Rapid Calcium Release and Proton Uptake at the Disk Membrane of Isolated Cattle Rod Outer Segments. 1. Stoichiometry of Light-Stimulated Calcium Release and Proton Uptake†

U. B. Kaupp,* P. P. M. Schnetkamp,‡ and W. Junge

ABSTRACT: We reported a rapid, light-stimulated release of calcium from isolated rod outer segments that is apparent only when both the disk membrane and the plasma membrane are made permeable to calcium by adding the ionophore A23187 [Kaupp, U. B., Schnetkamp, P. P. M., & Junge, W. (1979) *Biochim. Biophys. Acta 552*, 390–403]. In this paper, we have investigated the light-sensitive diskal binding sites and the calcium release mechanism in their dependence on the pH and the presence of mono- and divalent cations, including calcium itself. We have observed now that several different rod outer segment preparations (i.e., rod outer segments with an intact plasma membrane, broken cells, and sonicated material) possess a similar dependence of their calcium release on the ionic conditions, however, only if manipulated in a way that gives access to the outer conditions of sites within disks (namely, ionophore added in the case of intact rod outer segments). Monovalent cations, at concentrations between 20 and 40 mM, suppress light-induced calcium release. Divalent and trivalent cations are more efficient inhibitors by 1–2 and 2–3 orders of magnitude, respectively. These results suggest that calcium release is controlled by an interfacial potential. The optimum pH for calcium release is pH 6.3, and virtually no release occurs beyond pH 4.5 and 9. The drop for acidic pH is attributed to the pH dependence of calcium binding to disk membranes, and the drop for alkaline pH is attributed to the pH dependence of the metarhodopsin I/metarhodopsin II transition and the light-stimulated proton uptake. In general, calcium release parallels calcium binding as a function of pH and calcium concentrations, although the release saturates at lower calcium concentrations (K_D^{app} = 5 μM) than would be expected from the amount of calcium bound (K_D = 30–60 μM). The maximum stoichiometry is ~1 mol of calcium release per mol of rhodopsin bleached. Concomitant measurements of the light-stimulated uptake of protons by the disk membrane revealed a maximal stoichiometry of 2.8 mol of protons taken up per mol of rhodopsin bleached. We present an integrated description of light-stimulated calcium release, proton uptake, and changes of the interfacial potential at the disk membrane.

A number of studies of calcium fluxes in a variety of disk preparations and liposomes containing rhodopsin have now firmly established that light has some effect on the redistribution of calcium between disk vesicles and the suspending medium [see Smith et al. (1977) and Kaupp et al. (1979b)]. [A different phenomenon of calcium release in intact retinas has been described by Yoshikami et al. (1980) and Gold & Korenbrot (1980)]. Several authors interpreted their results in terms of a light-regulated change in the permeability of the disk membrane to calcium. The release stoichiometry from disk vesicles loaded with high calcium never exceeded 2 mol of calcium released per mol of rhodopsin bleached (e.g., Smith & Bauer, 1979). A stoichiometry of 20–90 mol of calcium/mol of rhodopsin bleached was obtained only with artificial rhodopsin–phospholipid recombinants containing only a few rhodopsin molecules per vesicle (O'Brien, 1979). When time resolution of Ca^{2+} release was achieved either by a rapid filtration technique (Smith et al., 1977), by spectrophotometry with the calcium-indicating dye arsenazo III (Smith & Bauer, 1979), or by employment of a calcium-sensitive electrode (Shevchenko, 1976), calcium release was found to occur on a minute time scale.

At this point, it has to be remembered that the physiological events in visual transduction occur on a time scale of some 100 ms–1 s. In the presence of a divalent cation ionophore (A23187), we observed a more rapid calcium release from binding sites with half-rise times of 300 ms (intact rod outer segments) and 15 ms (fragmented rod outer segments) by rapid flash spectrophotometry with the dye arsenazo III (Kaupp et al., 1979a,b, 1980).

Upon fragmentation of intact rod outer segments, either by lysis or sonication, the light-induced calcium release was no longer observable (Kaupp et al., 1979a,b), but this effect could be prevented by reducing the ionic strength of the final suspension medium (Kaupp et al., 1980). In the presence of A23187, the velocity of calcium release from sonicated disk vesicles in a low electrolyte medium is greatly enhanced ($\tau_{1/2} \simeq 15$ ms), as compared with the release in intact material ($\tau_{1/2}$ = 300 ms; Kaupp et al., 1980). Evidence has also been presented that the metarhodopsin I/metarhodopsin II transition is involved in the proper release step.

The disk membrane takes up protons upon illumination (McConnell et al., 1968; Emrich, 1971; Bennett, 1978). The uptake occurs within some 10 ms after illumination, and with a stoichiometry of one to three protons taken up per rhodopsin bleached (Emrich, 1971; Bennett, 1978; Kaupp et al., 1981). Recently a rapid, light-induced change of an interfacial potential at the disk membrane/water interface was reported (Cafiso & Hubbell, 1980). This change of an interfacial potential was attributed to the rapid uptake of protons into the "boundary" layer of the disk membrane.

In the light of these findings, it is tempting to speculate that the rapid uptake of protons after photoexcitation of rhodopsin could be related to the release of bound calcium from disk membranes. A similar calcium/proton exchange mechanism at the disk membrane was hypothesized several years ago (Reich & Emrich, 1976). We have investigated the mecha-

† From the Department of Biophysics, University of Osnabrück, D-4500 Osnabrück, Federal Republic of Germany. *Received February 2, 1981.* This work was financially supported by the Deutsche Forschungsgemeinschaft.

* Address correspondence to this author at the Department of Physiology and Biophysics, State University of New York, Stony Brook, NY 11794.

‡ Present address: Department of Chemistry, University of California, Berkeley, CA 94720.

0006-2960/81/0420-5500$01.25/0

nism of calcium release from binding sites with regard to the role of (1) proton uptake, (2) medium pH, (3) ionic screening effects, and (4) fragmentation of intact rod outer segments. In this paper, we will detail the properties of the calcium release and proton uptake stoichiometry, and in the following paper in this issue, the kinetic properties of both processes (Kaupp et al., 1981).

Materials and Methods

Preparation. Intact cattle rod outer segments were prepared as described previously (Schnetkamp et al., 1979) and stored as a concentrated stock suspension (150–200 μM rhodopsin) at 4 °C for not more than 3 days. No significant loss of either the calcium release or the proton uptake capacity was detected. Within this period of time, intact rod outer segments were lysed by an osmotic shock (40-fold dilution) with distilled water. Sonicated disk vesicles were obtained by sonication of intact rod outer segments with a Branson Sonifier (Model B-12 with a microtip) for 30 s at 40 W in an ice bath. The sonicated suspensions were used immediately.

The rhodopsin concentration was determined according to deGrip et al. (1972). The final suspension contained 600 mM sucrose, 0.25 v/v Ficoll 400, 30 μM arsenazo III, and 3–6 μM rhodopsin. The free calcium concentration was 3–5 μM with the exception of those experiments in which the calcium dependence of the release stoichiometry was studied. Intact rod outer segments were buffered with Tris-HCl[1] (20 mM) at pH 7.4. In fragmented material, Tris was replaced by Hepes. The deleterious effect of Tris on the release stoichiometry in either lysed or sonicated material is probably due to binding of this buffer to biological membranes (Eisenberg et al., 1979). The buffer concentration in all experiments with fragmented material was reduced to 2 mM to keep the ionic strength of the medium low. The proton buffering capacity was sufficient to suppress any light-induced pH changes in the suspension. For ΔpH measurements, external proton buffers were omitted, and the indicator arsenazo III was replaced by bromocresol purple (30 μM).

Calcium Measurements. Rapid changes in the free calcium concentration were followed optically by using the indicator dye arsenazo III (Brown et al., 1975) (Sigma, grade I) in the flash spectrophotometer (see below). Stock suspensions of rod outer segments were diluted to a final concentration of 3–6 μM rhodopsin, 1–3 μM free Ca^{2+} in the presence of arsenazo III, and 10 μM divalent cation ionophore A23187. The ionophore-mediated efflux of endogenous calcium from rod outer segments was followed in a conventional spectrophotometer via the absorption of arsenazo III at 655 nm. Complete equilibration was apparent from a stable absorbance of the suspension at 655 nm, usually within 2–3 min. The equilibrating efflux of the endogenous calcium raised the concentration of free calcium to a value of about 4–6 μM.

In order to relate the calcium-indicating absorption changes of arsenazo III to nanomoles of calcium released, aliquots of calcium were added to the same suspension that was used in the light-release experiments, and absorption changes were monitored (Scarpa et al., 1978; Kaupp et al., 1979b). This calibration procedure automatically accounts for the intrinsic calcium buffering capacity of the disk membranes, the dissociation constant of arsenazo III (K_D^{ars}), and the free calcium concentration. It is convenient that the knowledge of each of these parameters separately is not required. In experiments where external free Ca^{2+} concentration was varied systematically (e.g., Figure 5), the absorption changes of arsenazo III were recalibrated for each sample. This recalibration was necessary because of the calcium dependence of the differential sensitivity of arsenazo III (Kaupp et al., 1979b) and, to a minor extent, because of the calcium dependence of the intrinsic calcium buffer capacity of disks. As a control, we calculated the differential sensitivity of arsenazo III (Kaupp et al., 1979b), considering the intrinsic calcium buffering capacity.

Arsenazo III is responsive not only to divalent cations but also to protons. Both the molar extinction coefficient of the dye/calcium complex, ϵ_{CaA}, and its dissociation constant, K_D^{ars}, vary with pH. These variations of the spectral and binding properties of the indicator were accounted for in experiments designed to determine the pH dependence of the light-induced calcium release.

The calcium release stoichiometry at various concentrations of free calcium was determined according to the following protocol. Rod outer segments were treated with A23187 (10 μM) in the presence of EDTA (500 μM) to remove all endogenous calcium from the membranes. The treated suspension was centrifuged and resuspended in calcium-free standard medium, and the calcium concentration was adjusted by addition of aliquots of calcium standard solutions. A spectrum was recorded for the determination of the free calcium concentration, before and after excitation of the suspension in the flash spectrophotometer. After recording the calcium-indicating absorption changes, the flash-induced arsenazo III response was recalibrated as described above. The filter action of arsenazo III against the exciting light source at 530 nm under the variable calcium concentrations was accounted for. Free calcium concentrations were determined from the absorbance of the suspension at 655 nm, taking the steady light-scattering level of the suspension at this wavelength into account.

The effect of medium pH on the calcium release stoichiometry was measured by use of the following buffers at 20 mM: below pH 6.5, Mes; between pH 6.5 and 7.5, Mops; above pH 7.5, Tris. Mes and Mops buffers were adjusted with Tris and Tris buffers with HCl to the appropriate pH value. We determined the pH dependence of calcium binding to the disk membrane. Aliquots of calcium-depleted intact rod outer segments (rhodopsin concentration 20 μM) were suspended at the appropriate pH values, and calcium was added in the presence of A23187 (2 μM) to give a final concentration of 150–250 μM free calcium. The suspensions were centrifuged at 3500*g* for 30 min, and the calcium concentration in the supernatant was determined with arsenazo III. The calcium content of the pellet was measured in the presence of 0.5% (v/v) Triton X-100 (Triton X-100 greatly reduced the intrinsic calcium buffering capacity of rod outer segments, thereby releasing all calcium from binding sites on the membrane).

Proton Measurements. Rapid, light-initiated changes of the pH in a rod outer segment suspension were followed by using the pH indicator bromocresol purple in the flash spectrophotometer. Bromocresol purple (pK_a = 6.1) is suitable for experiments in the region between pH 5.6 and 6.6, and it does not bind to disk membranes to a significant extent (Bennett, 1980). The dye responded only to pH changes in the aqueous phase; a rod outer segment suspension buffered with Mes/Mops (20 mM) at pH 6.0 in the presence of bromocresol purple showed no "ΔpH-indicating" absorption change due to the dye. However, a small absorption change

[1] Abbreviations used: Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mops, 3-(*N*-morpholino)propanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,-*N*',*N*'-tetraacetic acid.

caused by the rhodopsin photochemistry was observed. Its amplitude was about 15% of the amplitude of the pH-indicating absorption changes of bromocresol purple in the absence of added proton buffer and was accounted for in the quantitative determinations of proton uptake stoichiometries. The procedure for calibrating ΔpH-indicating absorption changes of bromocresol purple into moles of H^+ taken up per mole of rhodopsin bleached was similar to the procedure adopted for the calcium calibration (see above). The pH of the rod outer segment suspension was adjusted to the desired value by adding aliquots of NaOH or HCl. The cuvette was excited by a flash of light, and the ΔpH-indicating absorption changes were recorded. The flash-induced absorption change was calibrated in the same sample. Usually a change of 1 μM in the total proton concentration yielded a change in the relative transmission $\Delta I/I = (7–9) \times 10^{-3}$ in a medium at pH 6.0 containing 3 μM gramicidin, 10 mM NaCl, 600 mM sucrose, and 30 μM bromocresol purple. The change in relative transmission was corrected for the dilution effect during H^+ injection (0.25%).

Rapid Flash Spectrophotometry. The principle and details of instrumentation of time-resolving flash spectrophotometry have been reviewed elsewhere (Junge, 1976). Following addition of the indicator to rod outer segments, the suspension was allowed to equilibrate for ~1 min in the presence of an appropriate ionophore. The suspension was excited by a laser flash (half-duration time 10 ns) at 530 nm; the calcium-indicating absorption changes of arsenazo III were monitored at 655 nm and the pH-indicating absorption changes of bromocresol purple at 592 nm. If required, the differential sensitivity of arsenazo III ($d[A]/d[Ca^{2+}]$) and of bromocresol purple ($dA/d[H^+]$) were determined in the same cuvette after excitation by light. The relative light-induced changes of the free calcium concentration or of the bulk pH in the cuvette were small ($dp[Ca]$ or $dpH = 10^{-2}$); therefore the conditions before and after the flash were almost identical with regard to the sensitivity of the dyes.

The number of rhodopsin molecules bleached per flash was determined from the absorption change at 382 nm. The molar extinction coefficient $\epsilon_{380} = 40\,000\ M^{-1}\ cm^{-1}$ was used to calculate the amount of metarhodopsin II produced from the absorption change. We note that the absorption changes at 382 nm and the Ca^{2+} or pH changes were recorded under identical conditions.

Results

Calcium Release as a Function of the Ionic Conditions. The stoichiometry of calcium release from fragmented rod outer segments is dependent upon the concentration of various ions in the suspending medium (Figure 1). The light-induced calcium release was greatly reduced in the presence of more than 10 mM of either NH_4Cl, NaCl, KCl, LiCl, or tetraethylammonium chloride (TEA^+Cl^-). The effect was greatest for NH_4^+ and smallest for the large cation TEA^+. The solid line in Figure 1 describes the predicted influence of the screening of the surface potential by a given monovalent cation concentration on the binding of calcium to sites located at the membrane surface (eq A14, Appendix I). We estimated a 2 mM contamination (~1.5 mM NaCl and 0.5 mM $Tris^+$) with cations from the rod outer segment preparations and Hepes buffer, and it was for this reason that the maximum stoichiometry in Figure 1 was ascribed to a concentration of monovalent cations, $c_{mono} = 2$ mM. Zwitterionic species, e.g., Hepes, were not considered, although they may also contribute to a screening effect.

Magnesium cations were almost 2 orders of magnitude more efficient than monovalent cations in their ability to inhibit

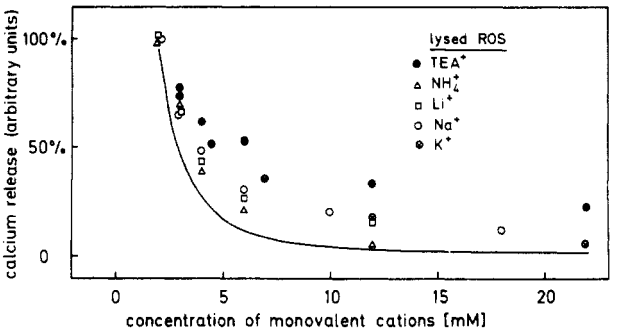

FIGURE 1: Calcium release stoichiometry in fragmented rod outer segments at various concentrations of different monovalent cations. Reaction medium contained 600 mM sucrose, 0.25% (v/v) Ficoll 400, 2 mM Hepes/NaOH buffer at pH 6.8–7.0, 30 μM arsenazo III, 10 μM A23187, 4–5 μM rhodopsin concentration and 3–4 μM free calcium concentration. Temperature 20 °C. The solid line represents the expectation of the decrease of the release stoichiometry by screening of the surface potential by monovalent cations. It has been calculated according to eq A14 of Appendix I. Note that the concentration of monovalent cations at which the calcium release stoichiometry was at maximum was set at $C_{mono} = 2$ mM. This approximately accounts for the residual concentration of monovalent cations which were introduced by the pH buffer, arsenazo III, and the stock suspension of rod outer segments. 100% corresponds to a stoichiometry of about 0.7 mol of Ca^{2+}/Rh*. Data were obtained at a bleaching percentage of about 3–4%. Each concentration point was determined independently from a fresh sample. Samples were taken from one preparation batch.

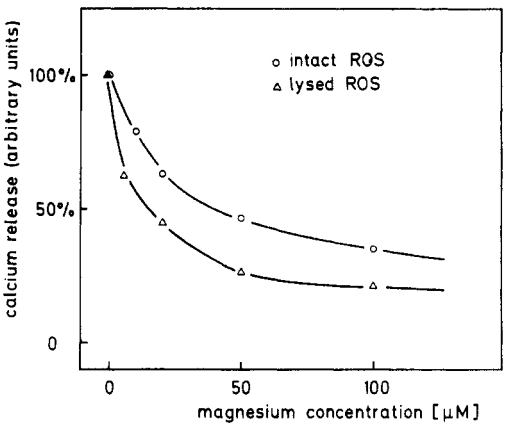

FIGURE 2: Calcim release stoichiometry at various concentrations of magnesium ions in intact (○) and fragmented (△) rod outer segments. Conditions as in Figure 1.

calcium release (Figure 2): 100 μM Mg^{2+} reduced the stoichiometry by the same degree as 5–10 mM Na^+. When trivalent cations were tested, a concentration of 10 μM was sufficient to abolish calcium release completely (Figure 7). Streptomyocin, a trivalent amino sugar, was chosen for these experiments because it does not interfere with arsenazo III (in contrast to La^{3+} which strongly interacts with arsenazo III). The suppression of calcium release by other ions in fragmented material was not dependent on the particular fragmentation procedure used; sonicated and lysed rod outer segments were equally susceptible to the presence of positively charged ions.

Unlike the release observed with fragmented material, calcium release in intact rod outer segments is not very sensitive to the presence of monovalent cations such as Na^+ or K^+ (Figure 3). In the presence of 16 mM NaCl, the maximum stoichiometry was reduced by about 25% whereas the same concentration of NaCl inhibited calcium release almost completely in lysed material. This result could mean that either the cation or anion alone or both were nonpermeant in

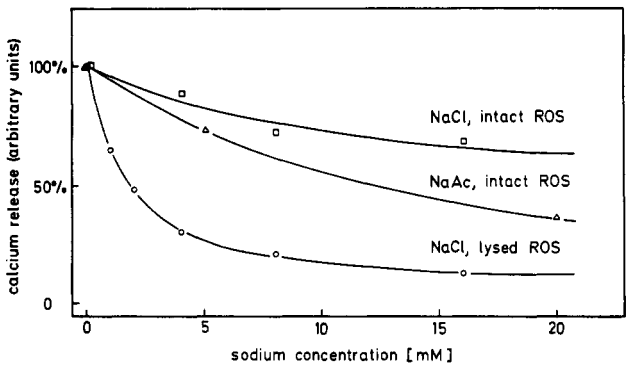

FIGURE 3: Comparison of the susceptibility of the calcium release stoichiometry to sodium ions in intact and fragmented rod outer segments. Medium conditions: intact rod outer segments + NaCl (□); intact rod outer segments + NaOAc (△); fragmented material + NaCl (○). Further conditions as in Figure 1.

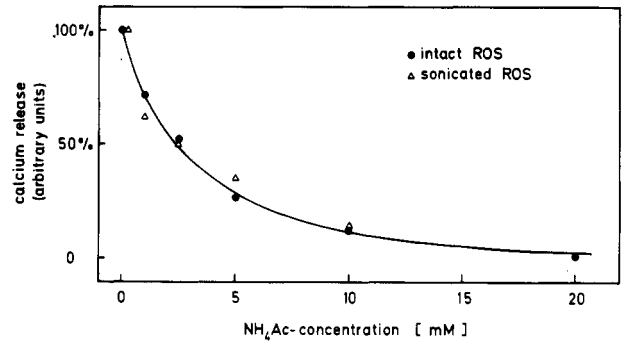

FIGURE 4: Comparison of the susceptibility of the calcium release stoichiometry to NH_4OAc (ammonium acetate) in intact (●) and fragmented (△) rod outer segments. Conditions as in Figure 1.

intact rod outer segments. It may be possible to discriminate between these possibilities by employing cation-selective ionophores and/or anions that are permeant for biological membranes, e.g., weak acids like acetic acid/acetate couple. Several control experiments indicate that the differences in the effects of monovalent cations on calcium release in the various preparations are a function of the permeability properties of the plasma and/or disk membrane in intact rod outer segments.

$Tris^+$ (20 mM) reduced calcium release in fragmented material nearly completely (not shown) but did not affect Ca^{2+} release in intact rod outer segments even after incubation times of 2–3 days (e.g., storage of stock suspension).

In the presence of a permeant anion, NH_4OAc, no differences between the release stoichiometry of Ca^{2+} from intact rod outer segments and from fragmented ones could be distinguished (Figure 4).

When experiments were performed in the presence of potassium acetate and gramicidin, the reduction of the release stoichiometry in intact rod outer segments was maximum if compared with KCl alone or KCl + gramicidin (Table I).

This evidence indicates that in order to reduce calcium release, ions must have access to the interior of intact rod outer segments. It is also evident, however, that some structural factors limit the screening ability of cations, e.g., stacking of disks; compare lysed and intact rod outer segments. This phenomenon can also be seen in Figure 2, where in the presence of Mg^{2+} (100 μM) and A23187 calcium release in intact rod outer segments was somewhat less reduced than in lysed rods.

The above data show that the accessibility of monovalent cations to calcium binding sites inside disks is hindered by the limited permeability of the outer envelope and/or the disk membrane for either these cations or for the anion (Cl^-), which results in a diffusion potential. Furthermore, the ionophore A23187 apparently did not contribute to the permeability of rod outer segment membranes for monovalent cations under these conditions.

Table I: Stoichiometry of Ca^{2+} Release in Intact Rod Outer Segments at Various Ionic Conditions

conditions	calcium release (%)	
	intact rod outer segments	fragmented rod outer segments
control	100	100
5 mM KCl	97	35
10 mM KCl	83	18
10 mM KCl + 3 μM gramicidin	53	18
10 mM KOAc + 3 μM gramicidin	40	18

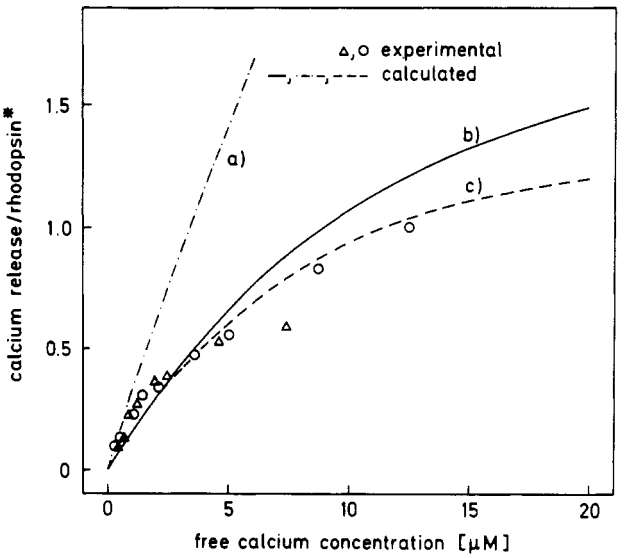

FIGURE 5: Calcium release stoichiometry at various calcium concentrations in intact (○) and fragmented (△) rod outer segments. Conditions as in Figure 1. Free calcium concentrations were calculated from the absorbance of the suspension at 655 nm under consideration of the steady light scattering level at this wavelength. (a) Expectation of the release stoichiometry by assuming model A of the release mechanism (see text). It was calculated according to eq 1 under the assumption that binding sites disappear completely upon photolysis of rhodopsin. The following parameters were used: K_D = 30 μM, $[BS]_0$ = 40 μM, and rhodopsin, 4 μM. (b) Same calculation as in (a) but according to eq A3 of the appendix and assuming that the change of the dissociation constant ΔK_D = 30 μM. the percentage of rhodopsin bleaching has been similarly accounted for as in (a), e.g., $\Delta[Ca]_b/[Rh^*] = [[Ca]_f[BS]_0/([Ca]_f + K_D^2)](1/[Rh])\Delta K_D$. (c) Expectation of the calcium release stoichiometry assuming model B of the release mechanism (see text). It was calculated according to eq A11b, assuming that the surface potential did not change with calcium levels within the concentration range of our measurements (i.e., $\tanh(ze\phi_s/2kT)$ = const). Furthermore, the amount of $\Delta[Ca]_b$ was related to the amount of rhodopsin bleached in order to obtain stoichiometries $\Delta[Ca]_b/Rh^*$. The following parameter was used: $\Delta K_D/K_D = 5 \times 10^{-2}$. Further conditions as in (a).

Calcium Dependence of Light-Triggered Calcium Release. When the concentration of free calcium in suspensions of rod outer segments was raised, a concomitant increase of the release stoichiometry was observed (Figure 5). The experimentally observed titration behavior of the calcium release shown in Figure 5 (circles and triangles) deviates from a simple binding isotherm. The curves were calculated according to different models discussed below. Two factors limited the range of free calcium concentrations within which accurate

and reliable measurements were possible, the decreased sensitivity of arsenazo III at high calcium concentrations (Kaupp, 1979) and the low occupation state of calcium binding sites at low calcium concentration, which allows little calcium release. It is for these reasons that the data shown in Figure 5 are restricted to concentrations ranging from 0.5 to 12.5 μM of free calcium, as determined from the absorbance of arsenazo III at 655 nm (see Materials and Methods). If it is assumed that the determination of the absorbance of arsenazo III at 655 nm in a rod outer segment/arsenazo III suspension is in error by $\Delta A = 3 \times 10^{-3}$, our calculations of the free calcium concentration are false by 20% at $[Ca]_f = 1$ μM and by 45% at $[Ca]_f = 15$ μM. Similar arguments apply to the empirical calibration procedure of the light-induced absorption changes at low and high calcium concentrations. We conclude that the deviations of the calcium release stoichiometry from what is expected by the amount of calcium bound cannot be attributed to an inherent inaccuracy in our spectroscopic measurements or to systematic errors.

The pH Dependence of Light-Triggered Calcium Release and Calcium Binding. The stoichiometry of the light-triggered calcium release is dependent on the pH in the medium. This is documented in Figure 6. At pH values below pH 4.5 and above pH 8.5, virtually no calcium release occurred. A maximum was observed at pH 6.2–6.5. However, the suppression of calcium release at alkaline pH is different in rods with an intact plasma membrane than in disintegrated rod outer segments lacking an intact envelope (Figure 6). It has been inferred previously that the plasma membrane of intact rods is relatively impermeable to protons on a time scale of seconds to minutes (Hagins & Yoshikami, 1977; Schnetkamp et al., 1979), and it was conceivable that a pH gradient existed between the outer suspending medium and the cell interior after the medium pH had been changed. When NH_4OAc (1 mM), a weak acid protonophore, was included in the medium, together with the appropriate pH buffers, the pH dependence of the calcium release in intact rod outer segments was not distinguishable from fragmented material (see Figure 6). This shows that the plasma and/or the disk membrane in intact rod outer segments is poorly permeable to protons; incubation times up to 10 min are not sufficient to allow complete equilibration. Consequently the pH sensed by the sites for calcium release was different from that determined in the outer aqueous phase. However, in the presence of NH_4OAc, any preexisting pH gradient is rapidly dissipated due to the protonophoric action of these weak acids [see also, for a recent review of protonophores, McLaughlin & Dilger (1980)].

The following controls were carried out to ensure that the pH dependence of calcium release is a true effect and not merely a reflection of the deterioration of the rod outer segments at pH values which differ from the physiological pH (pH 6.8–7.4). Rod outer segments were incubated for 10 min at pH 9 or 5, the pH was readjusted to pH 6.8, and the light-induced calcium release was recorded and compared with a sample not subjected to the incubation at extreme pH values. The release stoichiometry of the pretreated rod outer segments was reduced by $\leq$20% of the maximum value observed in rod outer segments that were not subjected to extreme pH values.

The observed pH dependence of calcium release could result from a combination of both pH dependence of calcium binding to the disk membrane and the pH dependence of the actual release step. We therefore determined the pH dependence of both calcium binding and the metarhodopsin I/metarhodopsin II transition which has been shown to be involved in the proper release mechanism (Kaupp et al., 1980). The variation of both

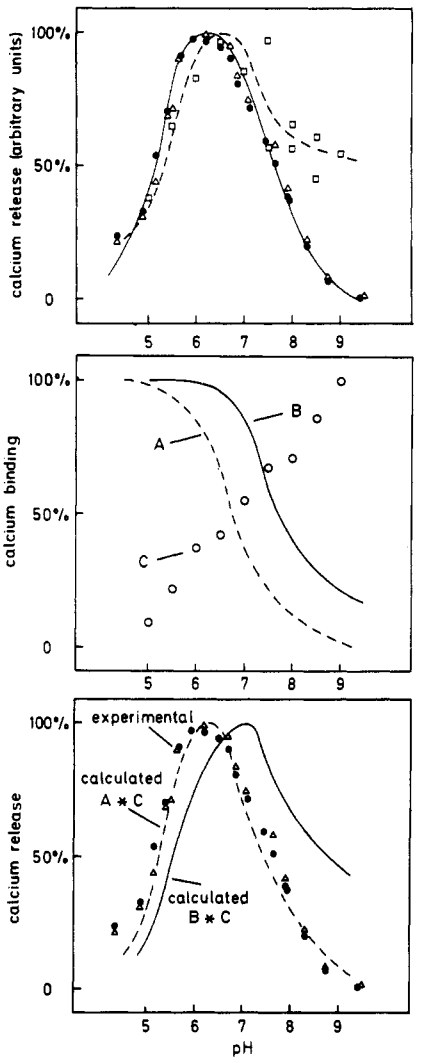

FIGURE 6: (Upper) pH dependence of the calcium release stoichiometry in intact rod outer segments (□), intact rod outer segments in the presence of 1 mM NH_4OAc (●), and sonicated rod outer segments (△). Conditions as in Figure 1 with the exception that 20 mM pH buffer was used in intact rod outer segments. Samples of intact rod outer segments without NH_4OAc were incubated for 10 min at room temperature (20 °C) at the appropriate pH. Sonicated rod outer segments and intact rod outer segments in the presence of NH_4OAc were used immediately after mixing. (Middle) pH dependence of the calcium binding to disk membranes in intact rod outer segments. The binding stoichiometry was determined as described under Materials and Methods. The reaction medium contained 600 mM sucrose, 0.25% (v/v) Ficoll 400, 20 mM respective pH buffer, 2 μM A23187, and 21 μM rhodopsin. The free calcium concentration varied from $[Ca]_f = 240$ μM (pH 5) to $[Ca]_f = 170$ μM (pH 9). (Solid line) pH dependence of metarhodopsin II formation, redrawn from Bennett (1978); (dashed line) pH dependence of light-induced change of an interfacial potential at the disk membrane, redrawn from Cafiso & Hubbell (1980). The pH dependence of the light-induced proton uptake is similar to that of the interfacial potential change and has been omitted for clarity. Data were normalized to their respective maximal values. (lower) Calculation of the calcium release stoichiometry at various pH values under the assumption that the stoichiometry is proportional to the amount of bound calcium and weighted by the amplitude of the proposed trigger steps [i.e., metarhodopsin I/metarhodopsin II transition (B) and a change in an interfacial potential or proton uptake (A)]. Calculated values have been renormalized to the maximal value. For convenience, the stoichiometry data from the upper part have been included (filled circles and triangles).

processes as a function of pH is depicted in Figure 6B. There was a continuous, almost 10-fold increase of calcium bound to membranes when the pH was raised from pH 5 to 9 whereas the metarhodopsin II formation decreased in amplitude by

about 4–5-fold (Figure 6B). A comparison of the upper and middle parts of Figure 6 indicates that the rise in the stoichiometry at pH 6.3 can be accounted for by the concomitant increase in calcium bound to the membrane (Figure 6, C). The drop at pH ≥ 6.5 cannot be attributed solely to the decay of the metarhodopsin II formation at these pH values.

Stoichiometry of the Light-Stimulated Proton Uptake. When intact rod outer segments were illuminated in standard medium without extra proton buffer in the presence of bromocresol purple (30 μM), a small absorption change was observed at 592 nm ($\Delta I/I = 1.5 \times 10^{-3}$ when 10% of the rhodopsin was bleached). This absorption change was completely abolished upon addition of Mes/Mops buffer (20 mM) to the suspension. This effect of 20 mM proton buffer was not due to an increase in ionic strength because addition of either KCl or NaCl (20 mM) did not prevent the absorption change. When either FCCP or gramicidin (3 μM) were included into the suspension, two effects were observed: the apparent proton buffering capacity increased by about 2–2.5-fold due to the access of buffering groups located inside and the pH-indicating absorption changes of bromocresol purple increased by a factor of 4–6. Thus the apparent stoichiometry of the proton uptake went from 0.28 (without protonophore) to 1.5–2.5 H^+/rhodopsin bleached (with protonophore).

Following fragmentation of intact rod outer segments, either by lysis or by sonication, the velocity of the "ΔpH-indicating absorption changes" increased in a manner similar to that previously observed for calcium release (Kaupp et al., 1980, 1981). However, the stoichiometry of the proton uptake remained constant, and no protonophores were required to give the maximum stoichiometry.

When calcium and A23187 were added to calcium-depleted rod outer segments (see Materials and Methods), the release of protons from the disk membrane was observed in the dark; the stoichiometry of this calcium/proton exchange was approximately 1:2 (not shown). We also investigated whether a similar exchange of protons and calcium occurs during light-stimulated processes. When the stoichiometry of proton uptake was determined under conditions of a high or low occupation state of the calcium binding sites (e.g., in the presence of excess calcium or in the presence of excess EGTA), similar results were obtained. We concluded that this rapid, light-triggered proton uptake is not very sensitive to the "loading status" of the disk membrane.

Compartmentation Behavior of the Calcium Release and Proton Uptake. Rod outer segments with either intact or leaky outer membranes gave no observable calcium release after excitation by a light flash unless the ionophore A23187 was added to the suspension medium (Kaupp et al., 1979a). This suggested that calcium is released from binding sites located on the lumenal side of the disk membrane. In sonicated or lysed rod outer segments, however, the situation seems more complicated: a substantial amount of calcium was released without A23187 present, independent of the fragmentation procedure (Figure 7). When the ionophore was included, an increase in calcium release was detected. These findings suggest that (1) some calcium is released into a compartment that is accessible only upon addition of A23187 and (2) light-triggered calcium release in the absence of A23187 probably involved a calcium pool either on the outside or at an accessible inner space of disk vesicles.

This possibility was tested by the following experiments. The trivalent cation streptomycin was added in micromolar amounts to a suspension of fragmented disks. In the absence

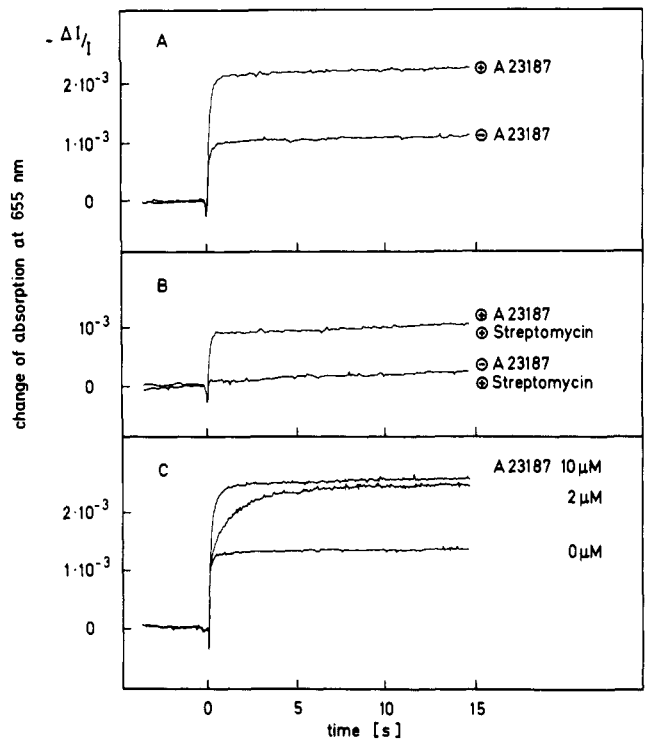

FIGURE 7: Compartmentation behavior of calcium release in sonicated rod outer segments. (A) A23187, 10 μM, further conditions as in Figure 1. (B) A23187, 10 μM, and streptomycin, 10 μM; further conditions as in Figure 1. (C) Concentrations of A23187 as indicated; further conditions as in Figure 1.

of A23187, the addition of streptomycin completely abolished calcium release (Figure 7). However, in the presence of both streptomycin and A23187 that fraction of calcium release made accessible by the ionophore could still be observed (Figure 7). Comparison of parts A and B of Figure 7 indicates that calcium release in the presence of streptomycin and A23187 is approximately equal in magnitude to the fraction observed in the presence of A23187 alone. The concept of two different calcium pools, one inside of closed vesicles and another, perhaps in the form of inside-out vesicles or open vesicles from which calcium is accessible to arsenazo III, is further supported by the dependence of the rate of calcium release on the A23187 concentration. No effect of A23187 on the release rate from the outer side was observed. The appearance of two kinetic components, observed in the presence of low concentrations of A23187, is a further indication of two distinct calcium pools that reside in different compartments in a suspension of fragmented disks. In similar experiments, we found that rapid proton uptake was not influenced by protonophores in fragmented material. In contrast to the experimental observations with Ca^{2+}, both the kinetics and the stoichiometry of proton uptake remained unaffected.

Discussion

Calcium Release is Sensitive to Changes in Surface Potential. The experiments presented here show that ions other than calcium can depress light-induced calcium release if they have access to a space within disks (e.g., through an appropriate ionophore). Particularly noteworthy in this respect is the observation that trivalent and divalent cations are respectively 1000- and 100-fold more efficient inhibitors than monovalent cations. (Technical considerations precluded the testing of a large number of different divalent and trivalent cations. The specific ratios between mono-, di-, and trivalent cations may vary with the ionic species.) This sequence for the efficiency of the screening of negative fixed charges has

been observed at the surface of other membranes. The magnitude of the surface potential as a function of the surface charge density and ionic screening is commonly described by the Gouy–Chapman theory [for a biologically directed review, see McLaughlin (1977)].

The efficiency by which monovalent cations decreased the light-induced calcium release from disks varied in the order $TEA^+ < K^+ \lesssim Na^+ \sim Li^+ < NH_4^+ \lesssim Tris^+$. Such a sequence is not predicted within the framework of the point-charge approximation of the Gouy–Chapman theory. A similar lyotrophic sequence was found in studies of the cation binding to phospholipid membranes (Puskin, 1977; Kurland et al., 1979; Eisenberg et al., 1979).

Mono-, di-, and trivalent cations could exert their effects on calcium release by one or more of the following processes: "screening" of the surface potential, reduction of the surface charge density by binding, and electroneutral competition for binding sites shared with calcium at the disk membrane. With monovalent cations, the first mechanism is probably the most important. The solid line in Figure 1 describes the predicted relative decrease of the stoichiometry if only "screening" were the mechanism for the release inhibition. It was calculated according to eq A14 of Appendix I. When plotted double logarithmically, a straight line with a slope of −2 is obtained. However, when plotted in this way, the data of Figure 1 yielded slopes ranging between −0.9 and −1.8 (not shown). A decreased screening efficiency was observed for manganese binding to liposomes in the presence of sodium (Puskin, 1977). This effect was later found to be due to manganese/proton competition for common binding sites (Puskin & Coene, 1980). A similar competition may exist in disk membranes between calcium and protons.

The influence of cations other than calcium on the release properties in intact ROS is governed by the accessibility of the calcium binding sites for these ions. When highly permeable cations and anions (e.g., $NH_4^+OAc^-$) were employed, intact and fragmented rod outer segments behaved similarly with respect to the screening efficiency of the monovalent cation (Figure 4).

Thus, inhibition of the rapid, light-induced calcium release from binding sites at the disk membrane by cations other than calcium can be qualitatively explained by the influence of these ions on the surface potential inside disks, which in turn modifies the apparent binding constant K_D of binding sites for calcium ions.

Calcium Release Reflects a Light-Sensitive Change of the Binding Constant K_D. At least two alternative release mechanisms are suggested by our results. (A) In the first mechanism, it is assumed that calcium binds exclusively to rhodopsin itself. Following the excitation of rhodopsin by light, these binding sites undergo a change, resulting in a drastic increase of the dissociation constant. Alternatively, (B) calcium could be bound to the phospholipid core of the disk membrane. Photolysis could then affect the binding properties of *all* binding sites at the membrane, via a decrease in the surface potential.

The release stoichiometry at various calcium levels expected from the first reaction scheme is depicted in Figure 5a,b. It was calculated according to a simple binding isotherm (for definition of symbols, see Appendix I)

$$[CaBS]/[Rh^*] = [Ca]_f[BS]_0/([Ca]_f + K_D)(1/[Rh]) \quad (1)$$

assuming that the binding sites disappear completely upon bleaching and, therefore, that the relase stoichiometry is directly proportional to the amount of bound calcium (K_D = 30 μM, $[BS]_0$ = 40 μM, and [Rh] = 4 μM). It is clear that both the maximum stoichiometry and the apparent binding constant of the observed "release isotherm" are different from those predicted by this model. This is also true if it is assumed that the dissociation constant K_D is only changed by ΔK_D = 30 μM (Figure 5b, calculated according to eq A3 in Appendix I and other parameters as in Figure 5a).

Alternatively, calcium release initiated by a small increase of the dissociation constant, K_D, due to a change in the surface potential was calculated according to eq A3 or A11b in Appendix I. A relative change of the dissociation constant $\Delta K_D/K_D = 5 \times 10^{-2}$ was assumed (Figure 5c, other parameters as used for Figure 5a,b; see also legend to Figure 5). This model fits the data much more satisfactorily. One major implication of this model is the delocalization of the calcium release events from the site of triggering. How can this occur? The primary electrostatic effect may be localized to a single rhodopsin molecule and extend only by some 20–30 Å due to screening by a Debye–Hückel cloud. However, the rapid lateral mobility of rhodopsin in the disk membrane could justify an analysis in terms of a smeared out and almost homogeneous surface charge density. The following considerations may justify the validity of this assumption. When 2–4% of the rhodopsin molecules are bleached, the area covered by one bleached rhodopsin molecule is about 10^{-11} cm^2 (r = 180 Å). The time required by a rhodopsin molecule to diffuse a distance Δr = 200 Å in a plane is estimated according to Einstein–Smoluchowski $\langle r^2 \rangle = 4Dt$ wherein $\langle r^2 \rangle$ denotes the mean square displacement of the molecule, D the translational diffusion coefficient, and t the time, respectively. Assuming a diffusion coefficient $D = 5 \times 10^{-9}$ cm^2 s^{-1} for rhodopsin in the disk membrane (Poo & Cone 1974; & Liebmann & Entine, 1974), a time = 200 μs is calculated for rhodopsin to move a distance Δr = 200 Å. The rapid lateral diffusion of phospholipids in the membrane provides additional support for this argument. Thus we are able to regard any previously localized change on the rhodopsin molecule proper or in its vicinity as being uniformly smeared out over the disk membrane surface within the time range at which calcium release occurs. The differences between the two models, outlined above, are the following: (1) In model A, the binding constant of only a fraction of the binding sites is affected by light (fraction of rhodopsin bleaching). In order to obtain the experimentally observed release stoichiometry (0.5–1.0 Ca^{2+} released/Rh*), a relatively large change of the dissociation constant, ΔK_D, is required ($\Delta K_D \geq 30$ μM). This is in contradiction with experimental results (Hemminki, 1975; Hendriks et al., 1977; Korchagin et al., 1978; U. B. Kaupp and R. Furrer, unpublished results). (2) In model B, a small change of K_D ($\Delta K_D/K_D \sim 10^{-1}$–$10^{-2}$) "seen" by *all* binding sites accounts for the observed release stoichiometry and requires no gross change of the binding constant. (3) At calcium concentrations $\geq K_D$, the release stoichiometry decreases again for model B (see eq A3 or A11b) but not for model A (see eq 1). This more rigorous distinction between models was not possible in our experiments because arsenazo III limited the concentration range of calcium within which accurate measurements were feasible.

Nature of the Change in Binding Constant K_D. The decrease of the surface potential as "seen" by the calcium binding sites and the concomitant increase of the apparent dissociation constant for calcium could be effected by the following mechanisms.

The light-induced uptake of positively charged ions could reduce the fixed negative surface charge. A similar effect would be observed if there is an increase of the surface area,

but the number of surface charges remains constant.

Alternatively, a change of the location of calcium binding sites with respect to an idealized diffuse array of negative surface charges within the plane of the membrane could alter the effective surface potential seen by these binding sites.

Quantitative assessments of these mechanisms are possible. For example, a change of K_D as small as $\Delta K_D = (1-2) \times 10^{-6}$ μM could be created by a decrease of the surface potential $\Delta\phi_s = 0.5$ mV (calculated according to eq A7 in Appendix I). At a surface charge density $\sigma = 2e$/rhodopsin (i.e., 1 electronic charge/2000 Å; Schnetkamp et al., 1981) of the disk membrane, this change in surface potential corresponds to the uptake of one positive charge/bleached rhodopsin at a bleaching percentage of 2–4% rhodopsin/flash (eq A11). It is noteworthy that the above "delocalized charge" model (B) predicts a linear relationship between the amount of calcium released and rhodopsin bleached (see eq A11a) in accordance with experimental results (Kaupp et al., 1979a,b). However, this is true only for small changes of the relative change of surface charge $\Delta\sigma/\sigma$, i.e., at nonsaturating bleaching levels ($\leq$20%). Second, for surface potentials $\phi_s \sim 60$ mV, the potential drop with distance from the membrane surface can be approximated by

$$\psi(x) = \psi_0 \exp(-\kappa x)$$

where ϕ_0 is the surface potential at $x = 0$ and κ is the Debye length [see Appendix I and McLaughlin (1977)]. Assuming a dielectric constant $\epsilon = 10$ at the location of the calcium binding sites and a concentration of monovalent cations $c_{mono} = 10$ mM, a change of the surface potential $\Delta\phi_s = 0.4$ mV requires a displacement of the binding sites from the plane of the idealized charges by $\Delta x = 0.2$ nm. Because we do not know the precise values of ϵ, ϕ_s, and σ, we do not want to overemphasize the quantitative aspects of this model. However, our calculations indicate that the "delocalized charge" model does not require unreasonable assumptions.

One attractive mechanism for a rapid charge transfer from the surrounding medium into the disk membrane (Cafiso & Hubbell, 1980) is the rapid light-triggered proton uptake which accompanies the metarhodopsin I/metarhodopsin II transition (Emrich, 1971; Bennett, 1980; Kaupp et al., 1981a,b). The involvement of a rapid proton transfer in calcium release mechanism is supported by the following observations: the kinetics and activation energies for both processes are very similar (Kaupp et al., 1981a,b), and at pH values >6.3, the stoichiometry of calcium release rapidly decays, although binding of calcium to binding sites in the disk membrane was increased [Figure 6, middle part; a similar increase of binding with increasing pH was observed for manganese binding to disk membranes (U. B. Kaupp and R. Furrer, unpublished observations)].

A similar decay of the stoichiometry as was observed for calcium release at pH values >6.3 was observed for the light-triggered proton uptake (Emrich, 1971; Emrich & Reich, 1974; Bennett, 1978; see also Figure 6, curve A). The amount of metarhodopsin II produced by excitation of rhodopsin is also lower at higher pH (Figure 6, curve B; Bennett, 1978). However, at room temperature (20 °C), the formation of metarhodopsin II is reduced less than proton uptake at higher pH values (Bennett, 1978). For example, at pH 8, the stoichiometry of rapid proton uptake is virtually zero (Bennett, 1978), whereas there is only a 70–80% reduction of metarhodopsin II formation (Figure 6, middle part; Bennett, 1978). From this it is inferred that the gradual inhibition of the metarhodopsin I/metarhodopsin II transition at pH >7 accounts only for a minor fraction of the inhibition of the calcium release in this pH range. Moreover, it can be concluded that calcium release appears to be triggered by a pH-dependent process that gradually decreases for pH values >5.

Recently, a change of both surface and "boundary" potentials [Cafiso & Hubbell, 1980; see McLaughlin (1977) for nomenclature] was detected in rod outer segments. It occurs within a few milliseconds after rhodopsin excitation and exhibits a similar pH dependence as is found for the light-triggered proton uptake. The relative stoichiometry of calcium release that is expected from the amount of calcium bound weighted by the amplitude of the proposed trigger steps (proton uptake, change of an interfacial potential, and metarhodopsin II formation) is depicted in Figure 6, lowest part. The pH dependence of the proton uptake is very similar to that of the interfacial potential change; therefore, only the latter curve is redrawn from Cafiso & Hubbell (1980). The calcium release data from Figure 6, upper part, are also included. The pH dependence of calcium release can be explained if it is assumed that the proton uptake/interfacial potential changes are the actual trigger. We cannot, however, rule out the possibility that metarhodopsin II formation mediates these processes. It is difficult to observe these processes independently because the metarhodopsin I/metarhodopsin II transition and the light-triggered proton uptake occur under conditions where calcium release is completely abolished (detergents and high ionic strength), but there are no experimental conditions where the reverse is true. Furthermore, our calculations show that coupling of a light-triggered proton uptake and calcium release could be brought forth by a change in the electrostatic potential at the disk membrane/water interface.

Site of Calcium Release and Proton Uptake. The majority of the calcium binding sites in cattle rod outer segments resides inside disks (Schnetkamp, 1979). In rod outer segments that have an "intact" envelope (i.e., they are not permeable to small solutes on a minute time scale), no light-triggered calcium release across the plasma membrane into the outer medium is observed (Kaupp et al., 1979a). Similarly with leaky rod outer segments [i.e., the outer membrane was made permeable to small solutes (Schnetkamp et al., 1977)], no calcium release is observed unless A23187 is added to the suspension (Kaupp et al. 1979a). From this we concluded that calcium release occurs from binding sites at the inner disk membrane into the internal space of disks, but calcium is not transferred across the disk membrane into the cytosol in this preparation (Kaupp et al., 1979a,b). This conclusion is corroborated by the results presented in this paper (Figure 7). However, one complication that occurred in sonicated or otherwise fragmented disks deserves a closer inspection, namely, the evidence for two different calcium pools, one accessible from outside and one shielded. This complication is discussed in Appendix II.

On the basis of our experimental results, it is difficult to make definitive conclusions about the site of proton uptake in rod outer segments. In leaky rod outer segments, proton uptake was maximal even in the absence of protonophores. However, the apparent velocity at which the proton uptake occurred in leaky rod outer segments was much lower ($\tau_{1/2}$ = 2–3 s) in the absence of protonophores than after addition of gramicidin or A23187 (Figure 8). We attribute this decrease of the apparent velocity to a translocation step of protons across the disk membrane into the disk interior. This interpretation requires two assumptions: first, it is assumed that the outer envelope in leaky rod outer segments is not a barrier to the free diffusion of protons from the outer medium into the cell; second, the disk membrane must have a permeability for protons that allows equilibration of light-induced pH

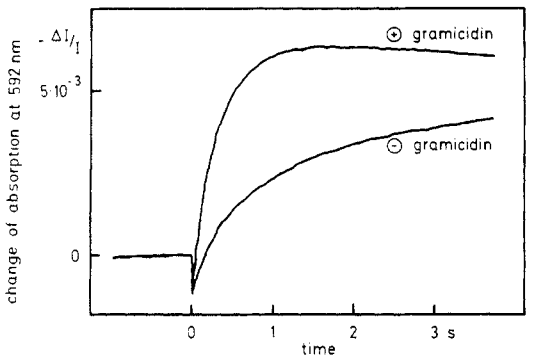

FIGURE 8: Time courses of proton uptake in leaky rod outer segments with and without ionophore (3 μM gramicidin). Medium conditions: 600 mM sucrose, 0.25% (v/v) Ficoll 400, 0.5 mM Tris-HCl at pH 5.95, 20 mM KCl, 30 μM bromocresol purple, 6–10 μM calcium concentration and 3.5 μM rhodopsin. Percentage of rhodopsin bleaching 10%.

gradients within a few seconds. The latter assumption is supported by the recent finding that the proton permeability of the disk membrane is low *only* in freshly prepared material; proton permeability is very high in disk membranes aged ≥3 h (Uhl et al., 1980). Our finding that the site of the pH sensitivity of the metarhodopsin I/metarhodopsin II transition most likely resides at the inner surface of the disk membrane also supports the above hypothesis (Schnetkamp et al., 1981).

On the other hand, other authors recently proposed that the rapid, light-stimulated proton uptake is responsible for the occurrence of electrostatic potential transients at the *outer* side of the disk membrane (Cafiso & Hubbell, 1980). Bennett (1980) arrived at the same conclusion by employing 9-aminoacridin. If this is true in our preparations, it implies that the uptake of protons at the outside of disks affects binding sites at the inner disk surface. Because electrostatic coupling between the cytoplasmic and the lumenal surfaces of the disk membrane is highly improbable (McLaughlin, 1977; Nelson et al., 1975), the effect of proton uptake must be transferred by some other means. Possible physical changes in the membrane could be (1) a change of the surface area and/or location of calcium binding sites relative to an idealized plane of the surface charges and (2) a change of the distance between the adjacent inner disk surfaces. Due to the close apposition of the two inner membrane surfaces (20–30 Å), calcium binding sites "see" the superposition of the interfacial potentials from both surfaces at the ionic strengths employed in our experiments. If the distance between these surfaces is increased by a light-induced structural change of the entire disk, then binding sites could experience a lower electrostatic potential.

Acknowledgments

We thank Professors L. H. Pinto and S. McLaughlin and Dr. H. W. Trissl for helpful discussions and reading of the manuscript. We gratefully acknowledge M. Offermann for preparing rod outer segments and drawing the figures. In particular, U.B.K. thanks Tazewell Wilson for giving him linguistic lessons while he was correcting the manuscript.

Appendix I

An expression is derived that allows calculation of the release of bound calcium $\Delta[Ca]_b$ after the surface charge density σ of the disk membrane has been changed by a small amount $\Delta\sigma$ by bleaching of rhodopsin. The following assumptions are made: (a) At the disk membrane, there exists only one homogeneous set of calcium binding sites with a maximum capacity $[BS]_0$ and a dissociation constant K_D. (b) When the surface charge density σ is changed by $\Delta\sigma$, the complete ensemble of binding sites responds uniformly by a change of the binding constant ΔK_D.

The following symbols will be used: $[Ca]_f$, free calcium concentration within disks; $[Ca]_b$, concentration of calcium bound to the membrane; $[BS]_0$, total concentration of calcium binding sites at the membrane; K_D, dissociation constant of calcium binding sites; ΔK_D, differential change of dissociation constant K_D; ϕ_s or ϕ_D, surface potential or Donnan potential, respectively; σ or σ_p, surface charge density at the disk membrane or polyanion concentration within the disk interior, respectively; C, concentration of monovalent cations in the suspending medium; z, electric charge; [Rh*], amount of rhodopsin bleached; [Rh], rhodopsin concentration; e, k, and T have their conventional meaning.

Change of Calcium Binding $\Delta[Ca]_b$. Our starting point will be the well-known isotherm for calcium binding:

$$[Ca]_b = \frac{[Ca]_f[BS]_0}{[Ca]_f + K_D} \tag{A1}$$

Differentiation of (A1) with respect to K_D gives

$$d[Ca]_b = -\frac{[Ca]_f[BS]_0}{([Ca]_f + K_D)^2} dK_D \tag{A2}$$

for small changes of dK_D and $d[Ca]_b$, the differentials can be replaced by differences.

$$\Delta[Ca]_b = -\frac{[Ca]_f[BS]_0}{([Ca]_f + K_D)^2} \Delta K_D \tag{A3}$$

Equation A3 relates a change in calcium binding to a change in the dissociation constant K_D, irrespective of the physicochemical nature of this change.

Change of Calcium Binding with Surface Charge Density σ. The local concentration of calcium ions at the disk membrane surface is given by the Boltzmann equation

$$Ca(x) = [Ca]_f(\infty) \exp(-2e\phi_s(x)/kT) \tag{A4}$$

wherein $[Ca]_f(\infty)$ denotes the calcium concentration in the bulk phase and $\phi_s(x)$ the surface potential at distance x from the membrane surface. A similar expression relates the dissociation constant K_D for Ca^{2+} to the presence of a surface potential $\phi_s(x)$:

$$K_D = K_D^0 \exp(2e\phi_s/kT) \tag{A5}$$

K_D^0 is an intrinsic dissociation constant in the absence of any electrostatic potential.

An infinitesimal change of $d\phi_s$ is related to a change of the dissociation constant dK_D by

$$dK_D = (2e/kT)K_D^0 \exp(2e\phi_s/kT)\, d\phi_s \tag{A6}$$

If eq A5 and A6 are combined differentials are replaced by differences, then

$$\Delta K_D/K_D = (2e/kT)\Delta\phi_s \simeq \Delta\phi_s/30\text{mV} \tag{A7}$$

If $\Delta\phi_s$ is caused by the uptake of protons into the disk membrane, it might be useful to express the surface potential change in terms of $\Delta\sigma$, the change in surface charge density. When the Poisson and Boltzmann equations are combined, the Gouy expression is obtained:

$$\sigma A/\sqrt{C} = \sinh(ze\phi_s/2KT), \quad z = 1 \tag{A8}$$

It relates the electrostatic potential in the aqueous phase just above the membrane surface ϕ_s to the charge density of the surface in the presence of C moles of any 1:1 electrolyte in

the bulk aqueous phase. [$A = 11/(8N\epsilon_\gamma\epsilon_0 kT)^{1/2}$, where N is Avogadro's number. ϵ_γ is the dielectric constant, and ϵ_0 is the permittivity of free space.] In mixed solutes which contain mono- and divalent cations, eq (A8) is only valid when (a) "screening" of the surface potential by divalent cations is negligible and (b) the charge density σ is not changed appreciably by specific adsorption, say of Ca^{2+}. Under the experimental conditions used in this study, both restrictions are fulfilled ($C^{2+}/C^{+} = 10^{-3}$; calcium is exchanged in an electroneutral way with protons at the membrane surface).

A small change in σ is related by, e.g., $\sigma A/\sinh$ by

$$d\sigma = (C^{1/2}/A)(ze/2kT)\cosh(ze\phi_s/2kT)\,d\phi_s \quad (A9)$$

$C^{1/2}$ in eq A9 can be substituted by, e.g., $\sigma A/\sinh(ze\phi_s/2kT)$ from eq A8 where

$$d\phi = (d\sigma/\sigma)(2kT/ze)\tanh(ze\phi_s/2kT) \quad (A10a)$$

or

$$\Delta\phi = (\Delta\sigma/\sigma)(2kT/ze)\tanh(ze\phi_s/2kT) \quad (A10b)$$

Combining eq A3, A7, and A12a, one finally arrives at

$$\Delta K_D = K_D(4/z)(\Delta\sigma/\sigma)\tanh(ze\phi_s/2kT) \quad (A11a)$$

and

$$\Delta[Ca]_b = -\frac{[BS]_0[Ca]_f K_D(4/z)(\Delta\sigma/\sigma)\tanh(ze\phi_s/2kT)}{([Ca]_f + K_D)^2} \quad (A11b)$$

From this expression, the amount of calcium released after changing the surface charge density by $\Delta\sigma$ has been calculated to fit the data in Figure 5. For surface potentials ≥ 60 mV, the factor $\tanh(ze\phi_s/2kT)$ becomes $0.83 < \tanh(ze\phi_s/2kT) < 1$, and therefore its contribution can be neglected at a supposed surface charge density of disk $\sigma = 1 - 8e/4000$ Å^2 and low ionic strength (1–10 mM monovalent cations).

Equation A11b can only be applied if the bulk concentration of calcium is low ($\leq 10^{-5}$ M). For higher calcium concentrations, a more general Gouy–Chapman equation is required in the presence of the 2:1:1 electrolyte (Abraham-Shrauner, 1975). In the presence of 4×10^{-5} M calcium and surface potentials of about 60 mV, estimates of the latter would be maximally 16% in error if the divalent cation is neglected (see Abraham-Shrauner, 1975).

Calcium Release and Monovalent Cation Concentration. An expression will be derived which relates the decrease in calcium release stoichiometry to the concentration of monovalent cations in the medium.

The dissociation constant for calcium binding K_d varies with the surface potential ϕ_s according to eq A5. The surface potential is related to the surface charge density and the concentration of monovalent cations by

$$\phi_s = (2kT/ze)\sinh^{-1}(A\sigma/C^{1/2}) \quad (A12a)$$

For a surface potential ~ 60 mV, eq A12a can be approximated by

$$\phi_s \simeq (2kT/ze)\ln(2A\sigma/C^{1/2}) \quad (A12b)$$

(an error of 10% for the estimation of ϕ_s is made by this approximation). Insertion of eq A12b into eq A5 gives [assuming a negative surface charge and $\tanh(ze\phi/2kT)$]

$$K_D \simeq K_D^0 \exp{-4\ln(2A\sigma/C^{1/2})} = C^2/[(\text{const})\sigma^4] \quad (A13)$$

After replacing K_D in eq A11 by eq A13 and considering that $[Ca]_f < K_D$ under our experimental conditions, one obtains

$$\Delta[Ca]_b \simeq \text{const}'/C^2 \quad (A14)$$

A similar expression has been derived by Puskin (1977).

Disk Interior as a Polyelectrolyte. The following considerations will show that a simpler description of calcium release in terms of Donnan potential change across the disk membrane yields similar results. The advantage of this approach is that the suppositions which enter into the calculations are more easily met by the conditions within a disk than those required for the application of the Gouy–Chapman formalism.

At low calcium concentrations and monovalent cation levels which are at least 3 orders of magnitude larger, the Donnan potential drop across the boundary polyelectrolyte/water depends predominantly on the concentration of monovalent cations.

$$\phi_D = (kT/e)\ln(\sigma_p/C) \quad (A15)$$

Provided the membrane is permeable (e.g., to divalent cations), calcium will equilibrate between the intradiskal and extradiskal space according to its electrochemical potential.

$$\phi_D = (kT/2e)\ln([Ca]_0/[Ca]_i) = (kT/e)\ln(\sigma_p/C) \quad (A16)$$

From eq A16 it follows that

$$[Ca]_{disk} = [Ca]_{bulk}\sigma_p^2/C^2 \quad (A17)$$

and therefore the concentration of bound calcium is

$$[Ca]_b = \frac{[BS]_0[Ca]_{bulk}(\sigma_p^2/C^2)}{K_D + [Ca]_{bulk}(\sigma_p^2/C^2)} \quad (A18)$$

Under conditions where $K_D \gg [Ca]_{bulk}(\sigma_p^2/C^2)$ (e.g., $[Ca]_{bulk} \ll K_D$ and $\sigma_p \lesssim C$), the intradiskal bound calcium varies inversely as the square of the monovalent cation concentration. Consequently the apparent dissociation constant of calcium binding sites inside disks is expressed by eq A5 if σ_s therein is replaced by ΔK_D. If the procedure going through eq A6, A7, and A10a is repeated with A15 and A16, one finally arrives at

$$\Delta K_D = -K_D(2/z)(\Delta\sigma_p/\sigma_p) \quad (A19)$$

From comparison of eq A11a and A19 and eq A14 and A17, respectively, it follows that calcium release due to a change of fixed negative groups within disks is only by a factor of 2 different if calculated by the two different theoretical approaches. Note that the above derivations contain some simplified assumptions and hold only for a certain range of the surface potential ϕ_s, the monovalent cation concentration C, and the calcium concentration Ca_f.

Appendix II

We have no independent information on the sidedness of the disk vesicles after sonication. It is therefore not possible to discriminate between a homogeneous disk suspension with calcium for one part bound to the inner and for the other part bound to the outer surface of the disk membrane and an inhomogeneous suspension consisting of "inside-out" and "right-side-out" vesicles but with calcium bound to only one surface. Both suspensions would appear similar in the above experiments. Formation of inverted but closed vesicles and creation of unsealed membrane patches are supposed to occur in sonicated, hypotonically suspended and freeze–thawed disks (Adams et al., 1979).

A possible discrimination between the above alternatives may be feasible by localizing the trigger step of calcium release. Unfortunately, from the type of experiments shown in Figure 7 for calcium, no definite conclusions can be inferred

for the uptake of protons. Under all conditions, the stoichiometry was independent from what protonophores were used and at what concentration. Second, unlike calcium release, the rapid proton uptake showed no compartmentation behavior. In analogy to the above reasoning, this could either mean that protons are exclusively taken up at one side of the disk vesicles in a homogeneous population or that the permeability of isolated disks is so high that a pH gradient due to the uptake of protons into the membrane phase at one side is instantaneously dissipated across a permeable disk membrane. The latter possibility is highly probable in view of the recent findings of Uhl et al. (1980).

References

Abraham-Shrauner, B. (1975) *J. Math. Biol. 2*, 333–339.

Adams, A. J., Tanaka, M., & Shichi, H. (1978) *Exp. Eye Res. 27*, 595–605.

Bennett, N. (1978) *Biochem. Biophys. Res. Commun. 83*, 457–465.

Bennett, N. (1980) *Eur. J. Biochem. 111*, 99–103.

Brown, J. E., Cohen, L. B., De Weer, P., Ross, W. N. & Salzberg, B. M. (1975) *Biophys. J. 15*, 1155–1160.

Cafiso, D. S., & Hubbell, W. L. (1980) *Biophys. J. 30*, 243–264.

deGrip, W. J., Daemen, F. J. M., & Bonting, S. L. (1972) *Vision Res. 12*, 1697–1707.

Eisenberg, M., Gresalfi, T., Riccio, T., & McLaughlin, S. (1979) *Biochemistry 18*, 5213–5223.

Emrich, H. M. (1971) *Z. Naturforsch. B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 26*, 352–356.

Gold, G. H., & Korenbrot, J. I. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5557–5561.

Hagins, W. A., & Yoshikami, S. (1977) in *Vertebrate Photoreception* (Barlow, H. B., & Fatt, P., Eds.) pp 97–138, Academic Press, London.

Hendriks, T. van Haard, P. M. M., Daemen, F. J. M., & Bonting, S. L. (1977) *Biochim. Biophys. Acta 467*, 175–184.

Junge, W. (1976) *Chem. Biochem. Plant Pigm., 2nd Ed.*, 233–333.

Kaupp, U. B. (1979) Thesis, Technical University of Berlin.

Kaupp, U. B., Schnetkamp, P. P. M., & Junge, W. (1979a) *Biochim. Biophys. Acta 552*, 390–403.

Kaupp, U. B., Schnetkamp, P. P. M., & Junge, W. (1979b) in *Detection and Measurement of Free Calcium in Cells*, (Ashley, C. C., & Campbell, A., Eds.) pp 287–308, Elsevier/North-Holland, Amsterdam.

Kaupp, U. B., Schnetkamp, P. P. M., & Junge, W. (1980) *Nature (London) 286*, 638–640.

Kaupp, U. B., Schnetkamp, P. P. M., & Junge, W. (1981) *Biochemistry* (following paper in this issue).

Korchagin, V. P., Berman, A. L., Skukolyokov, S. A., Rychkiova, M. P., & Etingof, R. N. (1978) *Biokhimiya 43*, 1749–1756.

Kurland, R., Newton, C., Nir, S., & Papahadjopoulos, D. (1979) *Biochim. Biophys. Acta 551*, 137–147.

Liebmann, P. A., & Entine, G. (1974) *Science (Washington, D.C.) 185*, 457–459.

McConnell, D. G., Rafferty, C. N., & Dilley, R. A. (1968) *J. Biol. Chem. 243*, 5820–5826.

McLaughlin, S. (1977) *Curr. Top. Membr. Transp. 9*, 71–144.

McLaughlin, S., & Dilger, J. P. (1980) *Physiol. Rev. 60*, 825–863.

Nelson, A. P., Colonomous D., & McQuarrie, D. A. (1975) *J. Theor. Biol. 50*, 317–325.

O'Brien, D. F. (1979) *Photochem. Photobiol. 29*, 679–685.

Poo, M., & Cone, R. (1974) *Nature (London) 247*, 438–441.

Puskin, J. S. (1977) *J. Membr. Biol. 35*, 39–55.

Puskin, J. S., & Coene, M. (1980) *J. Membr. Biol. 52*, 69–74.

Reich, R., & Emrich, H. M. (1976) *Pfluegers Archiv. 364*, 23–28.

Scarpa, A., Brinley, F. J., Jr., & Dubyak, G. (1978) *Biochemistry 17*, 1378–1386.

Schnetkamp, P. P. M. (1979) *Biochim. Biophys. Acta 554*, 441–459.

Schnetkamp, P. P. M., Daemen, F. J. M., & Bonting, S. L. (1977) *Biochim. Biophys. Acta 468*, 259–270.

Schnetkamp, P. P. M., Klompmakers, A. A., & Daemen, F. J. M. (1979) *Biochim. Biophys. Acta 552*, 379–389.

Schnetkamp, P. P. M., Kaupp, U. B., & Junge, W. (1981) *Biochim. Biophys. Acta 642*, 213–230.

Shevchenko, T. F. (1976) *Biophysics (Engl. Transl.) 21*, 327–330.

Smith, H. G., Jr., & Bauer, P. J. (1979) *Biochemistry 18*, 5067–5073.

Smith, H. G., Jr., Fager, R. S., & Litman, B. J. (1977) *Biochemistry 16*, 1399–1405.

Uhl, R., Kuras, P. V., Anderson, K., & Abrahamson, E. W. (1980) *Biochim. Biophys. Acta 601*, 462–477.

Yoshikami, S., George, J. S., & Hagins, W. (1980) *Nature (London) 286*, 395–398.