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CALCIUM IN DARK-ADAPTED TOAD RODS: EVIDENCE FOR POOLING AND CYCLIC-GUANOSINE-3′-5′-MONOPHOSPHATE-DEPENDENT RELEASE

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SUMMARY

1. We have used laser micromass analysis (l.a.m.m.a.) to investigate Ca uptake and release in intact 'red' rod photoreceptors in the dark-adapted retina of the toad, *Bufo marinus*.

2. With l.a.m.m.a. it is possible to measure separately the concentrations of each of the Ca isotopes. Rods normally containing almost exclusively ^{40}Ca can be incubated in Ringer solution containing the stable isotopes ^{42}Ca or ^{44}Ca. In this way, the movements of Ca into and out of the rod can be separately determined.

3. When rods are incubated in darkness in high ^{44}Ca (up to 20 mM), large amounts of ^{44}Ca accumulate in the outer segment at a rate which increases with increasing external ^{44}Ca concentration. However, this ^{44}Ca appears not to exchange with the ^{40}Ca originally present within the rod. This result suggests that the ^{40}Ca may be sequestered within a pool which normally exchanges slowly with external Ca.

4. We explored Ca exchange in high-Ca solutions in more detail with double-isotope labelling. In these experiments, rods were first pre-loaded with Ca of one isotope (^{42}Ca) and then incubated in Ringer solution containing another (^{44}Ca). We could then measure separately the rate of exchange of the pre-loaded ^{42}Ca with the ^{44}Ca in the Ringer solution and with the ^{40}Ca originally present within the rod in the sequestered pool.

5. These experiments show that the pre-loaded-Ca exchanges rapidly with Ca in the Ringer solution, at least in part by Ca–Ca exchange, but much more slowly with the Ca originally present within the rod. Thus Ca in the outer segments can exist in (at least) two pools: one which exchanges rapidly across the plasma membrane and is probably Ca free or loosely bound within the cytosol, and another which exchanges slowly and is probably Ca within the disks.

6. Although Ca sequestered within the outer segment normally exchanges quite slowly, it can be rapidly released if the extracellular free Ca is buffered to low levels with EGTA. The rate-limiting step for Ca release under these conditions appears not

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to be Na–Ca exchange, since the rate of Ca efflux is unchanged if the Na in the Ringer solution is substituted with choline.

7. Ca can also be released from the sequestered pool if rods are incubated in Ringer solution containing 100 or 500 μM-IBMX (3-isobutyl-1-methylxanthine). We speculate that the mechanism of release in both low-free-Ca solutions and IBMX solution involves the opening of cyclic-GMP-dependent channels in the disk membrane.

8. Rods incubated in IBMX show a dramatic increase in the rate of accumulation of Ca from the external medium. This is probably a direct result of the increase in the light-dependent conductance of the outer-segment plasma membrane in IBMX.

9. Under the assumption that the sequestered pool of Ca is within the disks, the rate of exchange of Ca across the disk membrane in darkness in physiological Ringer solution can be calculated to be no greater than 10^8 Ca cm^{-2} s^{-1} or about 100 Ca $disk^{-1}$ s^{-1}. This is more than three orders of magnitude smaller than the rate of Ca movement in darkness per square centimetre across the plasma membrane.

INTRODUCTION

The absorption of light by rhodopsin in a vertebrate rod produces a series of events which lead to the reduction of the conductance of the rod outer-segment membrane. Considerable progress has been made in understanding the mechanism of this process (see Chabre & Applebury, 1986; Yau, Haynes & Nakatani, 1986). The bleaching of rhodopsin is known to trigger its binding with a G protein (Kühn, 1980). The subsequent activation of the G protein then stimulates a cyclic guanosine 3′-5′ monophosphate (GMP) phosphodiesterase (Wheeler & Bitensky, 1977), which is presumed to cause a decrease in the free concentration of cyclic GMP (but see Goldberg, Ames, Gander & Walseth, 1983). Cyclic GMP has been shown to modulate a conductance both in excised patches of rod outer-segment plasma membrane (Fesenko, Kolesnikov & Lyubarsky, 1985) and in truncated-rod outer segments (Yau & Nakatani, 1985*b*), and this conductance appears to be the same as the one decreased by light (see Yau *et al.* 1986).

These experiments strongly support the hypothesis that the excitatory messenger or 'internal transmitter' of visual excitation is cyclic GMP. However, in addition to cyclic GMP, rod outer segments also contain large quantities of another putative messenger substance, Ca (Hagins & Yoshikami, 1975; Schnetkamp, 1979, 1980, 1986; but see Somlyo & Walz, 1985). We have shown that rods in the intact retina contain 4–5 mmol Ca/l tissue volume, or 1–2 Ca for each rhodopsin molecule in the outer segment (Schröder & Fain, 1984; Fain & Schröder, 1985). This result has been recently confirmed using a different method, for intact rods isolated in normal Ringer solution from frog retina (Nicol, Kaupp & Bownds, 1986). The great majority of the Ca in the rod exchanges very slowly with extracellular Ca (Fain & Schröder, 1985), probably because it is sequestered within the outer segment in the disks (Schnetkamp, 1979; Fain & Schröder, 1985). It can, however, be released from the rods by light with a gain as large as 10^4 Ca per bleached pigment molecule (Schröder & Fain, 1984). At present, the function of this Ca and its light-dependent release are unknown.

In an attempt to understand the role of Ca in the physiology of vertebrate

photoreceptors, we have used a recently developed method of microanalysis, called laser micromass analysis (or l.a.m.m.a.) in order to investigate the Ca economy of rods. With l.a.m.m.a., a small (4–5 μm diameter) area from a thin section of fast-frozen tissue can be vaporized and ionized with a sharply focused, high-power laser. Since detection with l.a.m.m.a. is based on mass spectroscopy, it is possible to identify and separately measure the signals of each of the isotopes of an element. This makes it feasible to use stable isotopes to study the movement of Ca into and out of the rod and between pools within the outer segment which exchange at different rates. Furthermore, all of these measurements can be made on rods within the whole retina, since it is not necessary to remove the outer segments for chemical analysis.

In this paper, we describe some features of Ca exchange and Ca pooling in toad rod outer segments in darkness. We show that, though most of the outer-segment Ca exchanges slowly with extracellular Ca, it is possible to accumulate large amounts of Ca within the outer segment in a pool which exchanges much more rapidly. We also show that the Ca which is sequestered can be released by treating the retina with Ringer solution containing either a low-free-Ca concentration or the cyclic GMP phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX).

Some of these experiments have been previously presented at the annual meetings of the American Biophysical Society (Schröder & Fain, 1986*a*) and the Association for Research in Vision and Ophthalmology (Schröder & Fain, 1986*b*).

METHODS

A detailed description of the methods used in this paper for isolation of the retina, processing of the tissue and l.a.m.m.a. analysis is given in Fain & Schröder (1985). In brief, dark-adapted toads were pithed and their retinas removed in the dark using an infra-red converter (Find-R-Scope, F.J.W. Industries, Mount Prospect, IL, U.S.A.) and a stereo microscope equipped with infra-red viewing. Retinas were mounted on a Millipore filter with the photoreceptors pointing upward. After isolation in this manner, retinas could be shown to have normal light responses, as monitored by the measurement of their electroretinogram. The retina together with its supporting filter was cut into eight wedge-shaped samples (as for a pie), and one piece was immediately shock-frozen and processed as a control. The other pieces were attached with insect pins to a 30 mm diameter disk of dental wax and were immersed in oxygenated Ringer solution.

The composition of the solutions used in these experiments is given in Table 1. Sodium methylsulphonate ($NaCH_3SO_3$) and potassium methylsulphonate (KCH_3SO_3) were made by titrating methane sulphonic acid to neutral pH with NaOH or KOH. Solution pH was adjusted to 7·8 by adding NaOH or (in the case of solution K) LiOH. The concentration of $NaCH_3SO_3$ in solutions I and J and of choline chloride in solution K was reduced by comparison to the NaCl concentration in normal Ringer solution (solution A) so that the osmolarity of these solutions remained the same as that of normal Ringer solution (Bastian & Fain, 1982). The concentration of cations was not diminished, however, since progressively more NaOH (or in the case of solution K, LiOH) had to be added to the EGTA-containing solutions to bring the pH to 7·8.

Stable-isotope labelling

Labelling was performed by replacing all the $CaCl_2$ in the Ringer solution (which contained approximately 97 % ^{40}Ca) by $CaCl_2$ containing predominantly the stable isotopes ^{42}Ca or ^{44}Ca. Stock solutions of labelled $CaCl_2$ were prepared by dissolving crystals of $^{42}CaCO_3$ or $^{44}CaCO_3$ (Rohstoffeinfuhr, Dusseldorf, F.R.G.) in HCl. The Ca labels we used were not pure: that is, the ^{42}Ca contained some ^{40}Ca and some ^{44}Ca, and similarly for the ^{44}Ca label. The composition of the labels was determined in the following way. Microdroplets of an aerosol produced from 20 μl of a stock solution of the label to be tested were sprayed onto a carbon support film. The isotope composition of the solution was then measured with l.a.m.m.a.

TABLE 1. Composition of solutions (in mM)

Solution	NaCl	$NaCH_3SO_3$	Choline chloride	$NaHCO_3$	KCl	KCH_3SO_3	$KHCO_3$	$MgCl_2$	$MgSO_4$	$^{40}CaCl_2$	$^{42}CaCl_2$	$^{44}CaCl_2$	EGTA	IBMX
A	106	—	—	0·13	2·5	—	—	1·5	—	1·8	—	—	—	—
B	106	—	—	0·13	2·5	—	—	1·5	—	—	—	1·8	—	—
C	106	—	—	0·13	2·5	—	—	1·5	—	—	—	5·0	—	—
D	106	—	—	0·13	2·5	—	—	1·5	—	—	—	10·0	—	—
E	106	—	—	0·13	2·5	—	—	1·5	—	—	—	20·0	—	—
F	106	—	—	0·13	2·5	—	—	1·5	—	—	20·0	—	—	—
G	—	106	—	0·13	—	2·5	—	—	1·5	1·8	—	—	—	—
H	—	106	—	0·13	—	2·5	—	—	1·5	1·8	—	—	1·815	—
I	—	100·5	—	0·13	—	2·5	—	—	1·5	1·8	—	—	2·296	—
J	—	90	—	0·13	—	2·5	—	—	1·5	1·8	—	—	12·67	—
K	—	—	100·5	—	—	2·37	0·13	1·5	—	1·8	—	—	2·296	—
L	106	—	—	0·13	2·5	—	—	1·5	—	—	—	1·8	—	0·1
M	106	—	—	0·13	2·5	—	—	1·5	—	—	—	1·8	—	0·5

EGTA is ethylene-bis-[oxyethylenenitrilol] tetraacetate. IBMX is 3-isobutyl-1-methylxanthine. All solutions contained 5·6 mM-glucose and 3·0 mM-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid). Solution pH was adjusted to 7·8 with NaOH or (for solution K) with LiOH.

The percentage composition of the two labels is given in Table 2. The values given in the row labelled '^{40}Ca' are the natural abundances for the isotopes of Ca, taken from the *Handbook of Chemistry and Physics* (Weast, 1970). The values given in the other two columns are the mean values of forty l.a.m.m.a. measurements from the stock solutions we actually used in our experiments. These did not differ significantly from the values of the isotope composition given by the supplier. We also measured the Ca isotope ratios in Ringer solutions containing one or another of the Ca labels, to exclude the possibility that the isotope ratios could have been altered by Ca introduced as an impurity, for example, of the NaCl in the Ringer solution. We found no significant difference between the ratios in the Ringer solution and in the stock solutions. The isotope ratios in the Ringer solution were unlikely to have been significantly altered by Ca coming from the cells in the retina

TABLE 2. Composition of Ca labels

Label	^{40}Ca (%)	^{42}Ca (%)	^{44}Ca (%)
^{40}Ca	96·97	0·64	2·06
^{42}Ca	22·1	74·1	3·86
^{44}Ca	2·9	0·7	96·4

Label compositions for ^{40}Ca are natural abundances, taken from Weast (1970). ^{42}Ca and ^{44}Ca compositions were measured with l.a.m.m.a., as described in the text.

during the course of an experiment, since the total volume of retina immersed in the incubation solutions was only a small fraction of the bath volume (0·1–0·3 %).

Since the labels that we used actually contained mixtures of isotopes, it was necessary to correct our measurements of Ca content for the actual isotope compositions given in Table 2. Consider the following example. Suppose a retina containing ^{40}Ca in its normal natural abundancy were incubated for a certain time in a Ringer solution containing the ^{42}Ca label. Provided some of the ^{42}Ca label entered the rod, the rod upon analysis would be found to have a certain concentration of ^{40}Ca, consisting partly of the ^{40}Ca entering the rod as a contaminant of the ^{42}Ca label. The rod would also contain some ^{42}Ca and ^{44}Ca, each also consisting partly of the Ca in the rod originally and partly of the new Ca accumulating during the incubation. When we measured the Ca composition of the rod, we determined the amplitude of the integral of the peaks for each of the isotopes. From these amplitudes and the composition of the Ca labels in Table 2, we could calculate the fraction of the Ca content of the rod which was due to Ca entering from the external solution. In the general case where we used both the ^{42}Ca and ^{44}Ca labels in the same experiment, it was necessary to solve three equations in three unknowns:

$$^{40}\text{Ca} = 0{\cdot}9697X_1 + 0{\cdot}221X_2 + 0{\cdot}029X_3, \quad (1)$$

$$^{42}\text{Ca} = 0{\cdot}0064X_1 + 0{\cdot}741X_2 + 0{\cdot}007X_3, \quad (2)$$

$$^{44}\text{Ca} = 0{\cdot}0206X_1 + 0{\cdot}039X_2 + 0{\cdot}964X_3, \quad (3)$$

X_1, X_2 and X_3 in these equations represent the concentrations of the Ca labels as if they were completely pure, as if normal Ca consisted only of ^{40}Ca, and the ^{42}Ca and ^{44}Ca labels had no contamination from the other isotopes. ^{40}Ca, ^{42}Ca and ^{44}Ca represent the mean values of the amplitudes of the integrals actually measured for the peaks of the three Ca isotopes. The coefficients are taken from Table 2.

For simplicity of presentation, we shall give eqns. (1) to (3) in matrix form by forming the vector **CA** whose components consist of ^{40}Ca, ^{42}Ca and ^{44}Ca, and the vector **X** whose components consist of X_1 to X_3. We then have

$$\mathbf{CA} = \mathbf{A} \times \mathbf{X}, \quad (4)$$

where **A** is the matrix whose components are the coefficients in eqns. (1) to (3), in the order given by these equations. This can be solved for **X** by inversion of **A**:

$$\mathbf{X} = \mathbf{B} \times \mathbf{CA}, \quad (5)$$

where **B** is the inverse of **A** (that is, $\mathbf{B} = \mathbf{A}^{-1}$). The elements of the vector **X** give the best estimates for the mean values of the three labels corrected for isotope impurity.

In order to calculate the standard deviations for the estimates of X_1 to X_3, it was necessary first

to calculate the covariance matrix for the measured values of the ^{40}Ca, ^{42}Ca and ^{44}Ca signals. This was done using standard methods (see for example Mood & Graybill, 1963) with the PRINCOMP Routine of SAS (SAS Institute Inc., Cary, NC, USA). We shall refer to this matrix as **COVCA**. The covariance matrix for **X (COVX)** can be shown to be given by:

$$\mathbf{COVX} = \mathbf{B} \times \mathbf{COVCA} \times \mathbf{B}^t, \quad (6)$$

where $\mathbf{B}^t$ is the transpose of the **B** matrix (from eqn. (5)). The standard deviations for X_1 to X_3 were then calculated as the square roots of the diagonal components of **COVX**.

In experiments using both the ^{42}Ca and ^{44}Ca labels (for example, Figs. 4 and 5), we have corrected the measured values of the means and standard deviations of the three Ca isotopes for contamination of the labels, according to eqns. (5) and (6). Although in these Figures and the corresponding text we refer to the concentrations of the three isotopes, it should be kept in mind that what we are actually plotting are the values of X_1 to X_3 and their respective standard deviations, since these more accurately reflect the Ca movements whose rates we were attempting to estimate. In experiments where only ^{44}Ca was used (for example Figs. 2, 8 and 9), only the means were corrected, since the ^{44}Ca was at least 96% pure and the effect of the corrections on the standard deviations was negligible.

Shock-freezing, tissue preparation and l.a.m.m.a.

As in previous experiments (Schröder & Fain, 1984; Fain & Schröder, 1985), each of the samples of retina supported by the Millipore filters was shock-frozen by quick immersion in melting Freon 22 (at −150 °C) and was then transferred to cryo-substitution tubes (at −194 °C). In all of the experiments in this paper, the retina was dehydrated by cryo-substitution in acetone–OsO_4 and embedded in an Epon–Araldite mixture, as described in detail in Fain & Schröder (1985).

Ca content was analysed with a LAMMA-500 (Leybold-Heraeus, Cologne, F.R.G.). A complete description of this instrument is given in Wechsung, Hillenkamp, Kaufman, Nitsche & Vogt (1979) and in Vogt, Heinen, Meier & Wechsung (1981). In brief, the LAMMA-500 uses two lasers, a continuous low-power He–Ne laser and a high-power Nd–YAG pulse laser, which are co-linear. In our experiments, 0·5 μm thick sections of retina supported on an electron microscope grid were placed in a vacuum chamber on the stage of a light microscope. The stage was moved so that the low-power laser beam fell onto a region of interest, and the high-power laser was then activated to vaporize a small area approximately 5 μm in diameter. Ions formed during vaporization were accelerated into the field of an electromagnetic lens and detected with a time-of-flight mass spectrometer. Spectra were recorded with a transient recorder, transferred to a Hewlett Packard HP 1000 minicomputer (Hewlett Packard Inc., Palo Alto, CA, U.S.A.) and stored on magnetic disks. A set of analytical programs first calibrated the positions of the peaks by comparison to standard spectra, of known composition. Then the Ca signals for the three isotopes used in this study were calculated by integrating within the limits $\pm$0·5 atomic mass unit (a.m.u.) of the peak for each isotope, such that all of the counts of the channels within these limits were summed. These limits were reduced to $\pm$0·3 a.m.u. of the peak in the experiments of Figs. 4 and 5, in which ^{42}Ca was used as a label. Our reason for doing this was that in some cases the ^{42}Ca signal within $\pm$0·5 a.m.u. was contaminated by adjacent peaks, in particular by the ^{41}K peak. We do not believe that the reduction in the peak width had a significant effect on our estimate of the percentage composition of the Ca in the rods, since most of the content of the Ca peaks fell within $\pm$0·3 a.m.u. As a check for this supposition, in some favourable cases it was possible to measure all three of the Ca peaks without interference from adjacent peaks using both the $\pm$0·3 and $\pm$0·5 a.m.u. limits, and the relative Ca isotope compositions calculated from these measurements were nearly identical.

All of the measurements in this paper were made from the so-called 'red' rods, which are maximally sensitive at 502 nm (Fain, 1976).

RESULTS

We have previously shown that, when retinas are incubated in darkness in Ringer solution containing the physiological Ca concentration, the exchange of outer-segment Ca for extracellular Ca is very slow. Since the plasma membrane of dark-adapted rods has been shown to be quite permeable to Ca under physiological

conditions (Yau & Nakatani, 1984*a*; Hodgkin, McNaughton & Nunn, 1985), this slow rate of exchange suggested to us that most of the Ca within the outer segment in intact rods must be sequestered.

To explore the dark exchange of Ca in rod outer segments in more detail, we have exposed rods to Ringer solutions containing elevated concentrations of ^{44}Ca. The

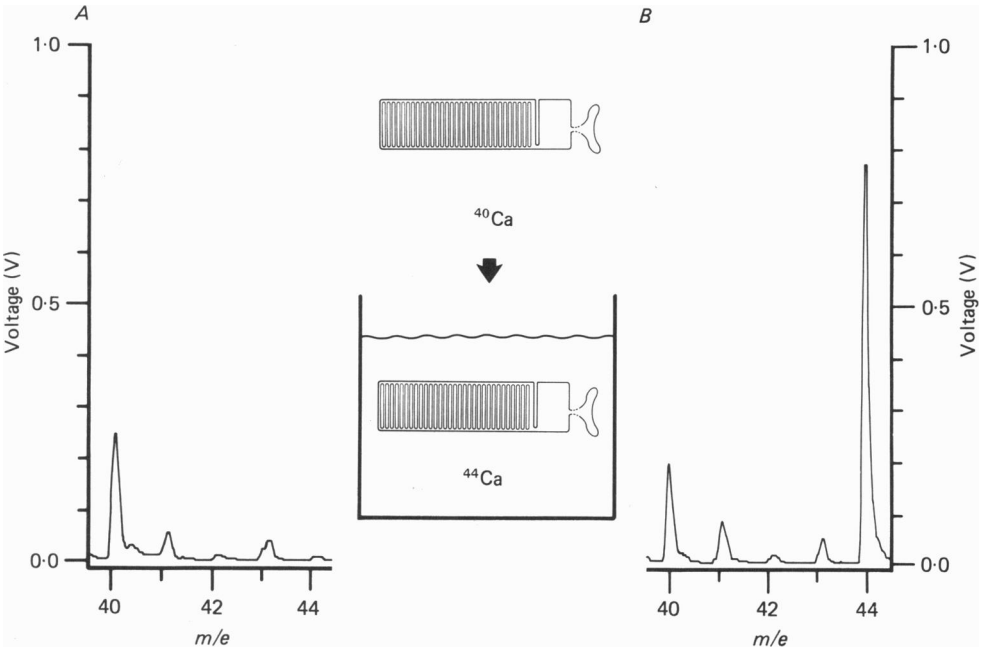

Fig. 1. Incubation of dark-adapted retina in high-Ca Ringer solution in darkness. Representative spectra. *A*, l.a.m.m.a. spectrum for $m/e = 40$ to $m/e = 44$ from rod outer segment of control piece of a dark-adapted retina. Piece was frozen immediately after the dissection. Ordinate gives the voltage signal from the secondary electron-multiplier tube of l.a.m.m.a. after amplification. Abscissa gives mass in a.m.u. divided by charge. This is equivalent to mass in a.m.u., since the microplasma produced by the high-energy laser of the l.a.m.m.a. has been shown to consist almost exclusively of singly charged particles (Wechsung *et al.* 1979; Vogt *et al.* 1981). Peak at $m/e = 40$ corresponds to approximately 4–5 mmol total Ca/l tissue volume (see Fain & Schröder, 1985). *B*, l.a.m.m.a. spectrum of rod outer segment from same retina as for *A* but from another piece which had been incubated for 30 min in darkness in a solution containing 20 mM-$^{44}CaCl_2$ (Table 1, solution E). Ordinate and abscissa as for *A*. Large peak at $m/e = 44$ represents approximately 12–15 mmol ^{44}Ca/l tissue volume which entered rods from external medium.

protocol for these experiments was as follows. A retina from a dark-adapted animal was cut into eight pieces, and one or two pieces were immediately shock-frozen as a control (see Methods). The other six were divided into three groups and placed two each into oxygenated Ringer solution containing 5, 10 or 20 mM-$^{44}CaCl_2$ (solutions C, D or E). They were incubated in these solutions in darkness and removed after 15 or 30 min to be immediately shock-frozen. Both control and experimental pieces were dehydrated and prepared for analysis as previously described (see Methods and Fain & Schröder, 1985).

Results representative of these experiments are given in Fig. 1. In *A* we show part of an l.a.m.m.a. spectrum from $m/e = 40$ to $m/e = 44$ for a rod outer segment from a control piece of retina (see legend to Fig. 1). There is a large peak at $m/e = 40$, which represents about 97 % of the Ca normally present in the rod. The much smaller peak at $m/e = 44$, representing the small amount of ^{44}Ca normally present in the rod, is

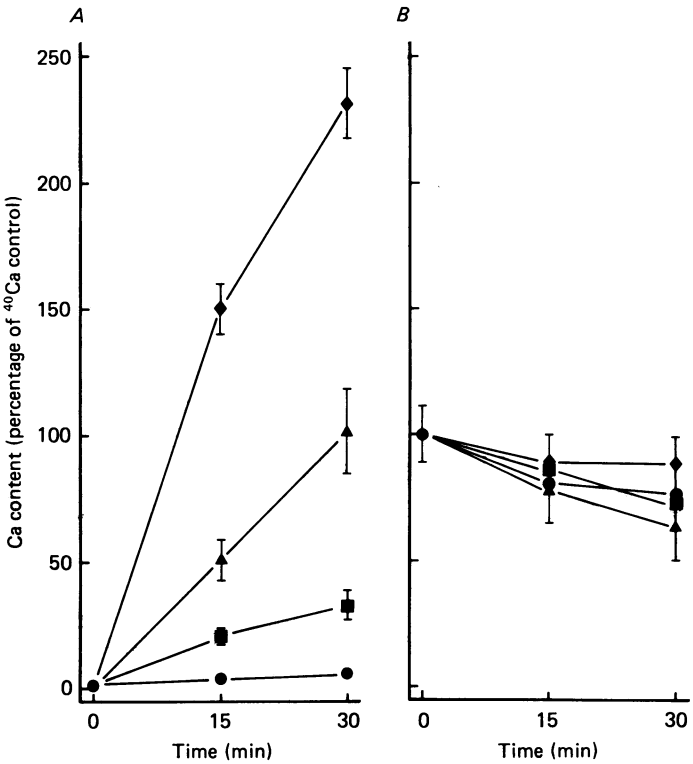

Fig. 2. Time course of change in Ca content of rod outer segments from dark-adapted retinas incubated in darkness in high-Ca solutions. Data points give the mean and one standard deviation for ^{44}Ca (*A*) or ^{40}Ca (*B*) content, averaged from ten outer segments each in three to five retinas and corrected for impurity in isotope composition (see Methods). Data are plotted as a percentage of the mean-corrected ^{40}Ca content of the control pieces, which were frozen immediately following the dissection. Pieces were incubated in Ringer solutions containing $^{44}CaCl_2$ at the following concentrations (mM): 1·8 (solution B, ●); 5 (solution C, ■); 10 (solution D, ▲); and 20 (solution E, ◆). Pieces were removed from these solutions after 15 and 30 min. Data points at $t = 0$ give the Ca content from the control pieces. Data in *A* and in *B* were taken from same spectra. Circles (except for controls) give the Ca content in 1·8 mM-$^{44}CaCl_2$ taken from Fig. 6 of Fain & Schröder (1985).

difficult to distinguish from noise in single spectra, but averages of many spectra show it to be 2–3 % of the amplitude of the ^{40}Ca peak (Fain & Schröder, 1985), in approximate agreement with its natural abundance (see Table 2). A more complete description of the l.a.m.m.a. spectra of dark-adapted rod outer segments can be found in our previous paper (Fain & Schröder, 1985).

In *B*, we show a spectrum from a rod outer segment incubated for 30 min in 20 mM-^{44}Ca Ringer solution (solution E). The large peak at $m/e = 44$ represents an accumulation of 12–15 mmol ^{44}Ca/l wet tissue volume, due to Ca entering the rod from the external solution. Notice, however, that there is still a substantial signal at $m/e = 40$. This is of some interest, since it indicates that much of the ^{40}Ca originally in the rod remains only slowly exchangeable even in the presence of a large accumulation of Ca from the extracellular milieu.

TABLE 3. ^{44}Ca accumulation in elevated-Ca solutions

	Rate of accumulation		
^{44}Ca in medium (mM)	^{44}Ca as percentage of ^{40}Ca control (min^{-1})	^{44}Ca rod^{-1} s^{-1}	^{44}Ca cm^{-2} s^{-1}
1·8	0·12	$1{\cdot}6 \times 10^{5}$	$9{\cdot}9 \times 10^{9}$
5	1·22	$1{\cdot}7 \times 10^{6}$	$1{\cdot}0 \times 10^{11}$
10	3·2	$4{\cdot}3 \times 10^{6}$	$2{\cdot}6 \times 10^{11}$
20	7·6	$1{\cdot}0 \times 10^{7}$	$6{\cdot}2 \times 10^{11}$

Rates estimated by linear regression from the data in Fig. 2*A*. Calculations assumed a rod outer-segment volume of 3 pl and a surface area of $1{\cdot}65 \times 10^{-5}$ cm^2.

A summary of the results from experiments of incubations in elevated $^{44}CaCl_2$ concentrations is given in Fig. 2. *A* and *B* give separately the ^{44}Ca and ^{40}Ca content, in both cases plotted as a percentage of the mean-corrected ^{40}Ca content of control retinas. The results in *A* show clearly that the rate of ^{44}Ca accumulation in the rod outer segment increases with extracellular ^{44}Ca concentration. This can be seen more clearly from the actual values of the rates of accumulation, estimated from the data of Fig. 2 by linear regression and given in Table 3. For the calculations of rates of accumulation in numbers of Ca atoms rod^{-1} s^{-1} (third column), we have made the assumption that the control ^{40}Ca content of a dark-adapted rod is 4·5 mmol/l wet tissue volume (Fain & Schröder, 1985), and that a rod outer segment is a cylinder 7·5 μm in diameter and 62·5 μm long (Fain, 1976), with a volume of about 3 pl. Notice that the rate of ^{44}Ca accumulation in normal Ringer solution is larger if expressed in units of Ca rod^{-1} s^{-1} than we previously estimated (Schröder & Fain, 1984; Fain & Schröder, 1985), because in Table 3 we have assumed a larger, and we believe more accurate, value for the total volume of the rod outer segment. For the calculation of rate of accumulation per square centimetre of plasma membrane (fourth column), we have used a value for the surface area of the outer segment of $1{\cdot}65 \times 10^{-5}$ cm^2. Had we included the area of the inner-segment plasma membrane, the rates of accumulation per square centimetre would have been of the order of 30 % smaller.

Although large amounts of ^{44}Ca accumulate in the outer segments of rods bathed in elevated Ca concentrations, there is little exchange between this ^{44}Ca and the ^{40}Ca originally in the rod. This can be seen from Fig. 2*B*, where we plot the ^{40}Ca levels from the same outer segments as for Fig. 2*A*. Although ^{40}Ca levels declined with time, they did so in all of the four extracellular Ca concentrations at a rate which was approximately constant and independent of the rate of the ^{44}Ca increase. These data confirm the impression given by the spectrum of Fig. 1*B*, that little of the ^{40}Ca exchanges even in the presence of high amounts of ^{44}Ca.

Double-isotope labelling experiments

The experiment of Fig. 2 suggests that the Ca in rod outer segments can exist in at least two pools. One, containing the great majority of the Ca originally present in the rod, exchanges slowly with extracellular Ca and is presumably sequestered

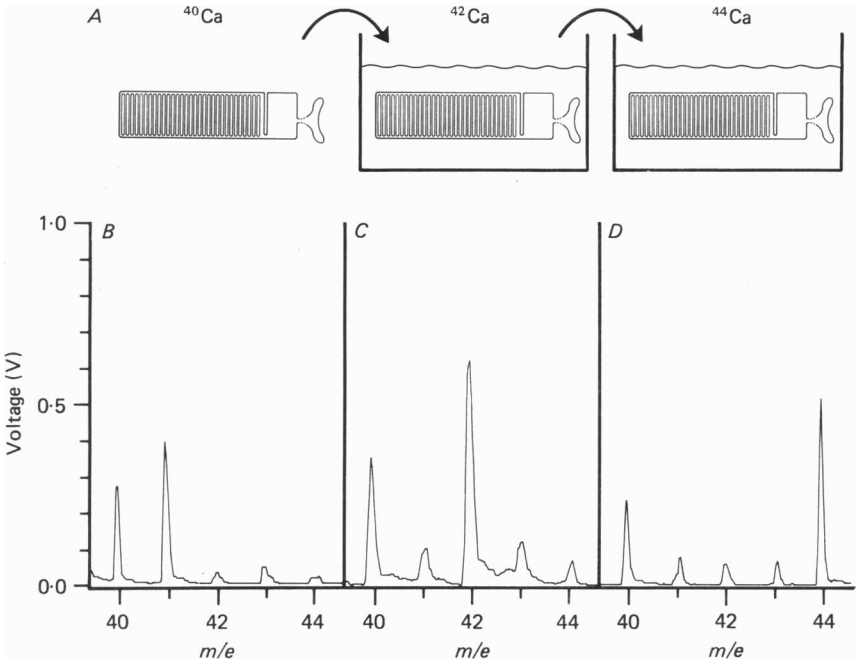

Fig. 3. Double-isotope labelling experiments. Protocol and sample spectra. *A*, flow chart for experiments. Dark-adapted retinas with rod outer segments containing mostly ^{40}Ca were incubated in darkness first in 20 mM-$^{42}CaCl_2$ Ringer solution to load rods with ^{42}Ca and then in solutions containing either 1·8 or 20 mM-$^{44}CaCl_2$. *B*, *C* and *D*, sample spectra. Abscissa and ordinate as in Fig. 2 (see legend). *B*, spectrum from outer segment of control piece of retina, frozen immediately after dissection. *C*, spectrum from outer segment of piece of retina incubated for 15 min in 20 mM-$^{42}CaCl_2$ (solution F). Note large amplitude peak at $m/e = 42$. Peaks at $m/e = 40$ and $m/e = 44$ were larger than in *B* because of impurity of ^{42}Ca label (see text). *D*, spectrum from outer segment of piece of retina incubated first for 15 min in 20 mM-$^{42}CaCl_2$ (solution F) and then for 1 min in 20 mM-$^{44}CaCl_2$ (solution E). Note that nearly all of the ^{42}Ca had left the rod apparently in exchange for ^{44}Ca.

within the disks (Fain & Schröder, 1985). The second, consisting of Ca entering the rod from the external solution, may on the other hand be Ca accumulating within the cytoplasm of the outer segment either free or loosely bound. However, the data of Fig. 2 do not exclude the possibility that the ^{44}Ca accumulating within the outer segment, instead of being free within the cytosol, also becomes tightly bound or sequestered, and that both the ^{40}Ca and the ^{44}Ca become trapped within the outer segment and unable to exchange with Ca in the external solution.

To test this possibility, we have exposed retinas to two stable isotopes, as illustrated in the flow chart of Fig. 3*A*. Rods from dark-adapted retinas whose outer

segments contained the normal complement of ^{40}Ca (Fig. 3*B*) were first exposed for 15 min to Ringer solution containing 20 mM-$CaCl_2$ consisting predominantly of the isotope ^{42}Ca (solution F). As in Fig. 1, exposure to the high-Ca Ringer solution caused a large accumulation of Ca within the outer segment, as can be seen from the large amplitude peak at $m/e = 42$ in the spectrum of Fig. 3*C*. This spectrum also shows increases in the peaks at $m/e = 40$ and $m/e = 44$, due to an influx of ^{40}Ca and ^{44}Ca into the rod as impurities of the $^{42}CaCl_2$ label in the Ringer solution (see Table 2, p. 365).

After having loaded the rod with ^{42}Ca, we next incubated the retina in Ringer solution containing $^{44}CaCl_2$. The spectrum in Fig. 3*D* is from a rod outer segment in a retina incubated for only 1 min in 20 mM-$^{44}CaCl_2$. Note that the peak at $m/e = 42$ has nearly disappeared and has been replaced by a large peak at $m/e = 44$. This result demonstrates directly that most of the Ca which enters the rod from the external medium in high-Ca Ringer solutions remains rapidly exchangeable across the plasma membrane. Note also that the ^{40}Ca peak is nearly the same amplitude in the spectrum of Fig. 3*D* as in Fig. 3*B*.

Complete results of the double-isotope experiments are given in Figs. 4 and 5. Although the experiments for these two Figures were done independently, the protocols were nearly identical. After freezing the control, the remaining pieces were incubated in darkness in oxygenated 20 mM-^{42}Ca Ringer solution (solution F) for 15 min. One of these pieces was then removed and frozen, and the others were left on the dental wax, which was patted dry with a Kimwipe to prevent cross-contamination of the isotope labels and then transferred as quickly as possible (10–15 s) into ^{44}Ca Ringer solution, either at 20 mM (solution E) or at the normal concentration (1·8 mM, solution B). The Ca content for each of the three isotopes was corrected for impurity in isotope composition, as described in the Methods, and plotted as a percentage of the mean-corrected ^{40}Ca value of the controls.

The results for incubation in 20 mM-^{42}Ca followed by 20 mM-^{44}Ca are given in Fig. 4. After exposing the rods for 15 min to 20 mM-^{42}Ca, large amounts of the ^{42}Ca label accumulated within the rod at a rate which can be estimated from the single data point at 15 min to be of the order of 2×10^7 Ca rod^{-1} s^{-1}. This is similar to the rate of entry in 20 mM-Ca given in Table 3. When the solution was then substituted with one containing 20 mM-^{44}Ca, there was at first a rapid exchange of ^{44}Ca for ^{42}Ca. Nearly all of the ^{42}Ca left the rod during the first minute at a rate of efflux which can be estimated to be at least $2–3 \times 10^8$ Ca rod^{-1} s^{-1}. After the rapid exchange of ^{42}Ca for ^{44}Ca, there followed a slower entry of ^{44}Ca at a rate ($1·5 \times 10^7$ Ca rod^{-1} s^{-1}) similar to that for ^{42}Ca loading.

The mean amount of ^{44}Ca entering the rods after 1 min was less than the mean loss of ^{42}Ca, so that the total Ca content of the rods decreased between 15 and 16 min. Since the Ca concentration in both the ^{42}Ca and ^{44}Ca solutions was the same (20 mM), this loss of total Ca from the rods was probably artifactual, perhaps occurring during the manipulations necessary to change solutions. It is possible that the drying of the dental wax containing the pieces of retina before re-immersion into the ^{44}Ca Ringer solution caused the pieces to become partially dehydrated.

In Fig. 5 we show results for incubation in 20 mM-^{42}Ca followed by 1·8 mM-^{44}Ca. There were several striking differences between the results of this experiment and that

in Fig. 4. In the first place, the rate of ^{42}Ca exit was slower. Since the only difference between the experiments in Figs. 4 and 5 was the external $^{44}CaCl_2$ concentration, the decrease in the rate of ^{42}Ca efflux suggests that Ca was exiting from the cell at least in part by Ca–Ca exchange. We shall return to this point in the Discussion (see p. 380).

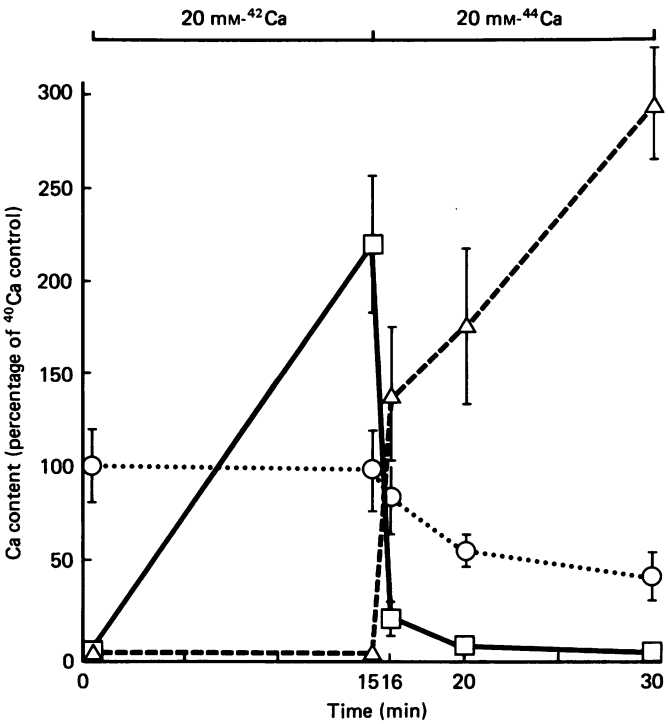

Fig. 4. Double-isotope labelling experiments: 20 mM-^{42}Ca to 20 mM-^{44}Ca. Time course of change in Ca content in rod outer segments from dark-adapted retinas incubated in darkness first in 20 mM-^{42}Ca and then in 20 mM-^{44}Ca. Data points give values for ^{40}Ca (○····○), ^{42}Ca (□—□) and ^{44}Ca (△---△). These have been corrected for isotope impurity as described in the methods, so that what are actually plotted are X_1, X_2, and X_3 from eqns. (1)–(3) (see p. 365). Means with one standard deviation are from ten outer segments from each of four or five retinas and are given as a percentage of the mean-corrected ^{40}Ca content of control pieces. Retinal pieces were incubated for 15 min in 20 mM-$^{42}CaCl_2$ Ringer solution (solution F) and then for 15 min in 20 mM-$^{44}CaCl_2$ Ringer solution (solution E). One piece from each retina was frozen immediately after the incubation in ^{42}Ca at $t = 15$ min. Additional pieces were frozen at 1, 5 and 15 min after transfer to ^{44}Ca. Data points at $t = 0$ give values for control pieces, frozen immediately after the dissection.

The second difference between the results in these two Figures is that, in Fig. 5, there was a much smaller entry of ^{44}Ca which appeared to cease at about $t = 20$ min. At this time, the exit of ^{42}Ca from the rod was also nearly complete. There was once again some loss in total Ca after the solution change, which may in part have been artifactual but may also in part have been caused by a net efflux of Ca as the result of the decrease in the external Ca concentration.

Although there was a rapid exchange of ^{44}Ca for ^{42}Ca in both Figs. 4 and 5, the

^{40}Ca content was much less affected. 1 min after the solution change at $t = 16$ min in Fig. 4, when nearly all of the ^{42}Ca had left the outer segment, the mean ^{40}Ca had declined by only 15%. A similar effect can also be seen at $t = 16$ min in Fig. 5. At later times, a significant decrease in the ^{40}Ca content could be seen in both Figs. 4 and 5. Although this decrease may seem to be larger in Fig. 5 than in Fig. 4, there

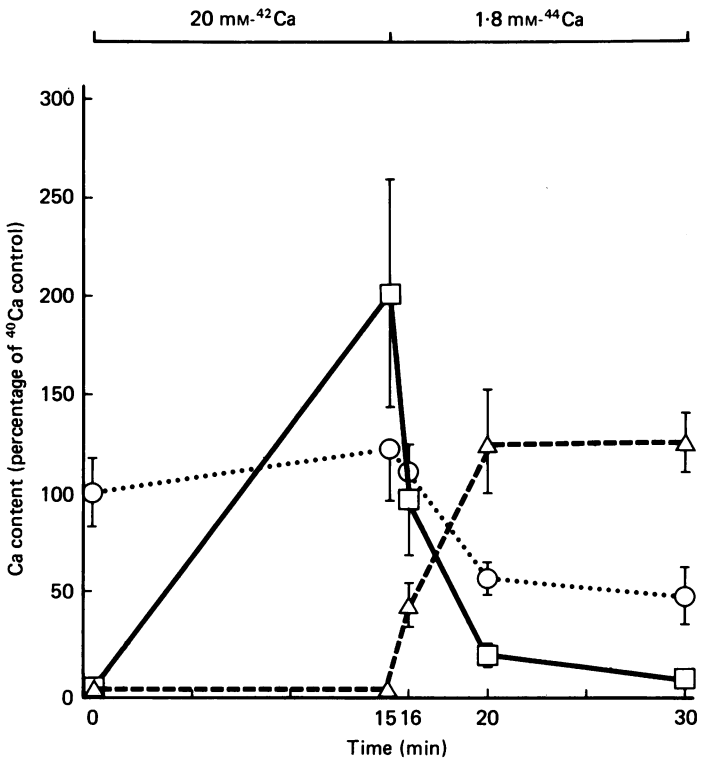

Fig. 5. Double-isotope labelling experiments: 20 mM-^{42}Ca to 1·8 mM-^{44}Ca. Time course of change in Ca content in rod outer segments from dark-adapted retinas incubated in darkness first in 20 mM-^{42}Ca and then in 1·8 mM-^{44}Ca. As in Fig. 4, data points give corrected means with one standard deviation from ten outer segments each from four or five retinas for ^{40}Ca (○ · · · ○), ^{42}Ca (□—□) and ^{44}Ca (△– – –△), plotted as a percentage of the mean-corrected ^{40}Ca control value. Protocol of experiment as for Fig. 4. Pieces were frozen immediately after the dissection (as controls), after a 15 min incubation in 20 mM-$^{42}CaCl_2$ Ringer solution (solution F), and then 1, 5 and 15 min after transferral to 1·8 mM-$^{44}CaCl_2$ Ringer solution (solution B). Data points at $t = 0$ give values for control pieces.

was in fact no significant difference in the ^{40}Ca measurements between these two sets of experiments. The decline of ^{40}Ca content in both these experiments was larger than previously observed for rods incubated in darkness in normal Ringer solution (Fain & Schröder, 1985). It was also larger than that seen in elevated Ca concentrations in Fig. 2*B*. It is possible that this loss of ^{40}Ca was also caused by the manipulation of the retinas during the solution change. It is also possible that, under the particular circumstances of the experiments in Figs. 4 and 5, some enhanced exchange of the

^{40}Ca pool may have taken place. We have at present no explanation for why this might have occurred.

Incubation in low-free-Ca Ringer solution

The results we have so far presented show that most of the Ca normally present in the rod exchanges slowly even when the outer segments have been loaded with high concentrations of Ca from the extracellular medium. This Ca can, however, be released if the rods are incubated in Ringer solution containing a reduced free-Ca concentration (Fig. 6). In these experiments, the rods were immersed in solutions for which the Ca was buffered with EGTA to a free concentration of 10^{-6}, $10^{-7.5}$ or 10^{-9} M (solutions H, I and J, respectively). These solutions also had a low Cl concentration, in order to reduce swelling of the outer segments caused by the entry of NaCl (Hagins & Yoshikami, 1977). Incubation in low-Cl Ringer solution containing the normal Ca concentration (solution G) did not by itself produce decreases in Ca much larger than those seen in normal Ringer solution. Perfusion of rods with low-Cl Ringer solution has been shown to produce small changes in rod dark resting membrane potential and rod light-response amplitude (Bastian & Fain, 1982), probably as the result of changes in conductance in the inner segment (Bader, Bertrand & Schwartz, 1982).

The data in Fig. 6 indicate that incubation in low-Ca Ringer solutions produces a depletion of the rod Ca at a rate which increases with decreasing extracellular free-Ca concentration. If the Ca depletion is assumed to occur as the result of a single rate-limiting step (for example, transport across the disk membrane), and if all (or nearly all) of the Ca in a dark-adapted rod can be assumed to be contained within a single compartment (for example, the disks), then the depletion of Ca from the outer segment should occur exponentially with a single time constant,

$$F = \exp(-t/t_0), \tag{7}$$

where F is the fractional ^{40}Ca content (^{40}Ca divided by the control ^{40}Ca), t is time in min, and t_0 is a constant. The data in Fig. 6 have been fitted to this equation using the Fletcher–Powell algorithm on a Hewlett Packard HP-71 calculator. This algorithm minimizes the gradient of F with respect to the free parameters and weights the data points according to their standard deviations. Note that in order to fit the data with eqn. (4), only one free parameter (t_0) was varied. Since the data were normalized to the Ca content of the control pieces, the Ca content at $t = 0$ was assumed to be 100 % in each of the low-Ca solutions. For consistency, the best-fitting curves were also constrained to pass through the point ($t = 0$, 100 %).

The best-fitting values for t_0 for the various Ca concentrations are given in Table 4, and the curves generated by these values have been superimposed upon the data in Fig. 6. The initial rates of Ca depletion are also given in Table 4, calculated as dF/dt at $t = 0$. The initial rates of depletion increase as the free-Ca concentration decreases and are of the same order as the rates of Ca accumulation in high-Ca Ringer solution given in Table 3.

Effect of Na substitution on Ca release in low-Ca Ringer solution

Our measurements of Ca release in low extracellular free-Ca solutions resembled those given by Schnetkamp (1980, 1986) for isolated bovine rod outer segments. We

were therefore curious to know whether the rate-limiting step for Ca efflux in our experiments was the same as for isolated bovine outer segments, that is, Na–Ca counter-transport across the plasma membrane. For this reason, we compared the rate of Ca efflux in low-free-Ca solutions for Ringer solutions containing Na and for Ringer solution in which all of the Na was substituted with choline.

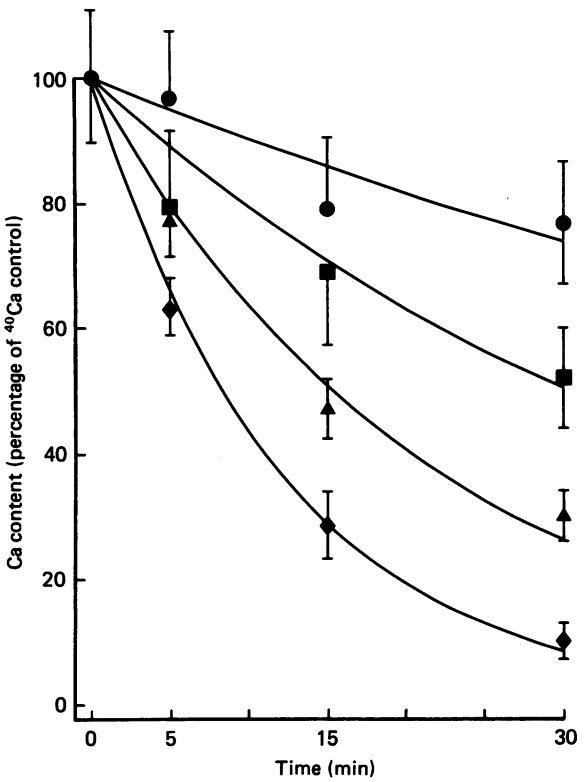

Fig. 6. Time course of decrease in rod outer-segment Ca content from dark-adapted retinas incubated in darkness in low-free-Ca Ringer solution. Data points give mean and one standard deviation for ^{40}Ca content for ten outer segments each from between three and five retinas. Pieces were incubated in Ringer solutions that had the following free-Ca concentrations: 1·8 mM (solution B, ●); 10^{-6} M (solution H, ■); $10^{-7.5}$ M (solution I, ▲); and 10^{-9} M (solution J, ◆). Pieces were removed from these solutions at 5, 15 and 30 min. Incubations in 10^{-6} and $10^{-7.5}$ M solutions were done as one series of experiments, and incubations in the 10^{-9} M solution were done separately as a second series. Data are plotted as a percentage of the mean ^{40}Ca content of the control pieces from the series of experiments in which the incubations were done. Standard deviation for the control pieces at $t = 0$ is the average of the standard deviations from the controls for the two series. Circles (other than control value) give the ^{40}Ca content in 1·8 mM free Ca taken from Fig. 6 of Fain & Schröder (1984). Data have been fitted with eqn. (7) as described in text using the values for t_0 given in Table 4.

The results of these experiments are given in Fig. 7. The time course of Ca efflux in $10^{-7.5}$ M-free-Ca solution containing Na (solution I) is given by the circles. These data are quite similar to those for $10^{-7.5}$ M-free-Ca given previously in Fig. 6, and the curve is in fact the same one used to fit the $10^{-7.5}$ M data in the previous Figure. Since

the experiments in Figs. 6 and 7 were done three years apart, the similarity of the time course is an indication of the repeatability of our measurements.

The squares give for pieces of the same retinas the time course of Ca efflux in choline Ringer solution (solution K). It is apparent that Ca release occurs at the same rate in $10^{-7.5}$ M-free-Ca solution whether Na is present or not. It is therefore unlikely

TABLE 4. Rate of Ca depletion

Free-Ca concentration (M)	t_0 (min)	Initial rate (Ca rod^{-1} s^{-1})	Initial rate (Ca cm^{-2} s^{-1})
1.8×10^{-3}	99.7	1.4×10^{6}	8.2×10^{10}
1.0×10^{-6}	43.4	3.1×10^{6}	1.9×10^{11}
$1.0 \times 10^{-7.5}$	22.3	6.1×10^{6}	3.7×10^{11}
1.0×10^{-9}	12.1	1.1×10^{7}	6.8×10^{11}

t_0 is the best-fitting time constant from eqn. (7) to the data in Fig. 6 for each of the Ca concentrations. Initial rates were calculated from eqn. (4) as dF/dt at $t = 0$, using the values for t_0 given in the table. We assumed a rod Ca concentration of 4.5 mmol/l wet tissue volume, and the same outer-segment volume and surface area as for Table 3 (see legend).

that Na–Ca exchange is rate limiting for Ca efflux in our experiments. Since exposing rods to low-Ca solutions has been shown to produce a large increase in the light-dependent conductance in the plasma membrane of intact rods (see for example Bastian & Fain, 1982; Hodgkin, McNaughton, Nunn & Yau, 1984), and since this conductance has been shown to be permeable to Ca (Yau & Nakatani, 1984*a*; Hodgkin *et al.* 1985), the movement of Ca through this conductance is also probably not rate limiting. This would suggest that the rate of release of Ca from the rods in low-Ca solutions is not limited by the rate of movement across the plasma membrane but rather by the rate of Ca efflux from the sequestered pool.

Effect of IBMX on Ca fluxes

On the assumption that the sequestered Ca is in the disks, our results suggested that exposure to low Ca might increase disk Ca permeability. Exposing rods to low Ca concentrations has been shown to increase the cyclic GMP concentration within the outer segments (Cohen, Hall & Ferrendelli, 1978). Furthermore, leaky rod outer segments and isolated disks release Ca upon exposure to cyclic GMP (Caretta & Cavaggioni, 1983; Koch & Kaupp, 1985; Puckett & Goldin, 1986). It therefore seemed possible that an increase in the outer-segment cyclic GMP concentration in low-free-Ca Ringer solution might open cyclic-GMP-dependent channels within the disk membrane and allow the Ca to be released from the outer segment.

To test this hypothesis, we attempted to increase the free-cyclic-GMP concentration within the outer segment without changing the extracellular Ca concentration. To do this, we incubated the retina in Ringer solution containing 1.8 mM-$^{44}CaCl_2$ and 100 or 500 μM-IBMX. IBMX has been shown to inhibit the light-activated phosphodiesterase of rod outer segments (Baehr, Devlin & Applebury, 1979) and might therefore be expected to decrease the rate of hydrolysis of cyclic GMP in intact rods. IBMX has also been shown to produce a large increase in the photoreceptor dark current (Cervetto & McNaughton, 1986).

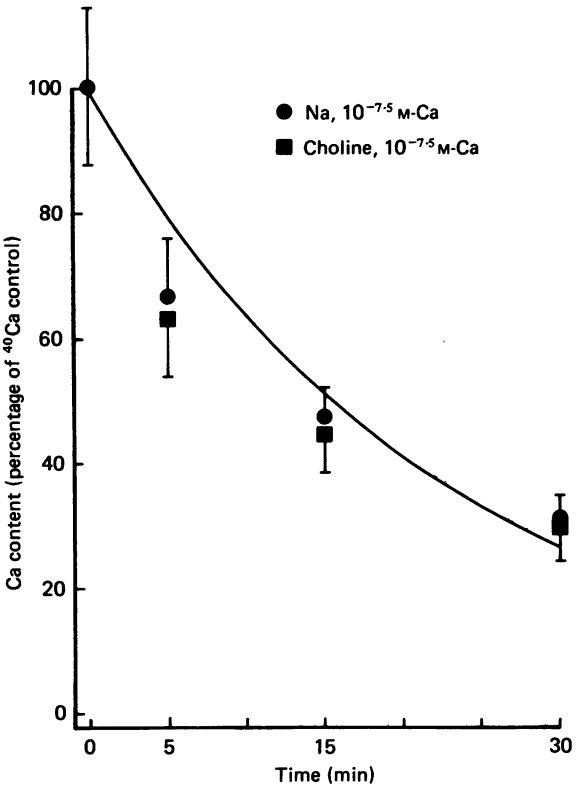

Fig. 7. Comparison of time course of rod outer-segment ^{40}Ca decrease in $10^{-7.5}$ M free-Ca Ringer solutions containing Na (●) or choline (■) as the principal cation. Pieces from dark-adapted retinas were incubated in darkness, first for 5 min in either low-Cl Ringer solution (solution G) or in normal Ringer solution (solution A). Pieces in low Cl were then transferred to a Na-containing Ringer solution (solution I) and those in normal Ringer solution to a choline-containing Ringer solution (solution K). Both of these solutions had the same free-Ca concentration ($10^{-7.5}$ M). We did not substitute Cl with methylsulphonate for the choline-containing Ringer solution, since the light-dependent conductance is impermeable to choline (Woodruff *et al.* 1982; Hodgkin *et al.* 1985), and rods would not be expected to swell even in the presence of Cl. No swelling of the outer segments was in fact observed in our preparations, even after 30 min in the choline chloride, low-free-Ca solution. This solution also contained approximately 5 mM-LiOH to neutralize the EGTA (see Methods). We chose Li for this purpose since, like choline, it is a poor substitute for Na in Na–Ca exchange (Schnetkamp, 1980; Yau & Nakatani, 1984*b*; Fain & Schröder, 1985). The transfer of the pieces into the Na and choline low-free-Ca solutions was staggered by 30 s, so that the pieces could be alternately removed from the two Petri dishes after precisely 5, 15 and 30 min. Data points give the means with one standard deviation, corrected for isotope impurity (see Methods), for ten outer segments each from between three and five retinas. ^{40}Ca content is given as a percentage of the mean-corrected ^{40}Ca content of control pieces frozen immediately after the dissection. Mean and standard deviation of controls are given at $t = 0$. The curve superimposed upon the data was calculated from eqn. (7) using the same value for t_0 (22·3 min) as for the $10^{-7.5}$ M free Ca data in Fig. 6.

Figs. 8 and 9 show separately the outer-segment ^{40}Ca and ^{44}Ca content from these experiments. The circles are from Fig. 6 of Fain & Schröder (1985) and show the decrease in ^{40}Ca and increase in ^{44}Ca in normal Ringer solution. The stars give the ^{40}Ca and ^{44}Ca contents from rods in the present experiments after 30 min in the control solution without IBMX and clearly are within the range of previous measurements.

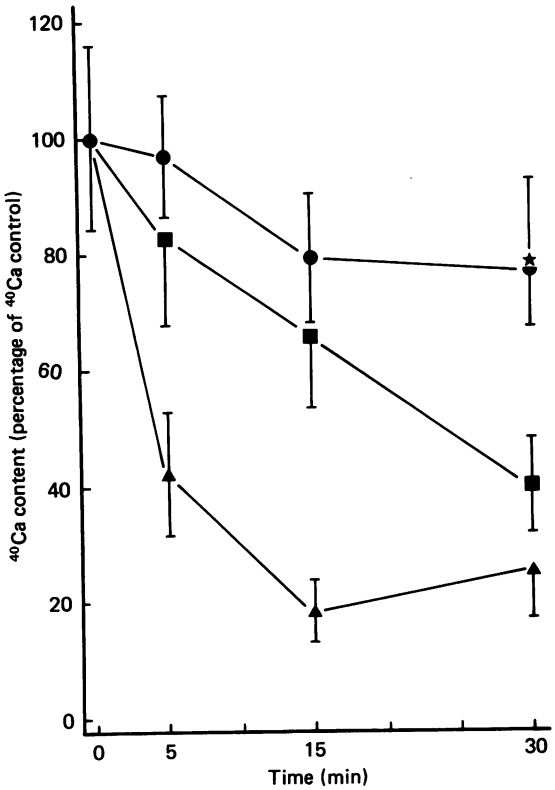

Fig. 8. Time course of ^{40}Ca decrease in IBMX. Pieces of a dark-adapted retina were incubated in darkness in normal Ringer solution containing 1·8 mM-$^{44}CaCl_2$ (solution B, ★), or containing 1·8 mM-$^{44}CaCl_2$ together with 100 (solution L, ■) or 500 μM (solution M, ▲) IBMX. Pieces were removed from normal Ringer solution at 30 min and from the IBMX solutions at 5, 15 and 30 min. Data points give the means with one standard deviation for the ^{40}Ca content corrected for isotope impurity (see Methods) from ten outer segments each from between four and seven retinas. Data are plotted as a percentage of the mean-corrected ^{40}Ca content of control pieces, frozen immediately after the dissection. Values for control pieces are shown at $t = 0$. Other circles are ^{40}Ca content taken from Fig. 6 of Fain & Schröder (1985).

IBMX produced a release of ^{40}Ca which increased with increasing IBMX concentration. This effect was similar to the one observed in low-Ca solutions (Figs. 6 and 7). In addition, IBMX produced a large increase in the rate of ^{44}Ca accumulation within the rod. A least-squares linear regression fitted to the data in Fig. 9 (with the line constrained to pass through the ^{44}Ca value at $t = 0$) gave best-fitting slopes for 100 and 500 μM-IBMX of $3{\cdot}9 \times 10^6$ and $8{\cdot}6 \times 10^6$ Ca rod^{-1} s^{-1}, respectively. These are similar in amplitude to the rates of entry in elevated-Ca Ringer solution (see Table 3).

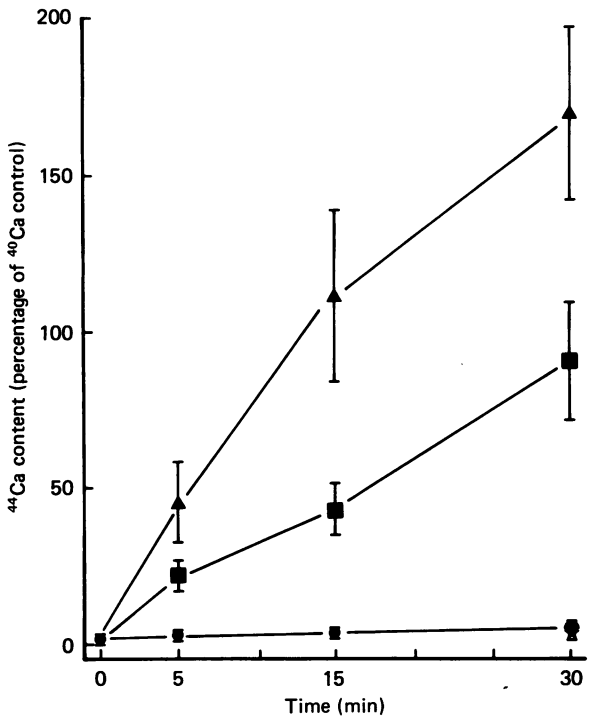

Fig. 9. Time course of ^{44}Ca increase in IBMX. Data were taken from the same spectra as for Fig. 8. Pieces from dark-adapted retinas were incubated in darkness in normal Ringer solution containing 1·8 mM-$^{44}CaCl_2$ (★) or in Ringer solution containing 1·8 mM-$^{44}CaCl_2$ together with 100 (■) or 500 μM (▲) IBMX. Data points give means with one standard deviation corrected for isotope impurity (see Methods) for ten outer segments each from between four and seven retinas, expressed as a percentage of the mean-corrected ^{40}Ca content of control pieces. Circles other than controls give the ^{44}Ca content in 1·8 mM-$^{44}CaCl_2$ Ringer solution taken from Fig. 6 of Fain & Schröder (1985).

DISCUSSION

The experiments in this paper show that the Ca within the rod outer segments can exist in at least two pools: one which is slowly exchangeable with the external solution, which we believe to be Ca contained within the disks; and a second, which is rapidly exchangeable, which we believe to be Ca either free or loosely bound within the cytoplasm. Under physiological conditions in darkness, most of the Ca is contained within the slowly exchangeable pool, and it is from this pool that Ca is released by illumination (Schröder & Fain, 1984). Ca can also be released from this pool by incubating the retina in low-free-Ca Ringer solution or in IBMX, probably as the result of the opening of cyclic-GMP-dependent channels within the disk membrane.

Pooling of Ca in rod outer segments

When the retina is placed in Ringer solution containing 5, 10 or 20 mM-$^{44}CaCl_2$, large amounts of ^{44}Ca accumulate within the rod (see Fig. 2). The mechanism of Ca entry into the rod is unclear. In the 5 mM and perhaps even the 10 mM-$^{44}CaCl_2$ solutions, some of the Ca may have entered through the light-dependent conductance,

which is permeable to Ca (Yau & Nakatani, 1984*a*; Hodgkin *et al.* 1985). However, this conductance is also blocked by high concentrations of extracellular Ca (Brown & Pinto, 1974; Hodgkin *et al.* 1984), and it is uncertain how much Ca could have entered through this pathway. Ca may also have come into the rod through Ca channels in the inner segment (Fain, Gerschenfeld & Quandt, 1980), but exposure to high Ca hyperpolarizes the rod (Brown & Pinto, 1974), and this would decrease the current through the Ca conductance (Bader *et al.* 1982). Finally, Ca could have entered by reverse Na–Ca exchange (Schnetkamp, 1986). This is, in our opinion, the most likely mechanism for Ca entry in high-Ca solutions. The rates of Ca accumulation given in Table 3 are much slower than the maximum rate of Na–Ca exchange or Ca transport across the rod plasma membrane (see Fig. 4 and Yau & Nakatani, 1984*b*, 1985*a*), but they probably represent differences between rates of influx and efflux in each of the high-Ca solutions.

Though large amounts of ^{44}Ca enter the rod in high-Ca solutions, the ^{44}Ca exchanges little with the pool of ^{40}Ca originally present in the outer segment (Fig. 2*B*). The Ca which enters the rod appears to enter a second pool which is freely exchangeable with the Ca in the Ringer solution (see Figs. 4 and 5). The exchange of Ca under these conditions appears to occur at least in part by Ca–Ca exchange. The evidence for this is as follows. When the rods were pre-loaded in 20 mM-$^{42}CaCl_2$ and then placed in 20 mM-$^{44}CaCl_2$ (Fig. 4), the entry of ^{44}Ca occurred at first very rapidly and with approximately the same time course as the exit of ^{42}Ca. The rate of Ca movement in both directions across the plasma membrane was of the order of 2×10^8 Ca rod^{-1} s^{-1}, which is nearly the same as the maximum rate of Na–Ca transport observed by Yau & Nakatani (1984*b*, 1985*a*). After 1 min, nearly all the ^{42}Ca was gone from the rod, and the rate of ^{44}Ca entry slowed to the same rate as we have previously observed for Ca entry in the absence of pre-loading (see Fig. 2). The similarity in the time course for the initial phase of ^{44}Ca entry and for the exit of ^{42}Ca suggests that the entry and exit of Ca during this phase were mutually dependent, as would be the case if Ca were exchanging directly across the plasma membrane.

Additional evidence for Ca–Ca exchange comes from the experiment of Fig. 5. We have previously shown that when rods which have not been pre-loaded with Ca are placed in ^{44}Ca medium at the normal (1·8 mM) Ca concentration, Ca accumulates in the rod very slowly, at about $1{\cdot}6 \times 10^5$ Ca rod^{-1} s^{-1} (see Table 3 and Fain & Schröder, 1985). If as in Fig. 5 the rods are first incubated in a high-Ca solution and then placed in 1·8 mM-^{44}Ca, the ^{44}Ca enters at a rate which is at least two orders of magnitude greater. Once again the time course of ^{44}Ca entry is similar to that of ^{42}Ca exit, and it seems likely that Ca is directly exchanging across the rod plasma membrane. Facilitated Ca–Ca exchange has also been demonstrated for isolated bovine rod outer segments (Schnetkamp, 1986). It is probably mediated by the same protein that mediates Na–Ca exchange (see for example Slaughter, Sutko & Reeves, 1983).

Incubation in low Ca and IBMX

When the retina was incubated in low-free-Ca Ringer solution, the total Ca content of the rod outer segments decreased (see Fig. 6). This result should be interpreted with caution since Ca loss can occur artifactually, either as a result of mishandling of the tissue or poor fixation (Ornberg & Reese, 1980). Nevertheless, we believe the

Ca release we have observed to be physiological, for three reasons. First, the rate of Ca loss was a clear function of the extracellular free-Ca level, increasing with decreasing free-Ca concentration. Secondly, the extent of Ca release was quantitatively repeatable (compare Figs. 6 and 7). Finally, depletion of outer-segment Ca in rods incubated in low-free-Ca Ringer solution has also been observed for isolated intact frog rods (Nicol *et al.* 1986), and in these experiments it was not necessary to fix the tissue. Incubation of rods in IBMX also produced a release of outer-segment ^{40}Ca (see Fig. 8), at a rate which was greater at 500 μM-IBMX than at 100 μM. Furthermore, though the rods in IBMX lost ^{40}Ca, they gained ^{44}Ca (see Fig. 9), and their total Ca content increased. These observations suggest that the release of ^{40}Ca in IBMX was probably also at least in part physiological.

For the ^{40}Ca to be released from the outer segment, it must first be released from the sequestered pool, presumably by passing across the disk membrane, and it must then permeate or be transported across the plasma membrane. Movement across the plasma membrane is unlikely to be rate limiting in our experiments since, in intact photoreceptors, both low-free-Ca solutions and IBMX produce large increases in the light-dependent conductance (Hodgkin *et al.* 1984; Cervetto & McNaughton, 1986), which is permeable to Ca (Yau & Nakatani, 1984*a*; Hodgkin *et al.* 1985). Two mechanisms have been described for Ca release from disks: Na–Ca exchange (Schnetkamp, 1980, 1986) and cyclic-GMP-dependent release (Caretta & Cavaggioni, 1983; Koch & Kaupp, 1985; Puckett & Goldin, 1986). Na–Ca exchange seems to us unlikely to have made a large contribution to Ca release, at least in our experiments with low-free-Ca solutions. Although in Fig. 7 Na was substituted with choline in the *external* medium, the Na content of the inside of the rod should also have been depleted in this experiment, since turn-over of rod Na in low-free-Ca solutions is rapid (Woodruff, Fain & Bastian, 1982). Our experiments seem more consistent with the possibility that the rate-limiting step for Ca release is the opening of cyclic-GMP-dependent channels in the disks. Since the cyclic-GMP-dependent mechanism in isolated disks shares many pharmacological and physiological similarities with the light-dependent conductance in isolated patches of plasma membrane, and since low-free-Ca solutions and IBMX increase the light-dependent conductance, it seems reasonable to suppose that these treatments also open the cyclic-GMP-dependent pathway in the disks. The exact mechanism by which this may occur is at present unknown.

Incubating rods in IBMX also produced an increase in ^{44}Ca accumulation in the rods (see Fig. 9). We presume this occurred simply as a result of an increase in the dark current (Cervetto & McNaughton, 1986). The increase in Ca influx together with the release of Ca from the disks are probably responsible for the IBMX-induced increase in the free-Ca concentration in the outer segment, which has recently been observed in rods filled with the Ca-detecting protein aequorin (McNaughton, Cervetto & Nunn, 1986).

Ca permeability of disks

If as we believe the majority of the rod Ca is normally contained within the disks, the slow exchange of outer-segment Ca which we have observed in normal Ringer solution in darkness (Fain & Schröder, 1985) suggests that the permeability of the

disk membrane to Ca in darkness must be rather low. Assuming a toad rod has about 2000 disks (Bastian & Fain, 1979), the rate of Ca movement across the disk membrane can be estimated from the rate of ^{44}Ca accumulation to be no more than 100 Ca $disk^{-1}$ s^{-1}, or about 10^8 Ca cm^{-2} s^{-1}. This is more than three orders of magnitude smaller than the rate of Ca movement which occurs in darkness per square centimetre across the plasma membrane (about 6×10^{11} Ca cm^{-2} s^{-1}; see Yau *et al.* 1986). However, the rate of ^{44}Ca accumulation which we measure is of the same order as the rate of cyclic-GMP-dependent Ca efflux from leaky-rod or isolated-disk preparations (Caretta & Cavaggioni, 1983; Koch & Kaupp, 1985; Puckett & Goldin, 1986). It is therefore possible that Ca exchange across the disk membrane in darkness normally occurs through partially open cyclic-GMP-dependent channels in the disks. However, other mechanisms such as Na–Ca exchange (Schnetkamp, 1980, 1986) or ATP-dependent Ca uptake (Puckett, Aronson & Goldin, 1985) may also contribute.

Although the Ca in the disks exchanges slowly in normal Ringer solution in darkness, large amounts can be released in low-free-Ca or in IBMX. Our experiments suggest that this may occur through an increase in the cyclic-GMP-dependent permeability of the disk membrane. Light also releases Ca from the disks (Fain & Schröder, 1985). Since, however, light is thought to *decrease* the intracellular free-cyclic-GMP concentration, the light-induced Ca release may not be mediated by the cyclic-GMP-dependent channels but rather by some other mechanism, as yet unknown. A detailed description of light-induced Ca release and re-uptake will be given in a subsequent publication (G. L. Fain & W. H. Schröder, in preparation).

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