OPTICAL PROBES OF INTRADISKAL PROCESSES IN ROD PHOTORECEPTORS
II: LIGHT-SCATTERING STUDY OF ATP-DEPENDENT LIGHT REACTIONS†

RAINER UHL‡ and HERBERT DESEL

Max-Planck-Institut für biophysikalische Chemie, Am Fassberg, D-3400 Göttingen (F.R.G.)

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Abbreviations

BICINE	*N,N*-bis(2-hydroxyethyl)glycine
CAPS	3-(cyclohexylamino)-1-propanesulphonic acid
DCCD	*N,N'*-dicyclohexylcarbodiimide
DIDS	4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid
DNP	2,4-dinitrophenol
EGTA	ethylene glycol-bis(*b*-aminoethylether)-*N',N',N',N'*-tetraacetic acid
FCCP	carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone
GTP	guanosine 5'-triphosphate
HEPES	(*N*-[2-hydroxyethyl]-piperazine-*N'*-[2-ethanesulphonic acid])
MI, MII	metarhodopsin I and II
MES	2-(*N*-morpholino)-ethanesulphonic acid
NMG	*N*-methyl-D-glucamine
PDE	phosphodiesterase
PIPES	1,4-piperazinediethanesulphonic acid
ROS	rod outer segment
TBT	tributyltin
$\Delta\mu(H^+)$	electrochemical potential difference of protons
$\Delta\psi$	transmembrane electrical potential

Summary

Rod outer segment (ROS) disks, either stacked or freely floating, respond to flash illumination to yield a specific, ATP-dependent, light-

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‡Author to whom correspondence should be addressed.

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scattering signal A_L. In broken ROS A_L signals occur only when A_D signals (see Part I) have preceded them. The degree to which the preceding A_D signal has been completed determines the amplitude of the following A_L signal. However, in freshly detached ROS from dark-adapted frogs A_L signals with maximal size can be obtained without pre-incubation with exogenous ATP. The energized state, which is restored in broken ROS with the help of ATP, appears to prevail in the living retina and must therefore be considered to be "physiological".

A_L signals require structurally intact disks. Neither peripheral ROS proteins nor connecting filaments between adjacent disks are necessary. Their structural origin is the same as that of the preceding A_D signal, *i.e.* osmotic disk swelling.

A_L signals consist of a single slow kinetic component (half-life 10 s at room temperature) and multiphase fast kinetic component (70 ms). The slow phase corresponds to a light-stimulated resumption of ATPase activity (this has been dealt with in a previous paper) whereas the fast component reflects an immediate response of the energized disk to the metarhodopsin I to metarhodopsin II transition. The latter effect is the subject of this paper.

A variety of experiments, using different ATPase inhibitors, ionophores and membrane-permeable salts, have been carried out; they are all consistent with the notion that A_L originates in the disk interior and probes the existence of a proton electrochemical potential difference $\Delta\mu(H^+)$ across the disk membrane.

A model is presented which can explain all given properties of A_L satisfactorily. According to this model the photolysis of rhodopsin causes a proton release in the disk lumen. This, in turn, results in osmotic swelling of the disks, provided that the internal buffer sites have been (at least partially) titrated with protons prior to the flash. Such conditions, *i.e.* a low internal pH, are provided by the proton transport across the disk membrane, which presumably takes place during the course of the preceding A_D signal.

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1. Introduction

In Part I of this series we characterized the ATP-dependent light-scattering signal A_D, which can be obtained both from ROS fragments and from isolated disks. It requires the hydrolysis of ATP and appears to reflect the translocation of ions, most probably hydrogen and chloride ions, across the disk membrane. We have provided evidence that the resulting proton electrochemical potential difference $\Delta\mu(H^+)$ is physiological, *i.e.* it prevails in the living retina. Present models of visual transduction do not require such an electrochemical potential difference $\Delta\mu(H^+)$; however, it is hard to imagine that it would be present without being of functional significance.

In this paper we describe rapid light-dependent processes within the photoreceptor cell, which rely on the ion gradients established during the production of A_D. They manifest themselves as a rapid light-scattering

transient (termed A_L) [1 - 5], and they provide a convenient probe for the hypothetical energized state of the disk. All properties of A_L are consistent with the existence of $\Delta\mu(H^+)$. A molecular model is developed which can account for the underlying processes of A_L. However, it is not known whether these processes, apart from being a convenient indicator, serve any physiologically significant function.

A_L signals consist of a single slow kinetic component and a multiphase fast kinetic component. The slow component has been shown to reflect a light-stimulated resumption of ATPase activity [6]. It is not dealt with in this paper. Instead we focus on the fast component which is a direct consequence of the previous disk energization.

2. Materials and methods

All experimental procedures have been described previously. The light-scattering apparatus was described in ref. 5, the preparation of ROS in ref. 7 and all other details in Part I of this series. The wavelength of the actinic light source was 500 ± 25 nm; the duration of the flash was 100 μs.

A_L signals have to be separated from other light-scattering transients if they are to be studied in detail. This can be done in three ways which all yield identical results. The methods of separation make use of the fact that all other light-scattering transients from ROS suspensions, except for the so-called N signal [8, 9], reflect processes that occur with the participation of peripheral disk membrane proteins. A detailed description of these signals and their separation has been given elsewhere [5]. Briefly, peripheral disk membrane proteins are removed by a hypotonic wash or by the addition of GTP-γ-S [10]. In most experiments, however, a third method was used that is even simpler. All G-protein-related signals saturate at low bleaches, *i.e.* they are no longer observed when more than 0.8% (in the presence of GTP) or 10% (in the absence of GTP) of the total rhodopsin has been bleached [11]. In contrast A_L signals, like N signals, exhibit amplitudes which are strictly proportional to the amount of unbleached rhodopsin present in the ROS [2]. Therefore, bleaching approximately 12% of the rhodopsin immediately prior to the measurement of A_L prevents interference from unwanted signals. A further advantage of this method is that the time consuming disk preparation procedure is avoided and frozen samples may be used, thus allowing the examination of many identical aliquots rather than measuring signals from a progressively aging preparation. Isolated disks cannot be frozen since this destroys their ability to produce A signals (see Part I).

The usual measurement procedure was as follows. Frozen ROS was thawed and incubated for 5 min with or without Mg-ATP, in the measuring buffer. Then, with a frequency of 2 min^{-1} four small flashes were applied, each bleaching 3% of the rhodopsin. N or A_L signals were subsequently produced by a fifth flash, which followed 30 s later and bleached approxi-

mately 25% of the rhodopsin [5]. When lysed ROS was used the four "pre-bleaching" flashes were omitted.

The amplitudes of the A_L signals given in this paper refer to the difference in amplitude of the signals obtained in the presence and absence of ATP, determined 500 ms after the flash where the fast kinetic component is almost complete and the slow component hardly contributes. However, when actual signals are displayed they usually represent the total light response, *i.e.* a superposition of A_L (fast and slow components) and N.

3. Results and discussion

3.1. A_L signals vs. N signals

When ROS or isolated disks are flash illuminated in the absence of ATP and peripheral disk membrane proteins, only one signal is observed, the so-called N or rhodopsin signal [5, 8, 9, 11]. This signal represents a decrease in turbidity, *i.e.* a decrease in scattered light intensity, whose structural and molecular origin has not been elucidated as yet. It can be obtained from any rhodopsin containing lipid vesicle or micelle, provided that it exhibits turbidity [8].

In the presence of ATP the N signal appears to be greatly enhanced (refs. 2 - 5 and Fig. 1). However, the angular dependence of the new ATP-dependent signal is different [5], pointing to a distinct signal with a different structural origin. Moreover, both the kinetics (Fig. 1) and the pH dependence (Fig. 2) of the two signals are distinct. It is conceivable that a slightly enhanced N signal contributes to the amplitude of A_L, since N signals directly reflect the MI–MII reaction [8] and the MI–MII equilibrium is shifted

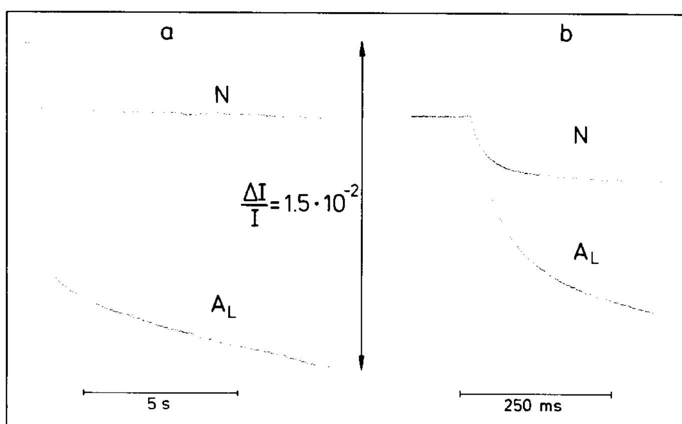

Fig. 1. N signal (in the absence of ATP) and A_L signal (in the presence of 1 mM ATP). Experimental conditions: scattering angle; 10°; temperature, 22 °C; the medium contained 60 mM Tris-HCl (pH 7.25) and 1 mM $MgCl_2$; ROS was incubated for 5 min; P signals were saturated with four flashes, each bleaching 3% of the total rhodopsin, and the above signals were produced by a fifth flash, bleaching approximately 25%; (a) and (b) show different time windows of the same signals.

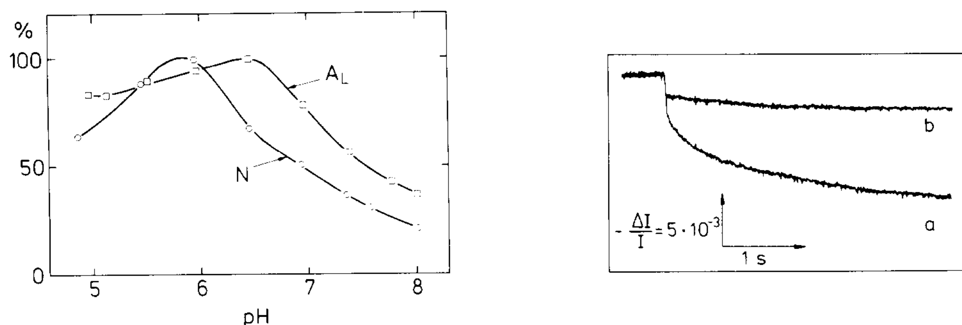

Fig. 2. Comparison of the pH dependence of A_L and N measured at a scattering angle of 10°. The medium contained 100 mM KCl, 1 mM $MgCl_2$, 1.5 μM rhodopsin and, depending on pH, one of the following buffers: pH 3.0 - 4.5, 5 mM citric acid; pH 5.0 - 6.0, 5 mM MES; pH 6.5 - 7.5, 5 mM PIPES; pH 8.0 - 9.0, 5 mM BICINE; pH 9.5 - 10.0, 5 mM CAPS. The temperature was 22 °C and the ATP concentration was 1 mM.

Fig. 3. A_L signal (a) and N signal (b) from freshly prepared frog ROS. A_L signals were obtained without addition of exogenous ATP and N signals after glasswool treatment of ROS and addition of apyrase. Experimental conditions as in Fig. 1.

towards MII in the presence of ATP [12]. However, this contribution is small and can be neglected [12].

3.2. A_L signals from freshly detached frog ROS

A_L signals have also been obtained from crude suspensions of freshly detached frog ROS. In this set of experiments less than 5 min is allowed to elapse between the death of the animal and the measurement of light-scattering transients. Under these conditions no A_D signals are observed, whether or not the plasma membrane is intact. However, on flash illumination A_L-like signals occur (Fig. 3(a)). This is not a contradiction. It suggests that the disks in these preparations are still energized. One way to destroy this energization is to keep the ROS on ice for approximately 30 min. This blocks the ATPase and allows the energization to be discharged (see also Part I). After rewarming of the ROS an A_D signal is observed. When the A_D signal is complete an indistinguishable A_L signal is observed. In order to destroy the energization completely and permanently, the ROS has to be forced through a glasswool-stuffed syringe needle in the presence of an ATP-destroying enzyme such as apyrase or hexokinase and glucose. Following this treatment only regular N signals are observed (Fig. 3(b)).

Subsequent removal of the apyrase and addition of exogenous Mg-ATP leads to the reappearance of both A_D and A_L. This clearly indicates that the energized state, established during A_D and probed by the appearance of A_L, is a physiological state of the photoreceptor disk compartment.

3.3. Structural origin of A_L and analogy between A_D and A_L

A_L signals, unlike N signals, require the structural integrity of the disk compartment. When the disk membrane is perforated by detergents or

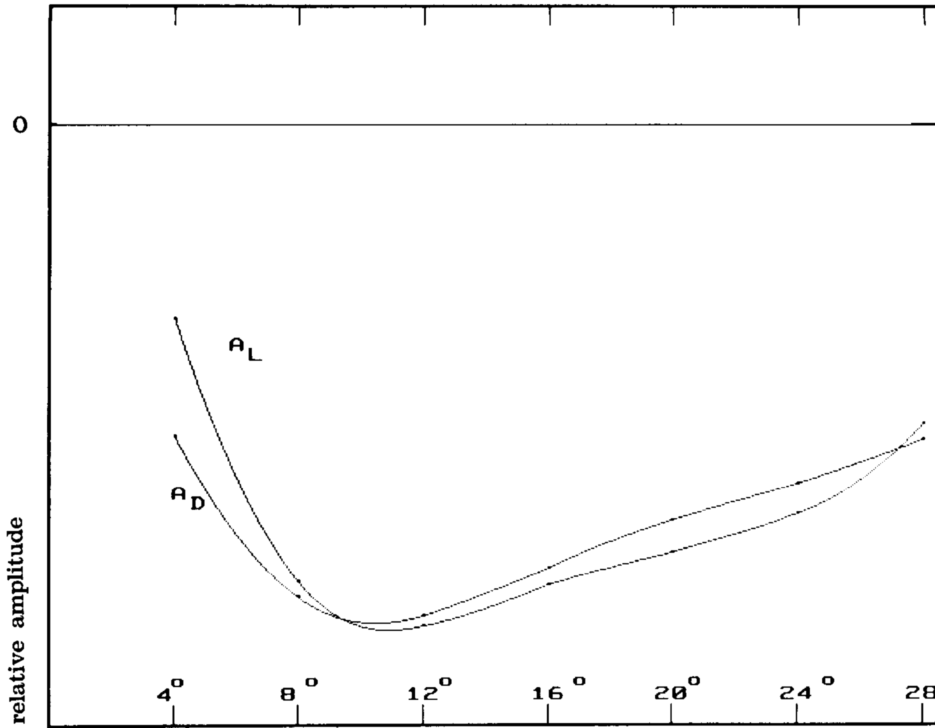

Fig. 4. Comparison of the angular dependence of A_D and A_L. Experimental conditions: room temperature; pH 7.2; the medium contained 100 mM KCl. 2 mM $MgCl_2$, 5 mM HEPES and 1 mM Mg-ATP.

lysolecithins, only N signals are observed. However, as in the case of the A_D signal the structural integrity of the disk stack is not required: both stacks of disks (in the presence and absence of GTP-γ-S) and isolated, freely floating disks exhibit A_L signals with similar kinetics (data not shown). The amplitude is usually smaller in the case of isolated disks. This may be due to the presence of some damaged disks or, as suggested by electron micrographs [7], to the presence of some small disk vesicles that contribute less to the scattering profile in the angular region under observation.

The angular dependence of A_L signals closely resembles that of the preceding A_D signals (Fig. 4), suggesting that both signals share a common structural origin: disks swell in the dark in the presence of ATP and they also swell in the light, provided that they have been pre-swollen in the presence of ATP.

3.4. *The "energized state" is a prerequisite for A_L*

In Part I we provided evidence for uptake of HCl during the production of A_D, leading to the development of an electrochemical potential gradient for protons $\Delta\mu(H^+)$ across the disk membrane. Disks are energized after completion of this process. The following results demonstrate that the

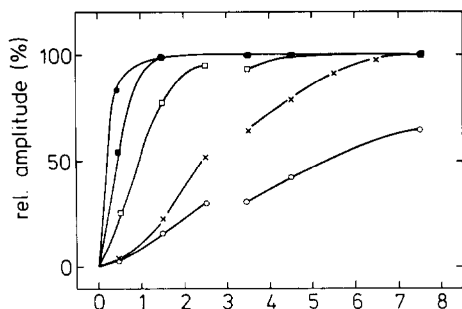

Fig. 5. Development of A_L signals over time as a function of ATP concentration. Amplitudes of A_L signals were determined at the indicated time intervals after the addition of five different ATP concentrations: ○, 4 μM ATP; ×, 10 μM ATP; □, 20 μM ATP; ■, 100 μM ATP; ●, 1 mM ATP. The medium contained 100 mM KCl, 1 mM $MgCl_2$, 1.5 μM rhodopsin and 5 mM PIPES (pH 7.0). The temperature was 22 °C. A ROS suspension was divided into two equal parts and both were incubated in the dark with varying amounts of ATP or, as a reference, with 5 U ml^{-1} of apyrase, an ATP-hydrolysing enzyme. One suspension was flash illuminated 0.5, 1.5, 2.5 and 7.5 min after incubation with ATP and the other after 3.5, 4.5, 5.5 and 6.5 min. The amplitude of the pure A_L signal was determined as the difference in the amplitudes of the signals obtained in the presence and absence of ATP. Maximal (100%) signals were obtained in the presence of 100 μM ATP after 6 min of pre-incubation. In this experiment P signals were not saturated before the first flash. However, subtracting signals obtained in the presence of apyrase provides a sufficient correction.

extent to which the energization has proceeded determines the amplitude of the subsequent A_L signal.

The temporal development of A_L signals follows the increase in amplitude of A_D, *i.e.* A_L signals increase with increasing incubation time in the presence of ATP. The more ATP that is present, the faster the increase (Fig. 5). The peculiar irregularities between 2.5 and 3.5 min are reproducible. They are due to the particular measurement procedure (see figure caption) and reflect the light stimulation of the ATPase responsible for A_D and A_L [6].

The close relationship between the dark process A_D and the subsequent light response A_L is also demonstrated by the following observations. Whenever A_D signals are enhanced, for instance in the presence of thiocyanate, iodide, imidazole or valinomycin + KCl, the corresponding A_L signals are also enhanced. Whenever A_D signals are attenuated or suppressed, for instance in the presence of sulphate, PIPES, glutamate or valinomycin + choline chloride, A_L signals are also suppressed (see also Table 1). This also holds for the action of the anion transport blocker DIDS, which blocks A_L just as it blocks A_D, provided that the transported ion is chloride. However, if lipophilic anions are used (Fig. 6; Part I, Fig. 10) no inhibition is observed. The action of DIDS must be specific and therefore our previous conclusion is further supported, namely that the disk membrane contains a DIDS-inhibited anion transport unit. Its purpose appears to be a buffering of $\Delta\mu(H^+)$. It allows much more energy to be stored in the disk than would be possible without it.

TABLE 1

Comparison of amplitudes of A_D and A_L in different reaction media

Conditions	A_D	A_L
KCl	0	0
KCl + ATP	1	1
KCl + NIG + ATP	2	2
KCl + VMC + ATP	1.5	1.5
KCl + FCCP + ATP	0.2 - 0.5	0.2 - 0.5
KCl + EGTA	1	1
KCl + EGTA + A23187	1	1
KCl + DIDS + ATP	0	0
KiA + ATP	0	0
KpA + ATP	2	2
KpA + DIDS + ATP	1.5	1.5
ImCl + ATP	1.5	1.5

Amplitudes (representing between two and ten experiments) are given in arbitrary units, with the amplitude in a standard medium (KCl + ATP) set to unity. Pure N signals have an amplitude of zero.

NIG, 1 μM nigericin; VMC, 1 μM valinomycin; FCCP, 10 μM FCCP; EGTA, 1 mM EGTA; A23187, 2 μM A23187; DIDS, 10 μM DIDS; DCCD, 20 μM DCCD; iA, impermeable anion (glutamate or PIPES); pA, permeable anion (thiocyanate or iodide); Im, imidazolium.

Lysed ROS was incubated for 5 min in a medium containing 100 mM of the particular salt, 5 mM NMG–HEPES (pH 7.2) and 2 mM $MgSO_4$. In some cases ionophores or inhibitors were also present during the incubation. After 5 min, 100 μM ATP was added and A_D and A_L signals were recorded following the usual measurement procedure.

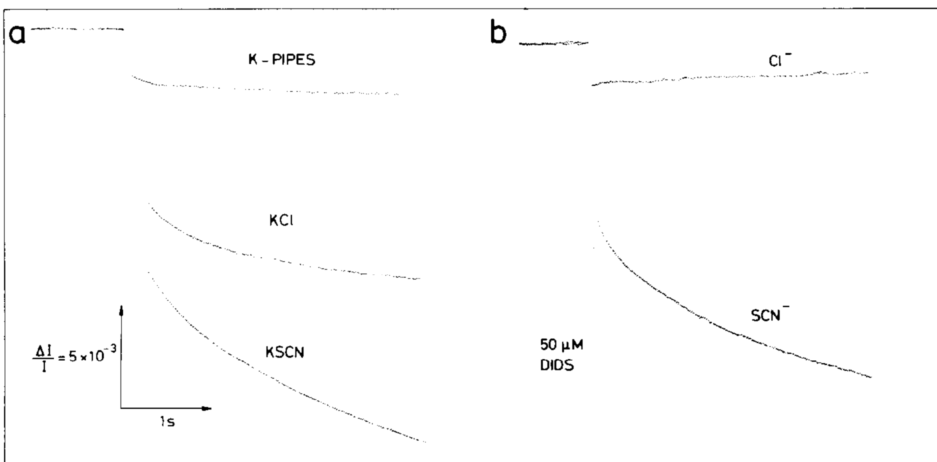

Fig. 6. A_L signals obtained in the presence of potassium salts of different anions: without 50 μm DIDS (a); with 50 μM DIDS (b). ROS was incubated in a DIDS-containing measuring buffer for 5 min before the Mg-ATP was added.

TABLE 2

Relative amplitudes of A_L in different resuspension media after the removal of ATP

Resuspension medium	A_L
KCl	f(*t*), half-life 10 min
KCl + FCCP	f(*t*), half-life 2 min
KCl + NIG + FCCP	0
KCl + NIG	0.5
KCl + TBT	0
ImCl	0.5
KpA	2
KiA	0
KCl + DIDS	0.8
KCl + DCCD	0

Abbreviations as in Table 1. Lysed ROS was incubated in a standard KCl medium in the presence of 100 μM ATP for 5 min (as in Table 1). The ROS was then pelleted in 1.5 ml Eppendorf vials in an Eppendorf centrifuge (1 min) and resuspended in the particular measuring medium, which did not contain any ATP. A_L signals were produced after 5 min of incubation.

3.5. Characterization of the "energized state"

If A_D reflects the development of an electrochemical potential gradient $\Delta\mu(H^+)$ across the disk membrane (as suggested in Part I), and if the amplitudes of A_D and A_L are strictly related (as shown above), A_L signals can be used to probe the size and stability of the supposed electrochemical potential gradient $\Delta\mu(H^+)$. We therefore carried out a number of experiments where energized disks were depleted of ATP by centrifugation and were subsequently resuspended in media of different composition (Table 2).

When the resuspension medium contains KCl and ATP, A_L signals are observed that are indistinguishable from the originals. Therefore centrifugation and subsequent resuspension have no detrimental effect. When ATP is omitted from the resuspension medium, a slow reshrinkage of disks occurs (half-life 10 min at room temperature) and A_L signals are attenuated accordingly, *i.e.* the signal amplitude decreases with the preceding reshrinkage. Within the framework of our model this reflects the leakage of HCl, driven by $\Delta\mu(H^+)$, which is consequently diminished. The observed rate of shrinkage coincides well with the leakage rate calculated from ATPase activity measurements (7% - 8% min^{-1}, see Part I).

The uncoupler FCCP accelerates the reshrinkage and hence the decay of the A_L amplitude: 10 μM reduces the half-life of the energized state to 2 min. Similarly, when FCCP is present during A_D, the swelling is attenuated and the development of subsequent A_L signals is retarded, but not completely suppressed (Table 1). The same is observed in the presence of 100 μM DNP and 2 μM SF6847, two other well-known uncouplers. It is peculiar that a potent uncoupler like FCCP should not be able to uncouple disks instantaneously and completely, and it would seem, at first sight, to contradict our

model. However, the dilemma may be resolved when certain facts, which are not immediately obvious, are taken into consideration.

(i) The disk interior is a small narrow compartment and a regular bulk phase cannot exist. This has been experimentally verified in the case of structurally preserved thylakoids [13] which bear a striking structural resemblance to the disk compartment. In this type of compartment the vast majority of the protons are membrane bound and have to be released before they can cross the membrane. Such release processes can be slow.

(ii) In our scenario $\Delta\mu(H^+)$ is buffered by $\Delta p(Cl^-)$, *i.e.* the rate-limiting step of uncoupling is not the transport of protons, but the flow of chloride ions throgh the putative chloride channel.

(iii) There is evidence from patch clamp experiments for rectification in this chloride channel (ref. 14 and Uhl *et al.*, [15]), allowing a faster chloride flux into the disk than out of it.

(iv) There may be a functional coupling between the proton ATPase and the chloride channel, as in other systems [16].

When these facts are combined it is not unusual that an uncoupler-mediated discharge of an existing electrochemical potential difference $\Delta\mu(H^+)$ can take minutes and that the presence of the uncoupler cannot prevent the build-up of $\Delta\mu(H^+)$, but only retard it.

In agreement with the above, it is observed that FCCP can uncouple ROS disks rapidly and completely when it is allowed to act synergistically with other ionophores. For instance, disks swell rapidly in potassium acetate, provided that both potassium and proton permeability exist. The former can be provided by valinomycin, the latter by FCCP [17]. Moreover, in conjunction with nigericin, FCCP exerts its uncoupling effect immediately: A_L signals are suppressed at once (Table 2). However, nigericin alone attenuates A_L, but it cannot suppress it completely.

TBT, a chloride–OH^- exchange carrier [17] which is capable of reversing the HCl uptake during A_D, destroys A_L. Imidazole, which can reduce the ΔpH component of $\Delta\mu(H^+)$, attenuates A_L considerably.

The observation that energized disks, resuspended in the presence of an impermeable anion, do not exhibit A_L signals seems to indicate that chloride (or some other permeable anion) is taken up during the production of A_L, just as in the production of A_D. However, the fact that DIDS causes only weak suppression of A_L when applied after the completion of A_D makes this unlikely. We therefore favour the following explanation: when disks are energized in a chloride-containing medium and are resuspended in a medium containing only impermeable anions, a large chloride diffusion potential develops, which, in turn, causes rapid proton leakage and hence a discharge of the energization.

The inhibition of A_L in the presence of DCCD (note that DCCD is added after the completion of A_D) is discussed in the context of our molecular model for A_L in the next section.

In summary, all results are consistent with the notion that $\Delta\mu(H^+)$ constitutes the driving force for the A_L signal. However, the mechanism through which $\Delta\mu(H^+)$ produces disk swelling during A_L is still unclear.

3.6. What happens during the production of A_L?

From the angular dependence of A_L we can conclude that disks, preswollen in the presence of ATP, respond to flashes of light by further swelling, the extent of which is proportional to the degree of previous swelling. During the course of the dark swelling it is probable that the gradients ΔpH, $\Delta\psi$ and $\Delta p(Cl^-)$ develop across the disk membrane. The fact that A_D and A_L have the same direction precludes simple models involving a light-induced discharge of existing gradients. Furthermore, the fact that the amplitude of A_L is strictly proportional to the amount of rhodopsin bleached per flash† and remains constant through a whole bleaching sequence (even longer in the presence of 11-*cis*-retinal [2]), clearly indicates that the gradients required for A_L remain constant during illumination. Whatever rhodopsin does in triggering A_L, every single rhodopsin molecule can do in exactly the same manner. Such a behaviour would be hard to reconcile if ciliary structures were involved in energization rather than ion transport across the disk membrane.

There are several light responses of the ROS which are fast, strictly proportional to bleaching and could serve as, or be related to, the trigger process of A_L.

(i) A proton uptake associated with the MI–MII transition [18 - 20] and, as a consequence, a change in interfacial potential [21].

(ii) A change in transmembrane electric potential, reflected by the fast photovoltage (early receptor potential). This is caused by a charge displacement within the rhodopsin molecule [22, 23].

(iii) A rapid calcium release in the disk lumen [20].

(iv) A rapid transient increase in cation conductance [23].

(v) A rapid increase in the rate of swelling which occurs when disks are suspended in a medium containing potassium acetate in the presence of nigericin [4, 24].

Concerning suggestion (i), the proton uptake undoubtedly occurs at the extradiskal surface [18, 19, 25]. There have been claims to the contrary [20], yet they have been convincingly disproved [19]. It has been demonstrated that disk vesicles, capable of retaining proton gradients over extended periods of time, show a rapid alkalinization which cannot be accelerated by protonophores. We have verified this using isolated disks or lysed ROS [12]. Moreover, Schleicher and Hofmann [25] have reported that a rapid alkalinization can be observed without delay if the disk stack of leaky ROS is perturbed by lowering the osmolarity to 30 mM salt. This does not change the permeability properties of the disk membrane. Therefore, the gramicidin-induced acceleration of the ΔpH signal [20] cannot reflect an accelerated proton transport across the disk membrane. The implications for our A_L signal are that a proton uptake, which occurs at the cytoplasmic surface of the disk, cannot be a trigger for A_L. The same is true for the change in

†This holds even for very small flashes, indicating that A_L does not reflect an artefact due to unphysiological high bleaches.

interfacial potential associated with the proton uptake. It should not be able to interact with an existing $\Delta\mu(H^+)$.

Concerning suggestion (ii), the charge displacement within the rhodopsin molecule, which makes the disk interior more positive, should increase $\Delta\mu(H^+)$ and hence drive the ATPase backwards. Instead, a light-induced stimulation of ATPase activity has been observed [6]. Moreover, the effect of the electric field produced by rhodopsin photolysis should not be altered by the previous energization of the disk. Therefore it cannot provide a plausible explanation for the A_L effect.

Concerning suggestion (iii), the calcium release itself cannot be the trigger of A_L, since A_L signals can be observed in the presence of A23187 and EGTA (Table 1), conditions which have been shown to suppress the calcium uptake completely [20]. However, calcium release and A_L could share a common trigger process as will be discussed below.

Concerning suggestion (iv), A_L signals are observed in the presence of impermeable cations such as choline or *N*-methylglucamine. Therefore, an increase in cation conductance cannot be essential for A_L.

Concerning suggestion (v), when the ionophore nigericin is added to leaky ROS, resuspended in a medium containing potassium acetate, a rapid swelling of the disks results [17, 24]. It is driven by an obligatory potassium–proton exchange. Protons leaving the disk can be rapidly replenished by the dissociation of acetic acid inside, since acetic acid (a small uncharged molecule) rapidly re-equilibrates. When a flash of light is applied during this nigericin-induced swelling, the swelling rate is rapidly and transiently increased [24]. Similarly, when a flash is applied after the completion of the swelling, *i.e.* when the ions have reached equilibrium concentrations, a further disk swelling is induced, closely resembling A_L. Since a swelling under such conditions requires obligatory potassium–proton exchange, the conductance increase described under (iv) cannot be responsible for this effect. Obviously the driving force for the exchange is increased by illumination. This could be brought about by an increase in external or a decrease in internal potassium concentration, or alternatively by opposite changes in proton concentration. The former two possibilities appear very unlikely and pH changes should only be possible in the disk interior, since the external medium is well buffered in our experiments. We therefore favour the assumption of a light-induced proton release in the disk lumen. It would explain the swelling observed in the presence of nigericin, it could be the trigger of the rapid calcium release [20] and it can explain all properties of our A_L signal, as is demonstrated below.

3.7. Molecular events during A_L: a model

Rhodopsin has a large number of proton binding sites which change their p*K* values on illumination [18]. Not all of these groups are accessible from the extradiskal space [18]. In our model (Fig. 7) at least one of these groups, located in the disk lumen, reduces its p*K* value dramatically on illumination. Consequently, a proton is released, provided that the group is

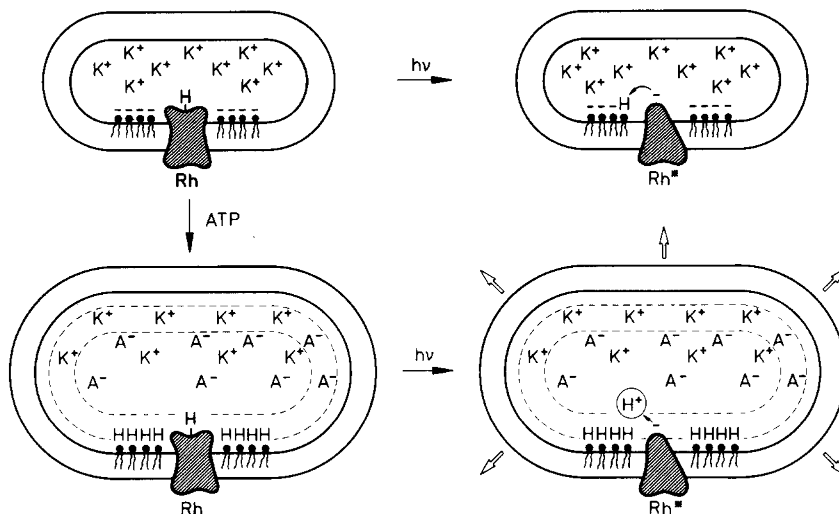

Fig. 7. Schematic representation of the proposed molecular events which occur during the production of A_L.

protonated prior to the flash. In turn, the proton can release calcium from nearby binding sites (see (iii), Section 3.6) or it can stimulate the potassium–proton exchange in the presence of nigericin (see (v), Section 3.6). In the presence of physiological sodium concentrations, only a few of these binding sites are loaded with calcium and at an internal pH of 7 most negative charges are compensated for by sodium and potassium. Proton release under these conditions does not lead to substantial osmotic swelling, since most of the released protons are buffered away by the internal proton or calcium binding sites, thus preventing them from being osmotically visible. However, under conditions of a large ΔpH as envisioned after the completion of A_D, these groups are saturated with protons and hence the released protons will have an osmotic effect. In addition, at a low internal pH the proton-releasing group of the rhodopsin molecule will be maximally protonated prior to the flash and this will cause maximal proton release when the pK changes.

A_L signals exhibit an enhanced amplitude when A_D signals are allowed to proceed in the presence of KCl and valinomycin or nigericin. Within the framework of our model this is easily understood. The gradient $\Delta\mu(H^+)$ established during the production of A_D does not depend on the presence of the ionophores. In the case of valinomycin, potassium distributes according to its Nernst potential. The light-induced swelling dilutes the internal potassium, which readily re-equilibrates, leading to further swelling. In the case of nigericin both the light-induced proton release inside and the resulting osmotic swelling increase the driving force for a further potassium–proton exchange, and this leads to additional swelling.

The enhanced amplitude of A_L in imidazolium chloride results from a similar effect, *i.e.* an uptake of more imidazole. In general, whenever ions or small molecules can equilibrate across the disk membrane, a primary swelling

step (due to the proton release) leads to a secondary step, which reflects re-equilibration of the permeable species.

The results of Table 2 are also readily understood in terms of the proposed model. Conditions which decrease the existing ΔpH value lead to a smaller (because fewer of the proton-releasing groups are protonated prior to the flash) and osmotically less efficient (because of internal buffering) proton release. Such conditions are as follows: a slow proton leakage in the absence of FCCP and a faster proton leakage in the presence of FCCP, the presence of nigericin, nigericin and FCCP, imidazole or TBT. Resuspending energized disks in KSCN leads to an enhanced A_L signal, since ΔpH should not be affected and the secondary swelling (see the paragraph above) should still be observed.

3.8. Is rhodopsin a proton pump?

There is a light-induced proton uptake by the cytoplasmic part of the rhodopsin molecule [19, 25] and there appears to be a concomitant proton release by its intradiskal part (this work). In bacteriorhodopsin, where similar effects have been observed, protons are physically translocated through the protein during the course of one photocycle. Therefore does the structural similarity between the two rhodopsin species (they both consist of seven transmembrane helices) lead to a functional similarity? The fact that DCCD (a covalently-binding inhibitor of proton-translocating ATPases) not only blocks the proton pumping in bacteriorhodopsin [26], but also A_L when added to energized disks (Table 2), seems to suggest that this is so. Moreover, on polyacrylamide gels radioactive DCCD labels not only the 160 kDa protein (thought to be the ATPase), but also rhodopsin [27]. There, however, the similarity ends. While bacteriorhodopsin causes a vectorial proton flux during each photocycle and can return to its original state without a reversal of the proton flux, rhodopsin requires an energy-consuming regeneration step in order to complete a photocycle and during this regeneration the previous proton movements are reversed.

4. Concluding remarks

From this and Part I it can be concluded that disk compartments, like many other cell organelles, possess the ability to create and maintain a $\Delta\mu(H^+)$ gradient across their boundaries. In the case of all other organelles this fact and its physiological significance have been known for many years. However, in the case of the disk membrane it has been exceedingly difficult to prove (when the calcium hypothesis of visual transduction was still in vogue, many laboratories were unsuccessfully seeking for disk energization processes) and the physiological significance is still unclear.

The fast highly amplified enzyme cascade leading to an increased PDE activity in the photoreceptor is relatively well understood. It requires neither a $\Delta\mu(H^+)$ gradient nor a fast proton release in the disk lumen. However the

fast shut off of this cascade is far from understood. Similarly, we know very little about the molecular basis of the so-called dark adaptation, *i.e.* the process that can shift the rod sensitivity over several orders of magnitude. This adaptation depends on the amount of bleached rhodopsin in the rod and it is still observed at very substantial bleaches. Whether or not the A_D- and A_L-related processes are involved in these deactivation or adaptation phenomena cannot be confirmed at present.

In addition, it is quite conceivable — and we presently favour this assumption — that the transport processes described above are somehow needed for the development and maintenance of the pronounced lipid asymmetry which exists across the disk membrane [28]. However, much more work will be required before these questions can be answered satisfactorily.

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