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ION SELECTIVITY OF THE CATION TRANSPORT SYSTEM OF ISOLATED INTACT CATTLE ROD OUTER SEGMENTS

EVIDENCE FOR A DIRECT COMMUNICATION BETWEEN THE ROD PLASMA MEMBRANE AND THE ROD DISK MEMBRANES

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Summary

The ion selectivity of cation transport through the plasma membrane of isolated intact cattle rod outer segments (rods) is investigated by means of ^{45}Ca-exchange experiments and light-scattering experiments. These techniques appear to provide complementary information: the ^{45}Ca experiments (^{45}Ca fluxes in rods) describe electroneutral antiport, whereas the light-scattering experiments (shrinkage and swelling of rods upon hypertonic shocks with various electrolytes) reveal electrogenic uniport. Electroneutral symport of ions (salt transport) does not take place without addition of external ionophores and application of salts of weak acids.

1. Intact rods recover from a hypertonic shock in the presence of FCCP when lithium, sodium and potassium acetate are applied, but not when ammonium chloride, calcium and magnesium acetate are used. This indicates that the plasma membrane of isolated intact cattle rods is relatively permeable to net transport of Na^+, Li^+ and K^+, and relatively impermeable to net transport of Cl^-, Mg^{2+} and Ca^{2+} under conditions that do not give rise to diffusion potentials.

2. Rapid ($t_{1/2} < 1$ min) efflux of ^{45}Ca from preloaded intact rods is observed when Na^+, Ca^{2+}, Sr^{2+}, and under certain conditions also Ba^{2+}, are added to the external medium. Li^+, K^+, Rb^+, Cs^+, Mg^{2+} and Mn^{2+} are ineffective in this

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Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

respect as well as protons at pH 7.4. It is concluded that ^{45}Ca efflux reflects electroneutral exchange diffusion of internal ^{45}Ca with external Na^+, Ca^{2+}, Sr^{2+} and Ba^{2+}, respectively.

3. All tested cations lower the rate of ^{45}Ca uptake. The latter can be described by a single rate constant indicating a homogeneous rod preparation and a homogeneous endogenous Ca^{2+} pool. However, only those cations which stimulate ^{45}Ca efflux from preloaded rods lower the final equilibrium of ^{45}Ca uptake. Except for the effects of K^+, Rb^+ and Cs^+ the reduction of the rate of ^{45}Ca uptake by external cations appears to arise from competition for a common site on the plasma membrane. The observed affinities for this site do not correlate with actual transport (as indicated by the ability to stimulate ^{45}Ca efflux).

4. K^+ increases the affinity of the exchange diffusion system to Ca^{2+} from 1 μM to 0.16 μM and changes the relative affinities with respect to Ca^{2+} for the other cations (Na^+, Mg^{2+}, Mn^{2+}, Sr^{2+}, Ba^{2+}). Furthermore, the maximal rate of Ba-Ca exchange is strongly stimulated by K^+, whereas the maximal rate of Ca-Ca exchange is reduced at saturating Ca^{2+} concentrations.

5. The exchange diffusion transport mode can be turned off by external Na^+ in a process that is not of a stochastic nature, which implies interdependence of individual transport entities and which results in an inhomogeneity of the endogenous Ca^{2+} pool. K^+ acts as antagonist of Na^+ in this effect.

The relevance of these findings is discussed in relation to the generally accepted view, that a diffusable transmitter in the rod cytosol communicates the photochemical event in the disk membrane to the electrical properties of the plasma membrane. It is argued that the exchange diffusion system present in the plasma membrane of isolated cattle rods has a number of properties in common with the system responsible for the dark current through the outer segment of a rod cell in the retina. It is concluded that the exchange diffusion transport mode of the cation transport system in the plasma membrane of isolated cattle rods has access to both the extracellular side of the plasma membrane and the disk interior. Under these conditions it behaves as a single system, which exchanges cations directly from the extracellular space to the disk interior, whereas the disk membranes do not appear to contain a separate Ca^{2+} transport system.

Introduction

Experiments on the electrical behaviour of the vertebrate retina have suggested that Ca^{2+} plays an important role in modulating the ion fluxes underlying the mechanism of visual excitation [1—6]. The excitatory mechanism of rod photoreceptor cells is localized in the rod outer segment and therefore the Ca^{2+} metabolism of these organelles has been investigated in this laboratory. For this purpose a new procedure was devised, which stabilizes the outer segment structure and allows purification of isolated cattle rod outer segments (rods) with either a functionally intact or leaky plasma membrane [7]. Using these preparations, it appeared possible to preserve and characterize a Ca^{2+} translocation system and a Ca^{2+}-binding capacity in a reproducible way [8].

It was found that the endogenous Ca^{2+} pool of the intact cattle rods is primarily bound to binding sites within the disks and exchanges with external Ca^{2+} by the operation of a translocation system which performs exchange diffusion without net transport [8].

In the present study the ion selectivity of this cation translocation system is investigated with a special emphasis on the interrelation between Ca^{2+} and Na^{+} ions. Because of the accuracy of the methods to assay and analyse ^{45}Ca fluxes, data on the latter are found to be very useful also in the study of the effects of other ions. The cation translocation system, described in this study, is shown to share a number of properties with the system responsible for the dark current of Na^{+} in the vertebrate retina.

Materials and Methods

Preparations and general procedures

All procedures with rod outer segments are carried out in darkness or in dim red light.

Stable cattle rod outer segments (rods) with either a leaky or an intact plasma membrane are prepared as described before [7]. Whenever the term rods is used, it always refers to the stable intact rod preparation. Other rod preparations are explicitely defined in the text. The rod preparations are stored in the dark at 4°C as a concentrated suspension (100—200 μM rhodopsin) in the standard medium. The standard medium contains 600 mM sucrose, 5% (w/v) Ficoll 400 and 20 mM Tris-HCl (pH 7.4). The sucrose-Ficoll solution is deionized by passing it over a mixed-bed ion-exchange column before use.

Intact rods remain intact as determined by the criteria used before [7] during all manipulations imposed on them in this study (i.e., electrolyte additions, osmotic manipulation, addition of various ionophores).

The Ca^{2+} content of the rod preparations is determined by the 'ionophore extraction' method described before [8]. The Ca^{2+} determinations are performed on a Pye Unicam SP 1950 double-beam atomic absorption spectrophotometer.

Rhodopsin determinations are performed according to the standard procedures of this laboratory [9].

^{45}Ca experiments

All ^{45}Ca experiments are performed at 25°C and use a final rhodopsin concentration of 15—25 μM in the standard medium. A preincubation time of 10 min is used to thermally equilibrate the suspension from 4°C to 25°C. ^{45}Ca fluxes in rods are assayed by applying the rapid filtration technique described before [10] to samples withdrawn from the incubated suspension at the indicated times. All ^{45}Ca contents described in this study refer to this assay. Throughout all the experiments the washing medium contains 600 mM sucrose, 20 mM Tris-HCl (pH 7.4) and 250 μM EGTA, which removes all adherent calcium [8,10]. Radioactivity is counted in 10 ml Aquasol (New England Nuclear, Boston, MA, U.S.A.) in a liquid scintillation counter.

The average total Ca^{2+} concentration in a rod suspension, containing 15—25 μM rhodopsin, amounts to 80—150 μM, of which 50—90 μM is endogenous

Ca^{2+} leaving an external Ca^{2+} concentration of 30—60 μM. This means an average total Ca^{2+} content of purified cattle rods of 5.7 mol Ca/mol rhodopsin of which about 60% (3.4 mol Ca/mol rhodopsin) is endogenous Ca^{2+}, localized within intact rods [8]. To this suspension of cattle rods the various tested cations (chloride salts) are added simultaneously with ^{45}Ca at the start of the incubation in the influx experiments, or are added after a prior 10-min equilibration with ^{45}Ca at the start of the efflux experiments. In order to avoid aspecific effects due to the rather precarious (in)stability of the rods under various medium conditions [8] the ^{45}Ca influx and efflux experiments are restricted to at the most 5 min. In view of the insensitivity of the rods to osmotic manipulation, electrolyte additions up to 120 mosM are not osmotically compensated for by leaving out sucrose. Deviations from the normal procedure are explicitly stated in the text.

Kinetic analysis of the ^{45}Ca experiments

When no net Ca^{2+} movements occur in rods, ^{45}Ca equilibration can be formally described by two opposing first order reactions. The resulting equations are:

$$\ln [X_f/(X_f - x)] = [k/X_f]\, t \tag{1}$$

and

$$v = k(1 - X_f)Ca_t \tag{2}$$

X_f is the fraction of the total radioactivity in rods after equilibration, x is the fraction of the total radioactivity in the rods at time t, k is the rate constant, v is the unidirectional flux of Ca-Ca exchange, and Ca_t is the total Ca^{2+} concentration in the suspension. In all preparations the V (maximal velocity) is used as a reference and is defined as the observed velocity of the unidirectional ^{45}Ca flux in a rod suspension without any additions, i.e., at an external Ca^{2+} concentration of 30—60 μM. This concentration is sufficient to saturate the transport system [8]. Suspensions with low free external Ca^{2+} concentrations are obtained by addition of various amounts of EGTA, and the resulting free Ca^{2+} concentrations are calculated according to Caldwell [11].

Osmotic experiments

Certain aspects of the permeability properties of the plasma membrane of intact rods are investigated by an osmotic technique. A hypertonic shock on osmotically active particles results in a shrinkage of the particles, which can be monitored by an increase of light-scattering at wavelengths smaller than the dimensions of the particle. With permeable electrolytes the shrinkage is transient and a recovery is observed. The measurements are made in a Pye Unicam SP1750 spectrophotometer or a Beckman UV5260 spectrophotometer at a wavelength of 700 nm. The cuvette is placed directly in front of the photomultiplier. In order to obtain sufficiently large apparent absorbance changes the starting osmolarity of the rod suspension is reduced 3-fold immediately before use.

The rhodopsin concentration ranges from 5—15 μM and the experiments are performed at room temperature. The hypertonic shock is obtained by increas-

ing the osmolarity of the suspension with 100 mosM by the addition of the various electrolytes. In order to induce a permeability for the various cations different ionophores are used. FCCP (final concentration 5 μM) is used as an exclusive protonophore, gramicidin D (final concentration 3 μM) performs electrogenic transport of protons and monovalent cations, and A23187 (gift of E. Lilly and Co., Indianapolis, IN, U.S.A; final concentration 5 μM) exchanges divalent cations and protons. The ionophores are added as an ethanolic solution (final ethanol concentration 0.5% (v/v)). Furthermore, the fact has been used, that NH_3 and acetic acid permeate rapidly through lipid bilayers.

With the procedure used, mixing artifacts cannot be avoided. Therefore, the fast kinetics of recovery (<1 min) cannot be evaluated properly and the uncertainty in the amplitude amounts to about 10% of the initial apparent absorption change observed.

Results

Effects of various cations on ^{45}Ca fluxes in isolated cattle rods

Addition of ^{45}Ca to a suspension of intact cattle rods results in a rapid ($t_{1/2}$ = 12 s) uptake of ^{45}Ca in exchange with endogenous ^{40}Ca and leads to a complete equilibration of the external and endogenous Ca^{2+} pools [8]. Stimulation of ^{45}Ca efflux after previous ^{45}Ca equilibration of the rods is a most sensitive test to establish which cations may replace Ca^{2+} in the exchange diffusion transport. Fig. 1 demonstrates that Na^+, Ca^{2+} and Sr^{2+} do stimulate ^{45}Ca efflux from pre-equilibrated rods, whereas the other tested ions (Li^+ may have a minor effect) do not evoke a significantly larger ^{45}Ca efflux than the control. From the data in a previous study [8] it can be concluded that also La^{3+} and protons (the latter at the pH used and in view of the very slow ^{45}Ca

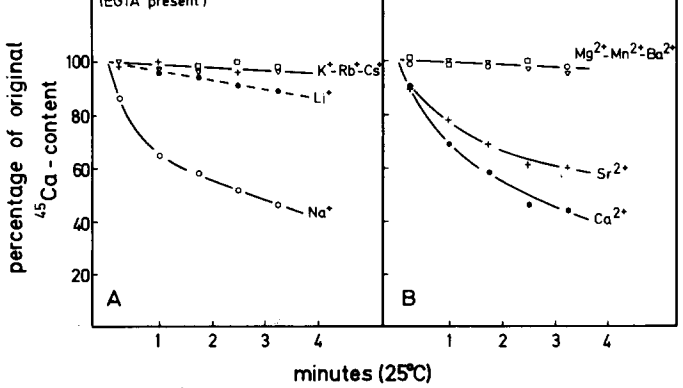

Fig. 1. Efflux of ^{45}Ca from preloaded rods in the standard medium (600 mM sucrose, 5% (w/v) Ficoll 400 and 20 mM Tris-HCl at pH 7.4) at 25° C. (A) In the presence of 250 μM external EGTA and after addition of the indicated chloride salts at the start of the incubation to a final concentration of 50 mM. LiCl (●- - - - -●); NaCl (○———○); KCl (+———+); RbCl (▽———▽); CsCl (□———□). (B) After addition at the start of the incubation to a final concentration of 1 mM: $MgCl_2$ (▽———▽); $CaCl_2$ (●———●); $SrCl_2$ (+———+); $BaCl_2$ (□———□); $MnCl_2$ (○———○). The data are expressed relative to the ^{45}Ca level after the previous equilibration. 100% represents a Ca^{2+} content of 4.6 mol Ca^{2+}/mol rhodopsin.

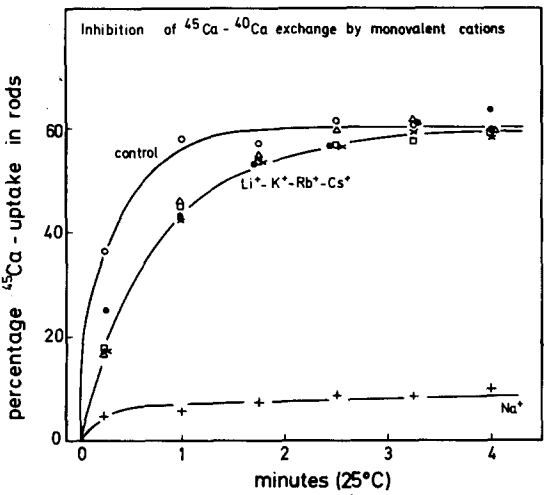

Fig. 2. Effect of monovalent cations on ^{45}Ca uptake in rods. In addition to the standard medium the indicated electrolytes are added at the start of the incubation at 25° C to a final concentration of 50 mM. No additions (○———○); LiCl (□———□); NaCl (+———+); KCl (△———△); RbCl (X———X); CsCl (●———●). The data are presented as the percent of total ^{45}Ca added. Total Ca^{2+} in the suspension amounted to 179 μM.

efflux, against external EGTA) are ineffective in this respect.

When ^{45}Ca is added to a rod suspension simultaneously with the tested cations ^{45}Ca uptake is retarded in all cases (Figs. 2 and 3). This means that the tested cations decrease the rate of ^{45}Ca-^{40}Ca exchange. In accordance with Fig. 1 the equilibrium level of ^{45}Ca uptake is not affected by those cations which do not stimulate ^{45}Ca efflux. As expected from Fig. 1, Na^+, Ca^{2+} and Sr^{2+}

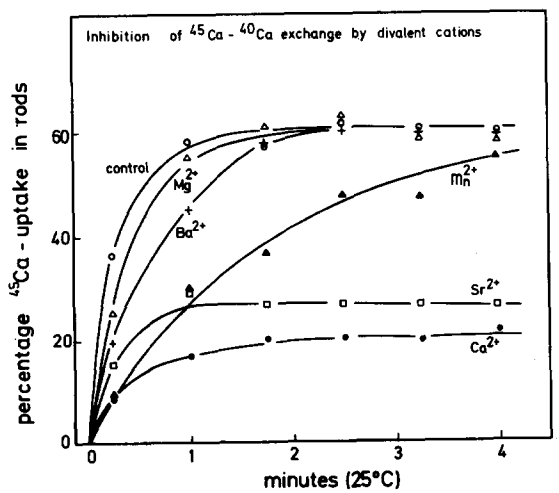

Fig. 3. Effect of divalent cations on ^{45}Ca uptake in rods. In addition to the standard medium the indicated electrolytes are added at the start of the incubation at 25° C to a final concentration of 250 μM. No additions (○———○); $MgCl_2$ (△———△); $CaCl_2$ (●———●); $SrCl_2$ (□———□); $BaCl_2$ (+———+). $MnCl_2$ (▲———▲). The data are presented as the percent of total ^{45}Ca added. Total Ca^{2+} in the suspension amounted to 179 μM.

do reduce the equilibrium level of ^{45}Ca uptake. The ^{45}Ca equilibrium levels reflect the ^{40}Ca distribution (expressed in mol ^{40}Ca/mol rhodopsin). Therefore, when Ca^{2+} and Sr^{2+} are added, the ^{45}Ca equilibrium levels observed in influx and efflux experiments such as shown in Figs. 1b and 3, respectively, differ by at most ten percent. Because of the presence of external EGTA a complete release of ^{45}Ca by Na^{+} could be expected, but does not occur (Fig. 1). This is investigated in more detail later on.

Single ion effects: divalent cations. Considering the cause of the difference observed in Fig. 1B between the two classes of divalent cations, the possibility that the selectivity of the rod binding capacity [8] determines this distinction rather than the selectivity of the rod transport system has to be investigated. Fig. 4 shows that in the presence of the added exchange carrier A23187, Mg^{2+} (and similarly Mn^{2+} and Ba^{2+}, not shown) affects the equilibrium level of ^{45}Ca uptake. The A23187 makes the binding sites which store endogenous Ca^{2+} aspecifically accessible to external divalent cations. This experiment demonstrates that the properties of the rod cation translocation system and not those of the binding capacity determine which divalent cation can promote ^{45}Ca efflux, most likely by being transported itself into rods in exchange for internal ^{45}Ca. Furthermore this experiment illustrates that the ionophore A23187 resides in both the plasma membrane and in the disk membranes, because in leaky rods Mg^{2+} does not stimulate ^{45}Ca efflux in the absence of A23187 [10].

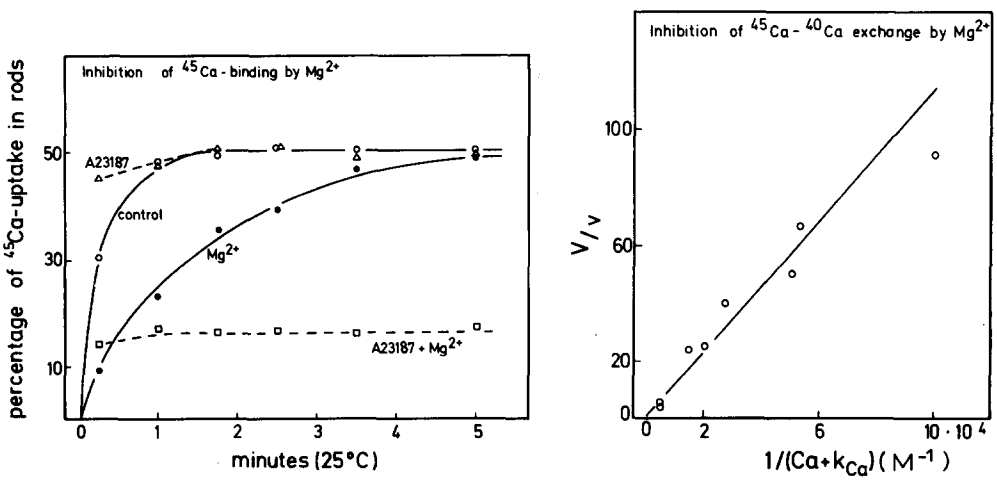

Fig. 4. Effect of Mg^{2+} on ^{45}Ca equilibration with and without the presence of A23187. In addition to the standard medium at 25°C: no additions (○———○); 500 μM $MgCl_2$ (●———●); 5 μM A23187 (△- - - - -△); 5 μM A23187 + 500 μM $MgCl_2$ (□———□). Mg^{2+} is added at the start of the incubation, A23187 is added at the start of the 10-min preincubation as an ethanolic solution (final ethanol concentration 0.5% (v/v)). The data are presented as the percent of total ^{45}Ca added. Total Ca^{2+} amounted to 37 μM, rhodopsin concentration was 12 μM. To obtain a clear picture the conditions were chosen so that ionophore addition did not change the final ^{45}Ca equilibrium level.

Fig. 5. Analysis of the inhibition of the ^{45}Ca-^{40}Ca exchange rate by Mg^{2+} in the standard medium at 25°C. The data points are obtained from kinetic plots similar as shown in Figs. 9 and 10, and are plotted according to: $V/v = 1 + (Mg^{2+} \times k_{Ca}/k_{Mg})/(Ca^{2+} + k_{Ca})$. This equation describes competitive inhibition of Ca^{2+} transport by Mg^{2+}. It is obtained by dividing the Michaelis-Menten expression for Ca^{2+} transport in the absence of Mg^{2+} (V) by that in the presence of Mg^{2+} (v). Same symbols as in Table I.

TABLE I

THE AFFINITIES OF THE EXCHANGE DIFFUSION SYSTEM FOR MULTIVALENT CATIONS

The affinities are obtained as inhibition constants of the ^{45}Ca-^{40}Ca exchange rate from plots as shown in Fig. 9 and are calculated according to the equation: $v/V = Ca^{2+}/[Ca^{2+} + k_{Ca} \times (1 + Mi^{2+}/k_{Mi})]$, where v is the observed rate of ^{45}Ca-^{40}Ca exchange, V is defined as the observed rate of ^{45}Ca-^{40}Ca exchange without additions to the rod suspension (see Materials and Methods), Ca^{2+} is the free Ca^{2+} concentration in the external medium, k_{Ca} is the affinity (dissociation constant) of the transport system for Ca^{2+} ions and a value of 1 μM is used [8], k_{Mi} is the affinity for the inhibiting cation, and Mi^{2+} is the external concentration of the inhibiting cation. In the case of $^{40}Ca^{2+}$ as inhibiting cation extra added $^{40}Ca^{2+}$ competes with Ca^{2+} already present in the suspension and results in an affinity, which is expected and found to be identical with k_{Ca}. The data of Table I refer to a rod suspension in the standard medium.

Cation	k * (μM)
Mg^{2+}	4.7 ± 0.6 (8)
$^{40}Ca^{2+}$	1.2 ± 0.2 (9)
Sr^{2+}	1.6 ± 0.2 (3)
Ba^{2+}	3.5 ± 0.2 (2)
Mn^{2+}	0.9 ± 0.2 (4)
La^{3+}	0.15 ± 0.03 (9)

* All values are means ± S.E. The number of observations is given in brackets.

Although Mg^{2+} (and similarly Mn^{2+} and Ba^{2+}) is apparently not transported by the exchange diffusion system, it does seem to compete with external Ca^{2+} for a common site. Higher Mg^{2+} concentrations give rise to a stronger reduction of the ^{45}Ca-^{40}Ca exchange rate. Fig. 5 shows an analysis of the unidirectional ^{45}Ca flux at an external Mg^{2+} concentration of 5 mM and as a function of the external Ca^{2+} concentration. A linear plot indicates competitive inhibition. Assuming competitive inhibition and using an affinity towards Ca^{2+} of 1 μM [8] the affinities (dissociation constants) of a number of tested divalent cations for the common site of the exchange diffusion system are calculated. The results are given in Table I and demonstrate that a high affinity (small dissociation constant) is not necessarily correlated to actual transport as indicated by the ability to stimulate ^{45}Ca efflux (Fig. 1B).

Single ion effects: monovalent cations. The observations in Fig. 1A could suggest that Na^+ and possibly Li^+ competes with Ca^{2+} for the common external site. Qualitatively consistent with this suggestion is the observation that reduction of the external Ca^{2+} concentration (but still $>>K_{Ca}$) in media containing Li^+ and Na^+, respectively, lowers the rate of ^{45}Ca-^{40}Ca exchange (Fig. 6) in a similar way as observed previously for the divalent cations (Fig. 5).

A different case appears to be represented by K^+, since it was previously found that a high concentration of K^+ does not reduce the (apparent) affinity of the exchange diffusion system for Ca^{2+} (see Ref. 8, Fig. 5). In agreement with those observations K^+ exposed to the rod suspension simultaneously with ^{45}Ca does not show competitive effects on the rate of ^{45}Ca-^{40}Ca exchange (Fig. 7). On the contrary, the rate of ^{45}Ca-^{40}Ca exchange is increasingly stimulated when the external Ca^{2+} concentration is lowered, and decreased when the external Ca^{2+} concentration is raised (Figs. 7 and 8). The Lineweaver-Burk presentation, shown in Fig. 8, analyses the relation between the unidirectional ^{45}Ca fluxes (as obtained from plots like the ones shown in Fig. 7) and the external free Ca^{2+} concentration. The control represents a rod suspension in the

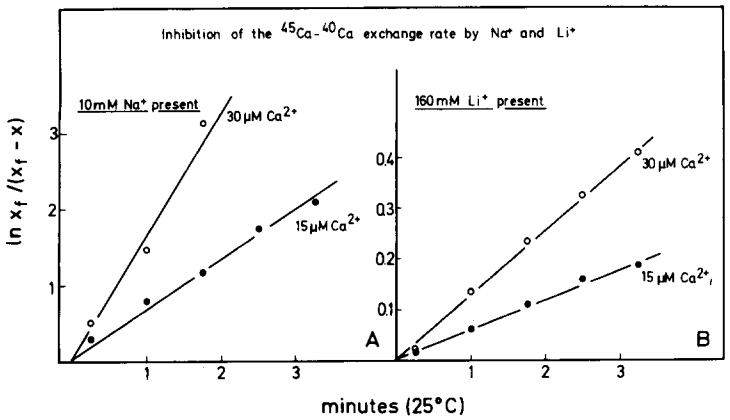

Fig. 6. Competitive effects of Na^+ and Li^+ on the ^{45}Ca-^{40}Ca exchange rate. The data are plotted according to Eqn. 1. Temperature 25°C. (A) In addition to the standard medium 10 mM NaCl and: external Ca^{2+} 30 μM (○———○); external Ca^{2+} reduced by addition of 15 μM EGTA (●———●) (see legend of Fig. 14 and the discussion of that figure). (B) 160 mM LiCl replaces 480 mM sucrose and 4% (w/v) Ficoll 400 in the standard medium. Further conditions and symbols is in (A).

standard medium (an extended plot from Ref. 8). The linear plot, obtained in this case, indicates a simple single-site saturation mechanism. No anomalies are observed, when the exchange system is either fully saturated (Fig. 8, left; external Ca^{2+} concentrations up to 300 μM as compared with an affinity for Ca^{2+} of 1 μM) or, when the exchange system is nearly completely unoccupied (Fig. 8, right; free external Ca^{2+} concentrations below 0.01 μM). Simultaneous addition of ^{45}Ca and K^+ apparently increases the affinity for Ca^{2+} from 1 μM to 0.16 μM concomitant with a reduction of the maximal rate of exchange at saturating Ca^{2+} concentrations. Upon prolonged exposure to high K^+ concentrations the original situation (i.e., similar to that in the absence of K^+) seems

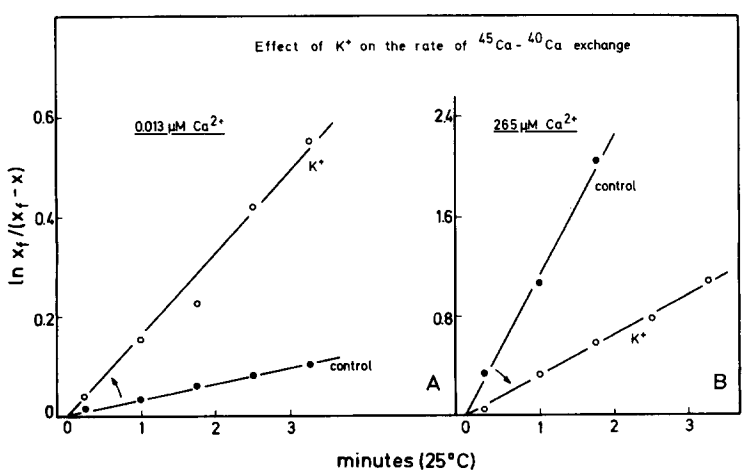

Fig. 7. Effect of K^+ on the ^{45}Ca-^{40}Ca exchange rate in the standard medium at 25°C. The data are plotted according to Eqn. 1. In addition to the standard medium: (A) Ca^{2+} = 0.013 μM; no KCl (●———●); +50 mM KCl (○———○). (B) Ca^{2+} = 265 μM; no KCl (●———●); +50 mM KCl (○———○).

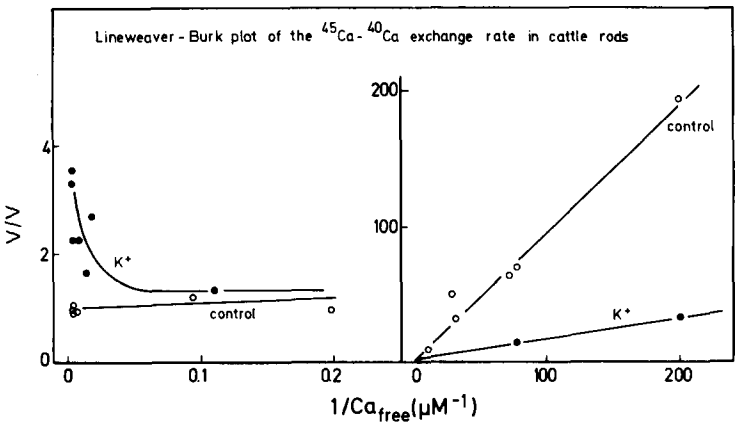

Fig. 8. Effect of K^+ on the Lineweaver-Burk plot of the unidirectional ^{45}Ca flux. Data points are obtained from kinetic plots as shown in Fig. 7 and are calculated according to Eqns. 1 and 2. The data are plotted according to the equation: $V/v = 1 + k_{Ca}/Ca^{2+}$, and the same symbols are used as in Table I. Open symbols: standard medium (data points from right-hand part of figure are taken from Ref. 8, Fig. 5). Closed symbols: in addition to the standard medium 50 mM KCl is added at the start of the incubations and simultaneously with ^{45}Ca. Temperature 25° C.

restored in both respects (see Ref. 8, Fig. 5), suggesting that the above described effects of K^+ are transient.

Effects of the combined addition of monovalent and divalent cations. The last part of the previous paragraph described that K^+ affects the exchange diffusion system of rods not in a competitive, but in a rather indirect way. Simultaneous addition of K^+ and ^{45}Ca in the presence of 250 μM external Sr^{2+} affects the rate of ^{45}Ca-^{40}Ca exchange in a way comparable to that in the presence of an additional 250 μM Ca^{2+} (Fig. 7B). In the presence of an external 250 μM Sr^{2+} the ^{45}Ca-^{40}Ca exchange rate is reduced to 23% by K^+ (four observations, S.E. = 2).

In the presence of divalent cations, incapable of stimulating ^{45}Ca efflux (Fig. 1B), K^+ has a different effect, opposed to that in the presence of the

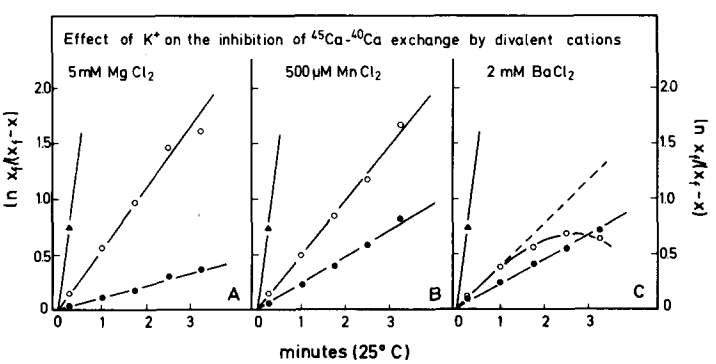

Fig. 9. Effect of K^+ on the inhibition of the ^{45}Ca-^{40}Ca exchange rate by divalent cations at 25° C. The data are plotted according to Eqn. 1. In addition to the standard medium: (A) no additions (▲——▲); 5 mM $MgCl_2$ (●——●); 5 mM $MgCl_2$ + 50 mM KCl (○——○). (B) no additions (▲——▲); 500 μM $MnCl_2$ (●——●); 500 μM $MnCl_2$ + 50 mM KCl (○——○). (C) no additions (▲——▲); 2 mM $BaCl_2$ (●——●); 2 mM $BaCl_2$ + 50 mM KCl (○——○). The external Ca^{2+} concentration amounted to 50 μM.

transportable ions Ca^{2+} and Sr^{2+}. Fig. 9 shows that K^+ increases the ^{45}Ca-^{40}Ca exchange rate in the presence of non-transportable divalent cations. This is consistent with the previous observation (Fig. 8) that K^+ increases the affinity (i.e., decreases the dissociation constant) of Ca^{2+} to the common site of the exchange diffusion system.

In a previous section it was shown that the rod cation translocation system discriminates cations into two classes, most probably representing ions transportable and non-transportable by exchange diffusion with Ca^{2+}. Fig. 10 reveals a second discriminatory criterion: K^+, Rb^+ and to a lesser extent Cs^+ all appear to change the relative affinities of Mg^{2+} and Ca^{2+} (as well as of the other divalent cations) for the common site on the exchange diffusion system, whereas Li^+, Tris (not shown) and all divalent cations are most likely ineffective.

A remarkable feature is observed in Fig. 9C. The kinetic analysis of ^{45}Ca equilibration in the presence of both K^+ and Ba^{2+} gives a linear plot only during the first minute of the incubation. The curious curvature of the plot is explained by the observations shown in Fig. 11. Whereas Ba^{2+} and K^+ separately do not stimulate ^{45}Ca efflux (see also Fig. 1), addition of both ions together results in a ^{45}Ca efflux, but at a slower rate than that observed for ^{45}Ca-^{40}Ca exchange. K^+ appears to bring about a conformational change of the exchange diffusion system, which then allows for transport of Ba^{2+}. The alternative interpretation, i.e., Ba^{2+}-stimulated K-Ca exchange is possible as well, but does not seem to be very likely.

Effects of Na^+: competition with Ca^{2+} for a common site. In a previous paragraph it was suggested (Fig. 6A) that Na^+ competitively inhibits the ^{45}Ca-^{40}Ca exchange rate. The ^{45}Ca-^{40}Ca exchange rate is inhibited by external La^{3+} [8] and external Mg^{2+} (Fig. 5). ^{45}Ca efflux, stimulated by external Na^+

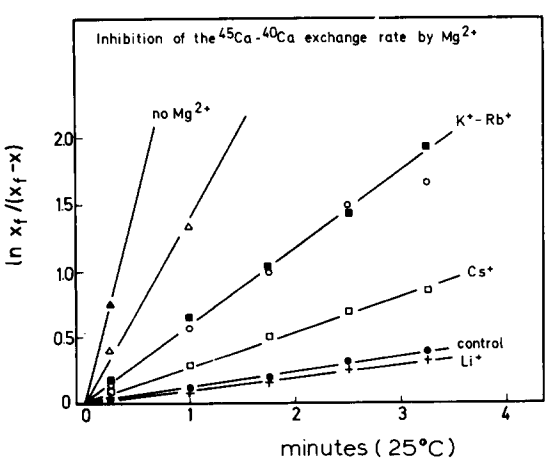

Fig. 10. Effect of monovalent cations on the inhibition of the ^{45}Ca-^{40}Ca exchange rate by Mg^{2+} at 25° C. The data are plotted according to Eqn. 1. In addition to the standard medium containing 5 mM $MgCl_2$: no additions (●———●); 50 mM LiCl (+———+); 50 mM KCl (○———○); 50 mM RbCl (■———■); 50 mM CsCl (□———□); $MgCl_2$ omitted (▲———▲); 160 mM KCl replaces 480 mM sucrose + 4% Ficoll 400 in the standard medium (△———△) and the rods are preincubated for 10 min in this KCl-medium before addition of Mg^{2+} and ^{45}Ca. The external Ca^{2+} concentration amounted to 50 μM.

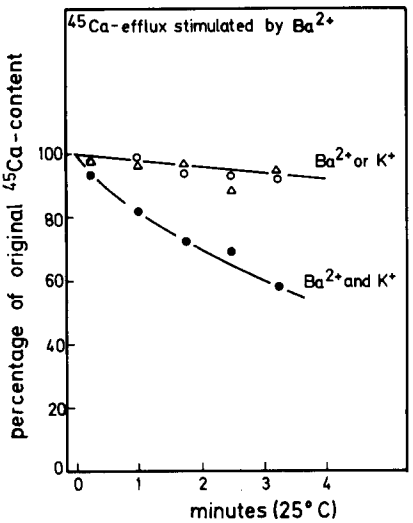

Fig. 11. Ba^{2+}-stimulated ^{45}Ca efflux from rods preloaded in the standard medium at 25°C. The efflux experiment is started by the addition of: 10 mM KCl (△———△); 5 mM $BaCl_2$ (○———○); 10 mM KCl + 5 mM $BaCl_2$ (●———●). The data are expressed as the percent with respect to the ^{45}Ca level after the previous equilibration.

(Fig. 1A), most likely represents Na-Ca exchange, by which external Na^+ and Ca^{2+} act on a common external site of the exchange diffusion system. These observations lead to the suggestion that La^{3+} and Mg^{2+} will reduce the rate of Na^+-stimulated ^{45}Ca efflux from rods, whereas addition of external EGTA is expected to increase the rate of Na^+-stimulated ^{45}Ca efflux from rods. The experiments shown in Fig. 12 confirm these expectations and are consistent with the fact that external Na^+ and Ca^{2+} compete for a common external site

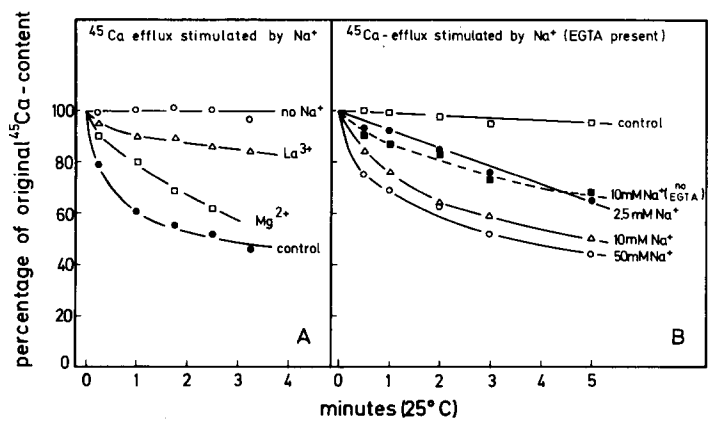

Fig. 12. Properties of Na^+-stimulated ^{45}Ca efflux from preloaded rods in the standard medium at 25°C. ^{45}Ca-efflux is started by addition of: (A) no additions (○———○); 50 mM NaCl (●———●); 50 mM NaCl + 250 μM $LaCl_3$ (△———△); 50 mM NaCl + 5 mM $MgCl_2$ (□———□). (B) 100 μM EGTA (□———□); 100 μM EGTA + 2.5 mM NaCl (●———●); 100 μM EGTA + 10 mM NaCl (△———△); 10 mM NaCl, no EGTA (■- - - - -■); 100 μM EGTA + 50 mM NaCl (○———○). The data are expressed as the percent of the ^{45}Ca level after the previous equilibration.

of the exchange diffusion system. Because of complications described in the next paragraph a precise kinetic analysis is not feasible. Tentative calculations on data as shown in Fig. 12 result in values for the affinity of Na^+ to the exchange diffusion system that range between 1 and 3 mM. For these calculations it is assumed that Na-Ca exchange is electroneutral (stoichiometry of 2 Na^+/Ca^{2+}).

Effects of Na^+: Na^+ makes endogenous Ca^{2+} inaccessible to rapid Ca-Ca exchange. It was previously found that external ^{45}Ca exchanges completely with endogenous Ca^{2+} in a process which could be described by a single rate constant [8]. A similar behaviour is also observed in this study as indicated by the linear plots in Figs. 7, 9 and 10. However, in spite of the presence of EGTA no complete ^{45}Ca efflux can be obtained with external Na^+ (Figs. 1A and 12B). After a rapid initial efflux-phase lasting approx. 1 min, ^{45}Ca efflux fades away, although no equilibrium can have been established at the common external site of the exchange diffusion system. This behaviour is investigated in detail in the experiment shown in Fig. 13. After prior equilibration with ^{45}Ca, Na^+ stimulates ^{45}Ca efflux from rods to a level which is not reached when ^{45}Ca and Na^+ are added simultaneously. A 3-min preincubation with Na^+ and subsequent addition of ^{45}Ca does not change the result (the reduction of the equilibrium level of ^{45}Ca uptake after preincubation with Na^+ as compared to that without preincubation is accounted for by the ^{45}Ca efflux during the preincubation period; Fig. 13A, broken line). These observations mean that in the presence of external Na^+ the endogenous Ca^{2+} pool is no longer homogeneous. Endogenous Ca^{2+} is now divided into two pools as opposed to the single pool observed under the Na^+-free conditions used in Figs. 7, 9 and 10 (see also Ref. 8, Figs. 4 and 6). One pool exchanges with external ^{45}Ca at a normal rate, whereas the other pool does not exchange with external ^{45}Ca at a noticeable rate within the time course of the experiment. When the rods are exposed to a medium with a

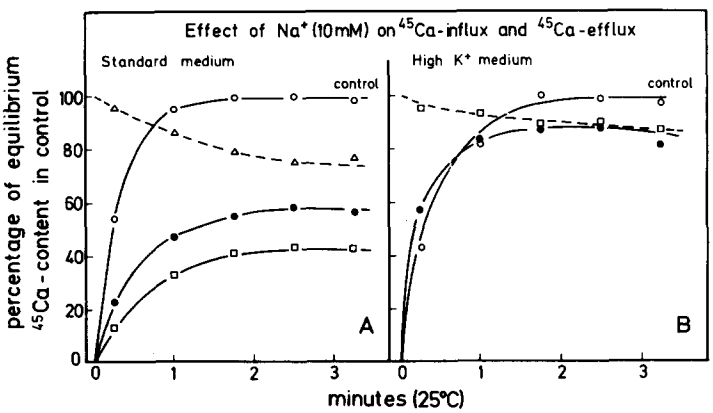

Fig. 13. Effect of Na^+ on the equilibrium ^{45}Ca-level at 25° C. In addition to the standard medium: (A) no additions (○———○); 10 mM NaCl (●———●); 10 mM NaCl added 3 min before addition of ^{45}Ca (□———□); 10 mM NaCl after previous equilibration with ^{45}Ca (△- - - - -△). (B) Same symbols as in (A) represent the same additions. 160 mM KCl replaces 480 mM sucrose + 4% Ficoll 400 in the standard medium and the rods are 10 min preincubated in this medium before the other additions start the incubations. The data are presented as the percent with respect to the ^{45}Ca level after equilibration without Na^+.

high K^+ concentration, the ^{45}Ca levels obtained in the influx and efflux experiment coincide without any indication for an inhomogeneity of the endogenous Ca^{2+} pool in spite of the presence of external Na^+ (Fig. 13B). In addition, K^+ seems to change the relative affinities of the exchange diffusion system for Ca^{2+} and Na^+, respectively. This is indicated by the reduced ^{45}Ca efflux, stimulated by Na^+ (10 mM) in the presence of K^+ (Fig. 13). This effect is consistently observed at Na^+ concentrations under 20 mM.

From the foregoing it is obvious that Na^+ and K^+ have complicated and interrelated effects on ^{45}Ca fluxes in rods. These effects become particularly prominent and unambiguous at elevated external Ca^{2+} concentrations. Under these conditions the competitive effects of Na^+ on ^{45}Ca uptake and therewith net Ca^{2+} transport are minimized. Fig. 14A shows that an increasing part of the endogenous Ca^{2+} in rods become inaccessible to rapid exchange with external ^{45}Ca when the external Na^+ concentration is gradually raised. The kinetic analysis shown in Fig. 14B demonstrates that the endogenous Ca^{2+} pool in rods, which still exchanges with external ^{45}Ca, does so with nearly the same rate, irrespective of the presence of Na^+. This indicates that under these conditions external Na^+ has only marginal competitive effects on the ^{45}Ca-^{40}Ca exchange rate.

Within the concentration ranges used, no other cation tested in this study is able to substitute for Na^+ with respect to the ability to induce inhomogeneity of the endogenous Ca^{2+} pool in rods. K^+, however, appears to act as antagonist of Na^+ and restores the accessibility of the endogenous Ca^{2+} pool to exchange with external ^{45}Ca (Fig. 14, see also Fig. 13).

In Figs. 7 and 8 it was shown that K^+ decreases the maximal rate of ^{45}Ca-^{40}Ca exchange in the presence of high external Ca^{2+} concentrations (200—

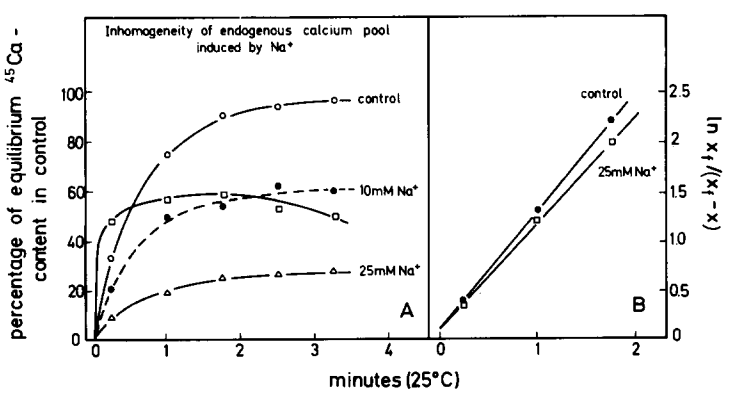

Fig. 14. Effect of Na^+ on the accessibility of endogenous Ca^{2+} to rapid exchange with external ^{45}Ca at 25° C. To a rod suspension in the standard medium external Ca^{2+} is added to a final external Ca^{2+} concentration of 250 μM. To this suspension are added: (A) no additions (○———○); 10 mM NaCl (●———●); 25 mM NaCl (△———△); 25 mM NaCl + 10 mM KCl (□———□). The data are expressed as the percent with respect to the ^{45}Ca-level after equilibration without added Na^+ or K^+. (B) Kinetic analysis of the ^{45}Ca-^{40}Ca exchange rate of exchangeable calcium with and without 25 mM NaCl. The data are plotted according to Eqn. 1 and represent the average of three determinations. In this case X_f represents the equilibrium ^{45}Ca level, which is not identical with the ^{40}Ca level, when Na^+ is present. No additions to the standard medium (●———●); 25 mM NaCl added simultaneously with ^{45}Ca at the start of the incubation (□———□).

300 μM). In Fig. 14A it is observed that the combined addition to the rod suspension of Na^+ and K^+ in the presence of a high external Ca^{2+} concentration increases the maximal rate of ^{45}Ca-^{40}Ca exchange considerably.

In the presence of external Na^+, ^{45}Ca-fluxes in rods are affected in three ways by external K^+. Firstly, the accessibility of endogenous Ca^{2+} to rapid exchange with external ^{45}Ca is restored (Fig. 14). Secondly, the maximal rate of Ca-Ca exchange is increased (Fig. 14). Finally, the relative affinities of the exchange diffusion system for Na^+ and Ca^{2+} are changed (Fig. 13, efflux curves). Li^+ and protonated Tris cannot substitute for K^+ in these effects.

Effects of Na^+: Na^+ makes endogenous Ca^{2+} inaccessible to rapid Na-Ca exchange. External Na^+ makes part of the endogenous Ca^{2+} in rods inaccessible to rapid exchange with external ^{45}Ca (previous paragraph). The observations shown in Fig. 15 indicate that external Na^+ induces a similar inaccessibility of the endogenous Ca^{2+} pool (previously marked by ^{45}Ca) to exchange with the same Na^+. At high external Na^+ concentrations the amount of endogenous Ca^{2+} accessible to rapid Na^+-stimulated Ca^{2+} efflux is increased upon addition of external K^+ in a simlar way as observed for Ca-Ca exchange in Fig. 14. At lower Na^+ concentrations (<20 mM) the change of the relative affinities, induced by K^+ and resulting in a preference for Ca^{2+} with respect to Na^+, predominates and causes a reduced rate of Na^+-stimulated Ca^{2+} efflux upon addition of K^+ (Fig. 13, efflux curves).

Osmotic experiments

An essential prerequisite for the interpretation of the effects of the various tested electrolytes on ^{45}Ca fluxes in rods is to establish which of the electro-

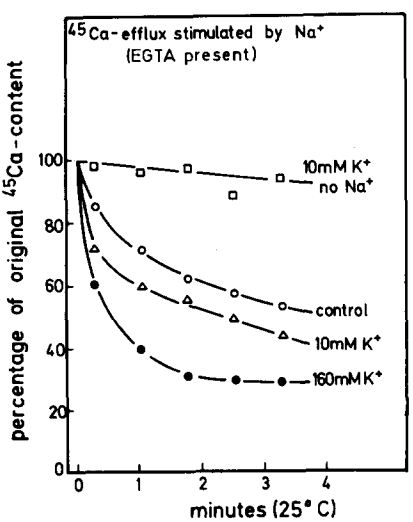

Fig. 15. Effect of Na^+ on the accessibility of endogenous Ca^{2+} to rapid Na^+-stimulated ^{45}Ca efflux at 25° C. Rods are equilibrated at 25° C in the standard medium with ^{45}Ca, or in one case in a medium, in which 160 mM KCl replaces 480 mM sucrose + 4% Ficoll 400 (●———●). The incubations are started by addition of 250 μM EGTA and: 10 mM KCl (□———□); 50 mM NaCl (○———○); 10 mM KCl + 50 mM NaCl (△———△); and to the rods in the KCl-medium: 50 mM NaCl (●———●). The data are expressed as percent with respect to the ^{45}Ca level after the previous equilibration.

lytes may permeate through the rod plasma membrane in another way than indicated by rapid exchange with endogenous Ca^{2+}. In other words: which externally applied electrolytes may accumulate to a considerable extent in the rod cytosol and therefore may act also on the disk membranes.

The turbidity of a rod suspension is used as an assay for osmotic shrinkage and swelling. With the optical geometry of the two spectrophotometers used, addition of 100 mosM (impermeable) electrolyte to a rod suspension in the 3-fold diluted standard medium results in a 10—20% increase of the apparent absorbance at 700 nm (ΔA 0.05—0.1) indicating a shrinkage of the rods. The first row of Table II shows that upon addition of permeable electrolytes like ammonium acetate or alkali cation acetates in the presence of gramicidin D the absorption changes are rapidly recovered. This result supports the validity of the method used.

The second row of Table II shows that alkali cation chlorides do not permeate (the origin of the slow recovery with NaCl is unclear). This result implies that the plasma membrane of the cattle rod preparation does not contain conductance pathways for both alkali cations and Cl^-. When the conductance barrier for the cations is removed by the addition of appropriate ionophores (gramicidin D, NH_4Cl in combination with FCCP) still no large recoveries are observed (third row in Table II). This suggests that the plasma membrane of rods is impermeable to chloride. The conductance barrier for anions

TABLE II

THE RECOVERY OF INTACT CATTLE RODS FROM A HYPERTONIC SHOCK, MONITORED BY LIGHT-SCATTERING CHANGES

A rod suspension in the standard medium is diluted immediately before use such as to yield a 3-fold reduction of the osmotic strength (final medium: 200 mM sucrose, 1.67% (w/v) Ficoll 400, 10—20 mM Tris-HCl at pH 7.4). Light-scattering is followed at room temperature 5 min preceding the hypertonic shock (increase by 100 mosM) and 5 min following the hypertonic shock. The data are presented as the percent of the initial increase of apparent absorption, when a relatively impermeable electrolyte is used. The rate of slow recovery is expressed as percent per minute and is presented as the mean ± S.E. and the number of observations is given in brackets.

Applied electrolyte (100 mosM)	Fast recovery (<1 min)	Comments
$CH_3CO_2NH_4$	100%	
CH_3CO_2Li, gramicidin D present (3 μM)	100%	
CH_3CO_2Na, gramicidin D present (3 μM)	100%	
CH_3CO_2K, gramicidin D present (3 μM)	100%	
LiCl	<10%	slow recovery: <1%/min
NaCl	<10%	slow recovery: 4.5 ± 0.5 (8)
KCl	<10%	slow recovery: <1%/min
NH_4Cl, FCCP present (5 μM)	20 ± 3 (3)	slow recovery: <1%/min
NaCl, gramicidin D present (3 μM)	23 ± 4 (3)	slow recovery: <1%/min
CH_3CO_2Na, KAc, LiAc	29 ± 3 (7)	further slow recovery
CH_3CO_2Li, FCCP present (5 μM)	55 ± 3 (3)	
CH_3CO_2Na, FCCP present (5 μM)	79 ± 9 (4)	
CH_3CO_2K, FCCP present (5 μM)	75 ± 9 (4)	
$(CH_3CO_2)_2Ca$, FCCP present (5 μM)	<10%	
$(CH_3CO_2)_2Mg$, FCCP present (5 μM)	<10%	
$(CH_3CO_2)_2Ca$, A23187 present (5 μM)	61 ± 7 (5)	slow increase of absorbance

can be removed by the use of acetates in combination with FCCP. Acetic acid permeates and the protons go back to the external medium via FCCP if the cation concerned permeates, resulting in a net transport of the cation acetate. The fourth row of Table II shows that a substantial to complete recovery is observed when alkali cation acetates are used in combination with FCCP, but not for earth alkali cations. In the latter case a recovery for calcium acetate is observed when in combination with the use of acetates the exchange carrier A23187 is included. In this case acetic acid permeates and the protons return to the external solution in A23187-mediated exchange for external divalent cations, resulting in net transport of calcium acetate across the plasma membrane.

Reproducibility and observations on stable leaky rods

During the course of the work, presented in this study, 43 intact rod preparations were used and 5 preparations of stable leaky rods. All effects shown in the figures of this report proved to be qualitatively almost always reproducible. Quantitative agreement is within a factor of two to three. Remarkable observations are obtained with the stable leaky rods. Qualitatively these leaky rods behave exactly similar to the intact rods when ^{45}Ca metabolism is concerned. Experiments similar to those shown in Figs. 9A, 11 and 14 yield identical pictures from simultaneously prepared leaky and intact rods.

In 2 of the 43 intact rod preparations anomalous behaviour was observed. Endogenous Ca^{2+} did not or very slowly exchanged with external ^{45}Ca ($t_{1/2} >$ 10 min as compared with the normal 12 s). Analogous to Fig. 14, external K^+ restores exchange, i.e., relieves the inaccessibility of the endogenous Ca^{2+} pool in these preparations. This effect is again specific for K^+. Neither Na^+, nor Ca^{2+} nor Mg^{2+} can substitute for K^+.

Discussion

Experiments on the electrical behaviour of the vertebrate retina have been until now the major source of information about the ion fluxes underlying excitation of vertebrate rod photoreceptor cells. Measurements by intracellular [4] and extracellular recording techniques [2,12] have indicated the presence of a cation translocation system in the rod outer segment plasma membrane, which is able to discriminate Na^+ from all other cations tested, and which can carry a net current of Na^+. Ca^{2+}, but not Mg^{2+}, can inhibit this Na^+ current [1,4], but cannot replace Na^+ as charge carrier. In addition, the rod outer segment plasma membrane presumably contains a conductance pathway for K^+ [1,5] and may contain voltage-dependent conductance pathways [13,14]. As compared to the permeability for Na^+ the permeability for Cl^- appears to be low [4,15]. To extend this information to the molecular basis of these electrical phenomena direct measurements of ion fluxes with well-defined rod outer segment preparations may be useful.

Following the strategy outlined in two previous communications [7,8], this study intends to give a general survey of the ion selectivity of the translocation system(s) present in the plasma membrane of isolated cattle rods rather than to provide a fully detailed picture. Furthermore, the necessity to impose rather

unphysiological conditions on isolated rod outer segments during purification and storage, requires caution. On the other hand, qualitative reproducibility and quantitative homogeneity of the rod preparations used strengthen confidence in the results obtained and do not indicate a significant contribution of contaminating material.

The two types of experiments described in this study appear to provide complementary information about the ion fluxes through the rod plasma membrane of the intact cattle rods used. The osmotic experiments reveal electrogenic uniport or electroneutral symport, whereas the ^{45}Ca experiments describe electroneutral antiport.

Osmotic experiments

The control experiments with the intact rods and the permeable electrolytes (first row in Table II) support the validity of the osmotic technique used. This implies that the rod cytosol is the osmotically active compartment observed. The plasma membrane of intact rods is found to be relatively permeable to net transport of Li^+, Na^+ and K^+, and relatively impermeable to net transport of Cl^-, Mg^{2+} and Ca^{2+}. Only in the presence of an external divalent cation carrier (A23187) does net Ca^{2+} transport occur in agreement with the ^{45}Ca experiments described before [8]. As a consequence of the relative impermeability of the rod plasma membrane to Cl^- as compared to the permeability to Li^+, Na^+ and K^+, chloride salts of the latter cations do not permeate through the plasma membrane to an appreciable extent, but may give rise to diffusion potentials across the plasma membrane *. The impermeability of the rod plasma membrane to chloride salts due to the absence of a Cl^- conductance pathway is in agreement with electrophysiological observations [4,15] and with observations on isolated frog rod outer segments by some authors [16,17]. Others however do report that isolated frog rod outer segments are permeable to Cl^-, Na^+ and Li^+, but not to K^+ [18—20]. The reason for this discrepancy is unclear.

^{45}Ca fluxes: interpretation of transport mode

An essential conclusion to be drawn from the osmotic experiments with cattle rods is that all the electrolytes (chloride salts) used in the ^{45}Ca experiments do not permeate to any extent through the plasma membrane during the incubation times used and are only exposed to the external side of the plasma membrane. Therefore, they may interfere with ^{45}Ca fluxes in intact rods only by acting on the exchange diffusion system in the plasma membrane either directly (competition for an external site) or indirectly by establishing a diffusion potential across the plasma membrane. No substantial amount of the externally applied chloride salts can accumulate inside rods otherwise than indicated by stimulation of ^{45}Ca efflux by exchange diffusion. Therefore, stimulation of rapid ^{45}Ca efflux inevitably reflects electroneutral exchange if

* At an external Na^+ concentration of 50 mM, net transport of $8 \cdot 10^5$ positive charges through the plasma membrane of a cattle rod would generate a diffusion potential of 160 mV (inside positive) concomitant with an increase of the rod cytosol Na^+ concentration of 0.08 mM. Dimensions of 1 × 20 μM and a volume of 16 fl are used for a cattle rod outer segment, and a membrane capacitance of 1 $\mu F/cm^2$ is assumed. A complete recovery from a hypertonic shock with 50 mM NaCl would require an influx of 10^9 particles (Na^+ or Cl^-) into the rod cytosol.

no other charge carriers are available (e.g. cytosol K^+) and in the absence of ion pumps [8]. In summary, it is concluded that the experiments shown in Figs. 1, 11, 12 and 15 represent, at least for the greater part, electroneutral Ca-Ca, Ca-Sr, Ca-Ba and Ca-2Na exchange. This conclusion has been explicitly confirmed previously [8] for the case of Ca-Ca exchange.

Properties of the exchange diffusion system in the rod plasma membrane

An analysis of the ^{45}Ca fluxes in intact isolated cattle rods reveals that the exchange diffusion system present in the rod plasma membrane contains at least four distinct functions:

1. A low-selectivity external binding site, which determines the affinity towards transport, but not the actual transport rate. The selectivity order is:

$La^{3+} > Mn^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+} > Mg^{2+}$ and $Na^+ > Li^+ > Tris^+$; $K^+ \approx Rb^+ \approx Cs^+ \approx 0$

This common site determines competitive inhibition of the ^{45}Ca-^{40}Ca exchange rate as shown in the Figs. 3, 5, 6, 9, 10 and 12. Further evidence that the low-selectivity site is an independent entity of the exchange diffusion system comes from the La^{3+} effects described before (see Ref. 8, Table IV). Apparently, this site can be disconnected from the actual transport function, in view of the loss of inhibitory effects by La^{3+} without a parallel loss of exchange transport.

2. The conformational state of the exchange diffusion system, which results from the binding of cations to the low-selectivity site subsequently discriminates with high selectivity which cation may actually be transported. There is clearly no correlation between the affinity for the common binding site (Table I) and actual transport (Figs. 1 and 11). Ca^{2+}, Sr^{2+} and Na^+ are transported at approximately the same maximal rate. Ba^{2+} and (possibly) Li^+ may be transported under certain conditions (Figs. 1—3 and 11), but at a lower rate, whereas for the other tested cations the actual transport rate appears to be practically zero.

3. The observations shown in Figs. 13—15 indicate a third function with a unique selectivity for Na^+. This function operates as an on-off switch for the exchange diffusion transport mode. Any model based on the reversible binding of Na^+ to a certain site which then operates as a switch would result in a stochastic closure and opening of all transport entities during the incubation time and consequently in a reduced, but uniform, transport rate. This is evidently not the case.

4. A fourth function, like the third not of competitive nature, is revealed by the observations shown in Fig. 10 and has a complementary selectivity as compared to the low-selectivity site. Fig. 10 and its discussion demonstrate that only K^+, Rb^+ and Cs^+, which appeared to have no affinity towards the common low-selectivity site, may modulate the exchange diffusion system. This has been extensively investigated only for K^+, but it is assumed that Rb^+ and Cs^+ behave similarly (as shown in Fig. 10). All effects of external K^+ are presumably due to this function, which is rather versatile. Firstly, the Lineweaver-Burk plot (Fig. 8) suggests that K^+ alters the absolute affinity of the exchange diffusion system for Ca^{2+}. Therefore the results shown in Figs. 9 and 13 (efflux curves) are consistent with the fact that the relative affinities for all cations are changed. Secondly, external K^+ may control the maximal transport rate of

Ca-Ca exchange (Figs. 7, 8 and 14) and the range of ions, which are transported (Fig. 11). Finally, external K^+ acts as antagonist of Na^+ with respect to the operation of the third function. The fourth function may indicate the existence of (a) K^+-sensitive site(s) on the exchange diffusion system. Alternatively, K^+ may be expected to establish a diffusion potential. This would mean that the first free functions described here are all dependent on the membrane potential.

In none of the experiments, described in this study, external energy sources are added. Thus, a direct and causal relation between hydrolysis of ATP and all the ^{45}Ca fluxes and transport functions described in this study can be excluded by an identical line of reasoning as used before [8]. Intact cattle rods, however, appear to contain sufficient high-energy phosphates [7] and a more indirect involvement seems possible, but proves difficult to substantiate (Schnetkamp, P.P.M., unpublished material).

Comparison of the rod exchange diffusion system with other exchange systems

Cation exchange systems, which are selective for i.a. Na^+ and Ca^{2+} are described for a number of tissues (for a review, see Ref. 21). Similar properties as described here for the first two functions of the rod exchange diffusion system are found for the Na-Ca exchange system, present in giant squid nerve axons [22,23]. Also the Ca-Ca exchange system, described for rat heart mitochondria [24,25], has similar properties as the rod exchange diffusion system. However, in the latter case a different ion selectivity has to be noted. Rat heart mitochondria do not discriminate between Li^+ and Na^+ and have a 10-fold lower affinity towards Ca^{2+} [25]. Furthermore, the mitochondrial Ca^{2+} pool is sustained by energy-requiring processes [25], whereas the rod Ca^{2+} pool is stored by binding [8].

Comparison of the exchange diffusion system with electrophysiological data

Although care should be taken to extrapolate data obtained from isolated cell organelles to the in vivo situation, the exchange diffusion system, present in the plasma membrane of isolated intact rods and described in this and the previous study [8], has a number of properties in common with the system responsible for the dark current of Na^+ in the vertebrate retina.

1. The exchange diffusion system resides in the plasma membrane of isolated cattle rods and responds to a disturbance of the external ionic conditions (in particular the Ca^{2+}/Na^+ ratio) by changing the intracellular concentrations of the respective ions with half-times in the order of 10—60 s (Figs. 1, 12—15). Similarly, the intact rod photoreceptor cell in the retina responds rapidly to changes in the external medium and inhibition of the ionic battery [1], which are interpreted by concomitant changes of the intracellular concentrations of the respective ions (notably Na^+ and Ca^{2+}, see Refs. 1 and 4).

2. Rod photoreceptor cells in the retina respond electrically quite different to external Li^+, Na^+ or K^+. At a normal external Ca^{2+} concentration (1.36 mM) Li^+ cannot replace Na^+ as charge carrier in the dark current. The hyperpolarization of the rod photoreceptor cell upon substitution of Li^+ for Na^+ indicates that the permeability of the rod plasma membrane for Li^+ is much lower than that for Na^+ and K^+ [2,4,12]. Variation of the external K^+ concentration

modulates both the dark membrane potential and the photoreceptor potential [4,12]. The exchange diffusion system of isolated rods also discriminates sharply between external Li^+, Na^+ and K^+. Na^+ is efficiently transported, Li^+ is mainly inert and K^+ may establish a diffusion potential, but in any case exerts strong effects not of competitive nature (Figs. 1, 8, 10 and 14).

3. In the retina, variation of the external Na^+ and Ca^{2+} concentration modulates the dark current, the membrane potential and the photoreceptor potential [1,2,4,6] in a way that can be understood in terms of competition between Na^+ and Ca^{2+} for a common site. Upon reduction of the external Ca^{2+} concentration a dark current can be measured with external Na^+ concentrations as low as 1 mM [2]. Likewise, Ca^{2+} and Na^+ appear to compete for a common site on the exchange diffusion system of isolated cattle rods (Figs. 6 and 12). At low external Ca^{2+} concentrations an affinity of the exchange diffusion system of 1—3 mM to Na^+ enables Na^+ transport at low Na^+ concentrations (Fig. 12).

4. The affinity of Ca^{2+} towards the system, responsible for the dark current of Na^+ in the retina has been calculated to be 1 μM [3]. The affinity of the exchange diffusion system of isolated cattle rods is found to be adjustable between 0.16 and 1 μM (Fig. 8).

5. The plasma membrane of isolated cattle rods is found to be able to carry a net current of Na^+, but not of Ca^{2+} (Table II). The dark current of Na^+ ions in the retina is found to be inhibited by Ca^{2+}, but Ca^{2+} cannot replace Na^+ as charge carrier [1,2,4,6].

6. The dark current of Na^+ in rods in the rat retina is measured to amount 20—70 pA/rod or $1-3 \cdot 10^{14}$ charges/cm^2 per s [2]. The maximal Ca-Ca exchange rate observed for isolated cattle rods in this study (conditions as in Fig. 14 in the presence of both Na^+ and K^+) amounts to $0.1 \cdot 10^{14}$ (Ca-Ca)/cm^2 per s or $0.4 \cdot 10^{14}$ charges/cm^2 per s.

7. In the absence of ion pumps in the outer segment [8,26] Na-Ca exchange across the plasma membrane, driven by a Na^+ gradient, would be sufficient for Ca^{2+} homeostasis in the rod cytosol (cf. Ref. 27 *).

One essential piece of information is obviously missing in this series. It is not shown that net Na^+ transport through the plasma membrane of isolated intact cattle rods is performed by the same system, which is responsible for exchange diffusion transport. Furthermore, in contrast with ^{45}Ca experiments the osmotic responses of rods do not discriminate qualitatively between Li^+, Na^+ and K^+, when acetates are used in combination with FCCP (i.e., under a sort of voltage clamp). A further discussion on these points has to await data on the diffusion potentials, established by chloride salts of the monovalent cations in the isolated intact rods, and on the kinetics of the recovery from hypertonic shocks by alkali cation acetates in the presence of FCCP. The minor effects of chloride salts of Li^+ and Tris on ^{45}Ca fluxes as opposed to the effects of KCl, seem to confirm the suggestion that the plasma membrane in isolated cattle rods is impermeable to Cl^-.

* A driving potential across the plasma membrane of 60 mV is required for Na^+ to maintain a cytosol Ca^{2+} concentration of 1 μM at an external Ca^{2+} concentration of 1.36 mM, when the exchange system operates with a stoichiometry of 3 Na/1 Ca.

Evidence for a direct communication between disks and plasma membrane

At this stage it is interesting to recall the surprising observations described previously [8]. Rods with an intact plasma membrane equilibrate external ^{45}Ca with the endogenous Ca^{2+} pool as fast as do rods with a leaky plasma membrane, although in the former case all ^{45}Ca first has to pass the plasma membrane (the plasma membrane makes up only a few percent of the total disk membrane surface area). The observations, shown in Figs. 1 and 11—15, demonstrate that endogenous Ca^{2+}, which normally behaves as a homogeneous pool and which is for the greater part localized within disks [8], can be mobilized (Figs. 1, 11, 12, 15) or affected (Figs. 13, 14) equally rapidly as ^{45}Ca equilibration and without a noticeable delay. However, these effects are caused by ions, which were shown in a previous paragraph to act only on the plasma membrane. The implications of these observations are best illustrated by the experiment shown in Fig. 14. In this experiment it is observed, that a gradual increase of the external concentration of Na^+ excludes an increasing amount of intradiskal Ca^{2+} from rapid exchange with external ^{45}Ca. This means that either a number of disks become inaccessible to rapid exchange with external ^{45}Ca, or that individual rods as a whole become inaccessible to rapid exchange with external ^{45}Ca. The latter possibility seems difficult to reconcile with the homogeneity displayed by the rod preparation with respect to other properties of ^{45}Ca-metabolism (i.e., the single rate constants observed in Figs. 7, 9 and 10; see also Ref. 8). The former alternative implies that internal Ca^{2+} is at the least 70% localized (compare in Fig. 14 ^{45}Ca uptake in the control with that in the presence of 25 mM Na^+) in discrete intracellular compartments. The latter are most probably identical with the disks. Thus, external Na^+ acts on a transport system, which has access to the extracellular space and in the presence of external Na^+ discriminates between Ca^{2+} localized in different disks. This can be understood if a separate system for every disk performs the observed exchange diffusion transport directly between the extracellular space and the intradiskal space. This would imply that single transport units form a material connection between the plasma membrane and those disks, which are accessible to rapid exchange diffusion. This conclusion explains the findings that stabilized rods with a leaky plasma membrane behave similarly to intact rods as far as ^{45}Ca metabolism is concerned. The 'Na^+- and Ca^{2+}-selective exchange carrier' localized in the disk membranes in a previous study [10] can also be attributed to the presence of a leaky plasma membrane (see discussion in Ref. 10).

Alternative explanations of the above-mentioned observations (notably those in Figs. 11 and 14) require the assumption that endogenous Ca^{2+} is predominantly localized in the rod cytosol and bound to the cytoplasmic side of the disk membranes. Furthermore the observations in Fig. 14 imply that endogenous Ca^{2+} can become so tightly bound that it is inexchangeable, even on a minute time-scale. These stipulations are incompatible with the existing experimental evidence: Ca^{2+} in intact, leaky and lysed rods is inaccessible to chelation by external EGTA, but can be chelated rapidly and completely by EGTA upon addition of the ionophore A23187 [8,10]. This demonstrates that in the rod preparations used in this and the previous studies [8,10] no binding sites exist, which can compete with EGTA for Ca^{2+} and which allow residence times of

bound Ca^{2+} larger than the subsecond range.

Not inconsistent with a material connection between the disk membranes and the plasma membrane are electron microscopic studies on the osmotic behaviour of rod outer segments in the intact retina [28,29]. In an electron microscopic study on isolated cattle rods a picture is shown of osmotically shocked rods, which give the impression that the disks are kept together by the plasma membrane (Ref. 30, Fig. 1). This observation is consistent with the observed resistance of the intact rods to osmotic lysis [8].

In the case of an ion transport system, which connects the disk and the plasma membrane, an electrical coupling resistance between disks and plasma membrane, which according to Penn and Hagins [31] is still permitted by the electrical properties of the plasma membrane, should exceed $2 \cdot 10^{11}$ ohm. Assuming that the driving potential for the dark current is 50 mV, that the intensity of the dark current amounts to 50 pA and that a rod contains 500 disks [2,31] a value of $5 \cdot 10^{11}$ ohm for each connection is calculated. Therefore, a single connection between each disk and the plasma membrane in the form of a cation transport system with the capacity of the dark current is not incompatible with the electrical properties of the rod plasma membrane.

An alternative to the concept of a diffusable transmitter in the rod cytosol

The arguments put forward in the previous paragraph appear to suggest that individual disks can be connected to the rod plasma membrane by a cation transport system, which is selective to Ca^{2+} and Na^+. The concomitant state of the transport system performs exchange diffusion transport and can be reversibly turned off and on by external Na^+ and K^+, respectively (Fig. 14). In the state, which does not perform exchange diffusion transport the intradiskal Ca^{2+} pool appears isolated from the external Ca^{2+} pool and possibly also from the cytosolic Ca^{2+} pool (the latter is suggested by the strongly reduced rate of ^{45}Ca uptake after lysis of intact rods, cf. Ref. 8). If this were true the suggestion is obvious that the 'off'-state of exchange diffusion transport represents the 'on'-state of (electrogenic) transport between the cytosol and the external medium. An intriguing property of the mechanism regulating the ratio of the respective states is the non-stochastic nature mentioned in a previous paragraph. The data of Fig. 14 indicate the presence of a titratable transition between the two states in dependence of the external Na^+ concentration. In the presence of a moderate concentration of Na^+ both states are populated, but individual transport entities do not statistically fluctuate between the two states (the latter would result in a slower, but complete ^{45}Ca equilibration). This can be understood if individual transport entities would act interdependently. This means, that the information about the (change of) state of an individual transport unit is intercommunicated by a certain number of transport units.

Continuing this line of reasoning the notion arises that the process of visual transduction does not need to be mediated by a diffusable transmitter in the rod cytosol. Bleaching of a rhodopsin molecule could be communicated within the disk membrane to the transport unit residing in that individual disk. The interdependence of individual transport units subsequently enables a transfer of this information to a certain number of other transport units. This would result

in a local spread of a quantal event in the length axis of a rod outer segment. Not inconsistent with such a scheme are observations by Hagins et al. [32] and by Jagger [33,34] that local illumination of a rod outer segment results in a response, which is confined to a limited spread from the illuminated zone. This means, that an intracellular transmitter does not diffuse freely in the length axis of a rod outer segment within the time course of a photoresponse. Therefore, the spread of a transmitter by diffusion in the length axis of a rod is expected to be a major constituent in the time course of a photoresponse. On the basis of kinetic (uniform responses to quantal events) and thermodynamic (heat of activation of photoresponses) arguments Baylor et al. [35,36] have reached the conclusion that diffusion of an intracellular transmitter is not the principal rate-limiting step in the generation of a photoresponse in both rod and cone photoreceptors. Combined, these observations seem difficult to reconcile with the concept of a diffusable transmitter in the cytosol communicating between the bleaching of a rhodopsin molecule in the disk membrane and the subsequent conductance change of the rod plasma membrane. This argument is enforced by the fact that a steady current of Na^+, which is assumed to flow through the outer segment, does not allow diffusion barriers in the length axis of an outer segment, which would effectively divide the cytosol in subcompartments.

Concluding remarks

Intact isolated cattle rods contain a cation selective transport system, which resides in the plasma membrane and has a number of properties in common with the system responsible for the dark current of Na^+ in the vertebrate retina. It combines a high ion selectivity (e.g., sharp discrimination between Li^+, Na^+ and K^+) with a low heat of activation (Q_{10} = 1.08; Ref. 8) and a flux, which is sufficient to turnover the complete Na^+ and Ca^{2+} content of a rod outer segment within 1 min. The system contains different functions with distinctive ion selectivity ranges, which appear much narrower than those of the common Na^+ and K^+ channels (for reviews on the latter, see Refs. 37—39). The transport system exists in two states, whose populations are controlled by a mechanism, which infers interdependence of individual transport entities. One state performs exchange diffusion transport directly between the intradiskal space and the external medium and appears to be remarkably leakproof (net transport of Ca^{2+} three orders of magnitude slower than exchange transport, see Ref. 8).

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