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PROTEINS OF ROD OUTER SEGMENTS OF TOAD RETINA: BINDING WITH CALMODULIN AND WITH GTP

A. Morelli, G. Damonte, I. Panfoli and I. Pepe *

Istituto di Chimica Biologica dell 'Università di Genova,
Viale Benedetto XV 1, 16132 Genova, Italy

*Istituto di Cibernetica e Biofisica del C.N.R.,
Via Dodecaneso 33, 16146, Genova, Italy

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SUMMARY: Proteins of purified rod outer segments from toad retina were analysed by electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate. The binding of proteins with calmodulin and with guanosine triphosphate was studied by electroblotting the proteins resolved by electrophoresis onto nitrocellulose sheets and by incubating the blots with labelled ligands. The results indicate that rod outer segments from toad retina contain nine proteins which bind to calmodulin and one protein, different from transducin, that binds to guanosine triphosphate.

The rod outer segments of vertebrate retinal photoreceptors are highly specialized cell-structures devoted to the transduction of light signals into electrical signals. The electrophoretic pattern of the ROS protein complement is relatively simple when compared to that of whole cells or other organelles. In one-dimensional polyacrylamide gel electrophoresis about 20 - 25 major protein bands can be easily detected, including three proteins that up to now have been isolated and functionally identified, namely: rhodopsin, transducin (G-binding protein) and cGMP-phosphodiesterase, which account respectively for 70%, 17% and 1.7% of total ROS protein content (1). Those proteins are involved in the enzyme cascade linking photon absorption by rhodopsin to cGMP hydrolysis by PDE stimulation, through the activation of transducin. In the present model of phototransduction, in fact, the electrical signal on rod plasma membrane is modulated by changes in the concentration of cGMP (2,3). There remains to investigate in detail the hypothesis that calcium ions play a key role in the cGMP metabolism. Indeed Ca^{2+} has been reported to increase PDE activity (4) and inhibit guanylate cyclase (5-7). Therefore an involvement of calmodulin - a protein known to act as a primary regulator

Abbreviations : ROS, rod outer segment; PDE, phosphodiesterase; G-binding protein, guanine nucleotide binding protein; CaM, calmodulin.

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of Ca^{2+} dependent enzyme activity in many cellular systems - must be postulated in phototransduction. As a matter of fact, the presence of CaM and CaM-binding proteins was demonstrated in frog ROS, mainly on disk membranes (8).

The model of phototransduction based on cGMP cascade seems nonetheless still incomplete. The inositol-triphosphate-induced reversible hyperpolarization found in Salamander rods (9), has in fact further complicated the matter introducing the possibility that inositol triphosphate cascade, regulated by a novel family of G-binding protein, G_p, might also be involved in vertebrate phototransduction.

We show here preliminary results indicating that the ROS of the toad retina contain at least nine CaM binding proteins and one GTP-binding protein different from transducin of about 22 kDa of molecular weight.

MATERIALS AND METHODS

Outer segments preparation. Rod outer segments were obtained from dark adapted eyes of toads Bufo-Bufo, enucleated under dim red light. The eyes were hemisected under infrared light and retinae were removed and gently shaken (for 90 sec and then discarded) in 35% of sucrose (w / w) in Ringer solution (115 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, buffered at pH 7.5 with 10 mM Hepes and tetramethylammonium hydroxyde). The rod outer segment suspension was centrifuged at 6000 rpm (3000 g). The pellet, containing contaminants, was discarded; the supernatant was diluted to 10% sucrose with Ringer solution and ROS were collected by centrifugation at 4000 rpm for 10 min. More purified rod outer segment preparations were obtained by gently agitating toad retinae in 6% Percoll (Pharmacia) in Ringer solution and then centrifuging the resulting ROS suspension in Percoll gradients according to the method described by Hamm and Bownds (1). Only the major red band sedimenting at the 45-68% Percoll and containing osmotically intact and highly purified ROS was used for the experiments. However, as no detectable difference resulted for the CaM and GTP binding to the proteins of ROS prepared with the two methods, we routinely used the former method which is faster and easier to perform in complete darkness.

Sample preparation. ROS from about 10 retinae were homogenized in a small potter with 500 μl of distilled water containing 2 μM leupeptin and centrifuged for 30 min at 100.000 g in a Beckman ultracentrifuge. This procedure was repeated two times and gave a final pellet containing mainly integral membrane proteins and two mixed supernatants containing soluble proteins and proteins peripherally bound to membranes. Samples for electrophoresis were prepared by boiling the samples in small volumes of sample buffer containing 7.5% SDS in 30 mM Na-Phosphate, pH 7.0, 30% glycerol (v/v), 10 mM dithiolthreitol and 0.05% (v/v) Bromophenol Blue and boiling for 5 min.

Calmodulin-free human erythrocyte membranes, used as a control, were prepared according to Niggli et al. (10) and were dissolved in sample buffer prior to electrophoresis.
Radioiodination of Calmodulin. Calmodulin purifed from bovine brain was a gift of Joachim Krebs (E.T.H., Zurich) and was iodinated with $Na^{125}I$ following the method of Morrison and Bayse (11), by means of lactoperoxidase and glucose-oxidase attached to an insoluble matrix (Enzymobead Radioiodination reagent -Bio Rad Laboratories) in the presence of glucose. The specific activity was 1.0 μCi/mg.
SDS-PAGE and blotting of proteins. Samples from ROS homogenates were analysed on SDS-PAGE through linear gradient of 5 - 14% of polyacrylamide with the discontinuous

gel system described by Laemmli (12). Samples to be used for blotting experiments were loaded in duplicate onto the gels, so that one half of each gel was used for Coomassie Blue staining (13) and the other for electroblotting.The gels were electroblotted onto nitrocellulose sheet following the method of Towbin et al. (14) for 3 hours at 80 V.

Incubation of blots with [^{125}I] CaM or with [^{32}P] GTP. Nitrocellulose blots were incubated overnight at 4^{0}C in buffer A (0.14 M NaCl, 10 mM Na-Phosphate, pH 7.2, containing 2% defatted milk powder). After changing once the buffer, nitrocellulose sheets were incubated for 3 hours at room temperature with [^{125}I] CaM (250 μCi) in the same buffer containing either 0.1 mM $CaCl_2$ or 2 mM EGTA. After 2 washes with buffer A containing $CaCl_2$ or EGTA, the sheets were removed, air dried and exposed to Kodak XAR-5 X-ray film at -80^{0}C for 3 days. In order to study the binding with GTP, nitrocellulose blots were incubated for 1 h at room temperature with 1 μCi/ml [α - ^{32}P] GTP in a buffer containing 20% methanol, 0.2 M glycine and an amount of Tris to reach pH 8.3. After washing with the same buffer, blots were air-dryed and radioactivity detected by autoradiography by exposing the films to a Kodak XAR-5 X-ray film at -80^{0}C for 7 days.

For the determination of molecular weight of transblotted samples, methylated [^{14}C]-protein standards (Amersham) were electrophoresed and blotted in parallel with the experimental samples.

RESULTS AND DISCUSSION

CaM-binding proteins in ROS fractions. The typical electrophoretic pattern of proteins from the ROS of toad retina is highly reproducible and very similar to that of frog retina (1). It consists of few protein bands easily recognizable, as shown in left panel in Fig 1. In lane 2 the membrane fraction (P) of disrupted ROS is shown. The three spreaded and intense bands with molecular weight of about 33, 54 and 80 kDa are the rhodopsin monomer, dimer and trimer respectively. Soluble and peripheral membrane proteins are contained in lane 3: one can easily recognize the doublet of alpha and beta subunits of PDE around 94 kDa and of alpha and beta subunits of transducin (40.0 and 36.5 kDa respectively). Furthermore, other two proteins are easily identified: the "48 K" protein described by Pfister et al.(15) at approximately 50 kDa and the gamma subunit of transducin at about 12 kDa (3).

Lane 1 shows the well-known electroforetic pattern of the red blood-cells membrane proteins (R). Their molecular weights, determined by the use of the calibration curve obtained with the standards (lane 4), were in accordance to the values from literature (16),enabling us to refer to the migration of alpha and beta subunits of spectrin as to a standard for the build up of the calibration curve in the higher molecular-weight region.

In order to study their binding with CaM, ROS proteins resolved by SDS-PAGE were blotted on nitrocellulose sheet and incubated with [^{125}I] CaM. The results of autoradiography are shown in the right panel in Fig 1: nine major membrane proteins (lane 6) bind to CaM at about 240, 150, 137, 108, 60, 55, 49, 45 and 38 kDa. Six out of

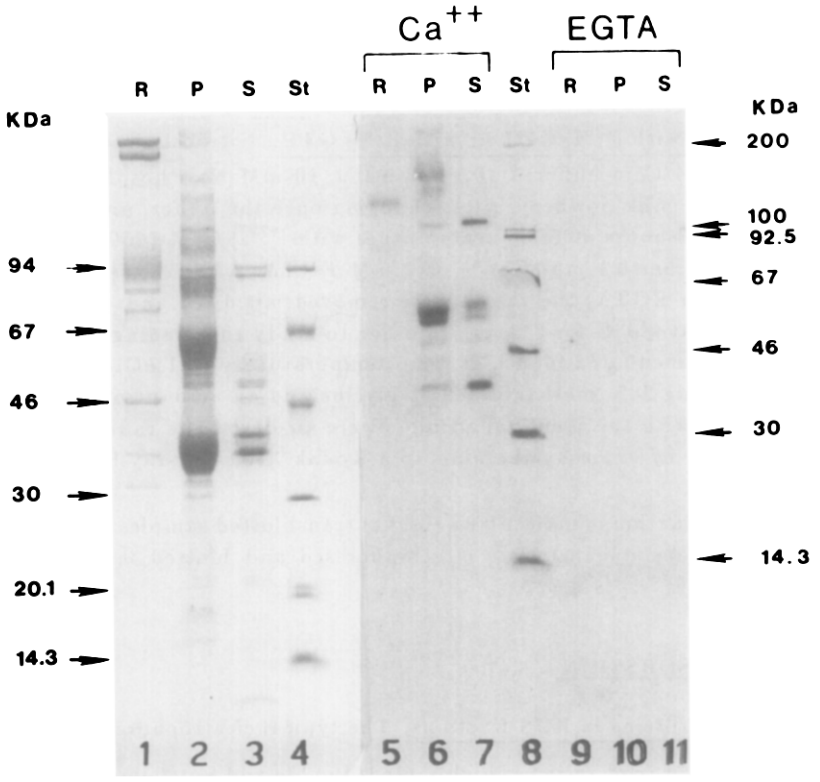

Fig.1. Electrophoretic pattern of proteins from the ROS of toad retina. Left panel (from lanes 1 to 4): protein bands on SDS/PAGE stained with Coomassie blue. Right panel (from lanes 5 to 8): autoradiography of nitrocellulose blots of ROS proteins resolved by SDS/PAGE and incubated with [^{125}I] CaM. Lanes 1 and 5: human erythrocyte membranes used as a control (R). Lanes 2 and 6: ROS membrane pellet (P). Lanes 3 and 7: ROS supernatant (S). For molecular weight determination, unlabelled (lane 4) and labelled (lane 8) protein standards were used.

those proteins are also present in the soluble fraction (lane 7 : 108, 60, 55, 49, 45 and 38 kDa) suggesting they are proteins peripherally bound to membranes that were not completely detached from disks by our fractionation method. The other three proteins of high molecular weight must be intrinsic membrane proteins as they are present only in lane 6. Apparently no soluble protein was found that binds to CaM. A possible reason for this is in that our ROS preparation might contain a low percentage of intact ROS and in that the amount of CaM-binding proteins in the soluble fraction may be very small in comparison to those embedded into the membrane fraction (this amount was less than 5% in frog ROS as reported by Nagao et al., ref.8).

The procedure is suitable to reveal the proteins binding to CaM, as shown by the electrophoretic pattern of proteins from human erythrocyte membranes used as a control (lane 5): the intense band around to 123 kDa is the CaM-activated Ca^{2+} ATP-ase (10).

For all the proteins labelled with ^{125}I, the binding with CaM was specific, as suggested by the complete desappearance of any protein band when incubation with [^{125}I] CaM was carried out in the presence of EGTA (lanes 9-11, Fig 1).

With regard to the problem of the identification of the CaM-binding proteins shown in Fig 1, the determination of molecular weight (see Methods) was only consistent with a preliminary analysis. In lane 6, the protein with higher molecular weight (240 kDa) might be the alpha subunit of fodrin, known to bind to CaM (16) (the doublet of supposed fodrin, the equivalent of erythrocytary spectrin, is seen in lane 2). The band at 150 kDa might be guanylate cyclase, known to be calcium-sensitive (5-7) in ROS. The smearing of the band may represent the formation of small amounts of the various dephosphorylated forms of the enzyme that has been proven to be highly phosphorylated in spermatozoan membranes of sea urchin (17). Consistently, the Authors (17) found values of 150 and 160 kDa for the two forms of the enzyme. Furthermore, Klumpp et al. (18) reported that the ciliary membranes of Paramecium tetraurelia contain a Ca^{2+}-regulated guanylate cyclase tightly bound to CaM. In lane 6, the protein of 137 kDa might be Ca^{2+}-ATPase, analogue to the form of 123 kDa found in the red blood cell, known to bind to CaM(19). The band around 60 kDa might be calcineurin, a protein abundant in nervous tissues, that in bovine brain has a molecular weight of 61 kDa (20). The intense protein bands at about 38 and 49.5 kDa might be the beta subunit of transducin and the "48 k" protein respectively, although no evidence can so far be found in literature that Ca^{2+} is involved in their activity. The visual pigment rhodopsin, whose band is contained in the lanes P of Fig 1, seems excluded from a binding with CaM, in agreement with previous results(8).

GTP-binding proteins of ROS fractions.

In order to detect GTP-binding proteins, nitrocellulose blots of ROS proteins resolved by SDS-PAGE were incubated with [^{32}P] GTP. The resulting autoradiography (see Fig 2) shows that after denaturing electrophoresis only a protein with apparent molecular weight of 22 kDa was able to bind GTP -lane 1,2 and 3-. As this binding appeared in both soluble (lanes 1-2) and particulate fractions (lane 3),the protein must be peripherally bound to disk membranes. The apparent small difference in the mobility of the two bands in lanes 1 and 3 should be ascribed to a disturbing action of the highly concentrated rhodopsin on the run of the integral membrane fraction, rather than to the presence of two different proteins.

The control with erythrocyte membranes shows a similar GTP-binding protein at 24 kDa already described (21).

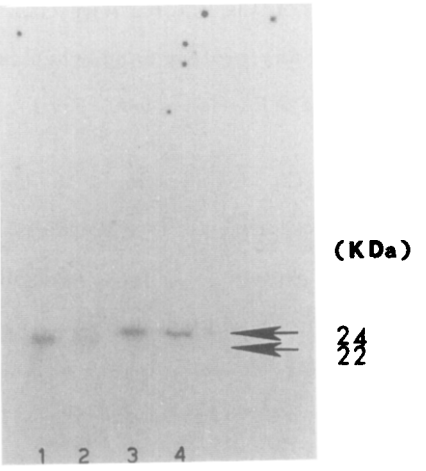

Fig.2. [^{32}P] GTP-binding of ROS proteins from toad retina resolved by SDS/PAGE. Lanes 1 and 2: ROS supernatant at two different concentrations (30 and 15μg respectively). Lane 3: ROS membrane pellet. Lane 4: human erythrocyte membranes used as a control.

The GTP-binding protein of ROS probably belongs to the 22-27 kDa "novel" G-binding protein family found in many systems such as placenta (22), bovine brain (23), platelets, red cells (21) and neutrophils (24). More experiments are needed to conclude that this GTP-binding protein, found for the first time in retinal photoreceptor cells, is involved in the release of inositol-triphosphate. The latter, besides the possibility of playing a central role in invertebrate phototransduction - parallel to that of cGMP in vertebrate (25,26) - is suspected to have an important role in the modulation of membrane voltage in vertebrate rods (9).

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