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Mono ADP-ribosylation of transducin catalyzed by rod outer segment extract

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Transducin is the retinal rod outer segment (ROS)-specific G protein coupling the photoexcited rhodopsin to cyclic GMP-phosphodiesterase. The α subunit of transducin is known to be ADP-ribosylated by bacterial toxins. We investigated the possibility that transducin is modified in vitro by an endogenous ADP-ribosyltransferase activity. By using either ROS, cytosolic extract of ROS or purified transducin in the presence of [α-^{32}P]nicotinamide adenine dinucleotide (NAD^+), the α and β subunits of transducin were found to be radiolabeled. The labeling was decreased by snake venom phosphodiesterase I (PDE I). The modification was shown to be mono ADP-ribosylation by analyses on thin layer chromatography of the PDE I-hydrolyzed products which revealed only 5'AMP residues. In addition we report that sodium nitroprusside activates the ADP-ribosylation of transducin.

Transducin; ADP-ribosylation; Rod outer segment; G protein; Rhodopsin; Retina

1. INTRODUCTION

Transducin, the retinal G-protein, plays a key role in the mechanism of phototransduction in rods of vertebrates [1]. One molecule of photoexcited rhodopsin catalyzes the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) of hundreds of transducin (T) molecules [2]. The GTP-bound form of transducin activates the cyclic guanosine monophosphate-phosphodiesterase (cGMP-PDE) by binding its γ inhibitory subunit [3]. The hydrolysis of about 10^3 molecules of $cGMP \cdot s^{-1}$ by each activated cGMP-PDE leads to a transient reduction of the intracellular cGMP level followed by the closure of cGMP-gated cationic channels [4]. Like most G-proteins, transducin is a heterotrimeric protein composed of α, β and γ subunits and the binding of GTP leads to the dissociation of the active Tα·GTP form from Tβγ. After hydrolysis of the GTP by its intrinsic guanosine triphosphatase (GTPase) activity [5], Tα returns to its Tα.GDP-Tβγ inactive form.

At present, little is known about post-translational modifications of transducin. It has been shown that Tγ can be farnesylated [6,7], modification essential for interactions among the three transducin subunits. In addition Tα and Tβ can be phosphorylated by protein kinase C [8,9] but the functional implications of these modifications remain to be established.

The α subunit of transducin has been shown to be mono ADP-ribosylated at different sites by cholera and pertussis toxins [10–13]. Mono ADP-ribosylation is a post-translational modification by which the ADP-ribose moiety of adenine dinucleotide (NAD^+) is covalently attached to the protein. Cholera toxin and pertussis toxin catalyze the ADP-ribosylation of an arginine residue of Gs or a cysteine residue of Gi or Go, in the adenylate cyclase system [14]. Recently, such ADP- ribosyltransferase acitivities have been attributed to eukaryotic proteins [15–17] and Watkins et al. [18] have shown that an enzyme isolated from turkey erythrocytes can ADP-ribosylate α and β subunits of transducin.

In this study we explored the possibility that ADP-ribosylation of transducin occurs in vitro without addition of any exogenous ADP-ribosyltransferase. The modification of transducin we observed was shown to be mono ADP-ribosylation. The modification rate seems to be slight and the functional consequences observed are rather weak.

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Abbreviations: DTT, dithiothreitol; cGMP-PDE, cyclic guanosine monophosphate-phosphodiesterase; GTPγS, guanosine-5'-*O*(3-thiotriphosphate); NAD^+, nicotinamide adenine dinucleotide; NEM, *N*-ethylmaleimide; PDE I, snake venom phosphodiesterase I; ROS, rod outer segments; Tα,Tβ and Tγ, α, β and γ subunits of transducin; TLC, thin layer chromatography.

2. MATERIALS AND METHODS

2.1. *Materials*

[α-^{32}P]NAD^+ was obtained from New England Nuclear. GTPγS was from Boehringer Mannheim. PDE I was from P-L Biochemicals, Inc., Milwaukee, WI 53 205. F 1440 cellulose plates were obtained

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from Schleicher and Schüll, Dassel, Germany. Other reagents were of the highest grade available.

2.2. *Transducin purification*

2.2.1. GTPγS active form of transducin (T·GTPγS).

ROS from 60 retinas isolated as previously described [19] were subjected to illumination for 2 min (250 W), suspended and pelleted (100,000 × *g* for 1 h) twice with 30 ml 10 mM Tris-HCl, pH 7.4. The pellet was washed once more with 66 mM sodium phosphate buffer pH 7 and incubated for 1 h at 37°C in 15 ml of the same buffer supplemented with 100 μM GTPγS. The transducin was extracted from the pelleted membranes (100,000 × *g* for 30 min) with 15 ml 10 mM HEPES, pH 7.4 by centrifugation (100,000 × *g* for 1 h). The supernatant was stored in the liquid nitrogen.

2.2.2. GDP inactive form of transducin (T·GDP).

The washed ROS pellet was incubated for 1 h at 37°C in 15 ml sodium phosphate buffer pH 7 supplemented with 100 μM GTP and transducin was extracted with 10 mM Tris-HCl pH 7.4.

2.3. *ADP-ribosylation assay*

ROS suspension (1 mg rhodopsin/assay), ROS cytosolic fraction (40 μg/assay) (obtained by centrifugation 100,000 × *g* for 1 h of ROS suspension) and transducin solution (20 μg/assay) were incubated in the dark at 30°C in a reaction mixture (500 μl) containing 10 mM HEPES, pH 7.4, 2.5 mM $MgCl_2$ and 1 mM (20 Ci·mol^{-1}) [α-^{32}P]NAD$^+$. After 30 min incubation, proteins were alkylated with *N*-ethylmaleimide (NEM) as in [20] and precipitated with cold ethanol/sodium acetate 0.1 M (2:1 v/v). After several washes with ethanol/acetate (3×), ethanol (1×) and ether (1×), the proteins were solubilized in loading buffer containing 1% SDS and 1% mercaptoethanol and subjected to SDS- PAGE according to the method of Laemmli [21]. Gels were stained with Coomassie brilliant blue R 250. Dried gels were submitted to autoradiography at −80°C using Kodak XAR-5 films.

2.4. *Hydrolysis of the ADP-ribosylated transducin*

After ethanol/acetate precipitation [^{32}P]ADP-ribosylated proteins (20 μg) were incubated in 50 mM Tris-HCl, pH 8.5, 15 mM $MgCl_2$ for 4 h at 37°C with or without phosphodiesterase I (1.5 U). Ethanol/acetate-precipitated proteins were submitted to SDS-PAGE and autoradiography.

2.5. *Thin layer chromatography (TLC)*

After 4 h PDE I hydrolysis of [^{32}P]ADP-ribosylated transducin, proteins were precipitated with trichloroacetic acid and pelleted by centrifugation. The supernatant was lyophilized and applied to a F 1440 celllulose plate for two-dimensional TLC according to Keith et al. [22]. AMP, UMP, CMP, GMP, ADP-ribose and NAD$^+$ were added to the assay as standards. The solvent systems were isobutyric acid/25% NH_4OH/H_2O (50:1.1:28.9, v/v) for the first dimension, and HCl/isopropanol/H_2O (15:70:15, v/v) for the second dimension.

2.6. *[α-^{32}P]GTP-binding assay*

Binding of [α-^{32}P]GTP to transducin was determined by adapting the method of Cook et al. [23]. Transducin (50–300 pmol) was incubated for 20 min at 23°C at room light in a total volume of 400 μl of medium containing 10 mM Tris-HCl pH 7.4, 120 mM NaCl, 3 mM $MgCl_2$ (binding buffer), 20 μM [α-^{32}P]GTP (500,000 cpm) and purified rhodopsin [24] (mol rhodopsin/mol transducin:10). The binding reaction was initiated by the addition of transducin. The assays were filtered through nitrocellulose filters (Millipore 0.45 μm) and washed eight times with 3 ml of ice-cold binding buffer and twice with 3 ml ethanol. The dried filters were dissolved in 5 ml scintillation fluid (Biofluor, NEN) for counting of the radioactivity.

2.7. *Protein assay*

Protein content was measured according to the method of Bradford [25] using the Bio-Rad protein assay reagent and bovine serum albumin as standard.

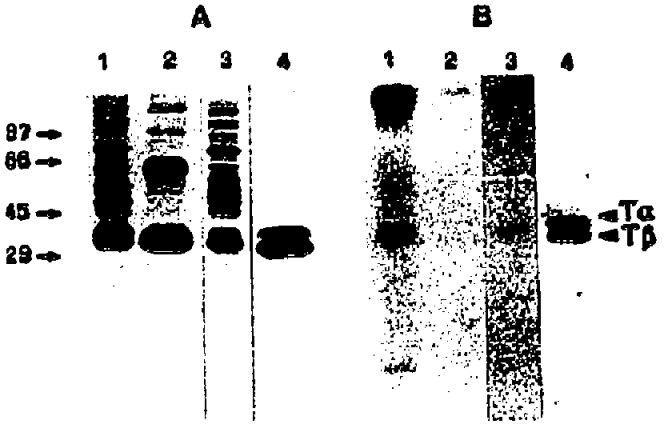

Fig. 1. SDS-PAGE and autoradiography of ADP-ribosylated transducin. (i) After incubation for 30 min at 30°C in 10 mM HEPES (pH 7.4), 2.5 mM $MgCl_2$, 1 mM [α-^{32}P]NAD$^+$ (20 Ci·mol^{-1}) cytosolic (lanes 1, 24 μg protein) and membrane (lanes 2, 170 μg protein) fractions of ROS suspension were separated by centrifugation at 100,000 × *g*. (ii) Membrane fraction from ROS suspension was separated from cytosolic fraction by centrifugation and discarded. Cytosolic fraction (lanes 3, 24 μg protein) was incubated as described in (i). (iii) Purified transducin solution (lanes 4, 8 μg protein) was incubated as in (i). Samples were alkylated with 2.5 mM NEM in the presence of 1 mM DTT and 2% SDS [20] and precipitated by ethanol/0.1 M sodium acetate (2:1, v/v). They were subjected to SDS-PAGE (12%) [21] and stained with Coomassie blue (A). Gels were exposed to Kodak XAR-5 film for autoradiography (8 days) (B).

3. RESULTS AND DISCUSSION

When a suspension of rod outer segments (ROS) was incubated in a reaction mixture containing 1 mM [α-^{32}P]nicotinamide adenine dinucleotide ([α-^{32}P]NAD$^+$) (as in section 2.3) without any additional source of ADP-ribosyltransferase activity, significant radioactivity was incorporated in the ethanol/acetate-precipitated proteins. These proteins were separated by SDS-PAGE (Fig. 1A) and the gels were autoradiographed. On the autoradiogram (Fig. 1B), soluble proteins of molecular mass of 37–39 kDa were clearly labeled (lane 1). Protein bands of molecular mass 37–39 kDa were also found to be labeled after incubation of ROS 100,000 × *g* supernatant (Fig. 1, lane 3). No protein labeling was observed in the membrane fraction (lane 2).

Since the apparent molecular weights of 39 and 37 kDa agree respectively with those of the α and β subunits of transducin, we prepared transducin according to Kühn [26]. The purified protein was characterized as transducin by its capacity to bind GTP in presence of photostimulated rhodopsin; under the conditions described in section 2.6, 17% of the protein was found to be able to bind GTP. The nature of the protein was also confirmed by its ability to stimulate cGMP-PDE activity; after addition of 15 mol of transducin (T·GTPγS) per mol of cGMP-PDE, the cGMP hydrolyzed by the hypotonic extract of ROS [27] was 2.5- to 3-fold that of basal activity. After incubation of purified transducin

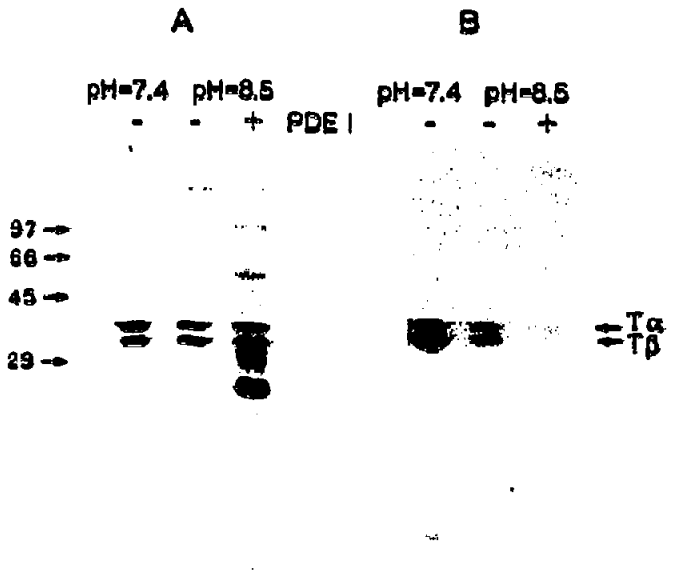

Fig. 2. Snake venom phosphodiesterase I (PDE I) hydrolysis of [^{32}P]ADP-ribosylated transducin. After ethanol/acetate precipitation, [^{32}P]ADP-ribosylated transducin prepared as described in Fig. 1 was incubated in 10 mM Tris-HCl pH 7.4 (control) or 50 mM Tris-HCl pH 8.5, 15 mM $MgCl_2$ for 4 h at 37°C with (+) or without (−) PDE I (1.5 U). Ethanol/acetate-precipitated transducin (6–8 μg protein/lane) was submitted to SDS-PAGE (A) and autoradiography (B) as in Fig. 1.

with [α-^{32}P]NAD$^+$, α and β subunits of transducin were labeled (Fig. 1, lane 4). Labeling of the α subunit has been observed for transducin incubated in presence of bacterial toxins [10–13]; the β subunit labeling has been previously described by Watkins [18], but only after incubation of transducin with ADP-ribosyltransferase from turkey erythrocytes.

To identify the nature of the modification of transducin, we extensively washed the labeled, precipitated transducin with ethanol/acetate supplemented with 10 mM NAD$^+$. The labeling of transducin was not affected by this treatment (not shown) suggesting that it is probably not due to NAD$^+$ binding but rather to a covalent modification. We never observed smears, either on gels or on autoradiograms, which could be indicative of different size ADP-ribose polymers linked to proteins. Snake venom phosphodiesterase I (PDE I) (EC.3.1.4.1) is commonly used to distinguish between poly and mono ADP-ribosylation [28]. PDE I cleaves pyrophosphate linkages and causes the release of phosphoribosyl AMP and AMP if the protein modification is poly ADP-ribosylation and only AMP in the case of mono ADP-ribosylation. After incubation with [α-^{32}P]NAD$^+$, the ethanol/acetate-precipitated transducin was treated (section 2.4) with PDE I and analyzed on SDS-PAGE (Fig. 2A,B). Densitometric scanning confirmed there is no loss of radioactivity by incubating the ADP-ribosylated transducin for 4 h at pH 8.5. The autoradiographic labeling of the second lane seems slight because of the difference of precipitation rate which led to a protein quantity lower then that of the first lane. PDE I hydrolysis for 4 h leads to a loss radioactivity associated with transducin of about 60%. The radiolabeled product released by this digestion was characterized by cellulose thin layer chromatography and autoradiography. It co-migrated with the 5'AMP marker (Fig. 3A,B). From these observations we can

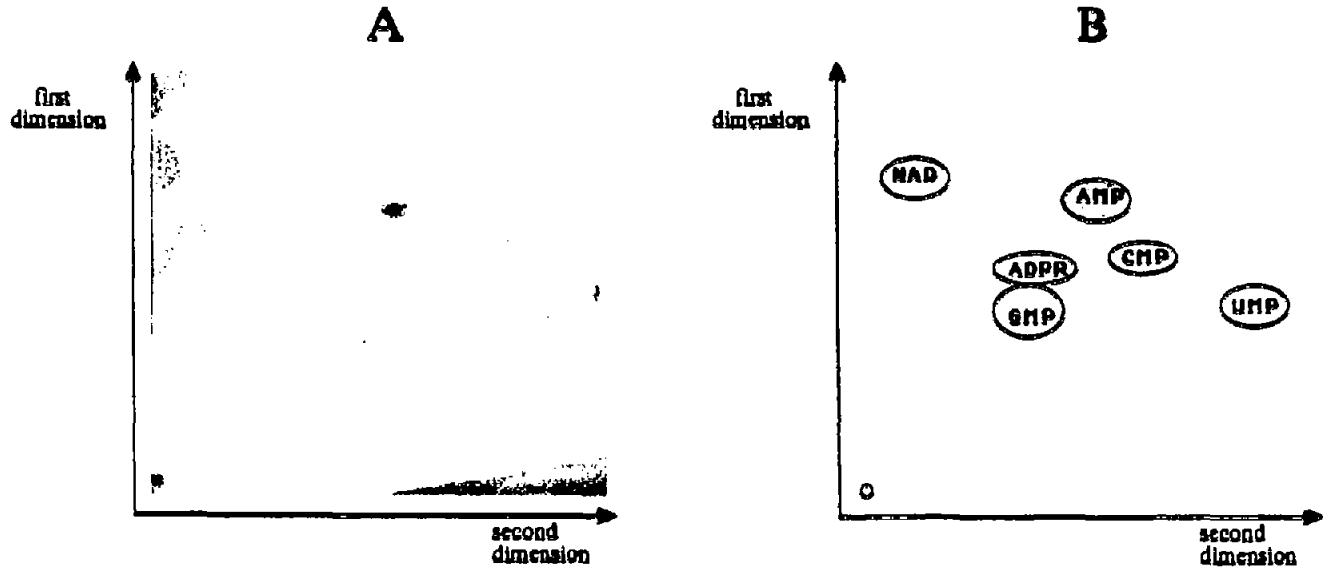

Fig. 3. Thin layer chromatography (TLC) of the acid-soluble fraction obtained after snake venom phosphodiesterase I (PDE I) hydrolysis of [^{32}P]ADP-ribosylated transducin. After 4 h PDE I hydrolysis of [^{32}P]ADP-ribosylated transducin, (20 μg) proteins were precipitated by TCA and pelleted by centrifugation. The supernatant was lyophilized and applied to a F 1440 cellulose plate for 2D TLC according to Keith et al. [22], together with AMP, UMP, CMP, GMP, ADP-ribose and NAD$^+$ as standards. Radiolabeled products were detected by autoradiography with Kodak XAR-5 film (A). Nucleotides were visualized with a U.V. lamp at 256 nm (B).

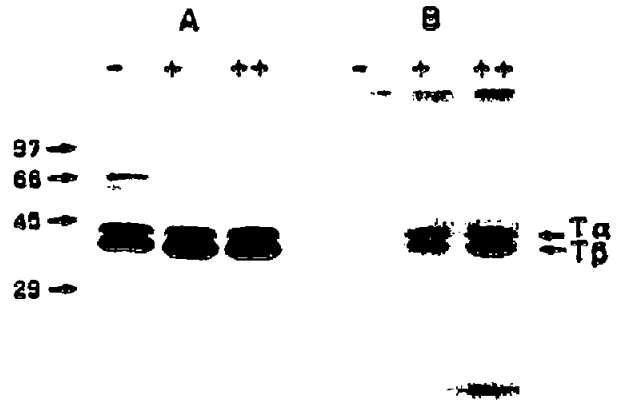

Fig. 4. Effect of sodium nitroprusside on [^{32}P]ADP-ribosylation of transducin. SDS-PAGE (A) and autoradiography (B) of purified transducin incubated (see legend of Fig. 1) in absence (−) or presence of 50 μM (+) or 100 μM (++) sodium nitroprusside (6–8 μg protein/lane).

conclude that the modification is likely to be mono ADP-ribosylation. We did not yet succeed in quantifying the endogenous ADP-ribosylation reaction of transducin, probably because of its very low rate.

We tested if sodium nitroprusside is able to enhance the modification of transducin as reported by Brüne et al. [29] for the mono ADP-ribosylation of acceptor proteins in different tissues. The radiolabeling of transducin was increased (Fig. 4, lanes (+) and (++)) by addition of 50 and 100 μM sodium nitroprusside in the ADP-ribosylation incubation medium. Densitometric scanning of the data shown in Fig. 4 revealed an increase of 135% and 222%, respectively, for 50 and 100 μM sodium nitroprusside present in the incubation medium. Sodium nitroprusside is known to activate soluble forms of guanylyl cyclase. It had apparently no effect on the ROS membrane enzyme and the concentration of sodium nitroprusside necessary to stimulate endogenous ADP-ribosylation of transducin is much lower than that used for activation of ROS solubilized guanylyl cyclase [30]. Nevertheless the observed increase of ADP-ribosylation of transducin by NO is of great interest because No can be produced endogenously by NO synthase present in the rod outer segment [31].

In this report we show that the α and β subunits of transducin in ROS membranes and in ROS-soluble fractions can be mono ADP-ribosylated in vitro by incubation with [α-^{32}P]NAD$^+$ in the absence of bacterial toxins. Moreover, these modifications occur in purified transducin and suggest that an endogenous ADP-ribosyltransferase activity may be copurified with transducin. Such a copurification of substrate and enzymatic activity has been previously reported by Fendrick et al. [32] for the elongation factor 2 (EF2).

Endogenous ADP-ribosylation of G proteins have already been discovered in some cell types, but their physiological consequences have not yet been completely elucidated. The very low rate of modification of transducin could explain the results of our preliminary [α-^{32}P]GTP binding assays. In conditions described in section 2.6, we observed 14.71 ± 1.90 pmol GTP bound for 100 pmol ADP-ribosylated transducin compared to 17.17 ± 0.58 pmol for the control. Because the decreasing of the binding capacity of ADP-ribosylated transducin is very weak, the results cannot be counted as significant although the diminution occurs for each experiment (n = 8). We found that the stimulation of the cGMP-PDE by transducin in hypotonic extract of ROS [27] was the same whether transducin was ADP-ribosylated or not.

The nature of the modified amino acid(s) has to be elucidated to establish if the endogenous ADP-ribosylation of transducin occurs at the same sites as with bacterial toxins [10,12] or at another residue. If it is confirmed that the functions and properties of transducin are modified in situ by an endogenous ADP-ribosylation, this could be an effective pathway for modulating visual transduction as suggested by Falk and Shiells [33]. They observed the reversal by nicotinamide of the block by pertussis toxin of the light response of salamander rods and according to the action of nicotinamide itself they proposed it could be due to the presence of an endogenous ADP-ribosyltransferase. Temporary inactivation of a pool of transducin molecules could as well provide a mechanism for terminating light stimulation. This will be resolved only by further studies including the characterization of the ADP-ribosylating and possibly hydrolyzing activities.

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