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# Interaction of small G proteins with photoexcited rhodopsin

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Bovine rod outer segment (ROS) membranes contain in addition to the heterotrimeric G protein transducin, several small GTP-binding proteins (23–27 kDa). Furthermore, these membranes contain two substrate proteins (about 22 and 24 kDa) for botulinum C3 ADP-ribosyltransferase known to ADP-ribosylate small G proteins in any mammalian cell type studied so far. Most interestingly, [$^{32}$P]ADP-ribosylation of ROS membrane small G proteins by C3 is regulated by light and guanine nucleotides in a manner similar to pertussis toxin-catalyzed [$^{32}$P]ADP-ribosylation of the α-subunit of transducin. These findings suggest that not only the heterotrimeric G protein transducin but also the C3 substrate small G proteins present in ROS membranes interact with photoexcited rhodopsin and thus contribute to its signalling action.

GTP-binding protein; Rhodopsin; Botulinum C3 ADP-ribosyl-transferase; Transducin; (Bovine rod outer segment)

## 1. INTRODUCTION

Signal transduction at the plasma membrane by a wide variety of hormone and neurotransmitter receptors, including the light receptor rhodopsin, is mediated by guanine nucleotide-binding proteins (G proteins) [1–4]. Members of this G protein family such as the adenylyl cyclase regulatory proteins $G_s$ and $G_i$ and the retinal transducins are heterotrimeric proteins composed of α-, β- and γ-subunits, with the guanine nucleotide-binding site at the α-subunit ($M_r$s of 40–50 kDa). These proteins are targets for the bacterial toxins, cholera and/or pertussis toxin, ADP-ribosylating the G protein α-subunits. This covalent modification can be regulated by the activity state of receptors interacting with the respective G proteins [5–9].

There is an additional large family of small G proteins ($M_r$s of 20–30 kDa), which include the ras proteins and about 20 other gene products [10,11]. Members of this G protein family have been detected in almost every mammalian cell type in both membrane and cytosolic forms. The exact cellular functions of these small G proteins, however, are unknown, although the widespread distribution suggests that they have important roles, e.g. in signal transduction at the plasma membrane or in mediating intracellular actions. Some of these small G proteins, including the rho A and C proteins, are substrates for botulinum C3 ADP-ribosyltransferase [12–14]. We report here that bovine rod outer segment (ROS) membranes contain, in addition to the heterotrimeric G protein transducin, several small GTP-binding proteins and also C3 substrates and provide evidence that the C3 substrate small G proteins interact with photoexcited rhodopsin.

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## 2. MATERIALS AND METHODS

### 2.1. *Materials*

[α-$^{32}$P]GTP and [α-$^{32}$P]ATP were from New England Nuclear, Dreieich, FRG. [$^{32}$P]NAD was prepared from [α-$^{32}$P]ATP as described [15]. Unlabelled nucleotides were from Boehringer Mannheim, Mannheim, FRG. Botulinum C3 ADP-ribosyltransferase was purified as in [16]. Pertussis toxin was isolated from the supernatant of *Bordetella pertussis* suspensions as in [17].

### 2.2. *Preparation of ROS membranes*

Untreated ROS membranes were prepared in dim red light from bovine retinas according to [18]. Transducin-depleted illuminated ROS membranes were obtained by repeated (3 ×) hypotonic elution of transducin in the presence of 100 μM GTP [19].

### 2.3. *Binding of [α-$^{32}$P]GTP to small G proteins*

Bovine ROS membranes (20 μg of protein) were solubilized in Laemmli's sample buffer [20] and heated for 3 min at 100°C. Proteins were then separated by SDS polyacrylamide gel electrophoresis (PAGE) with 13% (w/v) acrylamide in the separating gel. Thereafter, proteins were transferred electrophoretically onto nitrocellulose. The nitrocellulose paper was washed twice with 60 ml of buffer containing 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM $MgCl_2$ and 0.3% Tween 20. Then, the blot was incubated for 12 h at 25°C with 10 ml of the buffer including 12.5 nM [α-$^{32}$P]GTP (400 Ci/mmol). The paper was subsequently washed with the same buffer without [α-$^{32}$P]GTP until the buffer was free of radioactivity. Labelled proteins were visualized by autoradiography without intensifying screen.

### 2.4. *[$^{32}$P]ADP-ribosylation of ROS membrane proteins*

[$^{32}$P]ADP-ribosylation of bovine ROS membranes (15 μg of protein) by botulinum C3 ADP-ribosyltransferase was performed in a medium containing 50 mM triethanolamine-HCl, pH 7.4, 2 mM EDTA, 1 mM dithiothreitol, 0.5 mM ATP, 10 or 100 μM free $Mg^{2+}$, 0.1 μM [$^{32}$P]NAD (0.3–1 μCi/tube) and 0.2 μg purified C3 in a total volume of 50 μl for 15 or 60 min at 37°C. For [$^{32}$P]ADP-ribosylation by pertussis toxin, the toxin was preactivated for 10 min at 30°C in

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a buffer containing 100 mM Tris-HCl, pH 8.0, and 50 mM dithiothreitol. ROS membranes (15 $\mu$g of protein) were then incubated as described above for C3-catalyzed [$^{32}$P]ADP-ribosylation using 1 $\mu$g activated pertussis toxin instead of C3. Incubation was either in dim red or bright white light. For studies in bright light, membranes were illuminated before incubation for 5 min on ice. Reaction was terminated by addition of sample buffer [20] and boiling for 3 min. After SDS-PAGE with 10% acrylamide in the separating gel, labelled proteins were visualized by autoradiography without or with intensifying screens. For quantitative analysis, labelled bands were cut out from the gels and analyzed for incorporated radioactivity in a liquid scintillation spectrometer.

## 3. RESULTS AND DISCUSSION

As first reported by Bhullar and Haslam [21], the small G proteins, unlike the $\alpha$-subunits of heterotrimeric G proteins, retain their ability to bind GTP after polyacrylamide gel electrophoresis and transfer to nitrocellulose membranes. We used this method to study whether bovine ROS membranes also contain such proteins. As illustrated in Fig. 1, 4 distinct protein bands with $M_r$s of about 23–27 kDa but not the $\alpha$-subunit of transducin (39 kDa) were labelled when the ROS membrane proteins transferred to nitrocellulose paper were incubated with [$\alpha$-$^{32}$P]GTP.

Since G proteins of a molecular mass range of 21–24 kDa have been identified as substrates for botulinum C3 ADP-ribosyltransferase in almost every mammalian cell type studied so far, in both membrane and cytosolic forms [14], we examined whether bovine ROS membranes also contain such proteins. Incubation of dark-adapted ROS membranes with C3 and [$^{32}$P]NAD in dim red light resulted in labelling of two distinct bands with $M_r$s of about 22 and 24 kDa (Fig. 2). In most other tissues, also two proteins of about 21 and 24 kDa are labelled by C3 [14]. Most interestingly, when [$^{32}$P]ADP-ribosylation of ROS membrane proteins by C3 was performed in bright white light, the labelling of both protein bands was strongly reduced compared to labelling in dim red light. For comparison, [$^{32}$P]ADP-ribosylation of the $\alpha$-subunit of transducin by pertussis toxin was performed under identical conditions. As reported before [5–7], [$^{32}$P]ADP-ribosylation of the $\alpha$-subunit of transducin by pertussis toxin was also strongly inhibited by illumination. Incubation in bright white light reduced [$^{32}$P]ADP-ribose incorporation into the $\alpha$-subunit of transducin by about 90%. Labelling of the 22–24 kDa proteins by C3 was inhibited by illumination of the membranes by 60–70%. Thus, ADP-ribosylation of 22–24 kDa proteins by C3 and of transducin by pertussis toxin are regulated by light in a similar manner. The amount of radioactivity incorporated into the small $M_r$ proteins induced by C3 in dim red light was about 15% of the amount incorporated into transducin by pertussis toxin under the same incubation conditions. Similar as reported before in other membrane systems, e.g. of human platelets [16], [$^{32}$P]ADP-ribosylation of ROS membrane proteins by C3 was regulated by $Mg^{2+}$ in a biphasic manner. Maximal [$^{32}$P]ADP-ribosylation was observed at 10–100 $\mu$M $Mg^{2+}$, followed by a decreased incorporation of [$^{32}$P]ADP-ribose at higher $Mg^{2+}$ concentrations. Inhibition of [$^{32}$P]ADP-ribosylation by illumination was

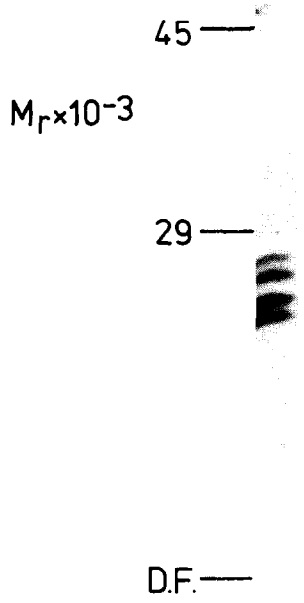


Fig. 1. Presence of small GTP-binding proteins in bovine ROS membranes. Bovine ROS membrane proteins were separated by SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose. The blot was then incubated with [$\alpha$-$^{32}$P]GTP as described in section 2. Shown is an autoradiogram with the molecular weight markers on the left. D.F., dye front.

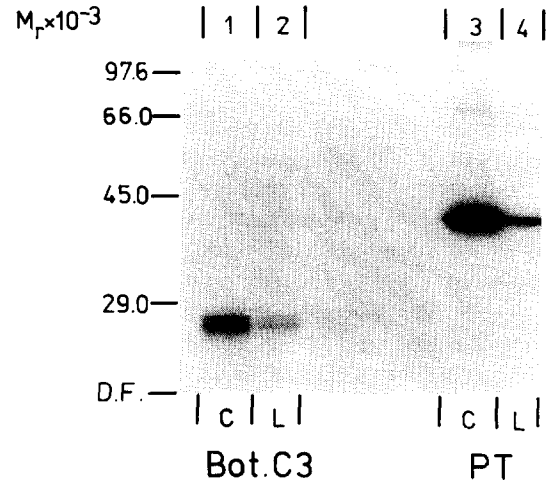


Fig. 2. [$^{32}$P]ADP-ribosylation of ROS membrane proteins by both pertussis toxin and C3 ADP-ribosyltransferase is regulated by light. Dark-adapted bovine ROS membranes were incubated either in dim red light (C, lanes 1 and 3) or in bright white light (L, lanes 2 and 4) with [$^{32}$P]NAD and either botulinum C3 ADP-ribosyltransferase (Bot.C3, lanes 1 and 2) or pertussis toxin (PT, lanes 3 and 4) as described in section 2. The [$^{32}$P]ADP-ribosylation was for 60 min with 100 $\mu$M free $Mg^{2+}$. After SDS-PAGE, labelled proteins were visualized by autoradiography for 4 h without intensifying screen.

observed at $Mg^{2+}$ concentrations between 10 $\mu$M and 1 mM (data not shown).

Transducin can be released from illuminated ROS membranes by treatment with GTP [22]. After release by GTP, the G protein is still an excellent substrate for pertussis toxin [4,6,7]. When [$^{32}$P]ADP-ribosylation by C3 was performed in such transducin-containing supernatants, there was no labelling detectable. Furthermore, while treatment of the ROS membranes with GTP reduced the amount of [$^{32}$P]ADP-ribose incorporated by pertussis toxin, C3-catalyzed labelling of the 22–24 kDa membrane proteins was essentially unaltered by this treatment (results not shown, see Fig. 3). These data suggest that, in contrast to transducin, the C3 substrates are rather integral membrane proteins.

Inhibition of pertussis-catalyzed ADP-ribosylation of transducin by illumination is apparently due to interaction of transducin with photoexcited rhodopsin, leading to dissociation of GDP from the G protein's $\alpha$-subunit [4]. This light-induced inhibition can be reversed by adding GDP and can even be increased by addition of hydrolysis-resistant GTP analogs such as guanylyl-5′-imidodiphosphate (GMPPNHP) or guanosine-5′-O-(3-thiotriphosphate) (GTP[S]) [5–7]. Interpretation of these data is that the preferred substrate of pertussis toxin is transducin·GDP, while both the nucleotide-free form and transducin bound with hydrolysis-resistant GTP analogs are poor toxin substrates. Thus, availability of transducin to pertussis toxin monitors the activity state of the light receptor rhodopsin, causing in its excited form dissociation of transducin-bound GDP and, in consequence, permitting binding of the GTP analogs. As illustrated in Fig. 3, [$^{32}$P]ADP-ribosylation of the $\alpha$-subunit of transducin by pertussis toxin in transducin-depleted illuminated ROS membranes was weak compared to the rather strong [$^{32}$P]ADP-ribosylation of 22–24 kDa proteins by C3. Note the different exposition times of the two parts of the autoradiogram. When GDP (10 $\mu$M) was added to these membranes, [$^{32}$P]ADP-ribose incorporation into the $\alpha$-subunit of transducin was largely increased. In contrast, in the presence of the poorly hydrolyzable GTP analogs, GMPPNHP and GTP[S] (10 $\mu$M each), the pertussis toxin-catalyzed labelling of the $\alpha$-subunit of transducin almost completely disappeared. In principle, similar data were obtained when [$^{32}$P]ADP-ribosylation of 22–24 kDa proteins by C3 was studied in these transducin-depleted illuminated membranes. Addition of GDP caused an increase in labelling. In contrast, in the presence of the poorly hydrolyzable GTP analogs, particularly of GTP[S], C3-catalyzed [$^{32}$P]ADP-ribosylation was strongly attenuated. Pretreatment of ROS membranes with unlabelled NAD and pertussis toxin had no apparent effect on subsequent [$^{32}$P]ADP-ribosylation of C3 substrates (results not shown).

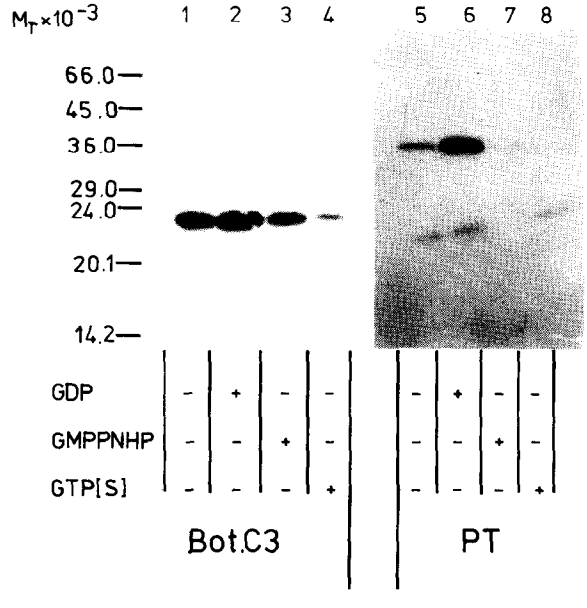


Fig. 3. [$^{32}$P]ADP-ribosylation of ROS membrane proteins by both pertussis toxin and C3 ADP-ribosyltransferase is regulated by guanine nucleotides. Partially transducin-depleted illuminated bovine ROS membranes were incubated with [$^{32}$P]NAD and either botulinum C3 ADP-ribosyltransferase (Bot.C3, lanes 1–4) or pertussis toxin (PT, lanes 5–8) in the absence (1, 5) and presence of either GDP (2, 6), GMPPNHP (3, 7) or GTP[S] (4, 8) (10 $\mu$M each). The [$^{32}$P]ADP-ribosylation was for 15 min with 10 $\mu$M free $Mg^{2+}$. After SDS-PAGE, labelled proteins were visualized by autoradiography, which was for 2 and 12 h for lanes 1–4 and 5–8, respectively, with an intensifying screen.

Taken together, evidence is presented that transducin is not the sole, although apparently the major, G protein in vertebrate ROS membranes, containing in addition several small GTP-binding proteins. Furthermore, two of such proteins, which may or may not be identical with proteins labelled by [$\alpha$-$^{32}$P]GTP, are in addition substrates for botulinum C3 ADP-ribosyltransferase. Most important, similar to transducin, these two small G proteins apparently interact with the receptor rhodopsin in an agonist (light)-dependent manner. The interaction of photoexcited rhodopsin with the C3 substrate proteins appears to occur independently of the presence or activity state of transducin. Although an effector protein(s) possibly regulated by these small G proteins in ROS membranes is not yet known, the demonstration of light-regulated availability of these proteins to C3 ADP-ribosyltransferase is, to our knowledge, the first clear evidence for an interaction of a small G protein with a membrane receptor and, thus, suggests that the C3 substrate small G proteins somehow contribute to the signalling action of these receptors.

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