Endocrine Journal 1994, **41** (5), 593–597

Rapid Communication

Cholera Toxin can ADP-Ribosylate Gs as well as Gi in ACTH-Unresponsive Human Adrenocortical Cancer

TETSUO NISHIKAWA, AKIKO YOSHIDA*, MASAO OMURA, HIRONOBU SASANO** AND MASATOSHI NODA***

*Department of Medicine, Yokohama Rosai Hospital, Yokohama 222, *Kanamachi Yoshida Clinic, Tokyo 125, **The Department of Pathology, Tohoku University School of Medicine, Sendai 980 and ***Second Department of Microbiology, Chiba University School of Medicine, Chiba 260, Japan*

Abstract. It is well known that cholera toxin (CT) stimulates ADP-ribosylation of Gs and also pertussis toxin (PT) does Gi. Each GTP-binding protein has its own action in the regulation of adenylate cyclase. A human non-functioning adrenocortical cancer tissue showed an unresponsiveness in adenylate cyclase to ACTH although ACTH and CT activated adenylate cyclase in a non-functioning adrenal adenoma tissue. CT ADP-ribosylated 40 kDa protein of the plasma membrane of the cancer tissue while CT and PT could ADP-ribosylate 43 kDa and 38 kDa protein in the adenoma tissue, respectively. Immunoblotting analysis of the cancer tissue demonstrated that 40 kDa protein was detected by anti-Gs antibody as well as by anti-Gi antibody. The present experiments demonstrated that CT could ADP-ribosylate Gs which has stimulatory action on adenylate cyclase and also Gi which inhibits adenylate cyclase. Thus it is suggested that CT can activate the ADP-ribosylation of Gs and also Gi in a human adrenocortical cancer tissue, partly resulting in abnormal regulation of adenylate cyclase which may be crossly related to ACTH-unresponsiveness.

Key words: Adrenocortical cancer, ACTH-unresponsiveness, Cholera toxin, Gs, Gi

(*Endocrine Journal* **41:** 593–597, 1994)

IT HAS BEEN recently reported that the major roles of guanine nucleotide-binding proteins (G-proteins) may transduce various intracellular signals by interacting between receptor molecules and effector enzymes [1]. It is well known that Gi and Gs are involved in adenylate cyclase inhibition and stimulation, respectively. If the interaction of these G-proteins is functionally impaired, this would certainly lead to dysfunction of the activation of the adenylate cyclase system, resulting in hypo- or unresponsiveness to trophic hormones. We had already reported that there seemed to be existed some abnormalities in G-proteins in ACTH-unresponsive adrenocortical carcinoma tissues [2]. Moreover it was reported that various concentrations of GTP and its analogue (GppNHp) could not enhance adenylate cyclase activity in the cell membrane prepared from non-functioning adrenocortical cancer tissue, while could in that obtained from adrenal cancer tissue presenting Cushing's syndrome [2]. Thus it is speculated that there may be existed some abnormality in G-protein in the cell membrane of non-functioning human adrenocortical cancer tissue. The present experiments were, therefore, performed in order to study the effect of cholera toxin (CT) on ADP-ribosylation in the cell membrane prepared from an adrenocortical cancer tissue showing unresponsiveness in adenylate cyclase activity to ACTH stimulation. The aim of those experiments was to

Received: August 18, 1994
Accepted: October 1, 1994
Correspondence to: Dr. Tetsuo NISHIKAWA, Department of Medicine, Yokohama Rosai Hospital, 3211 Kozukue-cho, Kohoku-ku, Yokohama 222, Japan

investigate abnormalities in the cell membrane functions including G-protein in the tissue of ACTH-unresponsive non-functioning adrenocortical cancer.

Materials and Methods

Materials

GTP, NAD, CT A-subunit and pertussis toxin (PT) were purchased from Sigma, and dithiothreitol (DTT) was obtained from Wako Chemical Company, Tokyo. [Adenylate-^{32}P]NAD (800 Ci/mmol) and anti-Gi antibody were purchased from Du-pont New England Nuclear. Anti-Gs antibody was kindly supplied from Dr. Millingan, Scotland. Anti-Gi antibody does not cross-react the protein of Gs at all, and the cross-reactivity of anti-Gs to Gi protein is precisely negligible [3]. CT A-subunit and PT were activated just before using.

Preparation of human adrenal membrane

Forty-five-year-old woman and 50-year-old man were diagnosed as non-functioning adrenocortical cancer and non-functioning adrenocortical adenoma, respectively. They were showing clinically no evidences of Cushing's syndrome, primary aldosteronism and adrenogenital syndrome. The adrenocortical tumors resected from these patients were used as soon as possible. The adrenocortical tissues were prepared and homogenized with 10 mM Tris-HCl buffer, pH 7.4 containing 0.25 M sucrose and 1 mM dithiothreitol as previously reported [2]. The homogenates were centrifuged at 800 × g in 4 °C for 10 min and the resultant supernatant was centrifuged at 20000 × g in 4°C for 30 min in order to prepare crude cell membranes as previously reported [2]. The membrane fraction was used for those experiments as described below.

ADP-ribosylation and immunoblotting

ADP-ribosylation was carried out as described by Ribeiro-Neto *et al.* [4]; the reaction mixture contained 1 μM [^{32}P] NAD (5–10 μCi/assay), 25 mM potassium phosphate buffer, pH 7.5, 3 mM $MgCl_2$, 1 mM thymidine, 3 mM DTT, 1 mM phosphatidylcholine (soy bean lecithin) and 100–200 μg of the crude membrane proteins in a total volume of 200 μl. The reaction was carried out for 30 min at 37 °C and terminated by adding 1.8 ml of 6% trichloroacetic acid (TCA). The precipitate was dried after centrifugation and suspended in the respective solubilization buffer for 10 min at 60 °C before being subjected to SDS-polyacrylamide gel electrophoresis. The gels were stained with 0.05% Coomassie brilliant blue R-250 in 40% methanol and 5% acetic acid. For autoradiography, gels were exposed to Kodak XAR-5 film at −80 °C. Confirmation or denial of the identity of polypeptide which was ADP-ribosylated in response to CT as Gs and Gi was performed by immunoblotting SDS/polyacrylamide gel electrophoresis-resolved membrane with anti-Gs and anti-Gi antibodies. An existence of Gs and Gi was assessed after SDS/polyacrylamide-gel electrophoresis and electroblotting on to nitrocellulose. The second antibody was goat anti-rabbit IgG coupled to horseradish peroxidase and the final reaction was detected by using Vecstatin ABC kit, obtained from VECTOR Laboratories.

Adenylate cyclase

The adenylate cyclase activity was estimated by incubating with the plasma membrane preparation (20–30 μg protein/each incubation) as previously reported [2].

Others

Total protein was assayed by the method of Lowry *et al.* [5] with bovine serum albumin as the standard. Statistical analysis was performed as Student's *t* test and results were expressed as mean ± SEM.

Results

ADP-ribosylation and immunological analysis

Immunoblotting SDS/polyacrylamide gel electrophoresis-resolved membrane of the adrenocortical cancer tissue with anti-Gs and anti-Gi antibodies was shown in Figs. 1 and 2 (lanes 3, 4). Figures 1 and 2 (lanes 1, 2) also demonstrated the results of ADP-ribosylation by using simultaneously the

same nitrocellulose membrane as used for immunoblotting. ADP-ribosylation of 40 kDa protein of the membrane by CT was observed (Figs. 1 and 2, lanes 1, 2). The same molecular masses were detected by immunoblotting of anti-Gs and anti-Gi antibodies (Fig. 1, lanes 3, 4 and Fig. 2, lanes 3, 4). We confirmed the identity of the 40 kDa polypeptide as forms of Gs and Gi by immunoblotting the cell membrane of the adrenocortical cancer tissue with anti-peptide antiserum. Moreover, lanes 3 and 4 of Figs. 1 and 2 demonstrated that there are one or two bands other than 40 kDa protein detected by anti-Gs and anti-Gi antibodies, suggesting that those proteins which are smaller molecular weight than 40 kDa protein seem to be the degradated products of Gs and Gi and/or to be some other protein(s) which is cross-reacted by anti-Gs and anti-Gi antibodies. On the other hand, CT (25 μg/ml of A-subunit) which is well known to only activate ADP-ribosylation of Gs ADP-ribosylated 43 kDa protein and 2 μg/ml PT which is usually believed to only ADP-ribosylate Gi showed ADP-ribosylation of 38 kDa protein in the cell membrane of the adenoma tissue (data not shown).

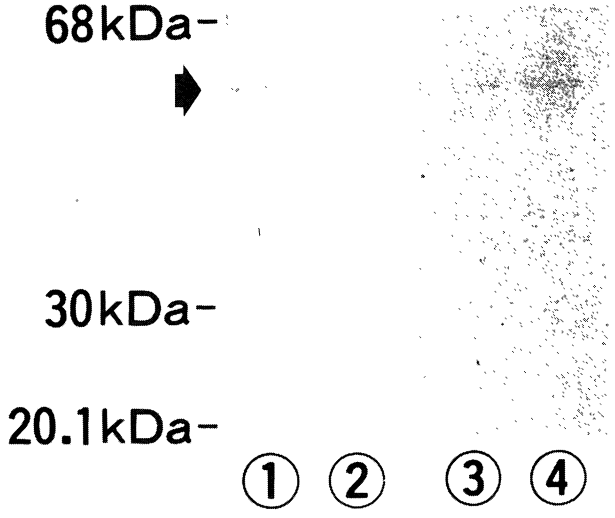


Fig. 1. Effect of CT A-subunit on ADP-ribosylation (lanes 1, 2) and immunoblotting SDS/polyacrylamidegel electrophoresis-resolved plasma membrane of the adrenal cancer tissue with anti-Gs. The plasma membrane preparation and [^{32}P] NAD were incubated with or without 25 μg/ml CT A-subunit as described in Materials and Methods. Immunoblot of the membrane proteins which were incubated as described above was performed as described in Materials and Methods. After being photographed, the same nitrocellulose membrane which was used for immunoblotting was exposed to the film for autoradiogram in order to detect the ADP-ribosylated proteins. Lane 1 and lane 2 show the autoradiograms of the SDS-polyacrylamide gel (5-15% linear gradient) of the labeled proteins when incubated with and without 25 μg/ml CT A-subunit, respectively. Lane 3 and lane 4 indicate the immunoblottings of each preparation which was incubated without and with CT A-subunit, respectively. Arrow indicates the ADP-ribosylated protein (40 kDa) which was also detected by anti-Gs antibody.

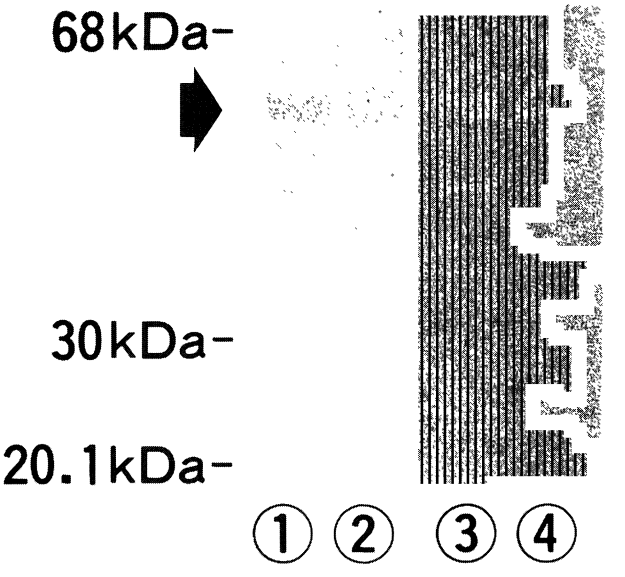


Fig. 2. Effect of CT on ADP-ribosylation (lanes 1, 2) and immunoblotting SDS/polyacrylamidegel electrophoresis-resolved plasma membrane of the adrenal cancer tissue with anti-Gi (lanes 3, 4). The plasma membrane preparation and [^{32}P] NAD were incubated with or without 25 μg/ml CT A-subunit. Immunoblot of the membrane proteins which were incubated as described above was performed as described in Materials and Methods. After being photographed, the same nitrocellulose membrane which was used for immunoblotting was exposed to the film for autoradiogram in order to detect the ADP-ribosylated proteins. Lane 1 and lane 2 show the autoradiograms of the SDS-polyacrylamide gel (5–15% linear gradient) of the labeled proteins when incubated with and without 25 μg/ml CT A-subunit, respectively. Lane 3 and lane 4 indicate the immunoblottings of each preparation which was incubated without and with CT, respectively. Arrow indicates the ADP-ribosylated protein (40 kDa) which was also detected by anti-Gi antibody.

Adenylate cyclase

Table 1 shows the effect of ACTH on the adenylate cyclase activity. ACTH could not enhance its activity in the adrenocortical cancer tissue. CT also did not activate its activity in the cancer tissue. However, ACTH and CT could activate its activity in the adenoma tissue, as shown in Table 1.

Discussion

The present experiments demonstrated that CT could stimulate ADP-ribosylation of 40 kDa protein of the cell membrane prepared from an adrenocortical carcinoma tissue. Thus it is suggested that there exists some protein ADP-ribosylated by CT in the cell membrane of the adrenocortical cancer tissue like as another plasma membranes [1]. It is also possible to consider that the regulation of adenylate cyclase in the adrenocortical cancer seems to be similar to that in the normal adrenals showing good response of adenylate cyclase to ACTH because of the presence of Gs which is a stimulatory G-protein for activation of catalytic unit of adenylate cyclase in the cancer tissue. From the present experiments it is clear that ACTH and CT could activate adenylate cyclase activity in the adenoma tissue (Table 1), which is similar to the response in the normal adrenal glands. Moreover, CT and PT ADP-ribosylated 43 kDa and 38 kDa proteins in the adenoma tissue, respectively. Thus it is suggested that CT may be able to activate only Gs because CT could not activate the ADP-ribosylation of 38 kDa protein which was done by PT. However, ACTH could not enhance the adenylate cyclase activity of the plasma membrane of the non-functioning adrenocortical cancer. We had already reported that there exists decreased activity of G-protein of cell membranes in human ACTH-unresponsive adrenocortical carcinoma although the catalytic unit of the adenylate cyclase seemed to be functionally intact [2]. The present experiments clearly demonstrated that the 40 kDa protein which was ADP-ribosylated by CT was immunologically the same protein as Gs and also Gi, suggesting that CT could ADP-ribosylate both Gs and Gi in the non-functioning adrenocortical cancer tissue. It had been already reported that CT can ADP-ribosylate not only Gs but also Gi in the HL-60 cell membranes [6], and they also demonstrated that CT ADP-ribosylated Gi appeared to be not only a less sensitive pertussis toxin substrate but also an efficient signal transducer between receptors and effectors [6]. Thus it is suggested that CT may ADP-ribosylate simultaneously Gs and Gi, resulting in attenuating the function of both Gs and Gi by increasing GTP-bound form. It is quite interesting that simultaneous activation of Gs and Gi by CT was not able to induce the activation of adenylate cyclase activity in this cancer tissue. The present experiments also demonstrated that ACTH could not enhance the adenylate cyclase activity, although we did no attempt to investigate whether or not ACTH receptor was present in this cancer tissue. Thus it is possible to speculate that cause(s) of unresponsiveness to ACTH seems to be lack of ACTH receptor and/or dysfunction of regulation in Gs and Gi. This is the first report describing that CT can ADP-ribosylate both Gs and Gi in a human cancer tissue.

In conclusion, CT ADP-ribosylated 40 kDa protein in cell membrane of an ACTH-unresponsive non-functioning human adrenocortical tissue. The present experiments also demonstrated that CT ADP-ribosylated both Gs and Gi, which may partly account for unresponsiveness to CT and ACTH.

Table 1. Effect of ACTH and CT on adenylate cyclase activity in the plasma membrane

Additives	adenylate cyclase activity (%control)	
	cancer	adenoma
none	100 ± 2	100 ± 5
ACTH, 1 μg/ml	98 ± 3	528 ± 10
ACTH, 100 μg/ml	101 ± 4	n.d.
CT*, 25 μg/ml	97 ± 3	318 ± 8

The adenylate cyclase activity was determined as described in Methods. The basal activities of adenylate cyclase in the cancer and adenoma tissues are 31.5 ± 2.1 and 25.6 ± 1.5 pmol cAMP/mg protein/10 min, respectively. *CT, A-subunit which was activated just before use. n.d., not determined. The results are expressed as mean ± SEM.

Acknowledgments

We are very grateful to Mrs. Kikue Tanuma for her skilled technical assistance with many of the experiments. Thanks are also due to Dr. Graeme

Millingan of Molecular Pharmacology Group, Department of Biochemistry, University of Glasgow (Scotland) for his gift of antiserum for Gs.

References

1. Taylor CW (1990) The role of G proteins in transmembrane signaling. *Biochem J* 272: 1–13.
2. Yoshida A, Nishikawa T, Tamura Y, Yoshida S (1986) Evidence for decreased activity of guanine nucleotide binding protein in adenylate cyclase of cell membranes in human ACTH-unresposive adrenocortical carcinoma. *Endocrinol Japon* 33: 891–899.
3. Ohisalo JJ, Millingan G (1989) Guanine-nucleotide-binding proteins Gi and Gs in fat-cells from normal, hypothyroid and obese human subjects. *Biochem J* 260: 843–847.
4. Ribeiro-Neto FAP, Mattera R, Hildebrandt JD, Codina J, Field JB, Birnbaumer L, Sakura RD (1985) ADP-ribosylation of membrane components by pertussis and cholera toxin. *Methods Enzymol* 109: 566–572.
5. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275.
6. Iiri T, Ohoka M, Ui M, Katada T (1992) Modification of the function of pertussis toxin substrate GTP-binding protein by cholera toxin-catalyzed ADP-ribosylation. *J Biol Chem* 267: 1020–1026.