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Small GTP-binding proteins of squid photoreceptor

Interaction with photoactivated rhodopsin

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Abstract

Squid photoreceptor membranes contain several small GTP-binding proteins (22–27 kDa) in addition to heterotrimeric G-proteins. In addition, these membranes contain several proteins which can be ADP-ribosylated by exoenzyme C_3 from *Clostridium botulinum* (C_3). These proteins are similar but not identical to C_3 substrates from bovine rod outer segments. It is shown that substrates of C_3 in squid photoreceptor interact with rhodopsin in a light-dependent manner. These data suggest involvement of small GTP-binding proteins in phototransduction processes in invertebrates.

Key words: GTP-binding protein; Rhodopsin; C_3 ADP-ribosyltransferase; Invertebrate vision

1. Introduction

In both vertebrates and invertebrates the process of photo-transduction is carried out through a G-protein-coupled receptor cascade. Heterotrimeric G-proteins (transducin in verteb-rates and G_q in invertebrates) are the main coupling proteins of the visual system. Recently, several small GTP-binding proteins were identified in bovine rod outer segment (ROS) membranes in addition to transducin. Two of them (M_rs 22–24 kDa) could be ADP-ribosylated by exoenzyme C_3 from *Clostridium botulinum*. ADP-ribosylation of these proteins is regulated by light and guanine nucleotides in a manner similar to pertussis toxin catalyzed ADP-ribosylation of the a-subunit of transducin [1].

We report here that photoreceptor membranes from squid also contain several small GTP-binding proteins. Furthermore, these membranes contain substrates for exoenzyme C_3 which can interact with photoexcited rhodopsin in a light-dependent manner.

2. Materials and methods

Photoreceptor outer segments were isolated from frozen retina of squid Loliqo pacificus by floatation on 40% (w/v) sucrose as described in [2], and were washed twice in 20 mM Tris-HCl (pH 7.5), containing 100 mM NaCl, 5 mM $MgCl_2$, 1 mM EDTA. Outer segment membranes (30 μg of protein in 10 μl) were solubilized in sample buffer for 15–30 min. Proteins were then separated by SDS-PAGE and transferred electrophoretically onto nitrocellulose membranes which were washed twice with 60 ml of buffer: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM EDTA, 0.3% Tween 20. Then, the blot was incubated for 1.5 h at 25°C_3 with 10 ml of the same buffer including 0.5 μCi/ml of [α-^{32}P]GTP (5,000 Ci/mmol). The membrane was subsequently washed with the same buffer without [α-^{32}P]GTP until the solution was free of radioactivity. Labelled proteins were visualized by autoradiography with an intensifying screen for 12 h at −70°C.

ADP-ribosylation was carried out in 25 μl of 50 mM Tris-Cl (pH 7.5), containing 2 mM MgCl, 1 mM EDTA, 1 mM DTT, 10 mM thymidine, 0.5 μCi of [^{32}P]NAD and 0.1 μg of C_3 exoenzyme purified as described in [3]. Assay medium usually contained 15 μg of membrane proteins. After 1 h incubation at 36°C_3 aliquots were electrophoresed in 12.5% (w/v) acrylamide by the Laemmli method [4]. The gels were dried and autoradiographed with an intensifying screen. Before chromatography on Con A-Sepharose photoreceptor membranes were solubilized in 20 mM Tris-HCl (pH 7.5), containing 1 mM MgCl, 1 mM DTT, 1 mM PMSF, 1% n-dodecyl-β-D-maltoside for 1 h at 4°C. Solubilized samples were applied to a Con A-Sepharose column (1.5 × 5 cm) equilibrated with the same buffer and then rhodopsin was eluted by 250 mM α-methyl-D-mannoside. For obtaining polyclonal antibodies, rabbits were immunized with 200 μg of purified squid rhodopsin every 14 days. Blood was taken on the 12th day after the fifth immunization. The immunoglobulins were purified by salting-out with a semi-saturated solution of ammonium sulphate and subsequent chromatography on DE-52 (Whatmann). The titre of antibodies was determined according to the ELISA method. For obtaining immunosorbent, 100 μg of the antibodies were added to 100 μl of Pansorbin (Calbiochem) suspension and incubated for 2h at 4 . Immunosorbent was washed twice in 20 mM Tris-HCl (pH 7.5), containing 1 mM MgCl , 1 mM DTT, 1 mM PMSF, 1% dodecyl maltoside and 100 mM NaCl. Solubilizates of the illuminated and unilluminated membranes (0.1 ml each) were [^{32}P]ADP-ribosylated by C_3 and incubated with 50 μl of immunosorbent overnight at 4°C. Then immunosorbents were washed twice with 2 μl of the same

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buffer to remove unbound proteins. Laemmli's sample buffer was added to sorbents and eluted proteins were analysed electrophoretically for detection of labelled proteins.

3. Results and discussion

We examined photoreceptor membranes from squid to identify proteins able to bind GTP after polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes. As illustrated in Fig. 1 three protein bands with M_rs 22–27 kDa were labelled when membranes tranferred to nitrocellulose were incubated with [α-^{32}P]GTP. As reported in [1], bovine ROS membranes also contain at least four proteins with similar properties. It is known that small GTP-ases of rac- and rho-families (rho A, rho C, rac 1 and rac 2) in mammals can be ADP-ribosylated by exoenzyme C_3 [5–9]. We have found that photoreceptor membranes of squid contain polypeptides with M_rs of 22, 24, 30, 45 and 80 kDa which can be labeled by C_3 in presence of [^{32}P] NAD (Fig. 2). Polypeptides 22 and 24 kDa are similar to substrates of C_3 detected in bovine ROS and other mammalians tissues [5–12]. The 45 kDa substrate has molecular weight similar to rhodopsin and co-migrates on electrophoresis but, unlike rhodopsin, the 45 kDa substrate adsorbed onto DEAE-Toyopearl from the membrane solubilizate in dim red light (data not shown). Proteins 30, 45 and 80 kDa has unusual molecular weight for substrates of exoenzyme C_3. There were no labelled polypeptides after incubation of membranes in ribosylation memedium with [^{32}P]NAD but without C_3. GTP-binding and GTP-ase activity of these proteins was not examined.

The principal G-protein of invertebrate visual system, G_q, is a membrane-bound and can be extracted from membranes by treatment with 2 M urea. We have found that all substrates of C_3 in squid retina are membrane

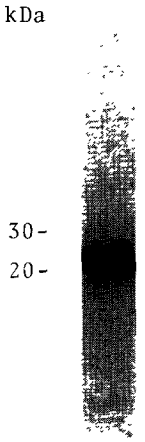

Fig. 1. Proteins from squid photoreceptor membranes which bind GTP after blotting on nitrocellulose membrane. Squid photoreceptor membrane proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane. The blot was then incubated with [α-^{32}P]GTP as described in Section 2.

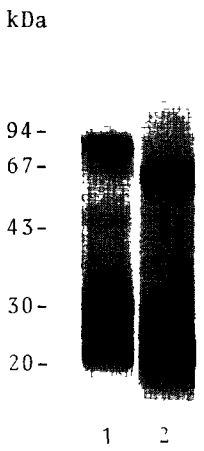

Fig. 2. Substrates of C_3 in squid photoreceptor membranes (1) and in bovine ROS membranes (2). Photoreceptor membranes were [^{32}P]ADP ribosylated in medium, containing [^{32}P]NAD and exoenzyme C_3 for 60 min as described in Section 2. After SDS-PAGE, labelled proteins were visualized by autoradiography for 16 h with intensifying screen.

localized. There was no labelling detectable in cytosol and in supernatants after treatment of membranes by hypotonic buffer, 400 mM KCl, 100 mM GTP or 2 M urea (data not shown).

We examined light-dependent [^{32}P]ADP-ribosylation of squid photoreceptor membranes by C_3 in comparison with [^{32}P]ADP-ribosylation of C_3 substrates in bovine ROS. The amount of radioactivity incorporated in 22–24 kDa proteins increased by 30% when ribosylation of squid photoreceptor membranes by C_3 was performed in bright white light (data not shown). In contrast, labelling of 22–24 kDa proteins by C_3 and of transducin by pertussis toxin in bovine ROS was inhibited by illumination [1].

It is known that invertebrate rhodopsin is a glycoprotein and can interact with Con A-Sepharose. We used this property to study light-dependent interaction of rhodopsin with substrates of C_3 in squid photoreceptor membranes. As illustrated in Fig. 3 when the solubilizate

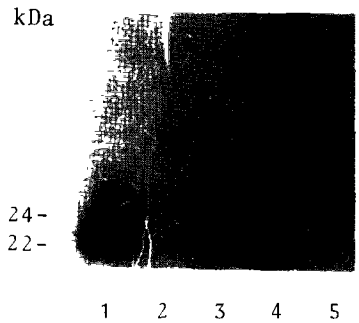

Fig. 3. Light-dependent interaction of substrates of C_3 from squid photoreceptor membranes with rhodopsin on Con A Sepharose. Membrane solubilizate (1). Proteins not absorbed (2) and absorbed (3) on Con A-Sepharose in complex with rhodopsin when the solubilizate was applied to column in bright white light. Proteins not absorbed (4) and adsorbed (5) on the same sorbent from solubilizate of dark-adapted membranes in dim red light.

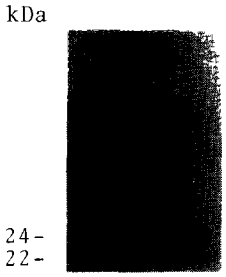

Fig. 4. Light-dependent precipitation of substrates of C_3 in complex with rhodopsin by immunosorbent to squid rhodopsin. Proteins which precipitated with rhodopsin from the solubilizates of dark-adapted (1) and illuminated (2) membranes, as described in Section 2, vere visualized by autoradiography.

was applied to Con A-Sepharose in bright white light 22–24 kDa C_3 substrates were detected together with rhodopsin after elution from column by α-methyl D-mannoside. But no substrates of C_3 were bound to rhodopsin after application of solubilizate of dark-adapted membranes onto Con A-Sepharose in dim red light. These data suggest that small GTP-binding proteins from squid photoreceptor membranes can interact directly or indirectly with rhodopsin in a light-dependent manner. This conclusion was confirmed by precipitation of rhodopsin in complex with C_3 substrates by immunosorbent to squid rhodopsin. As shown in Fig. 4 22–24 C_3 substrates were absorbed by the immunosorbent from solubilizate of illuminated membranes and were not absorbed from unilluminated solubilizate.

In addition to trimeric G-proteins there are a large number of 20–30 kDa GTP-binding proteins involved in control of a wide variety of cell processes such as signal transduction, growth, differentiation, cytoskeletal organization and vesicle transport [13–15]. It was shown earlier that these proteins can interact with rhodopsin in vertebrate visual system [1]. In invertebrate visual cells studied by biophysical methods, antibody binding and toxin labelling have indicated several α-subunits of G-proteins [16–18]. The data presented here show that squid photoreceptor membranes also contain small GTP-binding proteins. These proteins are membrane associated, like G-proteins, and can interact with photoactivated rhodopsin. Nothing is yet known about effector protein(s) regulated by these proteins in invertebrate photoreceptor cells. We propose that small GTP-binding proteins can couple rhodopsin to light-activated enzymes in a manner different from that in which effectors are coupled to rhodopsin by trimeric G-proteins.

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