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BIOCHEMICAL ASPECTS OF THE VISUAL PROCESS, XXXV

CALCIUM BINDING BY CATTLE ROD OUTER SEGMENT MEMBRANES STUDIED BY MEANS OF EQUILIBRIUM DIALYSIS

TH. HENDRIKS, P.M.M. VAN HAARD, F.J.M. DAEMEN and S.L. BONTING
Department of Biochemistry, University of Nijmegen, Nijmegen (The Netherlands)
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Summary

The calcium binding capacity of cattle rod outer segment membranes has been studied by means of an equilibrium dialysis technique. The binding is not affected by prior lyophilization of the membranes or by the presence of ionophore A23187, indicating that only passive binding to membranes is involved without active translocation.

The amount of calcium bound to the membranes is influenced by the ionic composition of the medium. Both Na^+ and K^+ decrease binding to about the same degree, but the size of the effects suggests a rather high specificity of the calcium binding sites on the membrane.

From Scatchard plots for the amount of calcium bound as a function of the free calcium concentration, it appears that two types of binding sites exist: high affinity sites which can accommodate 5 nmol calcium per mg protein (0.3 mol calcium/mol rhodopsin) and low affinity sites which can accommodate 195 nmol calcium per mg protein (13 mol calcium/mol rhodopsin). Depending on the medium composition, the high affinity sites show dissociation constants between 8 and 40 μM, and the low affinity sites between 0.3 and 1.6 mM.

Illuminated rod outer segment membranes show a slight decrease of calcium binding as compared to dark-kept membranes, but the effect is independent of the amount of calcium bound and does not appear to be significant.

From these findings and the assumption of a free calcium concentration of approx. 1 μM in the extrasaccular space in rod outer segments in vivo, it is concluded that mere passive binding to the rod sac membranes must be insufficient to explain the high calcium contents in rod outer segments.

Introduction

It has been proposed [1] that the calcium ion acts as a transmitter in the visual excitation of rods by bridging the gap between the photoreceptor mem-

brane and the rod outer membrane. Recently we have shown that rod outer segments contain a large amount of calcium [2]. The calcium-coupling hypothesis assumes that the bulk of this calcium is stored in or on the rod sacs and we have indeed found a large part of the calcium to be present in the rhodopsin-containing sedimentable fraction. However, it has not yet been established whether the calcium is mainly stored inside the rod sacs or is bound to the cytoplasmic side of the sac membrane.

Calcium binding to rod sac membranes has been investigated in a few cases, but a clear distinction between binding and translocation has not been made. Bownds et al. [3] have described an ATP-stimulated calcium accumulation in suspensions of frog outer segments, while Neufeld et al. [4] have reported calcium "binding" by cattle rod sac membranes, which is doubled by ATP. No effect of light has been reported by these authors. Hemminki [5] has measured "binding" to isolated outer segments by incubation with ^{45}Ca. He finds less "binding" after bleaching. Weller et al. [6] have measured "binding" to a hypotonically shocked outer segment preparation by means of the same technique as that of Hemminki. All these authors have used washing procedures with ^{45}Ca-free solutions in order to remove loosely adhering label, but which must also have caused some loss of bound ^{45}Ca.

Therefore, we have investigated the calcium binding capacity of lysed rod outer segment membranes by means of the equilibrium dialysis technique. This technique permits determination of passive binding of calcium without the danger of losing bound calcium by a washing procedure. The use of lysed membranes avoids complications due to the presence of an intact outer membrane and of possible soluble calcium-binding material located in the extrasaccular space.

Methods and Materials

Isolation of rod outer segment membranes

Cattle rod outer segments are prepared by means of sucrose gradient centrifugation, largely as described elsewhere [7]. The initial homogenization of the retinas is carried out in 0.16 M Tris · HCl, 1 mM EGTA (pH 7.4), and the upper layer of the gradient is diluted with an equal volume of the same buffer. After centrifugation (3000 × g, 15 min) each sediment derived from 15 retinas (approx. 0.5 ml) is washed once with 45 ml 15 mM EDTA, and twice with 45 ml ice-cold double distilled water (28000 × g, 15 min). The final sediment of lysed rod outer segment membranes is then taken up in 20 ml of the desired medium and immediately used for equilibrium dialysis experiments.

The rhodopsin content of these preparations is measured as described before [7]. Protein is routinely measured by the method of Lowry et al. [8], using bovine serum albumin as a standard. Combining the results of these two determinations, we find that our preparations contain 15 nmol or 560 μg rhodopsin per mg "Lowry" protein. This is considerably less than the values of 80—90% of total membrane protein commonly reported for pure rod outer segment membranes (cf. ref. 9). It is probably due to the fact that the Lowry et al. method gives erroneously high values in the presence of proteolipids [10] and that a small part (<10%) of the rhodopsin in our preparations is photolyzed.

The results in this paper are expressed per mg of "Lowry" protein and per mol true rhodopsin present. The calcium content of the isolated outer segment membranes, as determined by atomic absorption spectroscopy [2], is less than 0.3 mol calcium per mol rhodopsin present, or less than 10% of the calcium present in intact cattle rod outer segments after density gradient centrifugation.

Equilibrium dialysis

The isolated rod outer segment membrane preparations are suspended in the desired medium, usually 20 mM Tris · HCl (pH 7.4). The protein concentration ranges from 0.4 to 0.8 mg/ml. Samples of 5 ml are placed in dialysis bags consisting of Visking tube. The tube has previously been boiled in a solution containing 2 mM $NaHCO_3$ and 0.2 mM EDTA, and has then been thoroughly washed with double distilled water. The bags are closed in such a way that an air bubble is enclosed to allow mixing of the contents. The closed bags are placed in test tubes (1.8 × 8.3 cm) containing 10 ml of an identical buffer solution to which approximately 0.67 μCi ^{45}Ca ($^{45}CaCl_2$, 445 Ci/mol, Radiochemical Centre, Amersham, Nottingham, U.K.) is added. The stoppered tubes are attached to a disc, which rotates vertically at 1 rev./min. After dialysis for 40 h at 4°C in the dark, three 500-μl samples are taken from both the suspension inside and the solution outside the dialysis bag. The radioactive samples are mixed with 10 ml Aquasol and counted in a liquid scintillation analyzer (Philips). The total amount of calcium bound to the membrane fraction is calculated as follows:

$$\text{percent } {}^{45}Ca_{bound}\ (B) = \frac{(cpm_i - cpm_o) \times 10}{cpm_i \times 10 + cpm_o \times 20} \times 100\% \quad (1)$$

$$\text{percent recovery } {}^{45}Ca\ (R) = \frac{cpm_i \times 10 + cpm_o \times 20}{cpm_T} \times 100\% \quad (2)$$

$$\text{nmol } Ca_{bound} = \text{nmol } Ca_T \times \frac{R}{100} \times \frac{B}{100} \quad (3)$$

where cpm_i and cpm_o are the total number of counts in 500-μl aliquots from the dialysis bag and from the outside solution, respectively. The total amount of cpm present per tube (cpm_T) is calculated from an aliquot of the ^{45}Ca containing solution, taken before the start of the experiment. In this way the percent recovery of ^{45}Ca can be calculated (Eqn. 2). The recovery of ^{45}Ca in our experiments is always more than 90% and in most experiments even more than 95%, but always less than 100%. This is probably due to absorption to the dialysis bag and the glass tube. The number of nmol calcium bound can be calculated by means of Eqn. 3, where Ca_T is the total amount of calcium present. Ca_T is the sum of the calcium added to the dialysis solution (^{45}Ca plus ^{40}Ca) and the endogeneous calcium of the outer segment material, which is determined by means of atomic absorption spectroscopy [2].

Most experiments are carried out in dim red light up to removal of samples for counting radioactivity. In other experiments illumination is carried out at room temperature by a 75 W tungsten source through 3 mm KG1 and OG570

filters (Schott-Jena) at a distance of 20 cm for 15 min. Dark controls are kept at room temperature for the same time.

The ionophore A23187 is a gift from Eli Lilly and Company. It is dissolved in acetone (29 mg/ml) and then 9 vol. ethanol are added. Appropriate amounts of this solution are added to the dialysis medium.

Results

Methodological aspects

Our isolation procedure yields rod outer segment membranes, which are free from mitochondrial contamination (cf. ref. 7). The average rhodopsin yield is 10.5 (S.E.: 0.5, 23 determinations) nmol rhodopsin per retina. All experiments have been performed with freshly isolated outer segment material, which is depleted of endogenous calcium and lysed. Storage of the material overnight in a medium containing 100 mM NaCl and 20 mM Tris · HCl (pH 7.4), either at 4°C or at −70°C, does not change the calcium binding capacity of the preparation. Neither does lyophilization of the material have a significant effect.

In our binding assay equilibrium is reached in approx. 25 h of dialysis at 4°C, but 40 h was routinely used in all further experiments. Under these conditions there is no detectable loss of rhodopsin. Neither does the volume of the outer segment suspension inside the dialysis bag change, which has been determined by adding to the external dialysis solution dextran blue (M_r $2 \cdot 10^6$), for which the dialysis membrane is impermeable. There is no significant change in 620 nm absorbance before and after the dialysis experiment, indicating that the volume has not changed. Therefore we may apply Eqn. 1—3 for calculating the calcium binding capacity of the rod outer segment membranes. The protein concentration is not critical for this capacity within the range (0.4—0.8 mg/ml) used in our experiments.

In measuring binding of cations to macromolecules by means of equilibrium dialysis the occurrence of Donnan effects may cause errors, particularly when using low electrolyte concentrations. We have investigated the possible contribution of a Donnan effect in our binding studies by measuring the distribution of ^{22}Na, added to the external solution at the start of equilibrium dialysis. At a protein concentration of 0.88 mg/ml (equivalent to a rhodopsin concentration of about $13 \cdot 10^{-6}$ M), the resulting ratio $r = [Na^+]_o/[Na^+]_i$ is 0.993. If this is a Donnan ratio, then the Donnan ratio $[Ca^{2+}]_o/[Ca^{2+}]_i$ would have to be $r^2 = 0.986$. However, when using ^{45}Ca much lower ratio's, ranging from 0.77 to 0.59 for protein concentrations between 0.4 and 1.0 mg/ml, are obtained. This proves that under these conditions the Donnan contribution is negligible and that we are measuring the binding of calcium to the membranes.

Influence of medium composition

Table I shows the calcium binding capacity of cattle rod outer segment membranes in various media at a fixed calcium concentration of 10^{-5} M. When the dialysis medium contains only 20 mM Tris · HCl, 6.1 nmol calcium are bound per mg protein (0.4 mol calcium per mol rhodopsin). Addition of NaCl, 20 mM or 100 mM, decreases the calcium binding by about 60 and 75%, respectively. About the same amount of calcium is bound when the 100 mM NaCl is sub-

TABLE I

EFFECT OF MEDIUM COMPOSITION ON CALCIUM BINDING BY CATTLE OUTER SEGMENT MEMBRANES (IN THE PRESENCE OF 10^{-5} M Ca^{2+})

Dialysis medium	Calcium binding (nmol Ca^{2+}/mg protein)	Number of experiments
20 mM Tris · HCl (pH 7.4)	6.1 ± 0.4	12
20 mM Tris · HCl + 20 mM NaCl	2.5	1
20 mM Tris · HCl + 100 mM NaCl	1.4 ± 0.2	8
20 mM Tris · HCl + 100 mM NaCl + 5 mM $MgCl_2$	0.6 ± 0.1	4
20 mM Tris · HCl + 100 mM NaCl + 0.01 mM $LaCl_3$	1.4 ± 0.1	3
20 mM Tris · HCl + 50 mM NaCl + 50 mM KCl	1.2	1
20 mM Tris · HCl + 100 mM KCl	1.4 ± 0.4	2
20 mM Tris · HCl + 100 mM KCl + 5 mM $MgCl_2$	0.6 ± 0.1	3

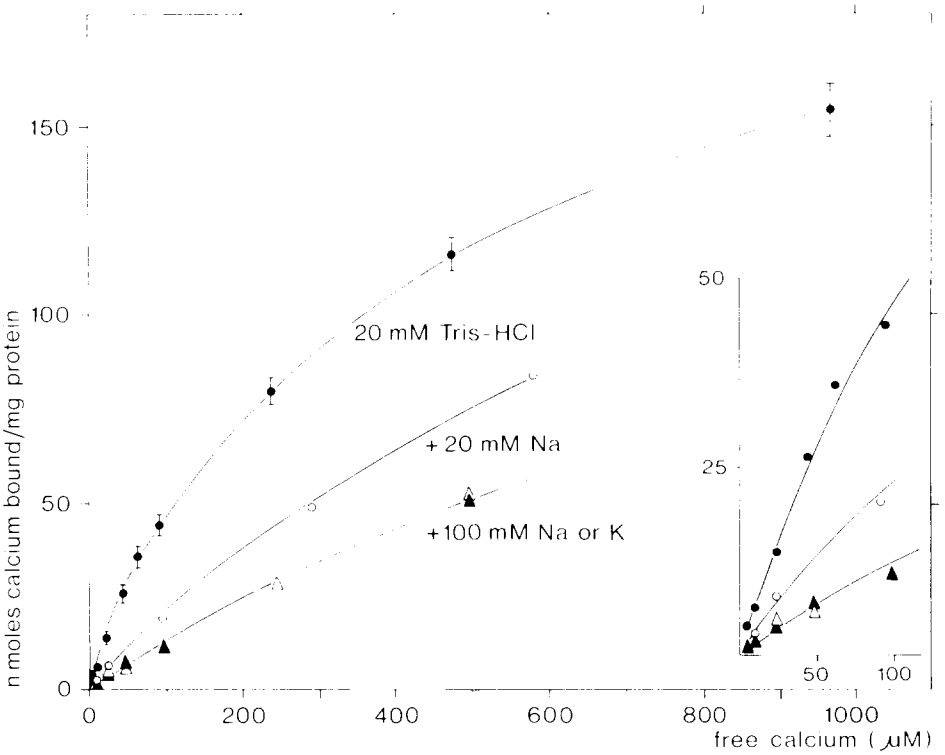

Fig. 1. Effect of Ca^{2+}-concentration on Ca^{2+}-binding by cattle ROS-membranes in 20 mM Tris · HCl (pH 7.4), ●; 20 mM NaCl/20 mM Tris · HCl (pH 7.4), ○ and 100 mM NaCl/Tris · HCl (pH 7.4), ▲ or 100 mM KCl/Tris · HCl (pH 7.4), △. Protein concentration as determined by the Lowry et al. method was about 0.35 mg/ml. Dialysis was carried out against 10 ml external solution.

stituted by 100 mM KCl or by a mixture of 50 mM NaCl and 50 mM KCl. Addition of 5 mM $MgCl_2$ to media containing either 100 mM NaCl or 100 mM KCl lowers the amount of bound calcium by a further 55%, but addition of 0.01 mM $LaCl_3$ has no influence.

Influence of free calcium concentration

The effect of the calcium concentration on the binding of calcium has been studied in four different media (Fig. 1). As expected, the binding of calcium increases at higher calcium concentrations. In media of higher ionic strength there is always less calcium bound. Calcium binding is equally affected by the presence of Na^+ and K^+. In 20 mM Tris · HCl and at 10^{-3} M calcium, 155 nmol calcium are bound per mg protein (10.3 mol calcium per mol rhodopsin).

The calcium binding data of Fig. 1 have been analyzed in Scatchard plots [11]. Fig. 2 shows that these Scatchard plots are biphasic, indicating the presence of (at least) two classes of binding sites. Linearized plots (Fig. 2; insert) are used to calculate the number of binding sites and their apparent dissociation constants, assuming two types of independent binding sites. The intercept on the ordinate (n) represents the number of binding sites per mg of membrane protein. The apparent dissociation constant is given by the slope of the linear segment. The results are presented in Table II, which indicates that the high

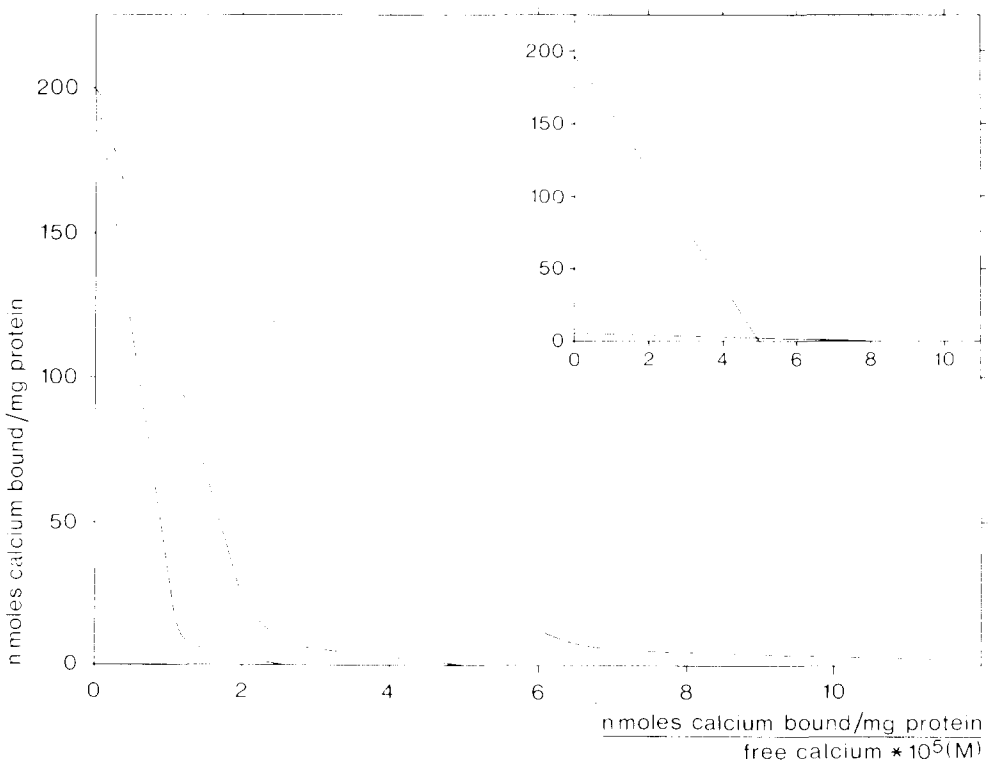

Fig. 2. Scatchard plots for the binding of Ca^{2+} by cattle ROS-membranes. The solid lines are calculated from the best fitting curves drawn through the experimental points presented in Fig. 1. The inserted figure shows an example of analyzing a Scatchard plot assuming two types of independent binding sites.

TABLE II

COMPARISON OF CALCIUM BINDING SITES

Values derived from Scatchard plots assuming independency of sites. n, amount of Ca^{2+} bound in nmol per mg protein; $K_{diss.}$, dissociation constant.

Assay conditions	High affinity		Low affinity	
	n	$K_{diss.}$ (mM)	n	$K_{diss.}$ (mM)
20 mM Tris — HCl, pH 7.4	5	0.008	195	0.3
20 mM NaCl + 20 mM Tris — HCl, pH 7.4	5	0.017	195	0.9
100 mM NaCl + 20 mM Tris — HCl, pH 7.4	5	0.040	195	1.6
100 mM KCl + 20 mM Tris — HCl, pH 7.4	5	0.040	195	1.6

affinity sites can accommodate 5 and the low affinity sites 195 nmol calcium per mg protein, or 0.3 and 13.0 mol calcium per mol rhodopsin, respectively. The high affinity sites have a dissociation constant ranging from 8 to 40 μM, the low affinity sites from 0.3 to 1.6 mM, depending on the medium composition.

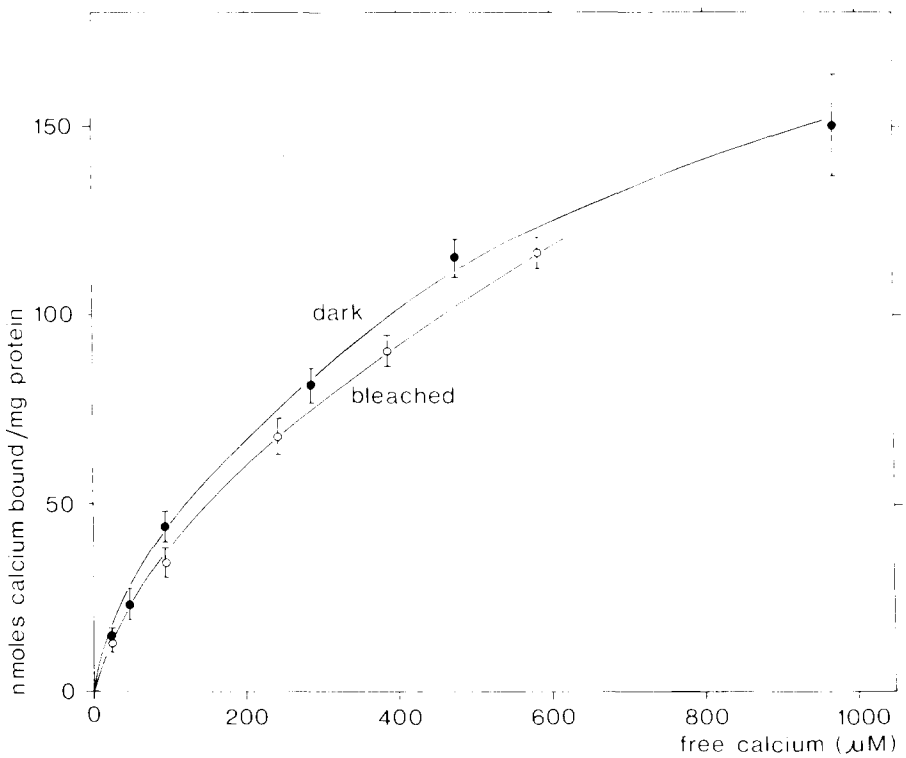

Fig. 3. Effect of illumination on Ca^{2+}-binding by cattle ROS-membranes in 20 mM Tris · HCl (pH 7.4). Dialysis was carried out in the dark, (●) or after the rhodopsin had been bleached for 15 min at room temperature, (○).

TABLE III

EFFECT OF IONOPHORE A23187 ON CALCIUM BINDING BY OUTER SEGMENT MEMBRANES IN 100 mM NaCl/20 mM TRIS · HCl (pH 7.4)

A23187 concentration (M)	Calcium bound (nmoles per mg protein)	
	10^{-5} M Ca^{2+}	10^{-3} M Ca^{2+}
0	1.5 ± 0.2	53 ± 0.3
10^{-7}	1.5 ± 0.02	53 ± 11
10^{-6}	1.6 ± 0.05	55 ± 3.7
10^{-5}	2.2 ± 0.23	67 ± 4.5

Influence of light

The effect of light on the binding of calcium by outer segment membranes has also been examined (Fig. 3). Each point of these curves is calculated from paired experiments with 4 different membrane preparations. The result suggests that illuminated membranes bind somewhat less calcium as compared to dark-kept controls. However, it should be noted that in 2 of the 4 experiments no difference in calcium binding has been observed between the "dark" and the "light" group. Our conclusion is that there is at most only a very minor effect of light on calcium binding.

Influence of ionophore addition

In order to investigate whether the calcium, associated with the outer segment material after 40 h of dialysis at 4°C, is accumulated actively within vesicles instead of merely being passively bound, we have used the ionophore A23187. In concentrations ranging from 10^{-7} to 10^{-5} M (i.e. up to a calcium-ionophore ratio of 1) this substance never decreases the amount of bound calcium (Table III). On the contrary, at the highest ionophore concentration an enhanced calcium binding appears to occur, which may be due to binding of calcium by ionophore molecules lodged in the membranes.

Discussion

The remarkably high calcium contents of rod outer segments previously found by us [2] have led us to investigate the process by which this accumulation may occur. In the present study the method of equilibrium dialysis has been used to investigate passive binding of calcium to isolated, lysed and calcium-depleted cattle rod outer segment membranes. Electronmicroscopy shows that the original rod outer segment structure has been lost completely and has been replaced by closed, swollen vesicles, as previously observed by Chen and Hubbell [12]. This also explains the relatively long period (approx. 25 h) required for complete calcium equilibration, since the half time for calcium efflux from liposomes prepared from rod outer segment lipids is 3—4 h [13]. The use of lysed membranes avoids complications due to the presence of an intact outer membrane and of possible soluble calcium-binding material located in the extrasaccular space.

The amount of calcium accumulated is fairly large, depending on the exter-

nal calcium concentration and the medium composition. The maximum binding capacity of 200 nmol calcium per mg protein or 13.3 mol calcium per mol rhodopsin exceeds the highest values we have ever found for the calcium content of freshly isolated cattle rod outer segments.

The process appears to be passive, since no ATP or metabolic energy is required, and since storage for 16 h at 4°C or lyophilization of the material prior to equilibrium dialysis does not affect the amount of calcium accumulated. Further support for this conclusion is obtained from the observation that addition of the calcium ionophore A23187 does not lower calcium binding. This substance is known to abolish calcium gradients [14], but it hardly affects ATP-independent calcium binding [15,16]. These findings strongly suggest that the accumulated calcium ions in these experiments are passively bound to the membranes and are not actively translocated into the vesicular structures.

The amount of calcium bound is affected by the composition of the incubation medium. Addition of 20 mM NaCl to the basic medium of 20 mM Tris · HCl (pH 7.4) lowers the amount of calcium bound considerably. Addition of 100 mM NaCl or KCl decreases calcium binding even more and equally, suggesting that this is merely an effect of ionic strength. The fact that even at a Na^+ or K^+ to Ca^{2+} ratio of about 10000 there is still a significant amount of calcium bound indicates the presence of rather specific calcium binding sites. Addition of 5 mM magnesium decreases calcium binding to less than half at low (10^{-5} M) calcium concentrations, but only by 10% at higher (10^{-3} M) calcium levels, again indicating the rather high specificity of the calcium binding sites.

The relation between amount of calcium bound and free calcium concentration, when expressed in a Scatchard plot, indicates the presence of at least two types of binding sites, high-affinity sites and low-affinity sites. The high-affinity sites have a capacity of only about 5 nmol/mg protein (0.3 mol/mol rodopsin), the low-affinity sites one of 195 nmol/mg protein (13.0 mol/mol rohodpsin). These results are in fair agreement with those of Hemminki [5], who reports capacities of 25 and 210 nmol/mg protein for the two types of sites. His values for the dissociation constants ($4.1 \cdot 10^{-4}$ M and $1.2 \cdot 10^{-2}$ M in 100 mM Tris · HCl, pH 7.4) are about 10-times higher than ours ($4.0 \cdot 10^{-5}$ M and $1.6 \cdot 10^{-3}$ M in 100 mM NaCl, 20 mM Tris · HCl, pH 7.4). This discrepancy may, at least in part, be due to loss of bound calcium in his millipore filtration procedure, since he washes three times with ^{45}Ca-free buffer solutions. This washing procedure must have removed a considerable fraction of bound ^{45}Ca in these experiments as well as in those of Bownds et al. [3], Neufeld et al. [4] and Weller et al. [6]. This will lead to apparently lower affinities than those derived from our experiments, where such a disturbance of the equilibrium state cannot occur.

Whereas we cannot offer any suggestion as to the identity of the high-affinity sites, we believe that the low-affinity sites may represent the polar headgroups of the membrane phospholipids and possibly rhodopsin, both of which bear negative charges at pH 7.4. In this respect, phosphatidylserine with its carboxylic group may be particularly important; there are about 10 molecules of this phospholipid per rhodopsin molecule in the rod sac membrane.

The high total binding capacity (13 mol calcium per mol rhodopsin) could at first sight suggest that the high calcium content of rod outer segments would be due merely to passive binding of this cation to the rod sac membrane with-

out active translocation into the rod sac interior. This appears, however, unlikely on the following two grounds. First, at the low cytoplasmic calcium concentrations found in various tissues ($\leqslant 10^{-6}$ M) and presumably also existing in the extrasaccular space [17], only the high affinity sites would be (partly) occupied. These sites have, however, a capacity of only 0.3 mol calcium per mol rhodopsin. Secondly, we find no significant decrease in the amount of bound calcium upon illumination. How could light then cause a release of calcium ions from the rod sacs, as shown indirectly by the experiments of Hagins [1] and more directly by the experiments of Hendriks et al. [2]? These considerations strongly suggest that the high calcium content of rod outer segments requires, in addition to passive binding, an active translocation of calcium into the rod sac interior. The search for this translocation process is the topic of a subsequent paper (Schnetkamp et al., Biochim. Biophys. Acta, in the press).

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