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PURIFICATION AND CHARACTERIZATION OF S-MODULIN, A CALCIUM-DEPENDENT REGULATOR ON cGMP PHOSPHODIESTERASE IN FROG ROD PHOTORECEPTORS

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S-modulin is a 26 kDa protein that regulates light sensitivity of cGMP phosphodiesterase in a Ca^{2+}-dependent manner in frog rod outer segments (ROSs). In the present study, we purified S-modulin by taking advantage of a hydrophobic interaction between Phenyl Sepharose and S-modulin at high Ca^{2+} concentrations. The yield was greater than 90 %. $^{45}Ca^{2+}$-binding experiment showed that S-modulin is a Ca^{2+}-binding protein. At high Ca^{2+} concentrations, S-modulin binds to ROS membranes. The binding target of the Ca^{2+}/S-modulin complex is possibly a ROS membrane lipid(s), but it was difficult to identify. The binding was observed mainly at > 1 μM Ca^{2+}. The amino acid sequence deduced from proteolytic fragments of S-modulin was approximately 80 % and 60 % identical to those of recoverin and visinin, respectively.

In rod photoreceptors, cGMP opens a cation channel and allows a current to enter the cell in the dark (1). On light absorption by rhodopsin, cGMP phosphodiesterase (PDE) becomes active to hydrolyze cGMP so that the channel closes to hyperpolarize the cell (2,3).

Rods are desensitized during light-adaptation. Since the electrical activity of rods is regulated by cytoplasmic cGMP concentration, the synthesis and the hydrolysis of cGMP must be modulated during light-adaptation. Recent work revealed that cytoplasmic Ca^{2+} concentration decreases during light-adaptation (4-6). This Ca^{2+} concentration decrease is thought to be at least one of the mechanisms of light-adaptation: when the decrease is blocked, photoreceptors do not show light-adaptation (7,8).

Our previous electrophysiological work showed that PDE activation is regulated by Ca^{2+} at concentrations in the physiological range: PDE light-sensitivity is higher and PDE light activation persists longer at 1 μM Ca^{2+} than at 30 nM Ca^{2+} (9). In addition, we isolated a 26 kDa protein, S-modulin that mediates the Ca^{2+} decrease signal to PDE: in the presence of S-modulin, PDE light-sensitivity was a function of Ca^{2+} concentration but not in its absence (9).

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In the present work, we further characterized S-modulin which was obtained by using a hydrophobic interaction between Phenyl Sepharose and a Ca^{2+}/S-modulin complex.

MATERIALS AND METHODS

Preparation of rod outer segment membranes

Frog (**Rana catesbeiana**) retinas were removed from pigment epithelium by forceps. Rod outer segments (ROSs) were brushed off the retinas and fragmented by passing through a #28 gauge needle several times in a solution containing 1 nM Ca^{2+} which was obtained by adding EGTA at a final concentration of 2.78 mM to a K-gluconate buffer solution (115 mM K-gluconate, 2.5 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM $CaCl_2$, 0.2 mM EGTA, 10 mM HEPES, pH 7.5). After centrifugation (15,000 x g, 5 min), the precipitate was washed with the K-gluconate buffer three times to eliminate endogenous S-modulin. The supernatant fraction obtained after the fragmentation at 1 nM Ca^{2+} (ROS low Ca^{2+} extract) contained S-modulin and was used for purification of S-modulin (see below).

Purification of S-modulin

Phenyl Sepharose CL-4B (Pharmacia-LKB) was equilibrated with the K-gluconate buffer containing 1 mM $CaCl_2$ (1 mM Ca^{2+}/K-gluconate buffer). By adding $CaCl_2$, the ROS low Ca^{2+} extract containing S-modulin (see above) was made 1 mM Ca^{2+} and then applied to a Phenyl Sepharose column (1 x 10 cm). After loading the extract, the column was washed with the 1 mM Ca^{2+}/K-gluconate buffer. Then the K-gluconate buffer containing 5 mM EGTA (5 mM EGTA/K-gluconate buffer) was applied to the column to elute proteins that bound to Phenyl Sepharose CL-4B at 1 mM Ca^{2+} but became soluble at 5 mM EGTA ($[Ca^{2+}] < 1$ nM).

DEAE-Sephadex A-25 (Pharmacia-LKB) was equilibrated with a 40 mM K-gluconate buffer which was made by reducing the K-gluconate concentration to 40 mM in the K-gluconate buffer while the other ingredients remained unchanged. S-modulin fractions obtained from the Phenyl Sepharose column were condensed in the 40 mM K-gluconate buffer and then applied to the DEAE-column (1 x 16 cm). The pass-through fraction was collected. Purity of S-modulin was greater than 90 %.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and $^{45}Ca^{2+}$-binding experiment

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (10) using 10-20% linear gradient acrylamide gel. $^{45}Ca^{2+}$ binding to proteins was examined by the $^{45}Ca^{2+}$-blot analysis. Proteins were transferred electrophoretically to Immobilon P membranes (Millipore) and the $^{45}Ca^{2+}$-blot was developed as described by Maruyama et al. (11).

Ca^{2+}-dependent binding of S-modulin to ROS membranes or liposomes

Liposomes were prepared in the following way. One mg of lipid(s) was dissolved in a chloroform-methanol (2:1) solution. After evaporating the solvent under a nitrogen stream, 1 ml of the K-gluconate buffer was added and the sample was exposed to ultra sonication for 1 min. The formed liposomes were collected by centrifugation (15,000 x g, 15 min).

To reduce the non-specific binding of S-modulin, ROS membranes or liposomes were first treated with 0.5 mg of bovine serum albumin. ROS membranes or liposomes were then washed three times by centrifugation (15,000 x g, 15 min) with 1 ml of the 1 mM Ca^{2+}/K-gluconate buffer each time. To the precipitated ROS membranes or the liposomes, 10 μl of 0.5 μM S-modulin was added. The sample was centrifuged after 20 min incubation at room temperature, and the supernatant fraction (high Ca^{2+} extract) was analyzed for measuring the amount of S-modulin that did not bind to the ROS membranes or the liposomes at a high (1 mM) Ca^{2+} concentration. Ten μl of the 30 mM EGTA/K-gluconate buffer was then added to the precipitate and the sample was centrifuged again. The supernatant fraction (low Ca^{2+} extract; $[Ca^{2+}] < 1$ nM) was analyzed for measuring the amount of S-modulin that became soluble at a low Ca^{2+} concentration.

When S-modulin binding to ROS membranes was examined as a function of Ca^{2+} concentration (Fig. 3), the membranes were washed with the K-gluconate buffer containing Ca^{2+} of desired concentration and then S-modulin was added. The Ca^{2+} concentration was buffered by using Ca^{2+}/EGTA buffering system as described (12).

Lipids used in the experiment shown in Table 1 were mostly purchased from Sigma. Phosphatidyl choline containing unsaturated fatty acids was a kind gift from Prof. T. Akino (Sapporo Medical School).

Amino acid sequence analysis

Approximately one nmole of purified S-modulin was digested with 10 pmoles of lysyl endopeptidase (EC 3.4.21.50; Wako) in 30 μl of a 20 mM Tris buffer (pH 9.2) containing 0.05 % SDS at 37°C for 6 h. Digested fragments were separated on a reversed phase HPLC column (Cosmosil $5C_{18}$-AR, Nacalai) using a 0-80% linear gradient of acetonitril over 60 min in 0.1% trifluoroacetic acid. Each fragment was analyzed with an automated gas phase protein sequencer (Model 477A, Applied Biosystems).

RESULTS AND DISCUSSION

Purification of S-modulin using Phenyl Sepharose and DEAE-Sephadex

S-modulin binds to ROS membranes at high Ca^{2+} concentrations, which suggested a possible hydrophobic interaction between a Ca^{2+}/S-modulin complex and membrane lipids (9). A similar Ca^{2+}-dependent hydrophobic interaction was observed in calmodulin (13,14) and this protein was purified using Phenyl Sepharose (15). Therefore, we tried to purify S-modulin using a similar method.

The ROS low Ca^{2+} extract was made 1 mM Ca^{2+} and applied to a Phenyl Sepharose column. The eluent was fractionated and analyzed on SDS-PAGE (Fig. 1A, lanes 1-20). Most proteins passed through the column (lanes 1-4). On application of the 5 mM EGTA/K-gluconate buffer, S-modulin (26 kDa) was eluted, which suggested that the Ca^{2+}/S-modulin complex bound to the phenyl residue of the column through a hydrophobic interaction.

The fractions containing S-modulin were pooled. In our previous work, S-modulin was found to be eluted at < 80 mM NaCl from the DEAE-Sephadex column (9). Therefore, the pooled fractions were diluted so that the K-gluconate concentration was 40 mM, and were applied to the DEAE-Sephadex column. The eluent which passed through the column was collected and analyzed by SDS-PAGE (Fig. 1B, lane 2). The purity of S-modulin was greater than 90 % judging from densitometry after Coomassie Brilliant Blue (CBB)-staining.

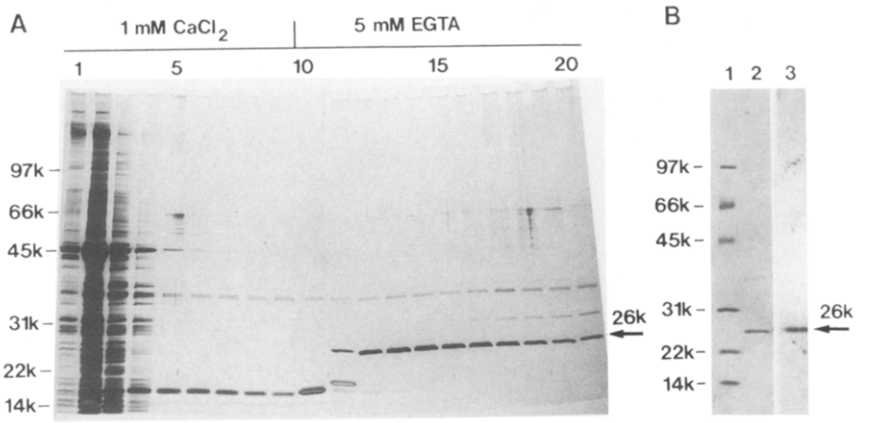

Fig. 1. **(A)** Purification of S-modulin with Phenyl Sepharose column chromatography and **(B)** $^{45}Ca^{2+}$ blot of S-modulin. **(A)** The ROS low Ca^{2+} extract obtained from 50 frogs were applied to a Phenyl Sepharose column. Ten μl of each fraction (3.5 ml) was applied to SDS-PAGE (silver stained). **(B)** **Lane 1**, CBB-staining of molecular markers transferred to an Immobilon P membrane; **lane 2**, CBB-staining of purified S-modulin (0.2 μg) transferred to the membrane; **lane 3**, $^{45}Ca^{2+}$ blot of the membrane shown in lane 2.

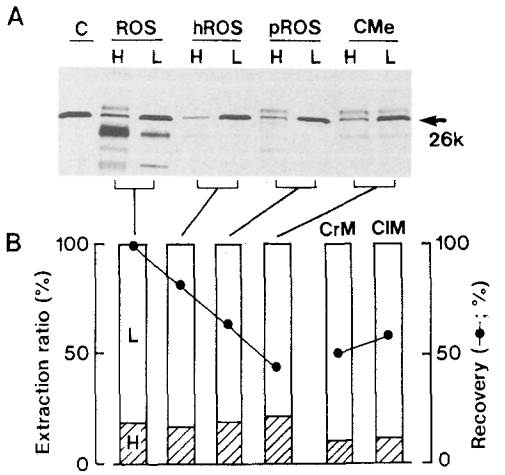

Fig. 2. Ca^{2+}-dependent binding of S-modulin to ROS membranes and liposomes.
(**A**) Silver staining of proteins in high ("H") and low ("L") Ca^{2+} extracts obtained after addition of 5 pmoles of S-modulin ("C") to 1 mg of variously treated ROS membranes (see text). (**B**) Extraction ratio of S-modulin in high ("H") and low ("L") Ca^{2+} extracts in (A) determined by densitometry. The amount of S-modulin recovered is also shown (closed circles).

The yield of S-modulin with the above method was much higher (over 90 % assuming that S-modulin and rhodopsin ratio is 1:140; ref. 9) than that with our previous method (10–15%). From 200 frogs we obtained 2.3 mg of S-modulin.

$^{45}Ca^{2+}$-binding to S-modulin

Purified S-modulin was electrophoresed and transferred to an Immobilon P membrane (Fig. 1B, lane 2). The membrane was incubated with $^{45}Ca^{2+}$, and the autoradiogram of the membrane (lane 3) showed direct binding of Ca^{2+} to S-modulin.

Ca^{2+}-dependent binding of S-modulin to ROS membranes and liposomes

S-modulin binds to ROS membranes at 1 mM Ca^{2+} (9), which is shown as a control in Fig. 2A. At 1 mM Ca^{2+}, purified S-modulin ("C" in Fig. 2A) was mixed with ROS membranes ("ROS" in Fig. 2A). The S-modulin content in the low Ca^{2+} extract (see MATERIALS AND METHODS; "L" under "ROS") was higher than that in the high Ca^{2+} extract ("H"). To search for the binding target of S-modulin in ROS membranes, the membranes were treated in several ways: ROS membranes were washed with a hypotonic buffer solution (5 mM HEPES, 2 mM EDTA, pH 7.5) to eliminate peripheral membrane proteins ("hROS"); hROS membranes were proteolyzed with trypsin (40 μg/ml) plus chymotrypsin (120 μg/ml) ("pROS"); ROS membrane lipids were extracted with a chloroform–methanol (2:1) solution and were reconstituted into liposomes ("CMe").

Despite the above treatments, S-modulin content in the low Ca^{2+} extracts was higher than that in the high Ca^{2+} extracts in all of the preparations examined (Fig. 2A). According to the densitometric measurement, the amount of S-modulin that bound to the membranes at 1 mM Ca^{2+} was about 80 % of the total extractable S-modulin (bars in Fig. 2B). The recovery of added S-modulin decreased when ROS membranes were treated (Fig. 2B, closed circles), but the reason for this is not known. In addition to ROS membranes, membranes prepared from mouse cerebrum ("CrM") and cerebellum ("ClM") both

Table 1. *Ca^{2+}-dependent binding of S-modulin to lipids*

Positive	Negative	
ROS membranes	Bovine brain extract	Phosphatidyl choline
Hypotonically washed	fraction 1	(dipalmitoyl)
ROS membranes	3	Phosphatidyl choline
Proteolyzed ROS	5	(containing unsaturated
membranes	6	fatty acids)*
ROS membrane-	7	Phosphatidyl ethanolamine
chloroform/methanol-	8	(dipalmitoyl)
extract	10	Phosphatidyl serine
Phospholipase C-	Cholesterol*	(dipalmitoyl)
treated ROS membranes	Docosahexaenoic acid	Phosphoinositides
Phospholipase A_2-	ethyl ester*	Phospholipase C-treated
treated ROS membranes	Galactocerebrosides*	phosphatidyl choline
	Phosphatidic acid	(dipalmitoyl)
	(dipalmitoyl)	Sphyngomyelin*
		Sulphatides*

* Liposomes were made as a mixture with phosphatidyl choline (dipalmitoyl).

bound S-modulin at a high (1 mM) Ca^{2+} concentration but not at a low (< 1 nM) Ca^{2+} concentration (Fig. 2B).

The result in Fig. 2, especially the S-modulin binding to the "CMe" liposomes suggested that the binding target of S-modulin might be membrane lipids. Therefore, we tested possible S-modulin binding to various types of lipids or ROS membranes (Table 1). However, S-modulin did not bind to the lipids examined unless the origin of the sample was ROS membranes. This result may imply that the real binding target of S-modulin is not lipids but a protein(s) that can be extracted with a chloroform-methanol solution and has a hydrophobic region(s) to interact with S-modulin. Further work is necessary to identify the binding target of S-modulin.

The S-modulin binding to ROS membranes was examined by varying Ca^{2+} concentrations (Fig. 3). The binding was obvious at Ca^{2+} concentrations above 1 μM. According to the result in Fig. 3, the

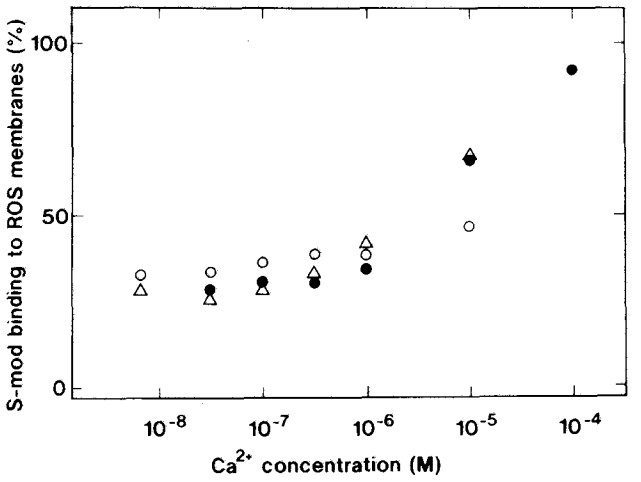

Fig. 3. S-modulin binding to ROS membranes as a function of Ca^{2+} concentration. Ten μl of 0.5 μM S-modulin (S-mod) and 1 mg (dry weight) of ROS membranes were mixed at a test Ca^{2+} concentration indicated on the abscissa. The percentage of S-modulin that bound to ROS membranes is plotted as a function of Ca^{2+} concentration tested. Different symbols indicate different experiments.

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                                                  ••••••••••••••••••••••••••••••
Visinin      1 MGNSRSSALSREVLQELRAS TRYTEEELSRWYEGFQRQCS DGRIRCDEFERIYGNFFPNS
               :::: : ::: : : ::    :  :::::: ::  :   :   :::   ::  ::  :::
Recoverin      MGNSKSGALSKEILEELQLN TKFTEEELSSWYQSFLKECP SGRITRQEFQTIYSKFFPEA
                                    ::: :::                    :  ::::
S-modulin                           KFTQEEL                    KQFESIYSK
                                    f1                         f2

                ••••••••••••••••••••••••••••••••                ••••••••••••••••••••
Visinin     61 EPQGYARHVFRSFDTNDDGT LDFREYIIALHLTSSGKTHL KLEWAFSLFDVDRNGEVSKS
              :  :: :::::::: : ::: ::: :: :::: :: :::   :::::::: ::: ::  ::
Recoverin      DPKAYAQHVFRSFDANSDGT LDFKEYVIALHMTSAGKTNQ KLEWAFSLYDVDGNGTISKN
                 ::::::::::::::: ::: :::::: :::::::: ::
S-modulin        KAYAQHVFRSFDANNDGT LDFKEYMIALHMTSSGK
                 f3                  f4

                ••••••••
Visinin    121 EVLEIITAIFKMIPEEERLQ LPEDENSPQKRADKLWAYFN KGENDKIAEGEFIDGVMKND
               ::::: :::::::  :     :::::: : ::: : :  :  :   ::  : ::: :   :
Recoverin      EVLEIVTAIFKMISPEDTKH LPEDENTPEKRAEKIWGFFG KKDDDKLTEKEFIEGTLANK
                        :::  :: :: ::::::::::   ::   :: :    :::: ::: :    :
S-modulin               KMINAEDXKH LPEDENTPEK   KIXVYFG K    KLTEGEFIQGIV  K
                        f5       f6             f7          f8            f9

Visinin    181 AIMRLIQYEPKK
                : :::: :: :
Recoverin      EILRLIQFEPQKVKEKLKEK KL
               ::::: :  :::
S-modulin      EILRLXQYXPQK
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Fig. 4. Partial amino acid sequence of S-modulin.
Amino acid sequences in 9 proteolytic fragments (f1 – f9) are compared with those of visinin and recoverin. Dotted lines indicate the putative Ca^{2+}-binding sites. Identical amino acid is indicated by a colon (:).

binding affinity of the Ca^{2+}/S-modulin complex to ROS membranes is not so high. Similar low affinity of S-modulin binding to ROS membranes was observed in our previous electrophysiological work: the S-modulin activity, and thus S-modulin itself, is lost during intracellular perfusion using an inside-out ROS preparation at 1 μM Ca^{2+} (9). The low affinity of the Ca^{2+}/S-modulin complex to ROS membranes may explain why the intracellular concentration of S-modulin is high (about 40 μM; ref. 9).

Amino acid sequence of S-modulin determined in proteolytic fragments

Using purified S-modulin, we first tried to analyze the amino acid sequence of intact S-modulin, but the result showed that S-modulin has a blocked N-terminus. Then, we analyzed the sequence in proteolytic fragments of S-modulin. By proteolysis with lysyl endopeptidase, we obtained 9 major fragments (f1 – f9 in Fig. 4). The amino acid sequence was analyzed in these fragments and 100 amino acid residues were determined. When compared with those of known proteins, S-modulin showed 65 % and 83 % sequence homology (identity) to those of visinin (16) and recoverin (17–20), respectively. The region indicated by dots in Fig. 4 is a putative EF-hand structure (Ca^{2+}-binding site) in visinin and recoverin deduced from the suggestion by Kretsinger (21). Two fragments (f3 and f4) showed high homology to the Ca^{2+}-binding site. This result together with the direct binding of $^{45}Ca^{2+}$ (Fig. 1)

indicated that S-modulin belongs to the EF-hand-type Ca^{2+}-binding protein family and forms a sub-family which includes S-modulin, visinin and recoverin.

S-modulin regulates PDE light-sensitivity in a Ca^{2+}-dependent manner (9). The present work suggests that this S-modulin action is mediated by a hydrophobic interaction between a Ca^{2+}/S-modulin complex and a lipid(s) (or proteins). Since PDE is known to be activated through an enzyme cascade (2,3), S-modulin probably acts on one or some of the components in this cascade through a hydrophobic interaction. Our attempt to identify the target in the cascade is now in progress.

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