Rapid Calcium Release and Proton Uptake at the Disk Membrane of Isolated Cattle Rod Outer Segments. 2. Kinetics of Light-Stimulated Calcium Release and Proton Uptake†

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ABSTRACT: By exciting isolated rod outer segments with a short flash of light, we have measured (1) light-induced calcium release (using the indicator arsenazo III and the ionophore A23187), (2) light-induced proton uptake (using bromocresol purple and protonophores such as gramicidin), and (3) the transition between metarhodopsin I/metarhodopsin II. The kinetics of both calcium release and proton uptake are highly susceptible to the structural integrity of rod outer segments. After sonication of rod outer segments with an intact envelope, the apparent rise times of both calcium release and proton uptake are accelerated by almost 2 orders of magnitude. Conversely, the time course of the metarhodopsin I/metarhodopsin II transition is virtually independent of the structural integrity of the isolated rod outer segments. Both the metarhodopsin I/metarhodopsin II transition and the proton uptake have similar half-rise times ($\tau_{1/2}$ = 5 ms at 20 °C) whereas the half-rise time of the calcium release is consistently smaller ($\tau_{1/2}$ = 13–18 ms at 20 °C) in sonicated rod outer segments. However, the energy of activation is similar for the three processes (E_a = 150 kJ/mol). The time courses of the three processes are complex and cannot be described by a single exponential. In intact rod outer segments, both calcium release and proton uptake are relatively slow ($\tau_{1/2}$ = 250–400 ms), which is different from the light-induced binding and debinding of these ions at the disk membrane, even at nonlimiting concentrations of ionophores. Moreover, their energies of activation are almost 4 times smaller (E_a = 43 kJ/mol) than in sonicated material (E_a = 150 kJ/mol), and the Arrhenius plot becomes nonlinear at temperatures < 10 °C. At these temperatures, the calcium release and the proton uptake in intact rod outer segments exhibit a predominant delay phase in its time course. These observations suggest that the appearance of calcium and the disappearance of protons in the outer aqueous medium involve an additional reaction step that is distinct from the redistribution reaction that occurs at the disk membrane between membrane-bound and free species. The data are discussed in terms of an interrelationship between light-triggered proton uptake and calcium release, respectively. However, the kinetic results do not allow us to postulate an unambiguous causality sequence: metarhodopsin I/metarhodopsin II–proton uptake–calcium release.

The stoichiometry of light-induced release of calcium from binding sites inside disks has been investigated under a variety of conditions in previous papers (Kaupp et al., 1979 a,b, 1981). It has been compared with the stoichiometry behavior of light-sensitive proton uptake by rhodopsin itself and probably also by proton buffering groups at the membrane surface. It has been proposed that calcium release is coupled to the light-generated proton uptake, which in turn follows the metarhodopsin I/metarhodopsin II transition. The present kinetic study is designed to question this proposed sequence of events.

Materials and Methods

Preparation. Intact rod outer segments were isolated on a sucrose density gradient (Schnetkamp et al., 1979). Sonicated disk vesicles were obtained by sonication of a suspension of intact rod outer segments for 30 s at 40 W (Branson Sonifier, Model B-12). During sonification, the sample was cooled in an ice bath.

Suspensions contained sucrose (600 mM), Ficoll 400 (0.1–0.25% v/v), arsenazo III (Sigma, grade I, 30 μM) Tris-HCl buffer at pH 7.0–7.4 (20 mM), rhodopsin (4–6 μM), and ionophore A23187 (10 μM). The free calcium concentration was always 3–6 μM. In sonicated material, Tris-HCl was replaced by Mes/Tris or Mops/Tris buffer (2 mM) at the indicated pH. When light-triggered proton uptake was measured, bromocresol purple (30 μM) instead of arsenazo III was used as the indicator, and the external proton buffer was omitted [for further details, see Kaupp et al. (1981)].

Flash Spectrophotometry. The time course of the light-stimulated absorption changes was recorded in a kinetic flash spectrophotometer (Junge, 1976). Changes in the free calcium concentration were recorded by using the absorption changes of arsenazo III at 655 nm, pH changes by using the absorption changes of bromocresol purple at 605 nm, and the metarhodopsin I/metarhodopsin II transition by using the absorption at 382 nm. Samples were excited by a flash from a Neodym/YAG laser at 530 nm (half-duration time of the flash $\tau_{1/2}$ = 10 ns).

When excited with visible light, arsenazo III alone undergoes intrinsic photochemical reactions (Kaupp et al., 1979b). Although the flash-induced absorption changes of arsenazo III itself are completely reversible within 20 ms, they may obscure absorption changes of arsenazo III in response to concentration changes of calcium in a suspension of rod outer segments. This is particularly relevant for sonicated disk vesicles where calcium release occurs with a half-rise time, $\tau_{1/2}$, of 10–20 ms. Signals due to the intrinsic photochemistry of arsenazo III were recorded separately and subtracted from the superimposed calcium signals in order to obtain undistorted recordings of the rapid calcium release in sonicated material. Subtracting the absorption changes attributable to the photochemistry of arsenazo III from the total calcium-indicating

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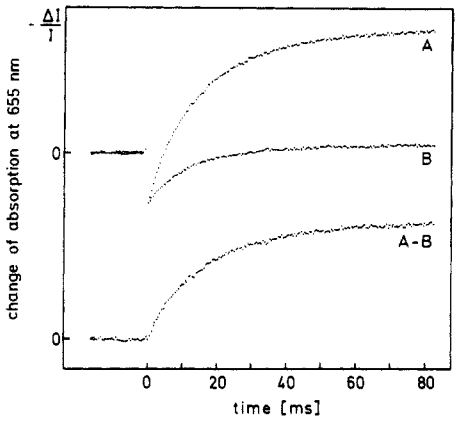

FIGURE 1: Correction procedure for the calcium-indicating absorption change of arsenazo III at 655 nm. (A) Time course of the light-induced absorption change at 655 nm of sonicated rod outer segments by a flash at $t = 0$ in the presence of arsenazo III and ionophore A23187. The suspension medium contained 600 mM sucrose, 2 mM Mes/Tris buffer, 0.5 mM Tris/HCl buffer at pH 6.33, 30 μM arsenazo III, 10 μM A23187, 3–4 μM free calcium concentration, and 6 μM rhodopsin. Cuvette path length was 10 mm, temperature 20 °C, excitation wavelength 530 nm, and half-duration of excitation flash 10 ns. Signals were obtained by averaging from two samples over six repetitions (three flashes applied to each sample). Time per address setting was 0.5 ms/address of the signal averager Tracor TN-1500. (B) Time course of the light-induced asborption change at 655 nm of sonicated rod outer segments in the presence of arsenazo III after extensive bleaching of the sample. Conditions were the same as in (A). (A – B) Difference signal obtained after subtraction of trace B from trace A. By means of the arithmetic facilities of the signal averager, signal B was weighted by a factor ranging between 0.8 and 1.0 in order to make the negative deflections occurring shortly after the flash equal in amplitude in both traces. This procedure was necessitated by the variability of the energy output of the laser which was checked by measurement with a photocell (~10%).

absorption changes in a suspension of sonicated disk vesicles was achieved in two different ways: (1) Calcium signals were inhibited by inclusion of 1 mM nitrilotriacetic acid as a calcium buffer. This virutally eliminated the calcium concentration changes in the suspension and left over the intrinsic absorption change of arsenazo III proper. (2) Light-triggered calcium release was recorded. Then the sample was extensively bleached until calcium-indicating signals could no longer be detected. Then, with the same sample, the intrinsic photochemical signal of arsenazo III was determined.

The respective control transient signal was subtracted from the original one by means of the arithmetic facilities of the averaging computer (Nicolet 1072 or Tracor TN-1500). An example of the correction procedure is shown in Figure 1. It is noted that the first control (inclusion of calcium buffer) accounts not only for the flash-induced photochemistry of arsenazo III but also for possible apparent absorption changes due to light-scattering transients and for true absorption changes resulting from the photochemistry of rhodopsin proper. It is noteworthy that the concentration of free calcium in the first control (with calcium buffer) was adjusted to the same value it had in the sample. During the second control, the change in free calcium due to rhodopsin bleaching was small ($dp[Ca] = 10^{-2}$), and therefore the reference signal was recorded under similar conditions to those used for the calcium-indicating arsenazo III response. Both methods yielded similar results.

Calcium release in intact rod outer segments is quite slow ($\tau_{1/2} = 300$ ms), and thus the rapid photochemistry of arsenazo III ($\tau_{1/2} = 20$ ms) did not severely interfere with the measurement of the time course of the calcium release. Therefore, calcium-indicating absorption changes from intact rod outer

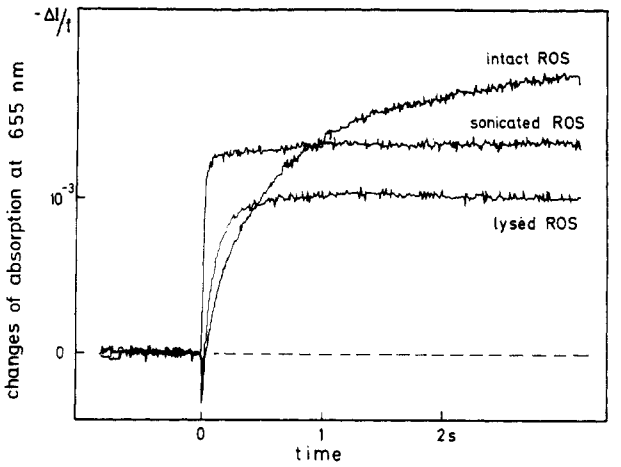

FIGURE 2: Time course of the light-induced absorption changes at 655 nm in intact and fragmented rod outer segments after excitation by a flash at $t = 0$ in the presence of arsenazo III and ionophore A23187. The suspension medium contained 600 mM sucrose, 2 mM Hepes/Tris buffer, and 0.5 mM Tris-HCl buffer at pH 7.0, 30 μM arsenazo III, 10 μM A23187, 3–4 μM free calcium concentration, and 3.5 μM rhodopsin. Fraction of rhodopsin bleached/flash was about 4–5%. Cuvette path length 10 mm, temperature 20 °C, and excitation conditions as in Figure 1. Signals were obtained by averaging from one sample over four repetitions at a frequency of 0.1 Hz. Time per address setting was 4 ms/address.

segments were not corrected for the flash-induced photochemistry of arsenazo III.

Unlike arsenazo III, bromocresol purple exhibited no flash-induced photochemistry of its own, and therefore no corrections were required. In some preparations, particularly in freeze–thawed and lysed rod outer segments, apparent absorption changes due to light-scattering transients were occasionally observed. They contributed no more than 30% to the total absorption changes. Procedure 1 alone automatically corrected for this error in experiments on calcium release. The ΔpH-indicating absorption changes of bromocresol purple were corrected in an analogous way by inclusion of a proton buffer (e.g., 20 mM Mes/Tris buffer at pH 6.3).

Results

Kinetics of calcium release and proton uptake are dependent on the structural integrity of rod outer segments. On a subsecond time scale, light-induced calcium release in intact rod outer segments can be observed only in the presence of calcium ionophore (e.g., A23187) [see Kaupp et al., (1979a,b)]. The "apparent velocity" of calcium release is the velocity of the reaction of released calcium with the water-dissolved indicator arsenazo III in the outer aqueous phase.

The apparent time course of light-stimulated calcium release differed in preparations with different integrity (Figure 2). The velocity of calcium release in rod outer segments with intact outer membrane [as defined by the low permeability to small solutes; see Schnetkamp et al. (1979)] was low ($\tau_{1/2}$ = 300 ms). It was greatly enhanced after rod outer segments had been lysed ($\tau_{1/2}$ = 50–100 ms) or sonicated ($\tau_{1/2}$ = 10–15 ms). The apparent velocity by which calcium was released was always maximal in sonicated disk vesicles whereas intermediate rise times were observed in rod outer segments which had been desintegrated by a hypoosmotic shock. On must note that for every experiment show in Figure 1, A23187 (10 μM) had been added to the suspension.

The calcium release properties of intact rod outer segments which had been freeze–thawed (to make the outer envelope permeable to small solutes but to leave the rod outer segment morphology apparently unaffected) and leaky rod outer segments [see Schnetkamp et al. (1977, 1979)] were kinetically

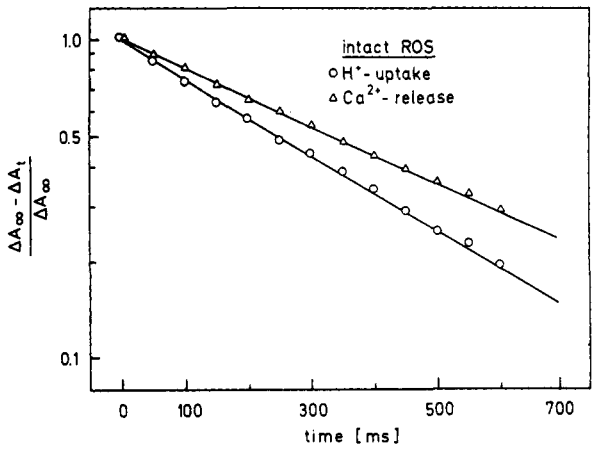

FIGURE 3: Semilogarthmic plot of the calcium-indicating and proton-indicating absorption change at 655 and 596 nm, respectively. Both signals were recorded at pH 6.45 and $T = 20$ °C. The calcium signal was measured in the presence of 20 mM Mes/Tris buffer, whereas during the pH measurement, any external buffer was omitted.

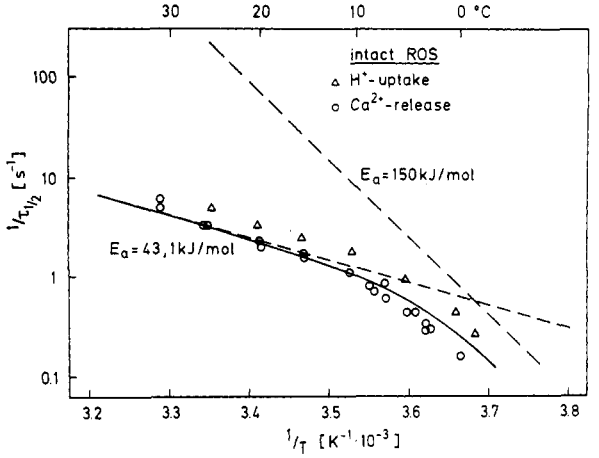

FIGURE 4: Arrhenius plot for the rate constants of calcium release (O) and proton uptake (Δ) in intact rod outer segments. Calcium release was measured at pH 7.4 and proton uptake at pH 6.0. Rate constants are expressed as the reciprocal of the respective half-rise time $(1/\tau_{1/2} = k(\ln 2)^{-1}$. The dashed lines represent calculated Arrhenius plots for the first-order reactions with activation energies $E_a = 150$ and 43 kJ/mol. They were used to reconstruct the Arrhenius plot for a consecutive reaction (solid line).

similar to those of untreated, intact rod outer segments. however, the release stoichiometry was somewhat reduced (10–30%).

The kinetic behavior of flash-induced proton uptake depended upon the structural integrity of the rod outer segments in a way that was similar to that of light-stimulated calcium release from binding sites: (1) In intact rod outer segments, proton uptake required the inclusion of protonophores (e.g., gramicidin, 3 μM and A23187, 10 μM, to be observable on a subsecond time scale) as previously observed by Emrich (1971). (2) In intact rod outer segments in the presence of protonophore (e.g., gramicidin, 3 μM), proton uptake is relatively slow ($\tau_{1/2}$ = 250 ms at 20 °C and pH 6.1). (3) After disintegration of intact rod outer segments by either lysis or sonication, the uptake velocity was accelerated in a way that was analogous to that of calcium release, but its stoichiometry remained constant. Again, proton uptake was most rapid in sonicated disk vesicles, while intermediate velocities were observed in lysed material.

Kinetic Parameters of Calcium Release and Proton Uptake in Intact Rod Outer Segments. The time course of calcium release in freshly prepared intact rod outer segments [procedure of Schnetkamp et al. (1979)] in the presence of A23187 (10 μM) and Tris–HCl buffer (20 mM, pH 7.4) was monoexponential with a half-rise time $\tau_{1/2}$ of about 300 ms (Figure 3). However, this uniphasic kinetic behavior was lost during treatments such as extensive washing, additional centrifugation steps, freeze–thawing, or soaking in high electrolyte medium (e.g., 120 mM Tris-HCl buffer). All these manipulations gave rise to additional faster kinetic components. Faster components of calcium release in "intact" rod outer segments always occurred together with additional light-scattering transients which were virtually absent in the most intact rod outer segment preparations. In addition, some calcium (~30%) was released without the aid of ionophore in the above preparations. Most likely the occurrence of faster kinetic components as well as calcium release in the absence of A23187 arises from the presence of broken cells (e.g., individual disks) in an otherwise intact rod outer segment suspension [see also Figure 7 in Kaupp et al. (1981)].

In intact rod outer segments, proton uptake was also monophasic with a half-rise time, $\tau_{1/2}$, of 250 ms at 20 °C and pH 6.45 (Figure 3). We checked that the alkalization observed with bromocresol purple was not rate limited by the protonophores at the concentrations that we used (e.g., A23187 or gramicidin ≥10 μM). The velocity of calcium release was, however, pH dependent. It was slower at pH 8 ($\tau_{1/2}$ = 600–800 ms) than at pH 5 ($\tau_{1/2}$ = 200 ms). From Figure 3 it becomes evident that the apparent velocities of the disappearance of protons and the appearance of calcium in the suspending medium are similar but not equal.

To elucidate the (diffusive?) process which slows these reactions down, we have investigated its temperature dependence. Figure 4 shows the Arrhenius plot. Both proton uptake and calcium release revealed a characteristic break at temperatures < 10 °C. This nonlinearity shifted the activation energy E_a from 43 kJ/mol at $T > 10$ °C to E_a = 100–150 kJ/mol for temperatures $T < 10$ °C (Figure 4, dashed lines). This suggests that the same conduction mechanism may slow down the migration of both protons and calcium in intact rod outer segments. Alternatively, it is conceivable that only one process (e.g., proton uptake) which is the precursor of the other (e.g., calcium release) is slowed down, thereby becoming rate limiting for the other.

An Arrhenius plot becomes nonlinear whenever the observed product (changes in the free concentration of protons or calcium) is formed (or removed) in a consecutive reaction

$$A \xrightarrow{k_1} B \xrightarrow{k_2} Pr$$

If the reactions A → B and B → Pr have different activation energies, one rate constant becomes rate limiting at high and the other at low temperatures. One consequence of a consecutive reaction scheme is the development of a delay phase for the formation of the product Pr. The delay of the rise of the product should be most easily observed at the temperature at which the rate constants k_1 and k_2 are of similar magnitude (intersection of the dashed lines in Figure 4 at $T \sim 0$ °C). Figure 5 shows the time course of the calcium-indicating absorption change at $T = 2.3$ °C. As can be seen in Figure 4, there was a pronounced delay in the appearance of calcium in the outer medium. The negative deflection occurring shortly after application of the laser flash reflects the photochemistry of arsenazo III at this temperature. The delay phase, although less distinct, could be observed up to $T \sim 10$ °C. Above $T > 10$ °C, it could no longer be detected. A similar delay could be also observed for the light-stimulated proton uptake at $T < 10$ °C (not shown).

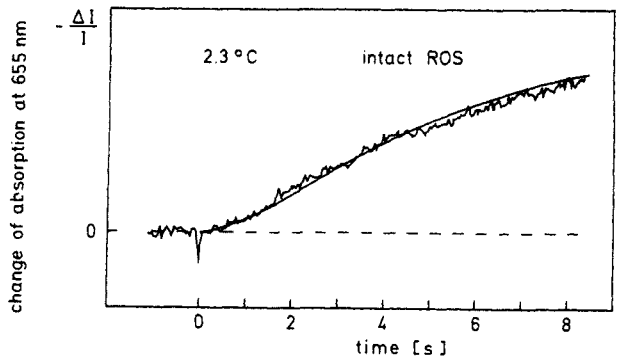

FIGURE 5: Time course of the calcium-indicating absorption change of arsenazo III in intact rod outer segments at $T = 2.3$ °C and pH 7.4. The signal was obtained by averaging from one sample over four repetitions at a frequency of 0.025 Hz. Time per address setting was 20 ms/address. The solid line was calculated under the assumption that calcium release follows a consecutive reaction scheme with rate constants $k_1 = 0.97\ s^{-1}$ and $k_2 = 0.35\ s^{-1}$.

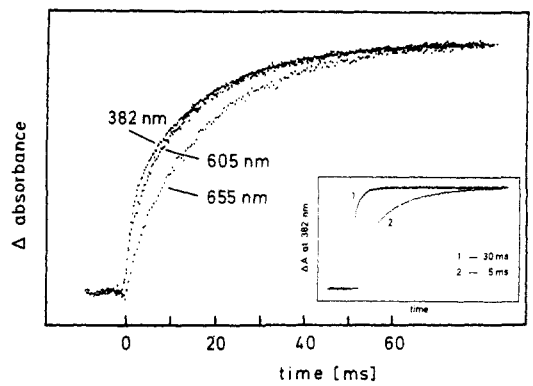

FIGURE 6: Time courses of the light-induced absorption changes at 382 nm (M I/M II), at 605 nm in the presence of bromocresol purple (proton uptake), and at 655 nm in the presence of arsenazo III (calcium release). Signals were recorded at pH 6.30 (M I/M II), 6.20 (proton uptake), and 6.35 (calcium release). Time per address setting was 0.3 ms/address for the absorption changes recorded at 382 and 605 nm and 0.5 ms/address for the absorption change at 655 nm. Further conditions as in Figure 1. The amplitudes of the signals have been normalized by the arithmetic facilities of the averaging device. (Inset) Each signal was recorded on a sufficiently large time scale in order to detect possible drifts of the absorbance or slow kinetic components. For a detailed kinetic analysis as shown in Figure 7, the signals were spread by means of the arithmetic facilities of the averaging computer. An example of the procedure is given for the absorption change at 382 nm (metarhodopsin I/metarhodopsin II transition).

Kinetic Parameters of Calcium Release and Proton Uptake in Sonicated Rod Outer Segments. In a previous paper we have shown that in sonicated disk vesicles calcium release occurs on the same time scale as the metarhodopsin I/metarhodopsin II transition (Kaupp et al., 1980). However, the signal-to-noise ratio of the calcium-indicating absorption changes in this study did not allow a more detailed comparison with this spectroscopic intermediate of the rhodopsin cycle. In the present study, we extend this comparison by (1) improving the signal-to-noise and (2) considering proton uptake. In sonicated rod outer segments, proton uptake and the formation of metarhodopsin II had very similary time courses (Figure 6). The half-rise time for proton uptake, $\tau_{1/2}$, was 6.6 ± 0.8 ms (five determinations), and, for the metarhodopsin I/metarhodopsin II (M I/M II) transition, $\tau_{1/2} = 4.2 \pm 0.5$ ms (five determinations, at pH 6.2–6.3 and $T = 20$ °C). Calcium release in sonicated disk vesicles was somewhat slower, $\tau_{1/2} = 18.3 \pm 3.3$ ms under the same conditions (Figure 6). We emphasize that the composition of the ionic milieu in all of the above experiments was similar, with the exception

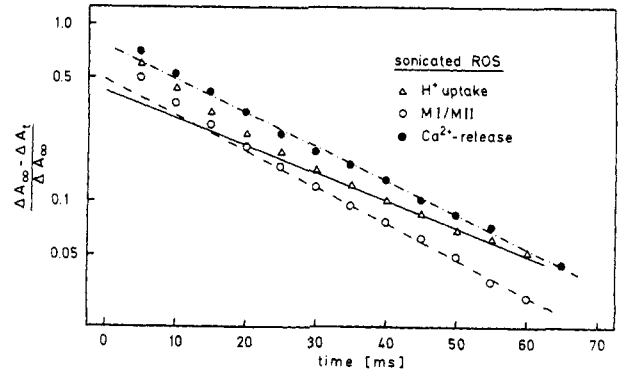

FIGURE 7: Analysis of the signals of Figure 6 in a semilogarithmic plot.

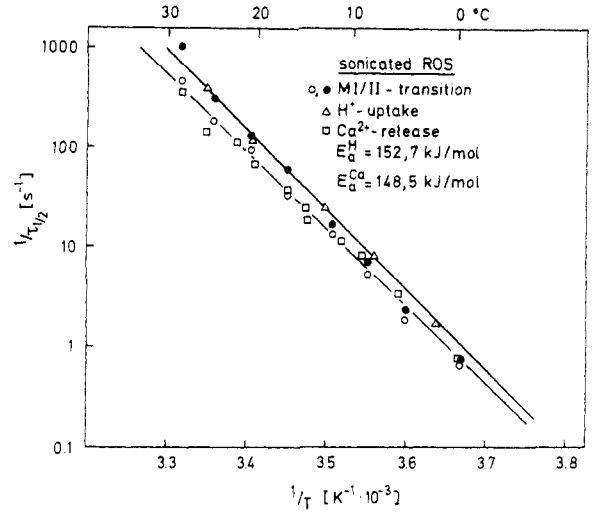

FIGURE 8: Arrhenius plot for the reciprocal half-rise of the metarhodopsin I/metarhodopsin II transition (○, ●), proton uptake (△), and calcium release (□) in sonicated rod outer segments. Metarhodopsin I/metarhodopsin II and calcium signals were recorded at pH 7.0 and proton uptake at pH 6.0. The reciprocal half-rise time of the two kinetic components of metarhodopsin I/metarhodopsin II have been determined in semilogarithmic plots as shown in Figure 7 and plotted separately: (●) reciprocal of the overall half-rise time; (○) reciprocal of the half-rise time of the slower kinetic component.

that the indicators and the external proton buffers were different for the proton uptake measurements.

The time course of the three signals (Δ[Ca], ΔpH, and M I/M II) are complex and cannot be described by a single exponential term (Figure 7). Both proton uptake and the metarhodopsin I/meterhodopsin II transition exhibited prominent biphasic kinetic behavior, consisting of a rapid component ($\tau_{1/2} = 1$ ms) and a slower one ($\tau_{1/2} = 15$ ms). The multiphasic kinetic behavior of the calcium signal was less pronounced (Figure 7). In particular, the calcium signal lacks a rapid kinetic component of similar velocity and amplitude to that observed for metarhodopsin I/metarhodopsin II or for proton uptake (Figure 7). It is also evident from inspection of Figure 7 that the slower kinetic phase of metarhodopsin I/metarhodopsin II and proton uptake more closely parallel the velocity of calcium release (irrespective of its possibly biphasic behavior, see discussion) than does the rapid kinetic component of these reactions.

In Figure 8, the temperature dependence of the respective rate constants is depicted. Rate constants were calculated from half-rise times according to the relationship $k(\ln 2)^{-1} = 1/\tau_{1/2}$ (irrespective of the complex time courses of the signals). Within experimental error, in sonicated rod outer segments, calcium release, proton uptake, and the formation of meta-

rhodopsin II had similar energies of activation (E_a^{Ca} = 148 kJ/mol and $E_a^{M\ I/II}$ and E_a^{pH} = 152 kJ/mol). An even better matching of the calcium data with those of the other two reactions was obtained when the two kinetic phases, e.g., of metarhodopsin I/metarhodopsin II, were considered separately (Figure 8, open and closed circles). Note that the difference between the overall half-rise time of calcium release and metarhodopsin I/metarhodopsin II becomes smaller with decreasing temperatures. Disregarding this subtlety it is evident from Figure 8 that the three reactions under investigation have very similar energies of activation.

Discussion

The stoichiometric aspects of calcium release and proton uptake were the subject of the preceding paper in this issue (Kaupp et al., 1981). The present study has compared the kinetics of metarhodopsin I/metarhosopsin II, proton uptake, and calcium release. We will discuss two aspects of the interrelationship between these reactions: (1) the difference in kinetic behavior of calcium release and proton uptake in the respective preparations and (2) the possibility that metarhodopsin I/metarhodopsin II and/or proton uptake is a precursor of rapid, light-stimulated calcium release. In intact rod outer segments, calcium release consists of two different processes. Calcium release in intact rod outer segments is relatively slow ($\tau_{1/2}$ = 300–400 ms) and not rate limited by the ionophore A23187 under our conditions. This is evident from (1) the investigation of the dependence of the calcium release velocity on the ionophore concentration (Kaupp et al., 1979a) and (2) the finding that in sonicated disk vesicles the ionophore translocates calcium which was released at the interior of disks, in $\tau_{1/2} \leq 10$ ms across the disk membrane into the outer medium (see Figure 7, Kaupp et al., 1981). Similarly, proton uptake in intact rod outer segments has a comparable rise time ($\tau_{1/2}$ = 250 ms) and is also not rate limited by the ionophore, as long as its concentration is $\geq 10\ \mu$M. When intact rod outer segments are fragmented, both calcium release and proton uptake are greatly accelerated. The temperature dependence of the rate constant of calcium release gives a nonlinear Arrhenius plot with a characteristic break at temperatures >10 °C. Concomitantly, in this temperature region, a delay phase in the rise time of the calcium-indicating signals appears (Figure 5). Both observations—nonlinear Arrhenius plot and delayed rise—are compatible with a consecutive reaction scheme for the appearance of calcium in the outer aqueous phase. The first reaction

$$A \xrightarrow[E_{a1}]{k_1} B$$

represents the proper release step whereas the second one

$$B \xrightarrow[E_{a2}]{k_2} Pr$$

represents an additional migration step of calcium from the place where it was released to the outer medium where the indicator is located. This hypothesis is further corroborated by the reconstruction of an Arrhenius plot for a consecutive reaction (Figure 4, solid line). It was assumed in this reconstruction that the rise times of calcium release in sonicated material reflected the actual velocity of the release mechanism. Consequently, rate constants k_1 were taken from Figure 8 (for convenience, the Arrhenius plot of calcium release has been redrawn in Figure 4, line with higher slope). Rate constants k_2 at various temperatures were directly calculated from Figure 4 with the aid of the activation energy E_a = 43 kJ/mol. As can ben seen from Figure 4, the break of the Arrhenius plot for temperatuers <10 °C is qualitatively reproduced by these assumptions. Our argument is further strengthened by the observation that only in the temperature region where the Arrhenius plot is nonlinear is a delay phase in the time course of calcium release detected. The initial rise of the calcium signal shown in Figure 5 can be satisfactorily simulated by assuming a consecutive reaction with rate constants k_1 = 0.97 s^{-1} and k_2 = 0.35 s^{-1} at T = 2.3 °C (compare with Figures 4 and 8).

The physical nature of the second reaction step B → Pr is unclear. Unfortunately, it cannot be inferred from our measurements. Nevertheless, the following mechanisms are possible.

(1) Free diffusion of calcium out of the photoreceptor is hindered by multiple rebinding of calcium to binding sites at the disk membrane. In the presence of A23187 (which makes all subcellular compartments accessible to calcium: Schnetkamp, 1979; Kaupp et al., 1979b), the location of these binding sites is irrelevant for slowing down the apparent velocity. A recent estimate suggests that the apparent diffusion constant of calcium in rods is lowered by at least 2 orders of magnitude if the calcium binding capacity of rod outer segment membranes is considered (McLaughlin & Brown, 1981). For example, calcium would reach the outer envelope within ~1 ms by diffusion if no calcium buffering groups existed and if the viscosity of the cytosol was similar to that of water. When the calcium buffering capacity of rod outer segment membranes is accounted for, the diffusion time would be in the order of several hundred milliseconds.

An apparent difficulty with this assumption is that the temperature coefficient is low for diffusion-controlled reactions which have activation energies in the order of a few kT (kT = 2.5 kJ/mol at 20 °C). However, when rebinding to buffering groups is allowed for, diffusion is controlled by two different terms, namely (1) the temperature dependence of the diffusion proper and (2) the temperature dependence of the change in enthalpy, ΔH, of the binding process. Therefore, an activation energy E_a of 43 kJ/mol, as observed for calcium release in intact rod outer segments, is not inconsistent with a diffusive migration step. Recently, it has been shown that calcium binding to PE/PS vesicles exhibits little temperature dependence, if any (McLaughlin et al., 1981). If these adsorption measurements can be extrapolated to disk membranes, they suggest that a diffusive step is less probable for slowing down the apparent velocity of calcium release in intact rod outer segments.

(2) Calcium release requires the occurrence of a trigger step which in its native environment is slow and therefore becomes rate limiting whereas reequilibration of calcium by diffusion is rapid. A possible trigger step for calcium release could be the uptake of protons from the cytosolic or intradiskal medium into the membranous phase. According to this model, proton uptake across the membrane/water interface is slow in an undisturbed stack of disks but is rapid ($\tau_{1/2}$ = 10 ms) in fragmented rod outer segments. This model is supported by the similar kinetic parameters of calcium release and proton uptake in intact rod outer segments and by the almost identical behavior of both reactions in response to alteration of the rod outer segment morphology. At the present time, an *unequivocal* attribution of proton uptake to calcium release is not feasible, and the above arguments should be taken as preliminary.

Is metarhodopsin I/metarhodopsin II and/or proton uptake a precursor of calcium release? In several systems, the rate by which calcium is exchange from simple ligands is in the

submillisecond time range. For example, the relaxation times of the antipyrylazo III–Ca and arsenazo III–Ca complexes are $\tau = 100$ μs and 1–3 ms, respectively (Scarpa et al., 1978). Haynes (1977) reported a relaxation time $\tau = 75$ μs for the exchange of calcium at a dimyristoyl-PC/PA membrane. The exchange of protons at proton buffering groups located at the membrane/water interface (e.g., lipid soluble pH indicator) occurs at even higher rates (Gutman et al., 1981). In analogy, the apparent velocity at which calcium release and proton uptake occur probably does not represent the proper dissociation or association rate of calcium and protons from their respective buffering groups in the membrane. More likely, the apparent velocity of both processes is determined by the rate at which the trigger step (precursor) takes place.

The time courses of the metarhodopsin I/metarhosopsin II transition and proton uptake are very similar (see Figure 6). In what follows both reactions will be considered as possible precursors of the calcium release. For the sake of simplicity, the difference between the time courses of these reactions will be ignored. Calcium release is 3–4 times slower ($\tau_{1/2} = 18.3$ ms) than the metarhodopsin I/metarhodopsin II transition or than proton uptake. If the latter reactions were precursors of calcium release, then a delay phase in the rise of the calcium signals should be observed. Such a delay phase for calcium release was never observed in our experiments with sonicated rod outer segments. At this point, it is emphasized that if the ratio of the rate constants k_1 and k_2 of the sequence

$$A \xrightarrow[k_1]{M\ I/II,\ H^{+}?} B \xrightarrow[k_2]{\Delta Ca^{2+}} Pr$$

becomes $k_1/k_2 < 0.1$, a delay would have escaped detection at the time resolution at which the calcium signal shown in Figure 1 was recorded. In this case ($k_1 \ll k_2$), however, the time course of calcium release should become equal to the time course of the precursor (e.g., metarhodopsin I/metarhodopsin II or protein uptake). Taken together from the above arguments, it appears as if calcium release, metarhodopsin I/metarhodopsin II, and proton uptake are independent from each other but accidentally occurring on the same time scale.

In conflict with this assessment are the following observations which are in favor of a connection of proton uptake and calcium release: (1) similar activation energies for both processes in sonicated as well as intact rod outer segments (compare Figures 4 and 8), (2) parallel decay of the calcium release stoichiometry and the metarhodopsin II production or proton uptake stoichiometry at pH >6.3 [see Kaupp et al. (1981)], (3) simultaneous inhibition of metarhodopsin I/metarhodopsin II and calcium release in the presence of 0.5% glutaraldehyde, and (4) similar sensitivity of calcium release and proton uptake to the structural status of the rod outer segments (Figures 2 and 3).

Finally, the reader is reminded that a proper assignment of one reaction to the other is hampered by the following points: (1) The time course of the signals can be described by at least two single exponentials. For example, several others have noted that the rise in the metarhodopsin II formation has two kinetic components, the nature of which has not been elucidated so far or is under dispute (Applebury et al., 1974; Stewart et al., 1976; Hoffmann et al., 1978; Bennett, 1978; Rapp, (1979). From Figure 7 it appears as if the slower component of metarhodopsin I/metarhodopsin II and proton uptake coincides with the rise of calcium release.

(2) There is some evidence that sonicated rod outer segments represent an inhomogeneous vesicle suspension [see Kaupp et al. (1981)]. It is conceivable that the local environments of proton uptake sites and calcium release sites slightly differ from vesicle to vesicle with a given sample, despite the presence of saturating concentrations of ionophore.

(3) The isolation of "true" time courses included correction procedures which may not completely compensate for distracting signals underlying those under investigation.

Finally, it is noted that the kinetic properties of the change of an interfacial potential ($\tau_{1/2} \sim 20$ ms, $E_a = 115$ kJ/mol; Cafiso & Hubbell, 1980) are similar to those of calcium release and proton uptake which were investigated in this paper. For corroboration of the above notion, the simultaneous measurement of the redistribution of ions across the membrane surface in response to changes of membrane electrostatics under similar conditions is highly desirable.

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.

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