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A 26 kd calcium binding protein from bovine rod outer segments as modulator of photoreceptor guanylate cyclase

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The resynthesis of cGMP in vertebrate photoreceptors by guanylate cyclase is one of the key events leading to the reopening of cGMP-gated channels after photoexcitation. Guanylate cyclase activity in vertebrate rod outer segments is dependent on the free calcium concentration. The basal activity of the enzyme observed at high concentrations of free calcium (>0.5 μM) increases when the free calcium concentration is lowered into the nanomolar range (<0.1 μM). This effect of calcium is known to be mediated by a soluble calcium-sensitive protein in a highly cooperative way. We here show that this soluble protein, i.e. the modulator of photoreceptor guanylate cyclase, is a 26 kd protein. Reconstitution of the purified 26 kd protein with washed rod outer segment membranes containing guanylate cyclase revealed a 3- to 4-fold increase of cyclase activity when the free calcium concentration was lowered in the physiological range from 0.5 μM to 4 nM. Guanylate cyclase in whole rod outer segments was stimulated 10-fold in the same calcium range. The activation process in the reconstituted system was similar to the one in the native rod outer segment preparation, it showed a high cooperativity with a Hill coefficient *n* between 1.4 and 3.5. The half-maximal activation occurred between 110 and 220 nM free calcium. The molar ratio of the modulator to rhodopsin is 1:76 ± 32. The protein is a calcium binding protein as detected with ^{45}Ca autoradiography. Partial amino acid sequence analysis revealed a 60% homology to visinin from chicken cones.

Key words: calcium binding protein/guanylate cyclase/light adaptation/vision

Introduction

Signal transduction in retinal photoreceptors is mediated by the light triggered amplified hydrolysis of cGMP (guanosine 3',5'-monophosphate) through an enzymatic cascade (Stryer, 1988) which leads to the closure of cGMP-gated cation channels in the plasma membrane (Kaupp *et al.*, 1988; Yau and Baylor, 1989). Concomitant with the decrease of cGMP is the decrease of the intracellular calcium concentration. The resting level of internal calcium in darkness was determined to be ~300 nM (McNaughton *et al.*, 1986; Ratto *et al.*, 1988; Korenbrot and Miller, 1989). Calcium ions enter the cell via the cGMP-gated channel and are extruded via the Na:Ca,K exchanger (Yau and Nakatani, 1985; Cervetto *et al.*, 1989). This balance of calcium influx and efflux in the dark state is disturbed by the action of light. After excitation and channel closure, calcium ions are now prevented from entering the cell but they are still extruded via the operation of the exchanger. The resulting decrease of calcium below the dark level of 300 nM after illumination is the signal for restoring the dark current and for light adaptation in rods and cones (Nakatani and Yau, 1988; Matthews *et al.*, 1988; for recent reviews see Lamb and Pugh, 1990; Fain and Matthews, 1990).

As derived from electrophysiological (Hodgkin and Nunn, 1988; Kondo and Miller, 1988; Rispoli *et al.*, 1988; Kawamura and Murakami, 1989) and biochemical experiments (Pepe *et al.*, 1986; Koch and Stryer, 1988) the main target of decreased calcium levels in photoreceptor cells is guanylate cyclase. A lowered intracellular calcium level leads to an increased production of cGMP. This stimulation of guanylate cyclase in a negative feedback loop is mediated by a calcium-sensitive protein with half-maximal activation around 100 nM free calcium in a cooperative way thereby increasing V_{max} about 5- to 20-fold (Koch and Stryer, 1988; Koch *et al.*, 1990). We here report the purification and initial characterization of a novel 26 kd calcium binding protein from bovine rod outer segments (ROS) which activates guanylate cyclase at nanomolar concentrations of free calcium.

Results

Bovine rod outer segments (ROS) contain a soluble factor which modulates (i.e. activates) guanylate cyclase activity in a calcium dependent way. The increased activity is 4- to 10-fold higher at nanomolar concentrations of free calcium (4 nM) than the basal activity observed at >0.5 μM free calcium. Washed ROS membranes which are depleted from soluble proteins show only the basal activity of guanylate cyclase. The calcium sensitivity can be partially restored by adding back an extract of soluble proteins in a reconstitution experiment (Koch and Stryer, 1988). We used this reconstitution experiment as a screening tool for the calcium dependent modulator of guanylate cyclase during purification. Different fractions obtained by the purification procedure (see below) were tested for the ability to stimulate guanylate cyclase at 4 nM free calcium compared to the activity at 0.5 μM of free calcium. The principal results of these reconstitution experiments are shown in Figure 1. Guanylate cyclase activity in whole ROS increased from a basal level (1.3–2.4 nmol cGMP/min/mg rhodopsin) at 0.5 μM free calcium to an elevated level of 10–14 nmol cGMP/min/mg rhodopsin at 4 nM free calcium (Figures 1A and 4A). Washed ROS have lost this calcium sensitivity and exhibit only the basal activity (Figure 1B). Reconstitution of washed ROS membranes with an extract of soluble proteins obtained after illumination of ROS membranes (see paragraph below) resulted in partial restoration of the initial

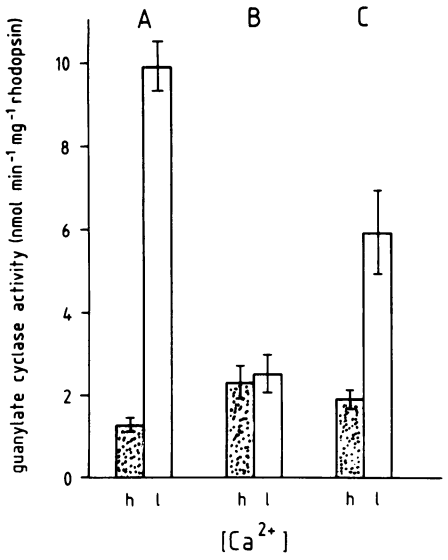

Fig. 1. Guanylate cyclase at high (h) and Low (l) free calcium concentrations ([Ca^{2+}]) in whole ROS (**A**), washed ROS membranes (**B**) and in a reconstituted system (**C**). High and low [Ca^{2+}] were 563 nM and 4 nM, respectively. Guanylate cyclase exhibited calcium sensitivity only in whole Ros (A) and in a reconstituted system (C). Washed ROS membranes (B) contained only the basal guanylate cyclase activity at high and low [Ca^{2+}]. Reconstitution was performed as described in Materials and methods with an extract of soluble ROS proteins obtained from bleached ROS ('light extract').

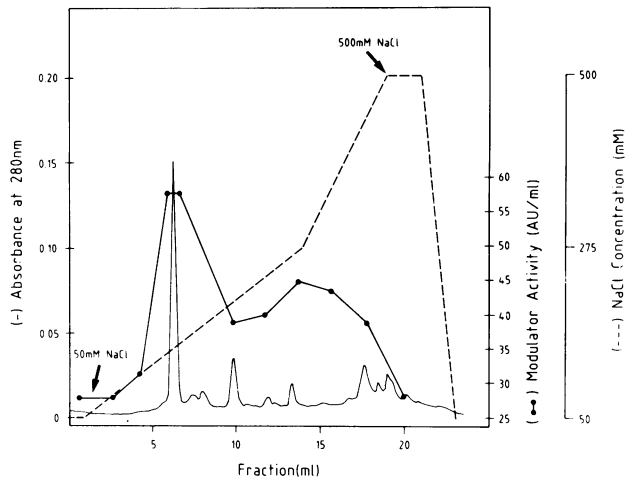

Fig. 2. FPLC anion exchange chromatography on a Mono Q column. The column was loaded with a boiled light extract of soluble ROS proteins. The peak eluting at 145 mM NaCl contained purified 26 kd protein. (−) absorbance at 280 nm, (--) gradient of NaCl, arrows mark start and end concentration of NaCl. (●—●) modulator activity (AU/ml).

calcium sensitivity (Figure 1C). By this strategy we isolated a novel calcium binding protein with a relative mass of 26 000 as the modulator of photoreceptor guanylate cyclase. We emphasize that the effect of the 26 kd protein is stimulatory and not inhibitory. Guanylate cyclase needs the presence of this protein and a low calcium concentration for an accelerated cGMP synthesis (Figure 1). For this reason we refer to the modulation of guanylate cyclase as the 'calcium dependent stimulation' of the enzyme.

Purification of the 26 kd protein

Purification was achieved by three steps: (i) *Bleaching of ROS and subsequent centrifugation*: Illumination of ROS at moderate ionic strength causes the light-induced binding of some soluble proteins, while other proteins are extractable (Kühn, 1980). We observed that ~35% of the soluble proteins became membrane-bound at moderate ionic strength after bleaching. The extract obtained after centrifugation ('light extract') contained four major proteins of 26, 40, 57 and 92 kd. Incubation of this extract with washed ROS membranes resulted in a pronounced stimulation of guanylate cyclase at low calcium concentrations (Figure 1C and Table I). An extract obtained from unbleached ROS ('dark extract') showed stimulation of cyclase to the same extent. (ii) *Heat denaturation*: The modulator of guanylate cyclase is a heat stable protein. Boiling for 4 min resulted in the loss of ~90% of the total protein content of the 'light extract'. Around 30% of the total activity was lost at this step compared to the total activity in the 'light extract'. The overall recovery was 55% (Table I). The boiled sample contained a 26 kd protein as a major protein band as analyzed with sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS−PAGE) (Figure 3 lane b) although there were still some other proteins visible. To obtain a nearly homogeneous fraction of the 26 kd protein, the boiled sample was applied onto a Mono Q FPLC anion exchange column. (iii) *Chromatography on a Mono Q anion exchange column*: At 50 mM NaCl the 26 kd protein was bound to the anion exchange column. The void volume contained almost no 26 kd protein (Figure 3c) and did not stimulate guanylate cyclase. Elution of bound proteins was performed with a NaCl gradient. At 145 mM NaCl a sharp protein peak appeared (Figure 2). One-third of the total activity in the sample loaded onto the Mono Q was recovered in the fractions corresponding to the first peak of modulator activity in Figure 2 (elution between 4.5 ml and 7.5 ml). A silver stained SDS gel showed a homogeneous protein (Figure 3e) with a relative mol. wt of 26 000. We used this purified fraction for further studies (see paragraph below). A second but smaller peak of activity appeared at a higher NaCl concentration (at 275 mM in Figure 2). Sometimes this second peak eluted at a higher NaCl concentration around 500 mM. Analysis of these fractions with SDS− PAGE showed the presence of the 26 kd protein and other proteins as contaminants. A typical protein composition of such a fraction is shown in Figure 3f. The stimulation of guanylate cyclase by these fractions was less pronounced than by fractions containing the purified 26 kd protein. A summary of the purification is presented in Table I. Purification was achieved with a 59-fold increase in specific activity with 18% recovery of total activity. Determinations from other preparations revealed an increase of 20- to 80-fold and 6−18% recovery of total activity.

Stimulation of guanylate cyclase by the 26 kd protein

Reconstitution of washed unbleached ROS membranes with the purified 26 kd protein resulted in restoration of the calcium dependent stimulation of guanylate cyclase activity. In Figure 4 the native system (whole ROS) is compared with the reconstituted system. Guanylate cyclase activity was around its basal level (1−2 nmol/min/mg rhodopsin) when free calcium was between 0.5 and 100 μM. At lowered calcium levels (100 nM) it increased to 10-fold higher values (Figure 4A). Only fractions containing the 26 kd protein (Figures 3 a, b, e and f) could restore the calcium sensitivity

Table I. Purification of 26 kd protein

Step	Volume (ml)	Total activity (AU)	Total protein (mg)	Specific activity (AU/mg)	Purification (x-fold)	Recovery (%)
Native system[a]	6.6	1026	9.9	104	1	
Light extract	6.6	847	7.5	113	1.1	82
Boiled light extract after PD-10	7.5	563	0.45	1251	12	55
Mono Q fraction	1.5	185	0.030	6167	59	18

[a]Guanylate cyclase activity measured in whole ROS; amount of protein is equivalent to the total cytoplasmic protein content determined from a 'dark extract' obtained after a centrifugation step (100 000 *g*, 15 min); AU, activator unit (for definition see Materials and methods).

of guanylate cyclase in washed ROS membranes (Figures 1C and Figure 4C). We used a purified sample of the 26 kd protein as shown in Figure 3E for the reconstitution experiment in Figure 4. Cyclase activity in the reconstituted system increased from 1.5 nmol/min/mg rhodopsin to 5 nmol/min/mg rhodopsin, similar to the value shown in Figure 1C (reconstitution experiment with the less pure 'light extract').

Analysis of the calcium/modulator dependent activation process (Figure 4A and C) in a Hill plot revealed a Hill coefficient of $n = 1.5$ and a half-maximal activation (EC_{50}) at 130 nM free calcium for whole ROS (Figure 4B) and $n = 1.4$ and an EC_{50} of 200 nM for the reconstituted system (Figure 4D). The similarity of the data indicates that the activation process in both systems is the same and that the 26 kd protein is a necessary component of the calcium dependent regulation of guanylate cyclase.

The free calcium concentrations of the experiments shown in Figure 4 were determined with rhodamin-2. Additionally we determined the free calcium concentration in our assay media with two other calcium indicators, fura-2 and fluo-3 and with a calcium buffer program. Analysis of our data revealed slightly different values for the EC_{50} and n depending on the indicator used. Differences probably emerged from partial titration of a second low-affinity binding site which was recently observed for fluo-3 (Meyer *et al.*, 1990). With fura-2, $EC_{50} = 140$ nM, $n = 3.3$ (whole ROS); $EC_{50} = 160$ nM, $n = 3.5$ (reconstituted system). With fluo-3, $EC_{50} = 80$ nM, $n = 1.7$ (whole ROS); $EC_{50} = 110$ nM, $n = 1.6$ (reconstituted system). With a calcium buffer program, $EC_{50} = 185$ nM, $n = 1.7$ (whole ROS); $EC_{50} = 220$ nM, $n = 1.6$ (reconstituted system). Thus, it is more suitable to give a range for the EC_{50} from 80 to 180 nM and for the Hill coefficient n from 1.5 to 3.3 (whole ROS) and EC_{50} from 110 to 220 and $n = 1.4-3.5$ (reconstituted system).

26 kd protein as a calcium binding protein

The strong calcium dependent switch of activity implicates that the modulator is a calcium binding protein. This was tested with ^{45}Ca autoradiography. As shown in Figure 5, a purified fraction of the 26 kd protein was labeled with ^{45}Ca. Calmodulin was used as control and is seen in the right lane. Partial amino acid analysis was performed with the purified 26 kd protein. Since the protein was N-terminally blocked it was digested with protease V8 from *Staphylococcus aureus* prior to Edman sequencing. The obtained partial amino acid sequence was compared with known calcium binding proteins. It shows ~60% homology to visinin (Figure 6) a calcium binding protein recently described in chicken cones (Yamagata *et al.*, 1990).

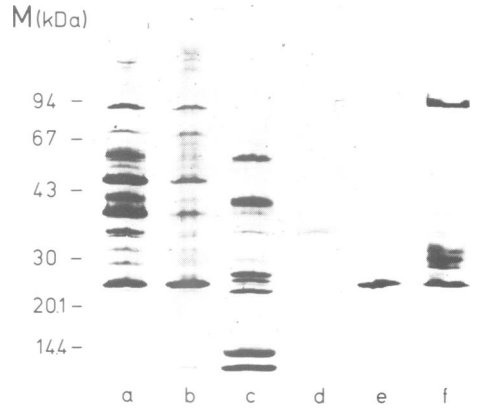

Fig. 3. SDS–PAGE summary of the purification of the 26 kd protein. Electrophoresis was performed with the Phast System of Pharmacia. The gel was silver stained. Lane a: light extract of soluble ROS proteins (1.5 μg protein); lane b: light extract after heat denaturation step (1 μg protein); lane c: eluted fraction in the void volume of Mono Q fractionation (1 μg protein); lane d: fraction after the void volume without activity (<0.01 μg protein); lane e: purified 26 kd protein (0.4 μg), pooled fractions of the peak eluting at 145 nM NaCl during run on Mono Q (Figure 2); lane f: fraction washed off the Mono Q column at high salt concentration (1 μg protein).

Discussion

Photoreceptor guanylate cyclase is highly sensitive to levels of free calcium in the submicromolar range (Koch and Stryer, 1988). We here present evidence that the calcium dependent stimulation of guanylate cyclase is mediated by a 26 kd calcium binding protein. This novel calcium binding protein can detect changes of calcium in the physiological range <300 nM and therefore transmits the signal of decreased calcium levels after illumination to guanylate cyclase.

There is now profound evidence that light adaptation is mediated by a change in cytoplasmic calcium concentration (see Introduction). Experiments designed to prevent or to minimize any changes of intracellular calcium have shown that the recovery phase of the photoresponse is markedly prolonged (Nakatani and Yau, 1988; Matthews *et al.*, 1988). This indicates that guanylate cyclase is kept at its basal activity i.e. the velocity of cGMP synthesis is not accelerated.

Stimulated cyclase activities at decreased calcium levels as shown in Figures 1 and 4 correspond to a cGMP production of $30-60\ \mu M\ s^{-1}$ in the ROS volume. These activities are probably achieved in 0.5–1 s after excitation when extrusion of calcium by the exchanger is driven to 10^{-7} to 10^{-9} M internal calcium concentration (Cervetto *et al.*, 1989) and can restore the cGMP dark level in less than a

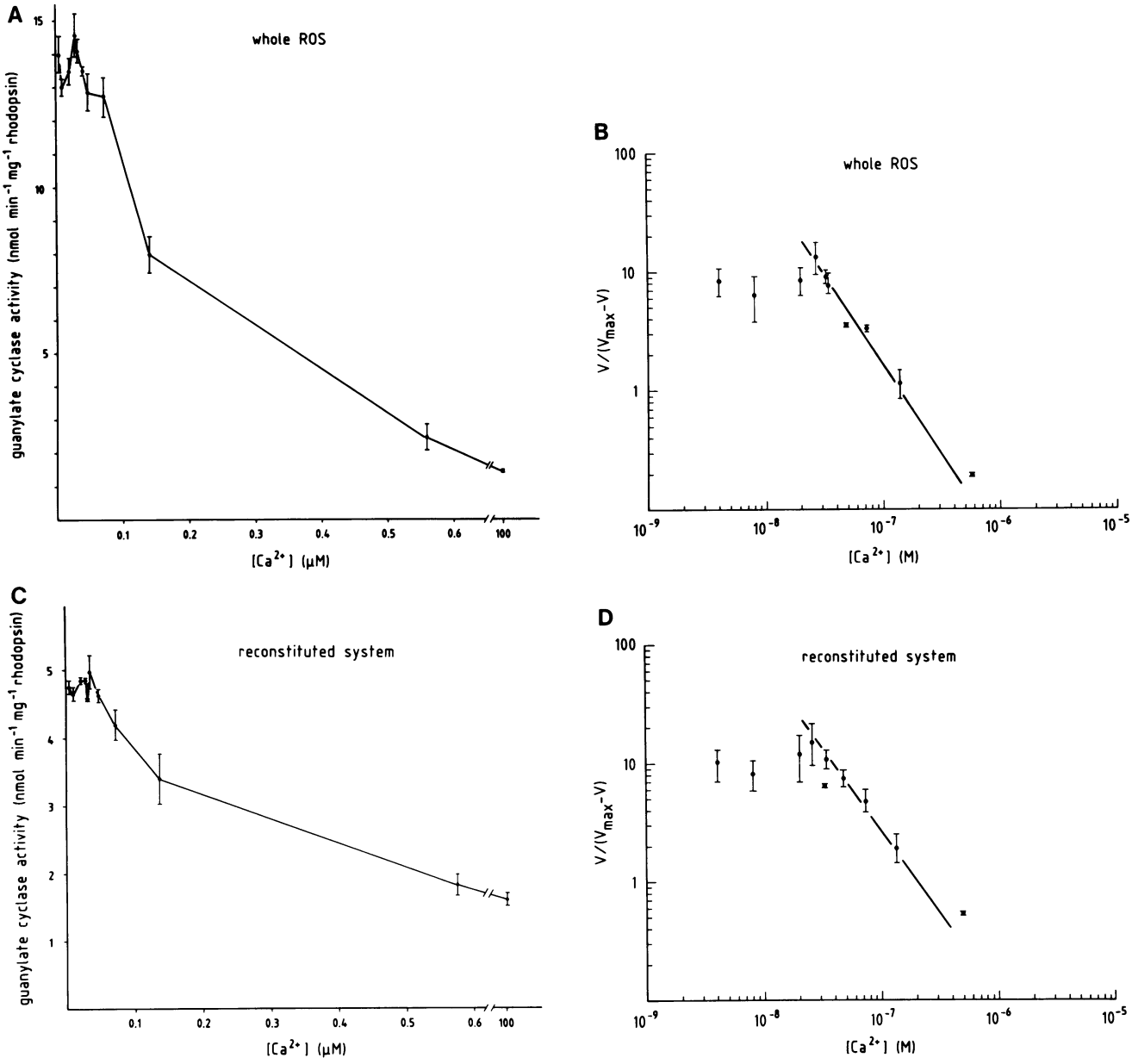

Fig. 4. Calcium dependence of photoreceptor guanylate cyclase in whole ROS (**A, B**) compared with the dependence in a reconstituted system (**C, D**). Hill plots (B, D) were derived from the corresponding data shown in A and C. Data points represent triplicates and error bars mark standard deviations.

second after dim flashes of light as also demonstrated by a mathematical model (Forti *et al.*, 1989). In addition it is necessary to shut off the excitation pathway rapidly. Recent experiments by Vuong and Chabre (1990) indicate that transducin is deactivated in the subsecond range. The phosphodiesterase activity is only influenced by calcium at unphysiologically high concentrations (i.e. 0.1 mM, Barkdoll *et al.*, 1989). An effect of calcium on the lifetime of one or more components of the transduction cascade was discussed by Torre *et al.* (1986), but until now the biochemical data are not sufficient to decide whether this influence of calcium occurs *in vivo*.

The increased cyclase activity at a low calcium level may also contribute to an altered sensitivity of the cell in the light adapted state. With background light a photoreceptor cell is desensitized, presumably having an internal calcium concentration below the resting dark state which would again correspond to an elevated production of cGMP. With acceleration of cGMP synthesis and a corresponding net increase in cGMP turnover rates as observed by Ames *et al.* (1986), higher flash intensities are needed to evoke the excitation signal. We suggest that the calcium dependent stimulation of guanylate cyclase via its modulator is probably the main biochemical basis for the recovery process after excitation as well as for light adaptation.

The data in Table I can be used to calculate the relative amount of the 26 kd protein compared to rhodopsin. About 18% of the total activity was recovered in 30 μg of a purified fraction after anion exchange chromatography on Mono Q. 100% of the activity would correspond to 167 μg in the starting material of 9.9 mg soluble protein extract. The corresponding amount of rhodopsin in the starting material was 28 mg (purification shown in Table I). This gives 6.4 nmol 26 kd protein to 700 nmol rhodopsin or a molar ratio of

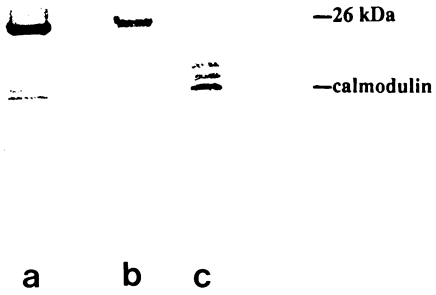

Fig. 5. ^{45}Ca autoradiography. Lane a: purified 26 kd protein blotted on nitrocellulose and stained with Amido Black; lane b: ^{45}Ca autoradiography of a sample like that in lane a, indicating calcium binding of the 26 kd protein; lane c: control, calcium binding of calmodulin. Several bands of calmodulin appear due to the presence of calcium in the electrophoresis buffer (Burgess *et al.*, 1980).

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26 kDa protein   --- Gln Gln Asn Asn Lys Phe Thr Glu Glu Glu
                                    .   .   :   :   :   :
visinin          --18Arg Ala Ser Thr Arg Tyr Thr Glu Glu Glu27

26 kDa protein       Leu Ser XXX Trp Tyr Gln XXX Phe XXX Leu ---
                     :   :       :   :   .       :
visinin          28Leu Ser Arg Trp Tyr Glu Gly Phe Gln Arg37--
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Fig. 6. Partial amino acid sequence of the 26 kd protein compared with a part of the known sequence of visinin from chicken cones (Yamagata *et al.*, 1990); : identical amino acids, · conservative substitution.

1:109 (1:76 ± 32 obtained from five different preparations). A calculation done for photoreceptor guanylate cyclase revealed a similar molar ratio of cyclase to rhodopsin ranging from 1:80 to 1:380 (K.-W.Koch, in preparation). From these data we suggest that guanylate cyclase and its modulator are present in equimolar amounts in rod photoreceptors. Interaction between both proteins is mainly influenced by calcium. As shown in the purification procedure the 26 kd protein did not shown any light induced binding to the membrane as it is known for other photoreceptor cytoplasmic proteins (Kühn, 1980). Beside its role as a stimulatory protein, the 26 kd protein may also serve as an internal calcium buffer. Its presumed binding affinity in the nanomolar range (EC_{50} from 110 to 220 nM) would complement the recently described calcium binding capacities of cytosolic proteins in the micromolar range (Huppertz *et al.*, 1990).

The observed homology of the 26 kd protein to visinin from chicken cones (Yamagata *et al.*, 1990) may point to a similar function of visinin in these cells since their light sensitivity is also regulated by changes of calcium (Nakatani and Yau, 1989; Matthews *et al.*, 1990). Interestingly, the 26 kd protein shows no homology to calretinin and calbindin, two other calcium binding proteins found in the chicken retina (Rogers, 1987). Thus it seems to belong to a new class of calcium binding proteins operating in sensory systems.

A preliminary form of this work was presented at an International Symposium about Signal Transduction in Photoreceptor Cells held in Jülich (1990). S.Ray *et al.* (1990) also reported similar observations made for a 26 kd protein.

Materials and methods

Preparation of rod outer segments

Rod outer segments (ROS) were prepared from freshly obtained bovine eyes according to Schnetkamp and Daemen (1982) with the following modifications: the 'light' medium contained 115 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES–KOH pH 7.5 600 mM sucrose and 1 mM dithiothreitol (DTT); the 'heavy' medium was the same, except that the sucrose concentration was 1.2 M. After gradient centrifugation the ROS-containing band was collected and washed with 115 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES–KOH pH 7.5. The pellet was resuspended in 10 mM Tris–HCl pH 8.0, 1 mM DTT and stored at −80°C. All steps were done in dim red light; pH was adjusted at room temperature. This preparation was used for the purification of the 26 kd protein.

Purification of the 26 kd protein

A suspension of ROS (5 mg rhodopsin/ml) was adjusted to 100 mM NaCl and bleached for 5–10 min at 37°C (Kühn, 1980). The suspension was centrifuged for 35 min at 35 000 r.p.m. (TI70, Beckman). The pellet was discarded, the supernatant ('light extract') was again centrifuged and desalted over a Sephadex-G25/PD-10 column, equilibrated with buffer A (50 mM NaCl, 20 mM Tris–HCl pH 8.0). After filtration, the sample was boiled for 4 min at 95°C and precipitated proteins were removed by centrifugation (15 min at 40 000 r.p.m.; TLA 45, Beckman). The clear supernatant was applied onto a Mono Q FPLC column (Pharmacia) equilibrated with buffer A. After elution of the fraction of non-bound proteins, retained proteins were eluted with a linear NaCl gradient (Figure 2), going from 50 mM to 275 mM NaCl in a total volume of 13 ml and from 275 mM to 500 mM in a total volume of 5 ml. Finally the column was washed with 2 ml of 500 mM NaCl. Fractions of 0.5 ml were collected. The flow rate was 0.5 ml/min. The calcium dependent stimulation of guanylate cyclase was determined in a reconstitution assay. For this purpose fractions were pooled prior to reconstitution; for the peak at 145 mM NaCl, three fractions of 0.5 ml each were pooled to give 1.5 ml; for other determinations, four fractions of 0.5 ml each were pooled.

Reconstitution and guanylate cyclase assay

ROS used in the reconstitution experiments were prepared as described previously (Koch and Stryer, 1988) in dim red light. Prior to the recombination with soluble protein fractions ROS aliquots (3–6 mg/ml rhodopsin) of 50 μl were diluted 5-fold with a low salt buffer (10 mM MOPS pH 7.1) and centrifuged. The supernatant was removed and the pellet was resuspended in 45 μl of the protein fractions obtained by the purification procedure described above. Ten μl of the resuspended, i.e. reconstituted ROS were incubated with 20 μl of assay buffer and 20 μl of a Ca/[ethylene-bis(oxyethylenenitrilo)]tetraacetic acid (EGTA) stock solution, the final free calcium concentration was in the range 10 nM–2 μM (see below). The assay buffer and the cyclase assay were as described previously (Koch and Stryer, 1988) and performed with slight modifications as specified in the following. The incubation reaction was stopped by adding 10 μl of 100 mM EDTA and boiling for 5 min. All steps were performed in complete darkness. After stopping the reaction, samples were handled in room light. Formation of cGMP was measured with an HPLC (high performance liquid chromatography) assay using a reversed phase HPLC column (250 × 4 mm, Hibar Lichrospher 100 RP 18, 5 μm, E.Merck, Darmstadt). Separation was achieved with a methanol gradient (0–60%) in 5 mM KH_2PO_4 pH 5.0. If necessary, values were corrected for cGMP hydrolysis. Hydrolysis of cGMP during a typical set of incubations was determined by incubation with [^{3}H]cGMP (Amersham). Nucleotides of the reaction mixture were separated on a TLC (thin layer chromatography) sheet; spots of cGMP were cut and counted. Hydrolysis was between 0 and 20%.

Calcium buffer

Stock solutions of Ca/EGTA consisted of 5 mM EGTA (Sigma) and a total calcium concentration between 0 and 4.5 mM. The following Ca/EGTA ratios were used: 0.9 (563 nM), 0.5 (137 nM), 0.3 (73 nM), 0.2 (48 nM), 0.15 (34 nM), 0.125 (33 nM), 0.1 (27 nM), 0.07 (20 nM) 0.03 (8 nM), no calcium 5 mM EGTA (4 nM). These values of free calcium (in parentheses) in the complete assay mixture were obtained with rhodamin-2 and are shown in the figures.

Measurement of the free calcium concentration in the assay mixture described above was performed with the fluorescent calcium indicators rhodamin-2 (K_D = 1000 nM; Minta *et al.*, 1989; Calbiochem), fluo-3 (K_D = 400 nM; Minta *et al.*, 1989; Molecular Probes) and fura-2 (K_D = 224 nM; Grynkiewicz *et al.*, 1985; Calbiochem). Fluorescence measurements were carried out with a SLM fluorimeter. Emission was collected at 576 nm (rhodamin-2), 526 nm (fluo-3) and 510 nm (fura-2). Excitation wavelengths were 556 nm (rhodamin-2), 506 nm (fluo-3) and 340 nm and 380 nm (fura-2).

Calculation of the free calcium concentration was done with a calcium buffer program (Fabiato, 1988) using constants from Martell and Smith (1974).

Definition of activator unit (AU)

One unit of modulator activity (AU) is defined as the two-fold increase of basal guanylate cyclase activity when a washed membrane pellet is reconstituted with 45 μl of a soluble protein extract. A control of washed membranes recombined with buffer solution was measured for every set of incubation and the x-fold increase of cyclase activity was determined. Values obtained with modulator containing fractions were divided by the value of the corresponding control.

Autoradiography

Detection of calcium binding by ^{45}Ca autoradiography was performed after Maruyama *et al.* (1984). After gel electrophoresis, proteins were blotted on a nitrocellulose membrane using a semi dry blot method. After the blot the nitrocellulose membrane was washed with a buffer containing 60 mM KCl, 10 mM imidazol pH 6.8, 5 mM $MgCl_2$ for 40–60 min with two changes of buffer. Afterwards the nitrocellulose membrane was incubated in the same buffer containing ^{45}Ca (0.5 Ci/mmol; Amersham) for 30 min at 37°C and 30 min at room temperature. Finally the membrane was washed three times with double distilled H_2O for 10 min, dried and exposed to Kodak X-Omat X-ray films for 48 h at −80°C using intensifying screens.

Other methods

SDS–PAGE was performed after Laemmli (1970) or alternatively with the Phast System of Pharmacia. Protein samples were dialyzed against 10 mM MOPS pH 7.1 and concentrated with a Speedvac Concentrator (Savant, Farmindale, NY) before electrophoresis. Protein concentration was determined with Coomassie Brilliant Blue and bovine serum albumin as a standard (Bradford, 1976).

Proteolytic digestion of the 26 kd protein was performed with protease V8 from *S.aureus*. The protease (Miles Laboratories) was added at a ratio of 2% (w/w) protease V8/26 kd protein. The solution was incubated for 24 h at 37°C with constant agitation. Samples were electrophoresed, blotted, stained with Amido Black and bands of interest were cut and sequenced with an automated sequencer.

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