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CALCIUM TRANSLOCATION AND STORAGE OF ISOLATED INTACT CATTLE ROD OUTER SEGMENTS IN DARKNESS

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Summary

Bovine rod outer segments (rods), isolated with an intact plasma membrane and a stable calcium exchange and storage capacity, contain 2—3 mol endogenous calcium/mol rhodopsin. By means of ^{45}Ca accumulation experiments and concomitant ^{40}Ca analysis, the calcium metabolism of these organelles has been studied with the following results:

1. The majority of endogenous calcium is localized within disks.
2. In the presence of the ionophore A23187 the intradiskal binding sites can be titrated with external calcium.
3. The Scatchard plot of calcium binding of rods indicates the presence of a single set of intradiskal binding sites with a maximal capacity of 8—9 mol calcium/mol rhodopsin and an affinity constant of 55 μM to calcium.
4. Without A23187 more than 99% of the rod calcium appears in a bound state in equilibrium with a free calcium concentration of 15—25 μM.
5. External calcium exchanges with endogenous calcium in a fast ($t_{1/2}$ = 12 s) process with a uniform rate constant, whereas net calcium transport is very slow ($t_{1/2} > 2$ h).
6. Intact rods contain a calcium translocation system, presumably located in the plasma membrane, which performs Ca-Ca exchange with a high unidirectional flux of $2 \cdot 10^6$ calcium ions/rod per s.
7. This translocation system can be saturated by external calcium (K_m = 0.5—1 μM) and has a low Q_{10} (1.08).

Both the calcium translocation system and the calcium binding system appear to depend on the structural integrity of the stacked disks and are very sensitive to the experimental conditions.

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The relevance of these findings is discussed in relation to the proposed role of calcium ions as the intracellular transmitter in vertebrate rod photoreceptor cells.

Introduction

Since Hagins (1) proposed Ca^{2+} as the intracellular transmitter in vertebrate rod outer segments (rods), a number of reports have appeared [2—5], which describe the endogenous calcium content of isolated rods and the effect of illumination on it. In another series of studies [6—14], rod fragments are loaded with exogenous calcium and a possible subsequent light-induced release is investigated. However, in both cases serious discrepancies are obvious. The calcium content may very over almost two orders of magnitude. With respect to a light-induced Ca release from rod disks, disagreement is even greater.

In view of this confusing situation we have focussed our attention on calcium storage and transport of isolated rods in darkness [15]. In a previous report [16] we established that cattle rods with a leaky plasma membrane contain a specific calcium translocation system in the disk membrane, sensitive towards Ca^{2+} and Na^{+}, but not to Mg^{2+} and K^{+}. In vitro this system does not behave as a $(Ca^{2+} + Mg^{2+})$-activated ATPase calcium pump, but under certain conditions it can be affected by ATP, resulting in net calcium uptake. The major ATP-independent transport modes are Ca-Ca exchange and Na-stimulated Ca efflux, presumably by Na-Ca exchange.

In addition to the translocation system cattle rod membranes also possess a calcium storage capacity, dependent on the structural integrity of the intact stack of disks and possibly depending on or generated by the hydrolysis of ATP [16]. The molecular basis of this storage capacity might be related to the presence of calcium binding sites on rod membranes, described by Hendriks et al. [17] and Hemminki [18].

Since the previously used rod preparations appeared to have a leaky plasma membrane and to be very labile in various respects [16], we have devised a new isolation procedure [19]. This yields cattle rods with an intact plasma membrane, in which the calcium translocation and storage systems are much better preserved. Moreover, the intactness of their plasma membranes can conveniently be manipulated without harming their stability. In this paper, some basic features of calcium translocation and storage in these rod preparations are described.

Materials and Methods

Standard procedures

All procedures with rods are carried out in darkness or in dim red light. ^{45}Ca translocation into rods is assayed by the rapid filtration method described before [16]. Throughout all experiments the washing medium contains 600 mM sucrose, 20 mM Tris-HCl (pH 7.4) and 250 μM EGTA, which removes all adherent Ca. Radioactivity is counted in 10 ml Aquasol (New England Nuclear, Boston, U.S.A.) in a liquid scintillation counter.

Calcium contaminations in the media are reduced by passing all sucrose and sucrose-Ficoll solutions over a mixed-bed ion-exchange column. The resulting calcium contamination is less than 1 μM. Tris (250 mM) does not contain a measurable calcium contamination, LiCl contains 15 μM calcium/100 mM LiCl and KCl contains 0.5 μM or 2 μM calcium/100 mM KCl (two different lots of KCl).

Rhodopsin determinations are performed according to the standard procedures of this laboratory [20].

Preparations

Three types of rod preparation have been used in this study. Stable intact rods are prepared in a sucrose-Ficoll medium according to the previous paper [19]. Their plasma membrane is intact, since it constitutes a permeability barrier for ATP, NADPH and protons. Whenever the term 'rods' or 'rod outer segments' is used in this paper without further qualification, it refers to this preparation.

Stable leaky rods are isolated in 0.16 M Tris-HCl buffer (pH 7.4) and finally resuspended in the sucrose-Ficoll medium. They lack the permeability barrier for ATP, NADPH and protons [19]. Both these preparations can be stored at 4°C as a concentrated suspension (100 μM rhodopsin). In the experiments rhodopsin concentrations between 10 and 20 μM have been used.

Finally rods, isolated according to the old procedure [16] in 0.16 M Tris-HCl buffer (pH 7.4) and resuspended in electrolyte media, have been used. These 'Tris-rods' are leaky, as measured by the criteria mentioned above [16]. The prefix 'depleted' (as opposed to non-depleted) refers to preparations, depleted of endogenous calcium having EGTA present throughout the isolation procedure.

^{40}Ca determination

Calcium is determined with a Pye Unicam SP 1950 double-beam atomic absorption spectrophotometer. All samples contain 0.5% $LaCl_3$ to overcome anionic interference. Calcium standard solutions are prepared from anhydrous $CaCO_3$, which is dissolved in 0.1 M HCl containing 0.5% $LaCl_3$.

In view of the conflicting results on the calcium content of rod outer segments between different investigators, three different methods of sample preparation have been compared. In the dry ashing method an aliquot (0.5 ml) of a rod suspension is placed in a quartz tube (10 × 80 mm), dried at 75°C and ashed at 520°C for 6 h. The residue is heated with 30% H_2O_2 to boiling until a clear and colorless solution is obtained. This is dried at 75°C and dissolved in 0.1 M HCl containing 0.5% $LaCl_3$. In the ionophore extraction method an aliquot (0.5 ml) of a rod suspension is incubated at 18—20°C for 15 min with 4 μM A23187 (gift of E. Lilly and Co., Indianapolis, U.S.A.) and excess EDTA. Ionophore is added as an ethanolic solution. The suspension is centrifuged and calcium is determined in an aliquot of the clear supernatant, using standards prepared in the same medium. The calcium content of the sediment remaining after ionophore treatment is below the detection limit. Finally, the previously described [2] acid digestion method has been used. The calcium contents of three rod outer segment preparations were independently determined by each

method in 4—8-fold. If the value obtained by the ionophore extraction method is set at 100%, the dry ashing method yields 98.2% and the acid digestion method 99.1%, with standard deviations of 1.9, 2.9 and 2.4%, respectively. Since these values are equal within the experimental error, the ionophore method has been used routinely in all further experiments in view of its convenience.

Results

Incorporation of ^{45}Ca in intact cattle rods

The average total calcium content of the rod pellet, immediately after the gradient procedure, is given in Table I. After subsequent resuspension of the rods in the virtually Ca-free standard medium (600 mM sucrose/5% Ficoll 400/20 mM Tris-HCl, pH 7.4) ^{45}Ca is added to the rod suspension. After equilibration a considerable fraction (50—60%) of the total ^{45}Ca is present in the rods, as determined by the rapid washing/filtration procedure (Table I).

If external ^{45}Ca is separated from the incorporated ^{45}Ca by passing the suspension over a Sephadex G-50 column, more than 90% of the radioactivity eluted with the rods remains on the filter after the washing/filtration procedure. This shows that nearly all rods are retained by the filter and that only few rods are disrupted by the washing/filtration procedure.

Excess EGTA, which buffers the external, free calcium concentration to below 10^{-2} μM, abolishes the uptake of ^{45}Ca (Fig. 1). La^{3+} (50 μM) apparently greatly decreases the translocation rate of calcium, but has no influence on the final equilibrium level (Fig. 1). These effects demonstrate that the washing/filtration procedure with 250 μM EGTA removes all adherent ^{45}Ca and ensures that translocation into the rods, rather than binding to the plasma membrane is measured.

Since the plasma membrane of our rod preparations constitutes a permeability barrier to ATP, NADPH and protons [19] ^{45}Ca must have been translocated through the plasma membrane. In view of the high rate of equilibration of ^{45}Ca (Fig. 1; $t_{1/2}$ = 12 s; S.E. 1; n = 13), this membrane appears to contain a highly active calcium translocation system.

Exogenous ^{45}Ca exchanges with at least 90% of the endogenous Ca. This has been determined in two ways.

TABLE I

CALCIUM CONTENT OF ISOLATED INTACT RODS

The endogenous calcium content of the rods is calculated by multiplying the total calcium content of the rod suspension with the fraction of the total radioactivity, incorporated in the rods after complete equilibration.

	Standard procedure	1 mM $CaCl_2$ omitted in isolation medium
Total calcium content (mol Ca/mol rhodopsin)	5.7 ± 0.6 (11)	3.2 ± 0.3 (23)
Percent of added ^{45}Ca incorporated after equilibration	59 ± 5 (10)	55 ± 2.5 (19)
Endogenous calcium content (mol Ca/mol rhodopsin)	3.4 ± 0.4 (10)	1.8 ± 0.2 (15)

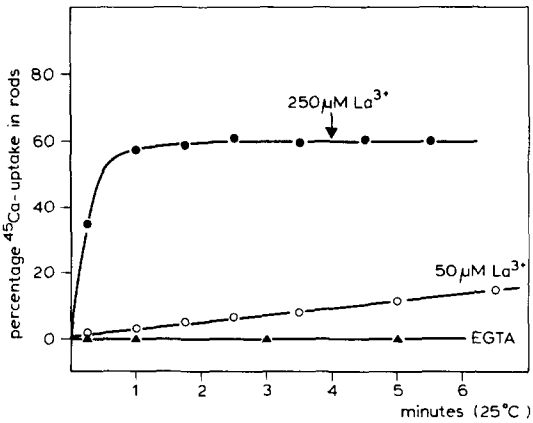

Fig. 1. Equilibration of ^{45}Ca between endogenous and exogenous calcium pools. Temperature: 25°C. Aliquots of the rod suspension are filtered over borosilicate glass filters. The radioactivity of the sediments is determined and expressed as percent of the total ^{45}Ca added. Medium: 600 mM sucrose, 5% w/v Ficoll 400, 20 mM Tris-HCl (pH 7.4). Triangles: in the presence of large excess EGTA (0.2 mM). Open circles: in the presence of 50 μM $LaCl_3$. Control level is ultimately reached. Closed circles: no additions at start, but after 4 min addition of 250 μM $LaCl_3$.

After addition of ^{45}Ca to a rod suspension, followed by equilibration and centrifugation, the ^{45}Ca/^{40}Ca ratios in supernatant and pellet differ by at most 10%. Addition of increasing amounts of ^{40}Ca decreases the ^{45}Ca incorporation proportionally to the decrease of the specific radioactivity as expected from a completely exchangeable calcium pool within the rods.

Reduction of external, free calcium by titration with EGTA causes changes in the rate of ^{45}Ca-^{40}Ca exchange (see Fig. 4 and its discussion) which allow an estimation of the initial external, free calcium concentration. The resulting values agree with those calculated from the ^{45}Ca distribution after equilibration and the total ^{40}Ca content of the suspension. Therefore, the endogenous calcium content of the rods can be obtained in good approximation by multiplying the total calcium content of the rod suspension with the fraction of the total radioactivity incorporated in the rods after equilibration (Table I).

When a rod suspension after the initial isolation is stored for varying times (up to 2 days at 4° C) before performing the ^{45}Ca uptake experiment, nearly the same ^{45}Ca uptake curve is observed as shown in Fig. 1. The final ^{45}Ca distribution between rods and external medium thus seems to be a very stable feature, despite the fact that the uptake of ^{45}Ca in exchange for endogenous ^{40}Ca is a very dynamic process.

Localization of endogenous calcium

In the previous paragraph it has been shown that exogenous ^{45}Ca exchanges with nearly all endogenous calcium in rods, resulting in a homogeneous distribution of ^{40}Ca and ^{45}Ca. Therefore, endogenous calcium can be quantitatively localized from the ^{45}Ca distribution after previous equilibration.

When leaky rods or lysed rods (disks) are used, the EGTA washing procedure is expected to remove all calcium except that which is located inside the disks [16]. An attempt to localize the calcium in intact rods is displayed in Fig. 2.

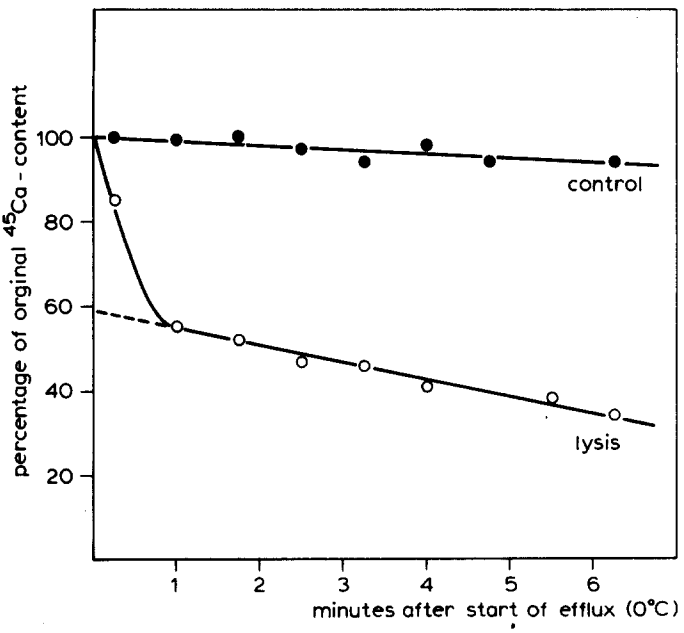

Fig. 2. Efflux of ^{45}Ca from pre-loaded rods in the presence of excess EGTA at 0°C. Rods, previously equilibrated with ^{45}Ca at 25°C, are ten-fold diluted with the indicated media. The data are expressed as percent with respect to the ^{45}Ca level after the previous equilibration. Closed symbols: control efflux in original medium: 600 mM sucrose, 5% w/v Ficoll 400, 0.2 mM EGTA, 20 mM Tris-HCl (pH 7.4). Open symbols: efflux after lysis in 5% w/v Ficoll 400, 0.2 mM EGTA. Results of three experiments with different preparations are averaged. The excess EGTA is sufficient to prevent re-uptake of ^{45}Ca.

Preloaded intact rods lose very little radioactivity upon dilution in an isosmotic, tracer-free medium containing excess EGTA. A ten-fold reduction in osmotic strength lyses the rods, but still at least 60% (extrapolation to zero time from the slow efflux phase) of the calcium remains inaccessible to EGTA and must, therefore, be located within the disks. The other 40% are lost in a fast efflux phase (Fig. 2), possibly due to rupture of part of the disks before or during the filtration procedure. This suggestion is strengthened by the observation that the contribution of the fast efflux phase strongly depends on slight variations of the conditions. Omission of Ficoll 400 from the lysis medium increases the fast efflux phase to 58%, whereas replacement of EGTA by La^{3+} (250 μM) in the lysis medium lowers it to 28%.

In agreement with these data obtained by ^{45}Ca analysis, the disk pellet after lysis (conditions as in Fig. 2) and subsequent centrifugation still contains about 80% of the ^{40}Ca, present in the non-lysed control. The 20% loss can roughly be accounted for by the slow efflux phase in view of the time elapsed between lysis and centrifugation.

These data indicate an exchange between external and intradiskal calcium. The participation of the calcium in the rod cytosol in this exchange is difficult to evaluate.

Properties of the calcium translocation system

Data on the net transport of calcium into rods after previous ^{45}Ca equilibra-

tion are summarized in Fig. 3. The half-times of net uptake (7 h with 1 mM Ca^{2+} outside) and net efflux (3 h with excess EGTA outside) are about three orders of magnitude slower than ^{45}Ca equilibration by exchange (Fig. 3 as compared to Fig. 1). This implies that the transport mode of the rapid calcium translocation system is a 1 : 1 exchange. Therefore, ^{45}Ca equilibration can be formally described by two opposing first order reactions. The unidirectional Ca flux of the exchange process can thus be determined by a kinetic analysis of ^{45}Ca equilibration. The appropriate equations are given in the legends of Fig. 4. The maximal unidirectional Ca flux, thus calculated, is surprisingly high and amounts to 3.85 mol calcium/mol rhodopsin per min (S.E. = 0.45, n = 15).

Titration of extracellular calcium with EGTA hardly affects the equilibration rate, until EGTA is in molar excess over total extracellular calcium. This indicates that the transport site is completely saturated with calcium at micromolar external calcium concentrations. With different amounts of EGTA, in excess over external calcium, various low free external calcium concentrations can be obtained. When ^{45}Ca uptake is restricted to a few minutes, no net calcium transport occurs and the data can be properly analyzed (Fig. 4). Free calcium concentrations are calculated according to Caldwell [21]. The linearity observed in these plots indicates a good homogeneity of the preparation, since all endoge-

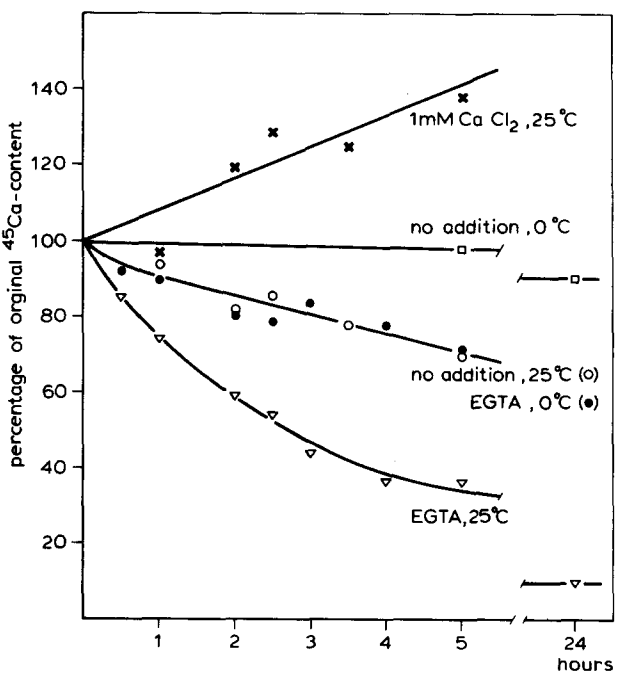

Fig. 3. Net transport in rods, previously equilibrated with ^{45}Ca. Medium: 600 mM sucrose, 5% w/v Ficoll 400, 20 mM Tris-HCl (pH 7.4). The data are expressed as percent with respect to the ^{45}Ca level after the previous equilibration. Crosses (X): 1 mM $CaCl_2$ added, temperature 25° C (previous equilibration in the presence of 1 mM $CaCl_2$ to adjust the specific radioactivity). Squares (□): no addition, temperature 0° C. Open circles (○): no addition, temperature 25° C. Closed circles (●): excess EGTA added (0.2 mM), temperature 0° C. Triangles (▽): excess EGTA added (0.2 mM), temperature 25° C. The excess EGTA (0.2 mM) is sufficient to prevent re-uptake of ^{45}Ca.

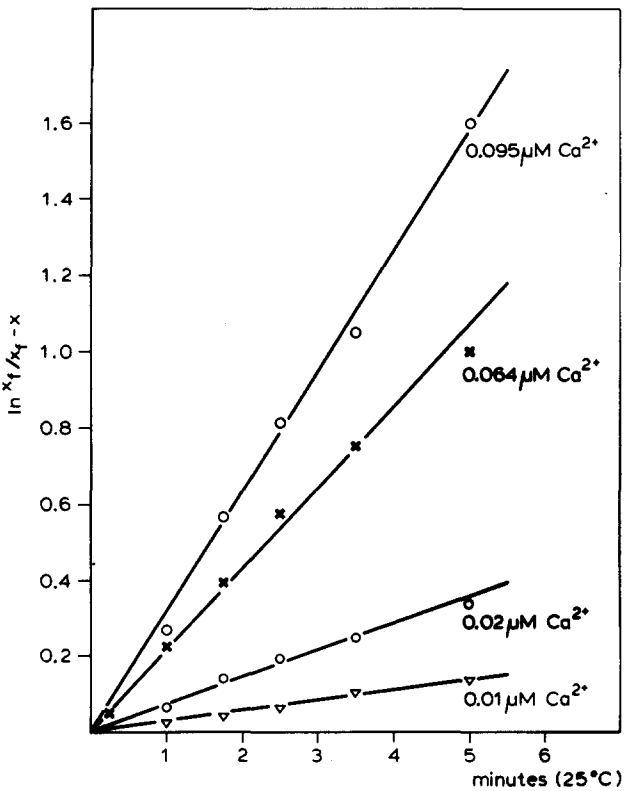

Fig. 4. Kinetic analysis of unidirectional ^{45}Ca fluxes. ^{45}Ca equilibration without net calcium transport can formally be described by two opposing first-order reactions, resulting in the equation: $\ln[x_f/(x_f - x)] = [k/x_f] \cdot t$, where x is the fraction of ^{45}Ca in the rods at time t, x_f is the fraction of ^{45}Ca in rods after equilibration, and k is the rate constant. The unidirectional flux is: $v = k(1 - x_f)Ca_t$, where Ca_t is the total calcium concentration, expressed as mol calcium/mol rhodopsin. Low free calcium concentrations are obtained by addition of various concentrations of EGTA, in excess over external calcium. Open circles: 0.095 μM Ca^{2+}; crosses: 0.064 μM Ca^{2+}, closed circles: 0.02 μM Ca^{2+}, triangles: 0.01 μM Ca^{2+}. The medium further contains 120 mM sucrose, 1.25% w/v Ficoll 400, 160 mM KCl, 20 mM Tris-HCl (pH 7.4). Temperature 25°C.

nous calcium pools exchange with the same rate constant.

The relation between external, free calcium concentration and the unidirectional ^{45}Ca fluxes can be further analyzed by transformation to a Lineweaver-Burk plot (Fig. 5). These plots appear to be linear from 0.01 μM Ca^{2+} to 10 μM Ca^{2+} (the latter is used as concentration with maximum velocity) and indicate saturation of a single type of transport site. The affinity of this transport site for calcium calculated according to this model and using the data points of Fig. 5 is 1.02 μM (S.E. 0.14; $n = 6$). Substitution of a major part of the sucrose by KCl does not greatly affect the result. The slightly higher apparent affinity for calcium in this case (0.52 ± 0.05 μM; $n = 5$) may be due to the effect of the electrolyte KCl on the association constants of EGTA. This effect may be comparable to the influence of various buffers [22].

The energetics of the Ca-Ca exchange has been investigated by determining its temperature dependence (Fig. 6). A Q_{10} value of 1.08 is found, indicating a heat of activation of only 1.3 kcal/mol. The Ca-Ca exchange can be maintained

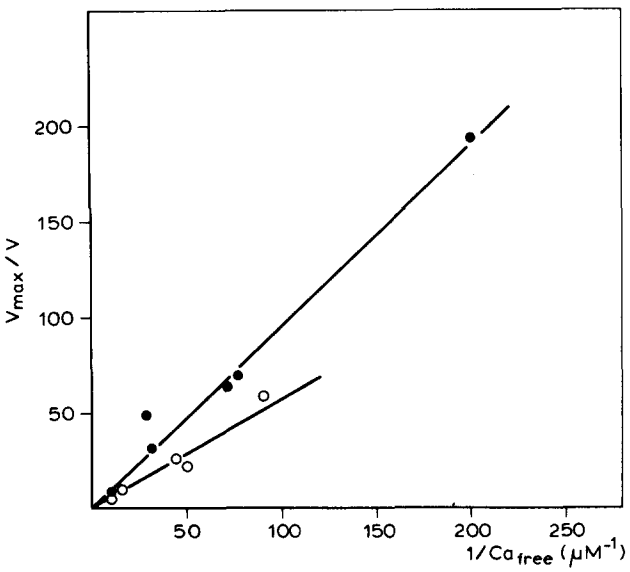

Fig. 5. Lineweaver-Burk plots of unidirectional ^{45}Ca fluxes. Open circles: data calculated from those in Fig. 4. Closed circles: same conditions as in Fig. 4, except for the medium: 600 mM sucrose, 5% Ficoll 400, 20 mM Tris-HCl (pH 7.4).

for a considerable length of time (several days at 4° C) without addition of exogenous energy sources.

With stable leaky rods [19] the unidirectional Ca flux of the Ca-Ca exchange is equal to that for intact rods within a factor of 2, and the kinetic analysis

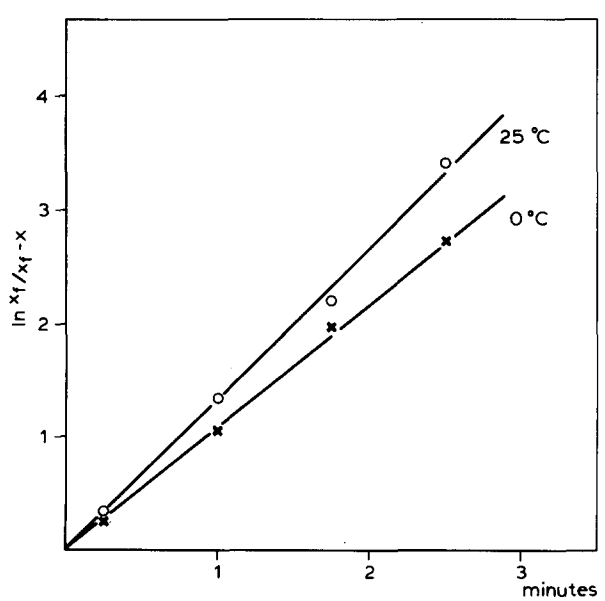

Fig. 6. Temperature dependence of unidirectional ^{45}Ca fluxes. The kinetic analysis is similar to that in Fig. 4. Open circles: 25° C, closed circles: 0° C. Medium: 600 mM sucrose, 5% w/v Ficoll 400, 50 mM KCl, 20 mM Tris-HCl (pH 7.4). The external calcium concentration (20 μM) is sufficient to saturate the transport system.

again yields linear plots. In the leaky 'non-depleted Tris rods' [16] ^{45}Ca equilibration by Ca-Ca exchange also takes place, but the unidirectional Ca flux of Ca-Ca exchange amounts to only 0.37 mol Ca^{2+}/mol rhodopsin per min (S.E. = 0.12, n = 6). This is a remarkable finding, since this flux is only 10% of that in intact rods, where calcium first has to pass the plasma membrane. The other properties of the Ca-Ca exchange in leaky 'Tris rods' are difficult to assess, because the kinetic analysis generally does not yield linear plots.

Properties of the calcium storage system

Stable intact rods lose only a small part of their endogenous calcium during storage at 4°C for up to 2 days (cf. Table II), while net efflux of calcium from rods down a calcium gradient (in the presence of 0.2 mM EGTA) has half-times in the order of hours, even at 25°C (Fig. 3). Therefore, as previously noticed for leaky Tris rods [16], the calcium content of intact rods appears to be virtually independent of the external calcium concentration.

When the divalent cation ionophore A23187 is added to the suspension, the calcium content of the rod becomes dependent on the external calcium concentration (Fig. 7A). The resulting linear Scatchard plot (Fig. 7B) suggests that a single set of binding sites is titrated with calcium. The maximal capacity of these sites amounts to 8—9 mol Ca^{2+}/mol rhodopsin and their affinity constant for calcium is 55 μM. The ionophore A23187 makes both the plasma membrane and the disk membrane fully permeable to calcium because endogenous calcium located within the disks becomes accessible to rapid complexation by EGTA (Fig. 8). When, after prior depletion, calcium is added back to the

TABLE II

EFFECTS OF VARIOUS MEDIA ON THE CALCIUM CONTENT AND ON THE Ca-Ca EXCHANGE

Results are presented as percent with respect to the calcium content and translocation rate of the rods in the standard medium. The calcium content is determined at 25°C by the ^{45}Ca level in the rods after equilibration (cf. Table I). The translocation rate is the maximal velocity of the unidirectional Ca flux of Ca-Ca exchange at 25°C. The half-time of ^{45}Ca-equilibration is the time required to reach half of the equilibrium ^{45}Ca level in rods at 25°C. It can be calculated with the equation given in the legend of Fig. 4. The calcium content in the presence of EGTA is determined after previous equilibration of ^{45}Ca at 25°C. The EGTA concentration is sufficient to prevent uptake of ^{45}Ca.

Medium	Storage conditions:						
	Calcium content				Calcium translocation rate		
	0 h	24 h 4°C	5 h 4°C + 0.2 mM EGTA	5 h 25°C + 0.2 mM EGTA	0 h	24 h 4°C	0 h $t_{1/2}$ of equilibration
Standard sucrose-Ficoll medium	≡100%	91	70	34	≡100%	95	≡1 (12 s)
KCl *	90	37	42	34	66	69	1.7
LiCl *	90	56	62	12	20	5	8.0
Tris-HCl *	76	24	n.d.	n.d.	35	11	3.2
KCl **	68	9	n.d.	n.d.	75	7	1.2

* 200 mM KCl, 200 mM LiCl, 230 mM Tris-HCl, respectively, with 20 mM Tris-HCl (pH 7.4) added, substitute 80% of the standard medium.

** Pellet resuspended in 200 mM KCl, 20 mM Tris-HCl (pH 7.4).

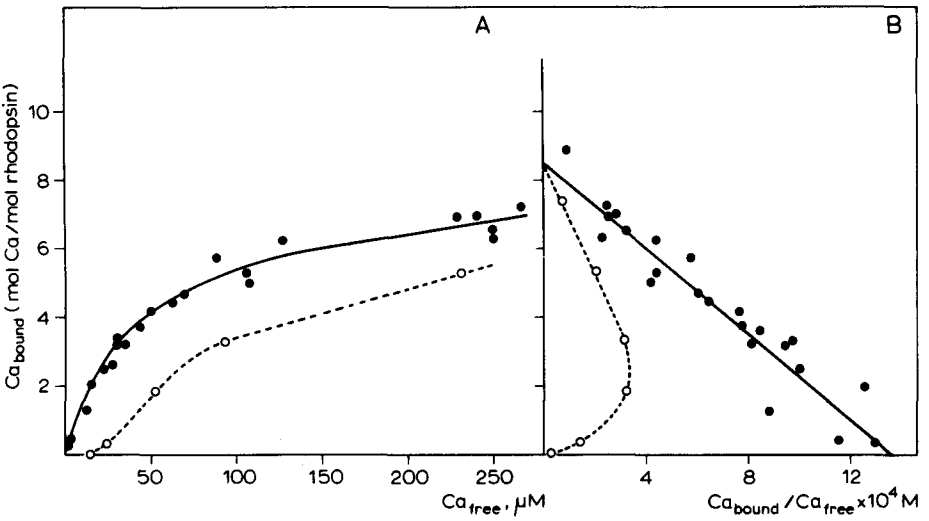

Fig. 7. Calcium binding by rods in the presence of A23187. Medium: 600 mM sucrose, 5% w/v Ficoll 400, 20 mM Tris-HCl (pH 7.4). A. Rods are equilibrated for 45 min at 25°C with ^{45}Ca in the presence of various amounts of added ^{40}Ca and EGTA (to obtain the lower calcium concentrations by reduction of endogenous calcium).Closed circles: Results of five different rod preparations (A23187 concentrations between 0.15 and 0.40 mol A23187/mol rhodopsin). Open circles: Rods are preincubated for 30 min at 25°C with 100 mM NaCl (replacing 200 mM sucrose) and excess EGTA (1 mM). After removal of NaCl and EGTA, the ionophore is added (0.2 mol A23187/mol rhodopsin). The further procedure is similar to that without preincubation. B. Scatchard plots from data of A. Same symbols used as in B.

medium in a concentration range of 5—400 µM free calcium, calcium is bound again to the intradiskal binding sites to almost the same values (102%, S.E. = 4, n = 18) as before depletion.

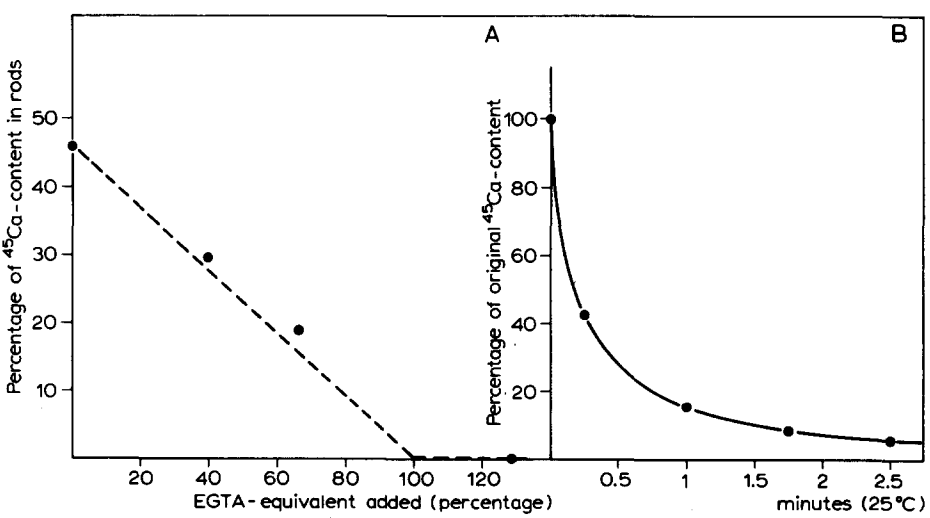

Fig. 8. Titration of ^{45}Ca from rods by EGTA. Medium: 600 mM sucrose, 5% w/v Ficoll 400, 20 mM Tris-HCl, pH 7.4. Temperature: 25°C. A. Rods are equilibrated with ^{45}Ca and various amounts of EGTA in the presence of A23187 (0.2 mol A23187/mol rhodopsin). EGTA-equivalent is expressed as percent of the total amount of calcium present. B. ^{45}Ca-efflux from rods, previously equilibrated with ^{45}Ca in the presence of A23187 (0.1 mol A23187/mol rhodopsin), by addition of excess EGTA (0.2 mM).

The Scatchard plot of calcium binding may show small deviations of linearity, because the optimal ionophore concentration varies somewhat with the free calcium concentration (not shown). Consistent deviations from linearity in the Scatchard plot may be observed upon isolation of rods in a low calcium medium (in the presence of EGTA). Upon exposure of rods to a medium containing EGTA and high Na^+, these deviations become more pronounced. The most extreme case observed is included in Fig. 7 (broken line) and shows a sigmoidal relation between Ca binding and the external calcium concentration.

Stability of the calcium storage and translocation system

The stability of both the calcium storage and translocation systems of rods in the sucrose-Ficoll medium contrasts with the observations for the leaky Tris rods [15], where electrolyte media were applied. Therefore the effect of partial medium substitution by various electrolytes has been investigated. NaCl has strong direct effects on Ca metabolism in isolated cattle rods, which will be presented in a subsequent communication and hence are omitted here.

When 80% of the sucrose-Ficoll medium is substituted by various electrolyte media, the rod outer membrane remains intact (no phosphorylation by exogenous ATP) and the Ca translocation and storage characteristics of the rods are only slightly affected (Table II). However, exposure to these media for some hours appears to decrease the stability of the rods more seriously (Table II). Tris and lithium ions affect both the Ca translocation rate and the calcium content (storage capacity), while K^+ mainly affects the latter. By resuspension of a pellet of intact rods in the indicated electrolyte media, the rods become leaky [19] and are much less stable (Tables III and V).

Table III shows the half-time of net ^{45}Ca efflux against excess external EGTA for various rod preparations. Net efflux increases by a factor of 35 going from stable rods with intact or leaky plasma membranes to the most maltreated rods: freeze-thawed depleted Tris rods. Concomitantly, the maximal velocity of the exchange system and also the sensitivity to La^{3+} are greatly decreased. Table IV demonstrates that the strong inhibition of Ca-Ca exchange by La^{3+} is gradually abolished in this series. This is not due to the fact that in the one case transport through the plasma membrane and in the other through the disk membrane is measured, since La^{3+} can also inhibit Ca-Ca exchange in lysed and leaky rods.

TABLE III

HALF-TIME OF ^{45}Ca EFFLUX AGAINST EXCESS EGTA FOR VARIOUS ROD PREPARATIONS

Preparation	Half-time (min; 25°C)
Intact rods in sucrose-Ficoll medium	150—180
Intact rods, 200 mM KCl substitutes 80% of sucrose-Ficoll medium	150
"Non-depleted Tris rods" * in K-medium (100 mM KCl, 2 mM $MgCl_2$, 20 mM Tris-HCl, pH = 7.4)	40— 50
"Depleted Tris rods" * in K-medium	10— 15
"Depleted Tris rods" * in K-medium after freeze-thawing	4— 6

* These preparations are defined under Materials and Methods and were used in a previous study [16].

TABLE IV

EFFECT OF LANTHANUM ON ^{45}Ca TRANSLOCATION IN VARIOUS ROD PREPARATIONS

^{45}Ca uptake after 1 min at 25°C in the presence of 100 μM La^{3+}, expressed as percent of uptake in a control suspension without La^{3+}. External calcium concentration: 15—30 μM.

Preparation	Percent ^{45}Ca uptake	State of the plasma membrane
Intact rods in sucrose-Ficoll medium	10	intact
Intact rods, 200 mM KCl substituting 80% of sucrose-Ficoll medium	11	intact
Lysed rods in sucrose-Ficoll medium	2	absent
Intact rods, resuspended in:		
200 mM KCl/20 mM Tris-HCl (pH 7.4)	1	leaky
200 mM KCl/20 mM Tris-HCl (pH 7.4) after 24 h at 4°C	67	leaky
"Non-depleted Tris rods" * in		
160 mM Tris-HCl, pH 7.4	21	leaky
160 mM Tris-HCl, pH 7.4 after 24 h at 4°C	100	leaky

* These preparations are defined under Materials and Methods and were used in a previous study [16].

The decrease of the calcium content of rods reflects a change of the calcium binding sites, which is analyzed by measuring the decay of the calcium binding capacity in different rod preparations in the presence of A23187 (Table V). With various external calcium concentrations up to five times the affinity of the binding sites, there is a proportional decay of calcium binding, suggesting a decrease in the capacity or a large shift of the affinity of the binding sites. These results explain the difference in response to A23187 of the intact rods as compared to the leaky Tris rods [16]. Whereas in the leaky rods addition of A23187, even in the absence of EGTA, gives a nearly complete release of calcium, no release is observed in intact rods under the same conditions. This difference cannot be explained by the presence or absence of an intact plasma membrane, since in the intact rods all calcium can be titrated stoichiometrically by EGTA in the presence of A23187 (Fig. 8A). In addition, the calcium binding capacity is largely retained in the stable leaky rods (not shown).

Effects of osmotic shock

Earlier observations [16] suggested that the loss of the stacked disk structure

TABLE V

DECAY OF CALCIUM BINDING IN DIFFERENT ROD PREPARATIONS

Calcium binding is determined in the presence of A23187 by Scatchard plots, similar as in Fig. 7.

Preparation	Half-time
Intact rods in sucrose-Ficoll medium, 0°C	>2 days
Intact rods in sucrose-Ficoll medium, 25°C	20 h
Intact rods, 50% of sucrose-Ficoll medium replaced by 200 mM KCl, 0°C	130 min
Intact rods, 50% of sucrose-Ficoll medium replaced by 200 mM KCl, 25°C	40 min
Intact rod pellet, resuspended in 200 mM KCl, 20 mM Tris-HCl (pH 7.4), 25°C	15 min

and the swelling of the disk are responsible for the decrease in stability described in the previous paragraph. To distinguish the immediate effects of disruption of the stacked disk structure from the subsequent ageing effects, the influence of osmotic shock on the Ca-Ca exchange have been investigated. Fig. 9 demonstrates that a shock with 3 vols. of water has almost no effect on Ca-Ca exchange. Under these circumstances the rod structure is preserved and the plasma membrane remains intact, according to the criteria used previously [19].

A shock with 6 vols. of water results in deformed rods, which are then visible as spherical objects under the light microscope. Similar observations with frog rods have been described by Hagins et al. [14]. After a shock with 10 vols. of water (rhodopsin concentration in the starting suspension should not exceed 15 μM), the turbidity of the suspension shows a steep decrease and no structure is visible any more under the light microscope. Most of the calcium is still present within individual disks (Fig. 2), but the rate of Ca-Ca exchange is greatly decreased (Fig. 9). The sensitivity to La^{3+} is not altered (Table IV). Net efflux of ^{45}Ca is still fairly slow (compare Fig. 2 with Table III), but the disks do not retain calcium upon addition of A23187, even in the absence of EGTA. This suggests that the binding sites for calcium are greatly altered.

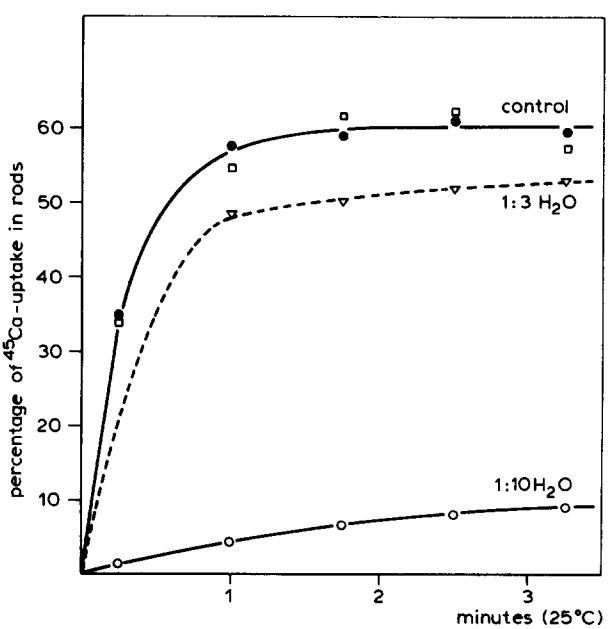

Fig. 9. Effect of osmotic shock on ^{45}Ca-equilibration. ^{45}Ca-uptake in rods is measured as a function of time at 25°C. Closed circles: Control, medium consists of 600 mM surcose, 5% Ficoll 400, 20 mM Tris-HCl (pH 7.4). Open circles: After previous lysis with 10 vols. of water, containing 5% w/v Ficoll 400. Final medium: same as control medium. Triangles: After previous shock with 3 vols. of water. Final medium: same as control medium. Squares: After a shock with 3 vols. of water and no change of medium. In the case of change of the medium (by centrifugation and resuspension) the data are related to a control sample, treated likewise.

Discussion

Calcium and rod outer segments

The intact isolated bovine rod outer segments (rods), used in this study and prepared according to the previous paper [19], contain 2—3 mol endogenous calcium per mol rhodopsin. Their higher calcium content, as compared to rods isolated in a calcium free medium (Table I), cannot be completely explained by uptake of calcium from the medium. Isolated rods do not accumulate appreciable amounts of calcium during a time interval (60 min) such as required for the isolation procedure. We rather presume that external calcium has a stabilizing effect on the rod structure, which results in better retention of endogenous calcium.

In the intact rods endogenous calcium appears to be mainly located within disks, since lysis in the presence of EGTA leaves the majority of the endogenous calcium in a compartment, which is not accessible to the EGTA in the washing medium. This is in agreement with the results of a histochemical localization technique [23]. The predominantly intradiskal location of endogenous calcium is consistent with the kinetic analysis of ^{45}Ca equilibration by Ca-Ca exchange, as shown in Figs. 4 and 6. The linearity of these plots indicates that all endogenous calcium pools exchange with the same rate constant. Although intact rods are generally believed to enclose two compartments, the cytosol and the intradiskal space, no indications for two consecutive reactions have been observed in our studies. In addition, ^{45}Ca equilibration in intact rods occurs with at least the same rate as in leaky rods. Thus we find no indication for the operation of two permeability barriers in these preparations. One interpretation of these results is that only a single barrier exists between intradiskal and external calcium. Alternatively, one must assume that in intact rods exchange across the disk membrane is much faster than observed in the various leaky or lysed rod preparations.

The endogenous calcium content of both intact rods (Fig. 3) and leaky rods [16] is essentially independent of the external calcium concentration. The predominant transport mode under the conditions used in this paper is therefore Ca-Ca exchange. This exchange is about three orders of magnitude faster than the net calcium transport. The half-time of net calcium efflux from rods (some hours) is comparable to that of the efflux of ^{45}Ca from liposomes, prepared from rod phospholipids [24]. Therefore, net calcium transport may reflect simple diffusion through the membrane bilayer down the calcium gradient, resulting from either a high or a very low external calcium concentration. Net calcium transport may als be governed by the transport rate (through diffusion) of the cotransported anion. The rod translocation system appears, under our conditions, restricted to exchange diffusion with a high unidirectional flux of 3.9 mol Ca^{2+}/mol rhodopsin per min. With the average rod dimensions of 1×20 μm and an overall rhodopsin concentration of 3 mM in the rod, a flux through the plasma membrane of 4.9 pmol Ca^{2+}/cm^2 per s or $2 \cdot 10^6$ Ca^{2+}/s per rod can be calculated.

The phenomenon of exchange diffusion and the presence of an external transport site, which exhibits saturation behaviour (Fig. 5), suggest the presence of a mobile carrier type translocation system [25]. The low Q_{10} value of

1.08 seems to plead against such a mechanism and is generally associated with a pore of channel type of mechanism [26]. Therefore, a neutral terminilogy (translocation system) is used, since a detailed molecular picture of the underlying system is not yet available.

The endogenous calcium content of intact rods, which is predominantly located within the disks, becomes dependent on the external calcium concentration upon addition of the ionophore A23187 (Fig. 7A). In isolated intact rods A23187 makes both the plasma and the disk membranes permeable to calcium (Fig. 8). The equilibrium between endogenous calcium and the external calcium concentration is then described by a linear Scatchard plot (Fig. 7b), which indicates the existence of a set of identical intradiskal binding sites with a maximal capacity of 8—9 mol calcium/mol rhodopsin and an affinity of 55 μM to calcium. A minor contribution of binding sites exposed to the cytosol cannot be excluded on the basis of the present data. The complete and reversible removal of endogenous calcium by EGTA is consistent with the notion that equilibration of free calcium concentrations between all compartments occurs in the presence of A23187. In view of the fact that A23187 exchanges calcium for protons, calcium can apparently be reversibly replaced by protons at the binding sites. Therefore, deviations of linearity in the Scatchard plot may be explained by the lack of sufficient exchangeable protons. From the binding curve and the endogenous calcium content of rods an apparent free intradiskal calcium concentration of 15—25 μM can be estimated for isolated intact rods without A23187. This means that more than 99% of the intradiskal calcium in rods must exist in a bound state.

Energetic considerations

Addition of A23187 results in a net efflux of calcium only when the external calcium concentration is below 15—25 μM. Complete efflux occurs when a strong chelator like EGTA is present externally (Fig. 8). The calcium content of rods is not affected by treatment with high concentrations of A23187 for several hours at 0°C, unless EGTA is added. This reinforces the conclusion that intact rods bind calcium, rather than sequester it by an energy requiring process. This is further strengthened by our observation that rods completely depleted of calcium in the presence of A23187 and EGTA, spontaneously rebind calcium to the original equilibrium levels when the external free calcium concentration is restored.

Furthermore, we observed that the calcium storage and the Ca-Ca exchange in rods are both preserved during storage of the rods at 4°C for up to 2 days. Under these conditions Ca-Ca exchange still persists (low Q_{10}) and no substantial amount of endogenous calcium is lost. On the other hand, the endogenous metabolic energy supply as measured by the ability to generate NADPH for the reduction of all-*trans* retinal following rhodopsin photolysis, is hardly affected by storage for two days at 4°C [19]. The following calculation shows that it is unlikely that hydrolysis of ATP is required for Ca-Ca exchange. From the unidirectional flux of 3.9 mol Ca^{2+}/mol rhodopsin per min, the Q_{10} of 1.08, a rhodopsin concentration of 3 mM and the assumption that approx. 1 mM ATP is present and is completely hydrolyzed in two days, it would follow that 29 000 mol Ca^{2+} would have been transported per mol ATP hydrolyzed. If,

alternatively, 2 mol Ca^{2+} would be transported per mol ATP hydrolyzed (the ratio found for the (Ca^{2+} + Mg^{2+})-ATPase system in sarcoplasmic reticulum and erythrocytes), the ATP supply would be exhausted in less than a minute. Neither is calcium storage sustained by an ATP-hydrolyzing system, since it is not affected by prolonged exposure (some hours at 0°C) to the combination of A23187 and a high (or intermediate) calcium concentration, which should maximally stimulate energy-requiring Ca-sequestering mechanisms. On the contrary, a substantial net uptake of calcium can be obtained in the presence of A23187 after previous depletion of calcium by EGTA (Fig. 7 and its discussion). Therefore, ATP is not required for net calcium uptake under these conditions. In addition, the ATP-effect on ^{45}Ca uptake, previously described, does not have the properties of the (Ca^{2+} + Mg^{2+})-activated ATPase pump system [16]. Finally, the decay of ATP has been reported to be fairly slow in frog rods under more physiological conditions [27,28]. In conclusion, none of the data presented in this paper indicate a significant involvement of metabolic energy in either the calcium translocation or the storage system. In agreement herewith purified rod membranes contain neither (Ca^{2+} + Mg^{2+})-ATPase [29, 30] nor (Na^+ + K^+)-ATPase [29,31].

From the present data the suggestion arises that in the rod outer segment ion fluxes occur primarily passively. The energy input to maintain asymmetrical ion fluxes could be supplied by the Na^+-K^+ pump, which is most likely located in the rod inner segment membrane. If the Na^+-K^+ pump is physically removed during isolation of the outer segments, it can be expected that new ionic equilibria are established within a few minutes, as suggested by rapid Ca-Ca exchange and Na-Ca exchange processes decribed in this paper and in Ref. 16. In support of this, the sodium dark current in the retina disappears with a half-time of 30 s, when the Na-K pump is specifically inhibited by ouabain [32]. These considerations supply the rationale for our use of a Na^+-free medium in isolating and maintaining the rod outer segments. In addition they indicate why experiments with these rod preparations should be restricted to short incubations after changing the external medium, since the exchange mechanisms might then rapidly establish a new equilibrium state. These points are illustrated for the intact retina by Sillman et al. [33], who have shown that after a 7 min exposure of the retina to ouabain in the absence of external Na^+, a receptor potential can still be recorded upon illumination, immediately after Na^+ is added back to the medium.

Comparison of different rod preparations

As discussed in the previous paper [19], it is essential for reproducible experiments with isolated rods to have a homogeneous preparation with well-defined properties. The various cattle rod preparations used in this paper illustrate how important this criterion is. Although the Ca translocation and storage systems are always present, characteristic parameters like rate constants and binding capacities vary considerably, and different responses to agents like La^{3+} (Table IV) and A23187 (Table V) are noted. Another critical point regards the stability of the preparation towards the medium conditions. For example, hypotonic shocks, sufficiently strong to abolish the stacked disk structure, appear to destroy the calcium translocation system and greatly affect the

calcium binding capacity, as indicated by Fig. 9 and by the calcium released in lysed rods upon addition of A23187. These observations are especially noteworthy since in most of the reports on a light-induced calcium release in isolated rods a hypotonically shocked or otherwise fragmented rod preparation has been used [6,7,10—13,17].

Therefore it seems essential to take into account the differences between the various preparations. In view of the consistent and reproducible experimental results obtained with stable intact and stable leaky rods, as defined in the previous paper [19], we prefer these preparations for studies of the calcium translocation and storage systems in rods. The sucrose-Ficoll medium does not seem to interfere with these systems. Substitutions of the major part of it by various electrolyte media has relatively minor effects at first (Table II), but after prolonged exposure, particularly after resuspension, large changes occur. This is illustrated by:

1. The half-time of the net Ca efflux, probably simple diffusion through the membrane bilayer, decreases from 165 min to 5 min (Table III).
2. The unidirectional Ca flux of the Ca-Ca exchange is greatly decreased (by lysis or in Tris rods).
3. The sensitivity of Ca-Ca exchange to La^{3+} is gradually abolished (Table IV).
4. The properties of the intradiskal calcium binding sites are drastically modified (Table V).

These changes must reflect modification of the membrane structure, probably by charge rearrangement, since they involve interactions with ions.

Concluding remarks

Intact cattle rods contain a calcium translocation system which can maintain high ion fluxes. This system bears some resemblance to the calcium exchange system described for nerve axon membrane [34,35], which will be discussed in a forthcoming communication on the ion selectivity of the rod translocation system.

The disks contain a large calcium binding capacity in equilibrium with free calcium concentrations in the micromolar range. Communication between external and intradiskal calcium occurs very rapidly by exchange. This conclusion is not easily reconciled with the calcium transmitter hypothesis of visual excitation [1]. Although the present data do not provide information on the calcium concentration of the rod cytosol, a crucial element in this hypothesis, there is no report that the disk membranes contain mechanisms to reduce the extradiskal, free calcium concentration to the required values of 1 μM or lower (cf. Ref. 36). Furthermore, in 'stable' rods, a fast light-induced shift of the intradiskal equilibrium of calcium binding appears to occur without detectable calcium release from disks [37].

The physiological relevance of the Ca-Ca exchange system in combination with the calcium storage capacity, described in this paper, remains to be established. In addition, we have to be aware of the limitations of isolated cell organelles in an artificial medium, when we wish to extend the conclusions from such preparations to the in vivo situation. When attempting to relate our observations to present views on the mechanism of visual excitation, these reservations should be kept in mind.

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