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Molecular Mechanism of S-Modulin Action: Binding Target and Effect of ATP[1]

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S-Modulin is suggested to increase the light sensitivity of rods by inhibiting phosphorylation of light-activated rhodopsin (Rh*) at high Ca^{2+} concentrations. The inhibition of rhodopsin phosphorylation was almost constant over a wide range of the Rh*/S-modulin ratio (10^{-4}-~10^{1}). A ^{125}I-labeled cross-linker that had been conjugated with S-modulin interacted with a protein of 60 kDa, a molecular mass close to that of frog rhodopsin kinase. These results suggested that the target molecule of S-modulin is rhodopsin kinase. To investigate the mechanism of the S-modulin action, we measured rhodopsin phosphorylation in the presence and absence of inhibition by S-modulin at various timings of ATP addition. The results suggested the following *in situ* mechanism of S-modulin action. After light-activation of rhodopsin kinase, the S-modulin/Ca^{2+} complex binds to the activated kinase and inhibits the phosphorylation of rhodopsin. The complex, however, does not affect the overall kinetics of the phosphorylation. The inhibition of the kinase by S-modulin is reversible in terms of the Ca^{2+} concentration. On the other hand, the kinase activity decreases as a function of time, probably *via* autophosphorylation.

Key words: calcium, phosphorylation, photoreceptor, rhodopsin kinase, S-modulin (recoverin).

In vertebrate rods, an inward current flows in the dark through a cGMP-gated cation channel. Light activates rhodopsin and the activated rhodopsin (Rh*) triggers a series of enzymatic reactions that ultimately leads to activation of cGMP phosphodiesterase (PDE) to result in the suppression of the inward current (for reviews, see Refs. *1-3*). While most of this current in the dark is carried by Na^+ (~70%), a small portion (~15%) is carried by Ca^{2+} (*4*). The Ca^{2+} that enters is pumped out by an Na^+-K^+, Ca^{2+} exchanger situated in the plasma membrane of the rod outer segment (*5, 6*). Since influx of Ca^{2+} is blocked while the Na^+-K^+, Ca^{2+} exchanger operates continuously in the light, the Ca^{2+} concentration decreases as a result of light absorption. This decrease in the Ca^{2+} concentration is one of the underlying mechanisms of light adaptation (*7, 8*).

S-Modulin [sensitivity-modulating protein (*9*)] found in frog rods inhibits rhodopsin phosphorylation at high Ca^{2+} concentrations and is believed to increase the light sensitivity of a rod by prolonging the life-time of Rh* (*10, 11*). Recoverin (*12*), a bovine homolog of frog S-modulin, has been shown to delay the recovery of a photoresponse (*13*) and the termination of PDE activation (*14*). The mechanism of the inhibition by an S-modulin/Ca^{2+} complex is not known and two possibilities have been proposed: the complex may bind directly to rhodopsin kinase to inhibit its activity, or it may bind to Rh* to inhibit the access of rhodopsin kinase to Rh*. In the present study, we first showed that the target molecule of S-modulin is rhodopsin kinase. Then, we further investigated the molecular mechanism of the S-modulin action by changing ATP conditions in the presence and absence of inhibition by S-modulin. The results afford a detailed insight into the mechanism of the S-modulin effect, in which Rh*, S-modulin, Ca^{2+}, and the kinase are involved.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP and Denney-Jaffe reagent (D-J reagent) were purchased from DuPont NEN; ATP and GTP from Sigma; Phenyl-Sepharose CL-6B, DEAE Sephadex A-25, and PD-10 column from Pharmacia LKB (Upsala, Sweden). All other chemicals were from Nacalai (Kyoto).

Purification of S-Modulin—The two transformants of *Escherichia coli* that express non-myristoylated S-modulin and myristoylated S-modulin, respectively, were kind gifts from Prof. Tokunaga (*15*). Recombinant S-modulin with or without myristoylation was overproduced in *E. coli* as described by Ray *et al.* (*16*). After induction of S-modulin, bacteria were collected by centrifugation, resuspended in a

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Abbreviations: D-J reagent, Denney-Jaffe reagent; D-J-S-modulin, S-modulin conjugated with D-J reagent; DTT, dithiothreitol; PDE, cyclic GMP phosphodiesterase; Rh, rhodopsin; Rh*, light-activated rhodopsin; ROS, rod outer segment.

lysis buffer consisting of 50 mM Tris, 50 mM KCl, 1 mM EGTA, 1 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride (pH 8.5), frozen at −80°C, and sonicated for 3 min. The precipitate, containing inclusion bodies of S-modulin, was resuspended in a solubilizing buffer (50 mM Tris, 50 mM NaCl, 4 mM EGTA, and 1 mM DTT, pH 8.5) containing 8 M urea. The solubilized S-modulin solution was dialyzed sequentially against the solubilizing buffer solutions containing 4, 2, 1, and 0 M urea, in this order. The solubilized S-modulin was finally dialyzed against a buffer solution containing 50 mM Tris, 200 mM NaCl, 1 mM EGTA, and 1 mM DTT (pH 8.5).

Purification of S-modulin was performed according to the method of Polans *et al.* (*17*) and Kawamura *et al.* (*18*) with slight modifications. After addition of $CaCl_2$ (5 mM; final concentration), the above solubilized S-modulin solution was applied to a Phenyl-Sepharose CL-6B column. S-Modulin bound to the Phenyl Sepharose column was eluted by a solution containing 10 mM Tris, 20 mM NaCl, 3 mM EGTA, and 2 mM $MgCl_2$ (pH 7.5). The eluate was then applied to a DEAE Sephadex A-25 column. S-Modulin was obtained as a single protein in the pass-through fraction. Using an ultrafilter (UFP2 LGC, Millipore), S-modulin was concentrated and the buffer solution was replaced with a potassium gluconate buffer (K-gluconate buffer; 115 mM potassium gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM $CaCl_2$, 0.2 mM EGTA, and 10 mM HEPES, pH 7.5).

Preparation of ROS Membranes—Frogs (*Rana catesbeiana*) were decapitated in complete darkness with the aid of an infrared image converter (NVR 2015, NEC). The photoreceptors were brushed off the retinas into the K-gluconate buffer. Rod outer segments (ROS) were fragmented by sucking into and ejecting from a thin needle (#28 gauge) 3 times vigorously. The resultant ROS membrane fragments were washed twice with the K-gluconate buffer to eliminate endogenous S-modulin. The membranes were stored overnight at 4°C in the presence of 0.1 mM ATP and 0.1 mM GTP and re-washed with the K-gluconate buffer just before use to eliminate these nucleotides.

Calcium Buffering—The Ca^{2+} concentration was buffered using a Ca^{2+}/EGTA buffering system as described elsewhere (*19*). Concentrated stock solutions (×5) were used throughout. The free Ca^{2+} concentrations were calibrated with Fura II at $<1\ \mu M$ Ca^{2+} or a Ca^{2+}-sensitive electrode (F2112Ca, Orion) at $>1\ \mu M$ Ca^{2+} using Ca^{2+} standard buffers (Calbuf-2, WPI).

Rhodopsin Phosphorylation—The procedure used for rhodopsin phosphorylation has been described previously (*10*). In brief, 25 μl of a reaction mixture containing (in final concentrations) rhodopsin (10 μM), [γ-^{32}P]ATP (0.1 mM; 24 MBq/μmol), and GTP (0.5 mM) was subjected to a light flash bleaching 0.115% of rhodopsin. The flash intensity was attenuated by inserting ND filters in the light path. When much higher bleaching was required, rhodopsin was bleached with a slide projector. The phosphorylation reaction was performed using endogenous rhodopsin kinase that was present in the washed ROS membranes. The reaction was terminated 2 min after the light exposure by addition of 150 μl of 10% (v/v) trichloroacetic acid. We used glass tubes for the phosphorylation experiment to minimize the effect of Cerenkov radiation. The reaction mixture was centrifuged at 10,000×*g* for 10 min, then the precipitate was washed once with 0.6 ml of the K-gluconate buffer and dissolved in the SDS sample buffer. The sample was subjected to SDS-PAGE, then the gel was stained with Coomassie Brilliant Blue and ^{32}P radioactivity was counted in rhodopsin bands.

Photoaffinity Labeling of the Target Molecule of S-Modulin—All labeling experiments were conducted at 0 to 4°C to minimize the degradation of Denney-Jaffe reagent (D-J reagent). S-Modulin conjugated with the D-J reagent (D-J-S-modulin) was prepared in the following way according to the manufacturer's recommendation (Fig. 1). An aliquot of the dried D-J reagent (23 pmol) having ^{125}I at its phenyl group (1.85 MBq) was dissolved in a 59 μl aliquot of 1 mg/ml of myristoylated or non-myristoylated S-modulin. The solution was stored at 4°C for 2 h to allow the derivation of primary amino groups of S-modulin with the carbonyl group at the *N*-oxysuccinimide ester bond of the D-J reagent. A 59 μl aliquot of 1 M glycine (pH 7.5) was then added to the tube to terminate the reaction, and the solution was kept at 4°C for 1 h. The resultant D-J-S-modu-

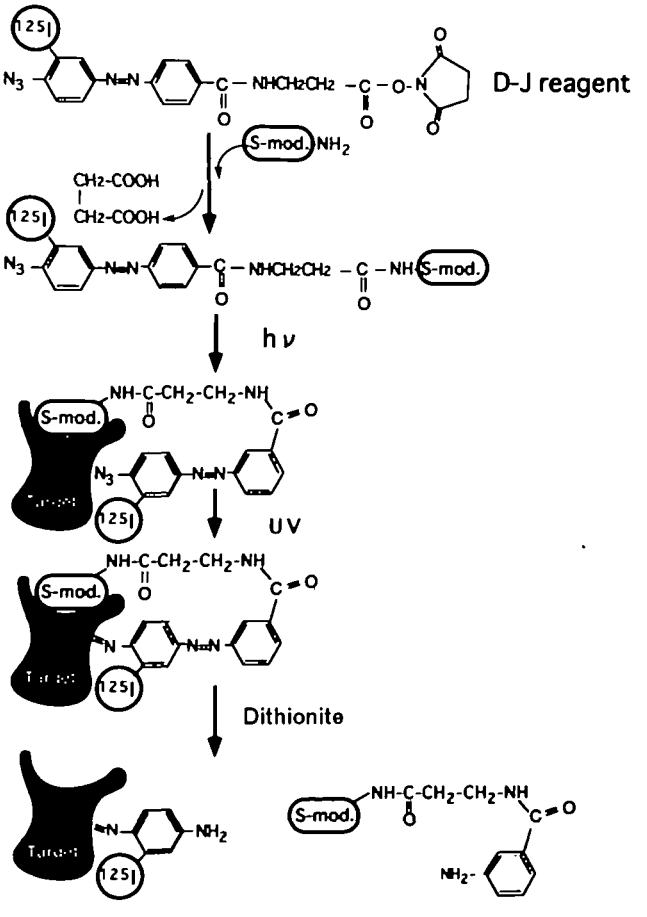

Fig. 1. **Preparation of D-J-S-modulin and mechanism of labeling of the target molecule.** D-J-S-modulin was prepared by conjugating the ^{125}I-labeled D-J reagent with purified S-modulin (see "MATERIALS AND METHODS"). The conjugate was mixed with ROS membranes in the dark, then UV light was applied to activate rhodopsin and also to introduce a covalent bond between the active azide group of the D-J-S-modulin and the target molecule. After extraction of the D-J-S-modulin/target molecule complex with detergent, the ^{125}I-labeled target was separated from S-modulin by reduction with dithionite.

lin was separated from the unreacted D-J reagent on a PD-10 column equilibrated with a phosphate buffer containing 115 mM potassium gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.2 mM EGTA, 16 mM K_2HPO_4, and 3 mM KH_2PO_4 (pH 7.5).

Photoaffinity cross-linking of D-J-S-modulin with a target molecule(s) was performed as follows (Fig. 1). ROS membranes containing 200 μM rhodopsin (50 μl) were mixed with 50 μl of the D-J-S-modulin (7.7 μM) in the dark, and then the mixture was exposed to a UV light (360 nm) for 15 min to allow the formation of a covalent bond between the azide group of the reagent and the target molecule(s). To avoid the degradation of S-modulin by irradiation, ovalbumin was added to the mixture at a final concentration of 0.1 mg/ml. The membranes were then washed 3 times with the K-gluconate buffer by centrifugation (10,000 $\times g$, 10 min) to eliminate free D-J-S-modulin. Membrane proteins, including those covalently linked with the D-J-S-modulin, were extracted with 30 μl of a K-gluconate buffer solution containing 1.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS). The diazo linkage in the D-J reagent was then cleaved by incubation with 12.9 μl of 1 M sodium dithionite at room temperature for 3 h. The extract was applied to SDS-PAGE and the gel was subjected to ^{125}I autoradiography to detect proteins that were labeled with ^{125}I.

RESULTS AND DISCUSSION

Target Molecule of S-Modulin—There are at least two candidate proteins as the target of S-modulin: one is Rh* and the other is rhodopsin kinase. To examine whether Rh* is the target molecule of S-modulin, we first measured the inhibition of rhodopsin phosphorylation by S-modulin with various values of Rh*/S-modulin ratio. If S-modulin binds to Rh* to hamper the access of rhodopsin kinase, the effect of S-modulin is expected to decrease when an excess of Rh* is present over S-modulin. The result (Fig. 2), however, showed that rhodopsin phosphorylation deviated from the expected curve (broken line) and was constant from a low level of Rh*/S-modulin ratio (approx. 10^{-4}) to the level where the amount of Rh* significantly exceeds that of S-modulin (approx. 7-fold). This result suggested that the target molecule is not Rh*. A similar result has been obtained by Klenchin *et al.* (*20*).

Then we tried to identify the target molecule with a ^{125}I-labeled cross-linker conjugated with S-modulin beforehand. In our previous studies (*11, 19*), we had always used washed ROS membranes and observed Ca^{2+}-dependent inhibition of rhodopsin phosphorylation by exogenously added purified S-modulin. These results indicated that the target molecule is present in washed membranes. One can therefore expect that the target molecule is membrane-bound and the S-modulin/Ca^{2+} complex interacts with it at high Ca^{2+} concentrations. For this reason, we mixed [^{125}I]D-J-S-modulin with washed ROS membranes at high and low Ca^{2+} concentrations to detect the binding target molecule(s) of S-modulin (see "MATERIALS AND METHODS"). After extraction with a detergent, the ^{125}I-labeled membrane proteins were analyzed by SDS-PAGE (Fig. 3).

Figure 3A shows the ^{125}I autoradiograph obtained with myristoylated S-modulin at low (47 nM) and high (10 μM) Ca^{2+} concentrations. A major band close to 60 kDa was found only at the high Ca^{2+} concentration (open arrow). The result indicated that this protein binds to S-modulin only at high Ca^{2+} concentrations, which suggests that it is the target molecule. Another major band of radioactivity was found at 26 kDa irrespective of the Ca^{2+} concentration. This

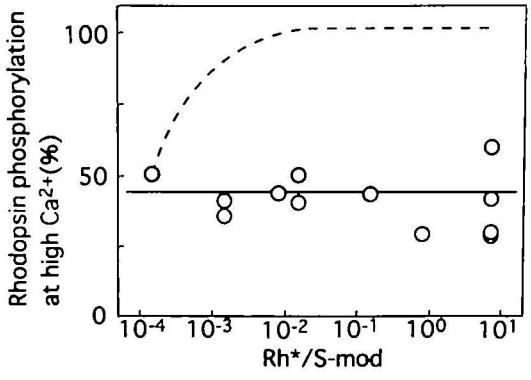

Fig. 2. **Inhibition of rhodopsin phosphorylation as a function of Rh*/S-modulin.** In the presence of S-modulin (10 μM), rhodopsin phosphorylation was measured at a high (10 μM) Ca^{2+} concentration by varying the Rh*/S-modulin ratio (Rh*/S-mod). Each data point shows a single determination and is normalized to the amount of phosphorylation observed in the absence of S-modulin at a low (47 nM) Ca^{2+} concentration at each Rh*/S-modulin level. To obtain Rh*/S-mod ratios from 1.5×10^{-4} to 0.75, light intensities were calibrated and the amounts of bleached rhodopsin were calculated. To obtain the Rh*/S-modulin ratio of 7, rhodopsin concentration was increased to 70 μM and rhodopsin was 100% bleached. The broken line shows the expected curve if the S-modulin/Ca^{2+} complex binds to Rh* to inhibit rhodopsin phosphorylation. The curve was plotted using the equation for inhibition by binding of an inhibitor to the substrate which is identical with the normal equation for simple competitive inhibition (*36*). We assumed that the inhibition is 50% at the Rh*/S-modulin ratio of 10^{-4}.

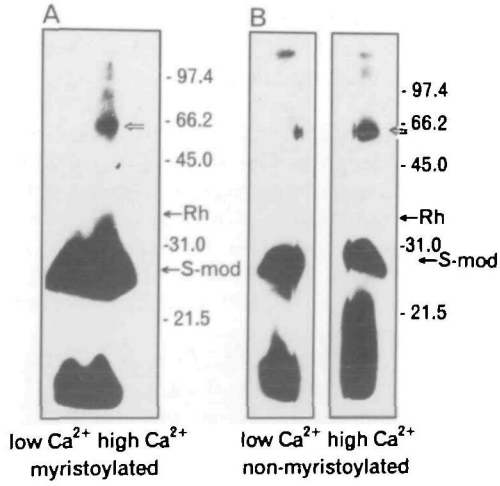

Fig. 3. **Autoradiograph of possible target molecule(s) labeled with ^{125}I in the D-J reagent.** The binding protein(s) of S-modulin was identified using D-J reagent (see "MATERIALS AND METHODS"). A: Autoradiograph obtained with myristoylated S-modulin at low (47 nM; left) and high (10 μM; right) Ca^{2+} concentrations. B: Autoradiograph obtained with non-myristoylated S-modulin at low (47 nM; left) and high (10 μM; right) Ca^{2+} concentrations. Molecular masses of the standards are indicated. The positions of molecular masses of rhodopsin and S-modulin are also indicated by arrows. A positive band near 60 kDa is indicated by an open arrow.

band is indistinguishable from the S-modulin band. We speculated that this band corresponds to [^{125}I]D-J-S-modulin somehow trapped in the ROS membrane non-specifically.

The Ca^{2+}-bound form of S-modulin is sticky (*21*) and for this reason the observed Ca^{2+}-dependent binding of myristoylated S-modulin to a 60 kDa protein could be non-specific. However, this possibility was excluded in the experiment using non-myristoylated S-modulin. Recoverin has been shown to be myristoylated at the N-terminal glycine (*22*). While myristoylated recoverin binds to ROS membranes in a Ca^{2+}-dependent manner, the non-myristoylated form does not (*23, 24*). Even so, the non-myristoylated form does have the ability to inhibit rhodopsin phosphorylation (*24, 25*). The non-myristoylated form, therefore, would be a more suitable form of S-modulin to detect specific interaction between D-J-S-modulin and the target molecule(s). Figure 3B shows that the non-myristoylated form of D-J-S-modulin also binds to a 60 kDa protein in a Ca^{2+}-dependent manner (open arrow). It should be mentioned here that we did not observe radioactivity in proteins whose molecular mass is close to that of rhodopsin (Fig. 3, A and B).

From the results in Figs. 2 and 3, we concluded that the 60 kDa protein is the target molecule of S-modulin. The molecular mass of this protein is close to that of rhodopsin kinase in frog retina (*20*). Our attempt to identify the kinase with anti-rhodopsin kinase antibody (a kind gift from K. Palczewski) was not successful, because this antibody was raised against a synthetic peptide of bovine rhodopsin kinase and did not react with frog rhodopsin kinase in our ROS membranes (data not shown). Previous studies showed that recoverin co-elutes in the presence of Ca^{2+} with a protein whose molecular mass (67 kDa) is close to that of rhodopsin kinase (*26*). Rhdopsin kinase solubilized with a detergent was identified as a binding protein on a recoverin-immobilized column (*27*). Our result is consistent with these findings and also with recent studies in which rhodopsin kinase was suggested to be the target molecule of S-modulin (*20, 27, 28*).

Both myristoylated and non-myristoylated S-modulin bind to a Phenyl Sepharose column in a Ca^{2+}-dependent manner (see "MATERIALS AND METHODS"), which indicated that both forms expose hydrophobic amino acid residues at their molecular surfaces on binding of Ca^{2+}. Rhodopsin kinase has also been reported to have a hydrophobic region (*29*). It is possible that their hydrophobic interaction is the underlying mechanism of their binding observed in the present study.

Time Course of Rhodopsin Phosphorylation in the Presence of ATP Throughout—In Fig. 4, the effect of S-modulin on the time course of rhodopsin phosphorylation was examined in the presence of ATP throughout. [γ-^{32}P]ATP was added 30 s before a light flash and the phosphorylation was terminated at the indicated times (open symbols). The phosphorylation increased as a function of time and reached a constant level at 3-4 min after the light flash both in the presence (high Ca^{2+}; open circles) and absence (low Ca^{2+}; open triangles) of inhibition by S-modulin. Though the overall phosphorylation was low in the presence of the inhibition, the time courses of the increase in the phosphorylation were similar in the presence and absence of the inhibition. This result indicated that the mode of the inhibition is simple: only the magnitude of the phosphorylation is affected by the S-modulin/Ca^{2+} complex and the time course of the phosphorylation itself is not affected.

The fact that the phosphorylation reached a constant level (Fig. 4) suggested that the phosphorylation reaction terminated at the constant level even in the presence of the inhibition where the phosphorylation was about 50%. This idea was supported in the following experiment where the inhibition by S-modulin was relieved during incubation.

In three samples, EGTA was separately added at 0.5, 2.5, and 10 min after a light flash to relieve the inhibition by S-modulin and the phosphorylation was quenched 2 min after the addition (closed circles in Fig. 4). The phosphorylation increased significantly at early times after the flash due to relief of the inhibition. The increases in the phosphorylation (broken arrows) were comparable with those observed in the absence of the inhibition (low Ca^{2+}; open triangles) during corresponding periods. The result indicated that the S-modulin effect is completely reversible in terms of the Ca^{2+} concentration. The increase in the phosphorylation, however, was not found at later times: at 10 min after a light flash, the relief of the inhibition did not induce additional phosphorylation. The result, therefore, indicated that the phosphorylation reaction terminated at 10 min after a flash not only in the absence of the inhibition (open triangles), but also in the presence of the inhibition (open circles). The termination of the phosphorylation was not due to consumption of ATP, since our HPLC measurement of ATP indicated that at least 84 μM ATP was present at 10 min after the light flash. One possibility to account for the termination of the phosphorylation is the conversion from Rh* to a photoproduct that cannot be phosphorylated, but this possibility was excluded. Instead, the termination was found to be due to deactivation of rhodopsin kinase (see below).

Recently, Senin *et al.* (*30*) measured a rhodopsin phos-

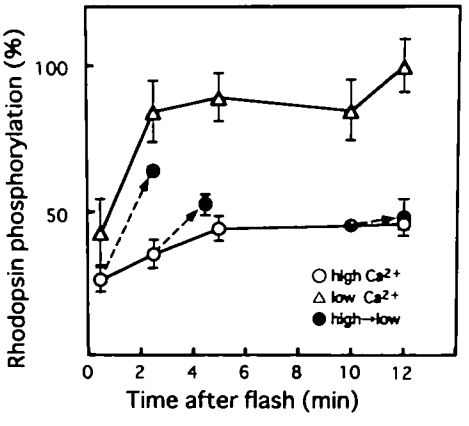

Fig. 4. Time course of rhodopsin phosphorylation in the presence of ATP. After addition of [γ-^{32}P]ATP, ROS membranes were illuminated with a light flash bleaching 0.115% of rhodopsin in the absence (47 nM Ca^{2+}; triangles) and presence (10 μM Ca^{2+}; open circles) of inhibition by 10 μM S-modulin. Each data point shows the phosphorylation obtained by incubation in the dark for the indicated period after the flash (mean±SD; n=3-5). In some measurements, the inhibition by S-modulin was relieved by reducing the Ca^{2+} concentration from 10 μM to 1 nM by addition of EGTA for 2 min as indicated.

phorylation time course in the presence of ATP in the same manner as above. Their result, however, is different from ours: rhodopsin phosphorylation at a low Ca^{2+} concentration was much slower than ours (~40 min to reach a steady level), and at a high Ca^{2+} concentration, there was a light-induced initial rapid increase in the phosphorylation (<1 min), but the phosphorylation stayed constant thereafter. The reason for this difference is not known. However, one reason might be the difference in the preparation. They used freeze-thawed bovine ROS membranes with purified rhodopsin kinase while we used fresh frog ROS membranes with endogenous membrane-bound kinase.

Time Course of the Rate of the Phosphorylation When ATP Is Omitted during Incubation—In the experiment in Fig. 4, ATP was present throughout and under this condition, rhodopsin phosphorylation terminated at 10 min after a light flash. In the experiment shown in Fig. 5, it is clear that rhodopsin phosphorylation can take place even 10 min after a light flash when ATP is omitted during incubation. In this experiment, a pulse of ^{32}P-ATP was given for 2 min at indicated times. The major difference in this experiment from that in Fig. 4 was that ATP was present for 2 min only during the measurement of the phosphorylation but not before this period. The result showed that the phosphorylation decreased as a function of time both in the presence (high Ca^{2+}; open circles) and absence (low Ca^{2+}; open triangles) of the inhibition. As expected, the phosphorylation was low in the presence of the inhibition. In some measurements, the Ca^{2+} concentration was kept high until ATP addition to inhibit the kinase, but was made low to relieve the inhibition during incubation with ATP (high→low; closed circles). The phosphorylation measured with this protocol was the same as that observed in the samples where no inhibition was present throughout (low Ca^{2+}; open triangles).

There were two noteworthy points in this experiment. First, the phosphorylation was not zero at 10 min after the light flash in the presence or absence of the inhibition. When the inhibition was relieved during incubation with ATP (closed circles), the phosphorylation was the same as that observed in the samples where no inhibition was present throughout (open triangles). These results showed that at the time of the ATP addition, the level of the substrate, namely the amount of non-phosphorylated Rh* was high and the same in the presence and absence of the inhibition. In other words, in the measurement in Fig. 5 where no ATP was present, non-phosphorylated Rh* was present at 10 min after the light flash even in the presence of the inhibition.

From the above consideration, the termination of the phosphorylation at 10 min after the light flash in Fig. 4 can be attributed to deactivation of the kinase. In Fig. 4, where ATP was present throughout, the constant level of phosphorylation was about half that in the presence of the inhibition. As far as the rhodopsin photoproducts are concerned, ATP is expected to contribute only to the phosphorylation reaction. Since non-phosphorylated Rh* can be present at 10 min after the light flash as shown above, the remaining half of non-phosphorylated species must be non-phosphorylated Rh*. Then, the inability to increase the phosphorylation at 10 min after the flash in Fig. 4 can be attributed to deactivation of the kinase. The deactivation is most probably a consequence of autophosphorylation (see below). Since the time courses of the phosphorylation were the same in the presence and absence of the inhibition in Fig. 4, the deactivation kinetics of the kinase must be independent of the inhibition by S-modulin.

The second noteworthy point in Fig. 5 was that the decrease in the phosphorylation was biphasic. One component showed a fast decay with a half-life of about 1 min and the other showed almost constant activity in the time range covered in this study. The 1 min half-life is close to that of the decay of metarhodopsin II in frog retina (*31*). The result therefore suggests that metarhodopsin II is either a preferred substrate for the phosphorylation or a more potent activator of the kinase than the later photoproducts. In the present study, we could not distinguish which is the case.

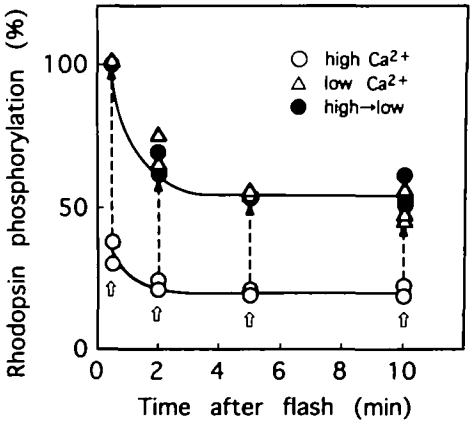

Fig. 5. **Time course of the rate of the rhodopsin phosphorylation in the absence of ATP.** ROS membranes were illuminated by a light flash bleaching 0.115% of rhodopsin and were kept in the dark for the indicated periods in the absence (47 nM Ca^{2+}; triangles) and the presence (10 μM Ca^{2+}; open circles) of inhibition by S-modulin. [γ-^{32}P]ATP was then added (open arrows) and the sample was incubated for 2 min to measure the phosphorylation. Each data point shows a single measurement. In some measurements, the inhibition by S-modulin was relieved by reducing the Ca^{2+} concentration from 10 μM to 1 nM by addition of EGTA during the period of the phosphorylation measurement (filled circles and broken arrows).

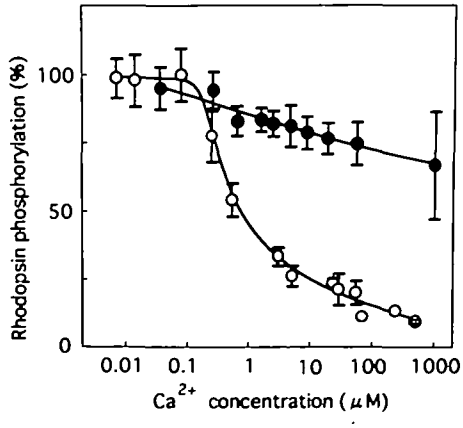

Fig. 6. **Effect of the Ca^{2+} concentration on the inhibition of rhodopsin phosphorylation by S-modulin.** Rhodopsin phosphorylation was measured at various calibrated concentrations of Ca^{2+} in the absence (closed circles) and presence of 10 μM S-modulin (open circles). Each data point is a mean ± SD (n=3-6).

Inhibition on Rhodopsin Phosphorylation as a Function of Ca^{2+} Concentration—In Fig. 6, rhodopsin phosphorylation was measured at various calibrated Ca^{2+} concentrations in the presence (open circles) and absence (closed circles) of 10 μM S-modulin. In the first report on the inhibition of rhodopsin phosphorylation by S-modulin, one of us described that a half effect was observed at 100 nM Ca^{2+} (*10*). Later studies done by other groups, however, indicated that the half effect could be observed at much higher Ca^{2+} concentrations (1-3 μM) (*20, 27*). We re-investigated this issue using calibrated Ca^{2+} buffer solutions. The result showed that the half effect was observed at about 600 nM, a slightly higher value than that which we reported previously. The discrepancy between our present and the previous values may be ascribed to whether the Ca^{2+} concentration was calibrated (present study) or not (previous study).

Possible In Vivo Mechanism of S-Modulin and Kinase Action—As shown above, rhodopsin kinase is active for more than 10 min in the absence of ATP (Fig. 5), but is deactivated in its presence (Fig. 4). This deactivation in the presence of ATP is possibly due to autophosphorylation of rhodopsin kinase. Autophosphorylated kinase has been suggested to be an inactive form of the kinase (*32*). Taking this mechanism into account, one can postulate the *in vivo* mechanism of the S-modulin and the kinase action to be as follows (Fig. 7).

When a rhodopsin molecule is activated by absorption of a photon, it activates both transducin (*33*) and rhodopsin kinase (*34, 35*). When the cytoplasmic Ca^{2+} concentration is high as in the dark-adapted state, the S-modulin/Ca^{2+} complex binds to the activated kinase to inhibit rhodopsin phosphorylation. The site of action of the S-modulin/Ca^{2+} complex must be on the activated kinase but not the activation reaction of the kinase. This is because even when the kinase was activated firstly in the absence of inhibition by S-modulin (low Ca^{2+} concentration), rhodopsin phosphorylation was still inhibited by the S-modulin/Ca^{2+} complex at increased Ca^{2+} concentrations (data not shown). The inhibition of the phosphorylation by the complex continues as long as the kinase is active. The activated rhodopsin kinase, however, undergoes autophosphorylation for deactivation. Therefore, at a certain period of time after the light flash, the kinase becomes deactivated. This process proceeds independently of the inhibition by the S-modulin/Ca^{2+} complex.

Unfortunately, the measurement of the phosphorylation on the 60 kDa protein (most probably rhodopsin kinase) was difficult in the present study. This was because the ^{32}P signal of rhodopsin dimer overlapped with that of the 60 kDa protein and the signal of the dimer masked the ^{32}P signal of the 60 kDa protein. Only when the rhodopsin phosphorylation level was very low at a very high Ca^{2+} concentration (0.1-1 mM) in the presence of S-modulin, could we detect phosphorylation on the 60 kDa protein (data not shown). Even though the mechanism shown in Fig. 7 accounts for our present result, further study is needed to confirm its validity.

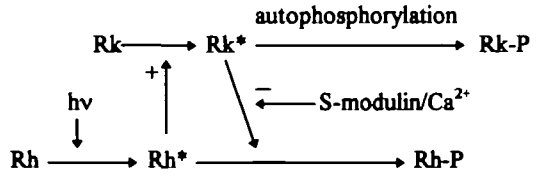

Fig. 7. **Mechanism of S-modulin and rhodopsin kinase action.** Light-activated rhodopsin (Rh*) activates rhodopsin kinase (Rk). Rh* is in turn phosphorylated by the activated rhodopsin kinase (Rk*). Rk* undergoes autophosphorylation for inactivation (Rk-P). S-modulin/Ca^{2+} binds to Rk* to inhibit it in a Ca^{2+}-dependent manner. The binding does not significantly affect the autophosphorylation of Rk*.

In the postulated mechanism shown in Fig. 7, autophosphorylation should be triggered after activation of rhodopsin. Even though we do not have direct evidence of it because of our inability to measure ^{32}P in the 60 kDa protein, our indirect evidence suggested that this is the case. In the present study, we used ROS membranes incubated overnight with ATP, which was washed away just before use (see "MATERIALS AND METHODS"). Therefore, any ATP effect observed in the present study, including the effect on rhodopsin kinase, should be dependent on activation of rhodopsin.

In the present study (Fig. 4), non-phosphorylated Rh* was shown to be present at 10 min after a light flash in the presence of both ATP and inhibition by the S-modulin/Ca^{2+} complex. However, the persistence of the presence of non-phosphorylated Rh* for such a long period may be due to very high light intensity used in the present *in vitro* experiment (0.115% bleach or $\sim 3.5 \times 10^6$ Rh*/ROS). In electrophysiological measurements of a light response, the light intensity necessary to evoke a photoresponse is in the range of 10^1-10^3 Rh*/ROS. Thus, under physiological conditions, all of Rh* would be phosphorylated before all of the rhodopsin kinase was autophosphorylated, or deactivated.

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