.6 mmol/l ATP was added to the disc suspension in a 1:2 ratio (v/v). Instead of ROS lysate an equal volume of hypotonic medium was added to control samples. The data presented are mean ± S.E.M. values of 3 experiments. IS — insignificant.

increases (from 0.2 to 0.3 μmol/l) while that for sodium decreases (from 3.2 to 0.8 mmol/l).

In order to obtain more detailed information on photoregulation of Ca^{2+} uptake, the dependence of apparent K's for Ca^{2+} and Na^+ on the degree of rhodopsin bleaching in the disc membrane was studied in separate experiments. The main changes in K's seemed to occur at low percentages of bleaching. The maximal effect was observed at about 5% rhodopsin bleaching (Fig. 5*A* and 5*B*). Both curves show a complex shape without the expected saturation. For K_i^{Na} the minimum (0.2 mmol/l) is followed by an increase in the value, not reaching, however, its initial level; for K_D^{Ca}, after the maximum (0.4 μmol/l) a partial decrease is observed.

The fedback between the photopigment and the system of Na^+-dependent Ca^{2+} uptake was the subject of special experiments on the regeneration of bleached rhodopsin with exogenous 11-cis-retinal. Rhodopsin regeneration did not lead to restoration of the initial parameters of the exchanger in washed photoreceptor discs (Table 3). Full restoration of K_i^{Na} for regenerated disc membranes was observed only in the presence of cytoplasmic ROS components with 0.2 mmol/l ATP. Without ATP, ROS lysate did not enhance the K_i restoration under the same conditions of rhodopsin regeneration.

Discussion

The data presented in Fig. 1 and 2 and Tables 1 and 2 suggest that Ca^{2+} uptake by photoreceptor discs is actually due to Na^+—Ca^{2+} exchange. It is evident that

the exchange efficiency is determined by extra- and intradisc Na^+ concentration. As can be seen from Table 1, the change in the ratios of sodium concentration on both sides of the disc membrane can change the Ca^{2+}-accumulating capacity by more than one order of magnitude (from 0.3 to 4 nmol/mg protein). The critical importance of the sodium gradient for Ca^{2+} accumulation follows from the data in Table 2. Elimination of the sodium gradient from the disc membrane using monensine resulted in a complete inhibition of Ca^{2+} uptake. Moreover, sodium ions induced calcium release from Ca^{2+}-overloaded discs (Fig. 2).

The results discussed here are consistent with the data of Schnetkamp and Bownds (1987) obtained in experiments with truncated rods, intacts and leaky ROS and which confirmed the operation of Na^+—Ca^{2+} exchange in the ROS discs.

Since the Na^+—Ca^{2+} exchanger together with rhodopsin is a part of the disc membrane providing for selective absorption of visible light quanta to activate the system of phototransduction, it seems quite possible that the exchanger activity is regulated by light. Indeed, light inhibits the steady-state level of Ca^{2+} uptake in the discs 1.5 times at 85% rhodopsin bleaching (Fig. 3). To investigate the reasons of photoregulation of the Na^+—Ca^{2+} exchanger, the influence of light on the exchanger affinities for sodium and calcium ions was determined. Rhodopsin bleaching was accompanied by an increase in affinity of binding sites for Na^+ and a decrease in that for Ca^{2+}.

It should be borne in mind that physiological effects of light occur at low percentages of rhodopsin bleaching (Stryer et al. 1981).

The changes in affinities of the high-affinity Ca^{2+}-binding sites observed at low degrees of rhodopsin bleaching point to the regulatory influence of light on the Na^+—Ca^{2+} exchanger. This is a new finding in the field of photoreception; as could be expected, the regulatory action of light was reversible. However, the process of dark restoration of initial properties of the Na^+—Ca^{2+} exchanger at rhodopsin regeneration with 11-*cis*-retinal was implemented with the participation of soluble ATP-dependent cytoplasmic component(s) of ROS.

It should be mentioned that the affinity of Ca^{2+}-binding sites of the Na^+-Ca^{2+} exchanger is 1—2 orders of magnitude higher than those of the exchangers from other membrane systems (Gill et al. 1981; Morel and Godfraind 1984; Shamoo and Ambudcar 1984). Therefore one may suppose that the Na^+—Ca^{2+} exchanger of the photoreceptor disc, due to its high affinity for calcium ions, is involved in the regulation of cytoplasmic Ca^{2+} concentration both in resting and in excited states of the rod photoreceptor cell.

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