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C-TERMINAL MODIFICATIONS OF PERTUSSIS TOXIN-SENSITIVE G-PROTEIN α-SUBUNITS DIFFERENTIALLY AFFECT IMMUNOREACTIVITY

EVIDENCE AGAINST ENDOGENOUS ADP-RIBOSYLATION IN HUMAN HEART, LUNG, THROMBOCYTES AND ADIPOSE TISSUE

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Abstract—Immunochemical detection of pertussis toxin-sensitive guanine-nucleotide binding proteins has been suggested to represent the most direct approach to quantitate the protein than pertussis toxin-catalysed [^{32}P]ADP-ribosylation. The latter technique is potentially hampered by pre-existing covalent modification of the C-terminus. However, limited data exist as to whether and in what way modifications of the C-terminus affect immunoreactivity of Giα (α-subunit of the inhibitory G-protein of adenylyl cyclase). Membranes from human myocardium, thrombocytes, adipose tissue and lung were treated with pertussis toxin or *N*-ethylmaleimide. Both, conditions prevented high affinity agonist binding to *m*-cholinoceptors and inhibited [^{32}P]ADP-ribosylation by pertussis toxin consistent with the notion that the modifications took place at the C-terminus. Pertussis toxin treatment increased immunoreactivity to different antisera raised against the C-terminal decapeptide of transducin α (KENLKDCGLF, DS 1–4, AS). *N*-Ethylmaleimide reduced immunoreactivity towards all antisera studied. Pertussis toxin reduced the mobility of Giα on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) depending on the presence of the toxin and sensitivity to inhibition of ADP-ribosylation by nicotinamide. In native membranes from none of the tissues studied, immunoreactive material comigrating with pertussis toxin-modified form of Giα was detected. It is concluded that modification of the C-terminus by pertussis toxin or *N*-ethylmaleimide resulting in the same functional consequence, i.e. prevention of high affinity agonist receptor binding, is capable of producing opposite changes of immunoreactivity. Pertussis toxin treatment reduces the electrophoretic mobility on SDS–PAGE. Separation of the native and pertussis toxin-modified form of Giα on SDS–PAGE demonstrates that endogenously ADP-ribosylated Giα is lacking in membranes from human myocardium, thrombocytes, lung and adipose tissue.

The α-subunits of one family of heterotrimeric G-proteins (guanine-nucleotide binding proteins§) are subject to ADP-ribosylation by the ADP-ribosyltransferase activity of pertussis toxin S_1-subunit (for review see Ref. 1) at a cysteine residue at the fourth position from the C-terminus [2]. Following separation of membrane proteins on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography, Giα (α-subunit of the inhibitory G-protein of adenylyl cyclase) can be identified and quantified, when [^{32}P]-NAD is used as a substrate in the pertussis toxin-catalysed [^{32}P]ADP-ribosylation reaction [3, 4]. Since covalent modifications of the α-subunit C-terminus and biophysical membrane properties could interfere with the pertussis toxin labeling of Giα, immunodetection was used to quantify the amount of Giα more directly by using antisera raised against native G-proteins [5] or synthetic peptides [6]. Accordingly, we recently reported differences in the quantification of Giα in membranes from various human cells and tissues with the pertussis toxin catalysed [^{32}P]ADP-ribosylation and immunochemical quantification of Giα [7]. However, there are no studies addressing the effect of covalent modifications on the immunoreactivity of Giα or investigating the presence of potentially occurring covalent modifications such as endogenous ADP-ribosylation in native membranes from human cells or tissues. Herein, we report that treatment of membranes with pertussis toxin or alkylation with *N*-ethylmaleimide (NEM), both result in prevention of high affinity agonist binding to *m*-cholinoceptors. Both modifications are accompanied by differential changes in the immunoreactivity of Giα, i.e. an increase of the pertussis toxin-modified form and a decrease of the alkylated form of Giα. The lack of immunoreactive material comigrating with pertussis toxin-modified Giα provides evidence against the idea that ADP

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§ Abbreviations: G-protein, guanine-nucleotide binding protein; Giα, α-subunit of the inhibitory G-protein of adenylyl cyclase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NEM, *N*-ethylmaleimide; QNB, quinuclidinylbenzylate; the amino acid sequences are given in one letter code.

ribosylates Giα in the human cells and tissues studied.

MATERIALS AND METHODS

Materials. The C-terminal decapeptide of transducin α (KENLKDCGLF) was kindly provided by Prof. U. Weber (University of Tübingen, Germany). ATP and GTP were from Boehringer (Mannheim, Germany). Sodium deoxycholate, gelatin type B, Tween 20, Triton X-100 and bovine serum albumin were purchased from the Sigma Chemical Co. (Deisenhofen, Germany). Reagents for SDS–PAGE (acrylamide, *N'N'*-methylenebisacrylamide, *N, N, N', N'* - tetramethylethylenediamine, ammonium persulfate and Coomassie Brilliant Blue R 250) and dithiothreitol were purchased from Serva (Heidelberg, Germany), and molecular mass standards were from Sigma. Pertussis toxin was from List Biological Laboratories (Campbell, CA, U.S.A.). Antisera AS and DS 1–4 were raised against the C-terminal decapeptide of bovine retinal transducin. AS was kindly provided by Dr A. M. Spiegel (National Institute of Health, Bethesda, MD, U.S.A.). The second antibody was affinity-purified goat anti-rabbit IgG (alkaline phosphatase-labeled), from Kirkegaard and Perry (Gaithersburg, MD, U.S.A.). Transfer nitrocellulose membranes were from Schleicher and Schüll (Dassel, Germany). All other compounds used were of analytical grade or the best grade commercially available. Only deionized and double-distilled water was used throughout.

Human membranes. Human thrombocytes were kindly donated by the blood bank of the University of Munich (Klinikum Großhadern, Munich, Germany). Thrombocytes were isolated with a cell separator (Fenval-Baxter, Munich, Germany). Human myocardial tissue was obtained during cardiac transplantation in four patients with severe heart failure due to dilated cardiomyopathy. Tissue pieces were suspended in ice-cold cardioplegic solution (NaCl, 15 mmol/L; KCl, 10 mmol/L; histidine HCl, 180 mmol/L; tryptophan, 2 mmol/L; mannitol, 30 mmol/L; potassium oxoglutarate, 1 mmol/L) and were delivered within 5 min from the operation room to the laboratory, where they were frozen in liquid nitrogen. Human adipose tissue was obtained during abdominoplasty. Approximately 10–20 g (wet weight) of peripheral tumor-free human lung tissue was obtained during thoracic surgery for bronchial carcinoma. Human adipose and human lung tissues were immediately frozen in liquid nitrogen and stored at −80° until membrane preparation was carried out.

Membrane preparations. For the preparation of membranes of human thrombocytes, cells were suspended in Tris–HCl (10 mmol/L) EDTA 1 mmol/L)/dithiothreitol (1 mmol/L), pH 7.4 containing sucrose (250 mmol/L) and disrupted with an Ultra-Turrax (Jahnke and Kunkel, Staufenbreisgau, Germany), and then homogenized with a glass–Teflon homogenizer. This suspension was centrifuged at 4000 *g*, and the pellet was resuspended and washed three time in the above-mentioned buffer. Myocardial, adipose or lung tissue was thawed on ice and chilled in 30 mL of ice-cold homogenization buffer (Tris–HCl, 10 mmol/L; EDTA, 1 mmol/L; dithiothreitol, 1 mmol/L; pH 7.4). Connective tissue was trimmed away and tissues were minced with scissors, and then membranes were prepared using a motordriven glass–Teflon homogenizer for 1 min. Afterwards, the membrane preparations were homogenized by hand for 1 min with a glass–glass homogenizer. The homogenate was spun at 484 *g* (Beckman HA 20 rotor) for 10 min. The supernatant was filtered through four layers of cheesecloth. Myocardial membranes were diluted with an equal volume of ice-cold KCl (1 mmol/L) and stirred on ice for 15 min. This suspension was centrifuged at 100,000 *g* for 30 min. The pellet was resuspended in 50 vol. of buffer (Tris–HCl, 50 mmol/L; $MgCl_2$, 10 mmol/L; pH 7.4) and homogenized for 1 min with a glass–glass homogenizer. This suspension was re-centrifuged at 100,000 *g* for 45 min. The final pellet was resuspended in buffer (50 vol.) and was stored at −80°. Storage did not alter the results.

Immunoblotting. Immunoblotting techniques were performed according to Gierschik *et al.* [5]. The polyclonal antisera DS 1–DS 4 were raised in rabbits against the C-terminal decapeptide of retinal transducin (KENLKDCGLF) coupled to keyhole-limpet haemocyanin, as described by Goldsmith *et al.* [6]. The antiserum AS was raised against the same peptide, but in a different laboratory. After electrophoretic separation, proteins were transferred from the SDS–polyacrylamide gel (10%, 16 cm length) unless otherwise indicated to nitrocellulose (125 mA, 12 hr, Bio-Rad Transblot apparatus). Under these conditions, one immunoreactive Giα band was detected. The sheets were immersed in 100 mL of 3% gelatin in Tris-buffered saline (TBS) buffer (Tris–HCl, 20 mmol/L; NaCl, 500 mMol/L; pH 7.5) and shaken for 1 hr at room temperature. Then they were incubated in the first antibody solution (e.g. DS 4) containing 100 μL of antiserum in 50 mL of 1% gelatine in TBS (24 hr, room temperature, shaker) to block non-specific binding. After two washings with 100 mL of TBS containing 0.05% Tween 20 for 10 min, the paper was incubated with the second antibody solution (5 μL of alkaline-phosphatase-labeled goat anti-rabbit IgG in 60 mL of 1% gelatin in TBS) for 1 hr. After repeated washings with 0.05% Tween in TBS, the sheets were transferred to 33 mg of nitro blue tetrazolium and 15 mg of 5-bromo-4-chloro-3-indolyl phosphate in 100 mL Tris–HCl (0.1 mmol/L) containing NaCl (100 mmol/L) and $MgCl_2$ (5 mmol/L) at pH 8.5. Color development was stopped after 10 min by rinsing with water, and nitrocellulose was dried between two sheets of filter paper. Under the conditions used, this serum was strongly reactive against transducin α, Giα_1 and Giα_2.

[^{32}P]*ADP-ribosylation by pertussis toxin*. [^{32}P]-ADP-ribosylation of Giα by pertussis toxin was performed for 12 hr at 4° in a volume of 50 μL containing Tris–HCl (100 mmol/L) (pH 8.0 at 20°), dithiothreitol (25 mmol/L), ATP (2 mmol/L), GTP (1 mmol/L), [^{32}P]NAD^+ (50 nmol/L; 800 Ci/mmol) and pertussis toxin (20 μg/mL) that had been activated by incubation with dithiothreitol (50 mmol/L) for 1 hr at 20° prior to the labeling

reaction. Samples were subjected to SDS–PAGE [10% (w/v) acrylamide, 16 cm total gel length]. Gels were stained with Coomassie Blue and dried before autoradiography was performed.

Pertussis toxin and NEM treatment of membranes. Pertussis toxin treatment was performed under the same incubation conditions as used for [^{32}P]ADP-ribosylation except that [^{32}P]NAD was replaced by 3 mmol/L NAD in the reaction. NEM treatment was performed according to Shinoda *et al.* [8]. In brief, membranes were prepared in Tris–HCl (100 mmol/L) and EDTA (1 mmol/L) at pH 7.4 at a concentration of 20 mg/mL membrane protein. Membrane suspensions were treated for 10 min with 0.5 mmol/L NEM at 4°. The reaction was stopped by adding dithiothreitol at a final concentration of 1 mmol/L to the reaction mixture. Control membranes were treated identically except that dithiothreitol was added before NEM. After two washings, membranes were subjected to [^{32}P]ADP-ribosylation or immunoblotting.

Miscellaneous. Protein was determined according to Lowry *et al.* [9] using bovine serum albumin as standard. SDS–PAGE was performed as described by Laemmli [10].

RESULTS

Receptor–G-protein coupling and [^{32}P]ADP-ribosylation by pertussis toxin

[^{3}H]QNB bound to *m*-cholinoceptors in human myocardial membranes in a concentration-dependent manner. Binding was saturable and transformation of the binding data according to Scatchard [11] revealed one class binding sites (not shown) with B_{max} values of 201 ± 44 fmol [^{3}H]QNB/mg protein and K_D values of 0.5 (0.39–0.64) nmol/L [12]. Agonist competition experiments were performed in pertussis toxin- (Fig. 1A) and NEM- (Fig. 1B) treated membranes. In control membranes, carbachol competed for [^{3}H]QNB binding in a concentration-dependent manner. Analysis of the binding data revealed that the agonist bound to one high and one low affinity class of binding sites. Gpp(NH)p at 100 μmol/L shifted the concentration–response curve to the right and abolished high affinity binding. Both in pertussis toxin- and NEM-treated membranes binding to only one class of low affinity sites was observed. Pertussis toxin and NEM treatment of myocardial membranes markedly reduced the effects of 100 μmol/L Gpp(NH)p on agonist binding of carbachol to *m*-cholinoceptors. In summary, both pertussis toxin and NEM suppressed high affinity binding of agonists.

In the next experiments, membranes from human heart, thrombocytes, lung and adipose tissue were treated either with pertussis toxin and non-radioactive NAD or with NEM and then subjected to pertussis toxin-catalysed [^{32}P]ADP-ribosylation. The autoradiographs shown in Fig. 2 demonstrate that pertussis toxin-induced [^{32}P]ADP-ribose incorporation into Giα was markedly attenuated by pertussis toxin plus NAD (a) and NEM (b) treatment. Note that the attenuation of [^{32}P]ADP-ribosylation by pertussis toxin and by NEM was less pronounced in lung and adipose tissue compared to myocardium

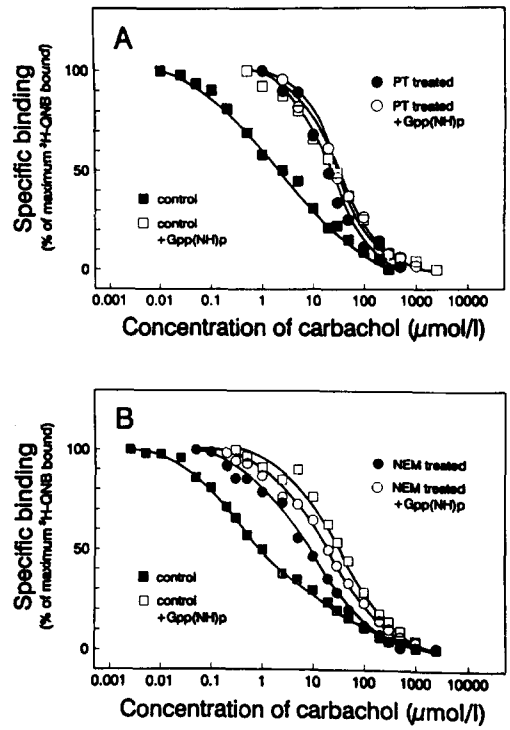

Fig. 1. Competition of carbachol for [^{3}H]QNB binding in human myocardial membranes treated with pertussis toxin plus NAD (3 mmol/L) (A) or NEM (B) and control membranes. Agonist-competition experiments were performed in the absence and presence of 100 μmol/L guanylylimidodiphosphate [Gpp(NH)p]. Each value represents the mean of triplicate observations.

and thrombocytes. Taken together, pertussis toxin and NEM treatment of membranes from human heart, lung and adipose tissue as well as thrombocytes led to a functional uncoupling of *m*-cholinoceptors from G-proteins and to a reduction of pertussis toxin-catalysed [^{32}P]ADP-ribosylation of Giα.

Immunoreactivity and Giα mobility on SDS–PAGE

The results outlined above are consistent with the notion that pertussis toxin and NEM treatment modified the cysteine residue of the C-terminus [2, 13], as shown by the inhibition of pertussis toxin-catalysed [^{32}P]ADP-ribosylation. The functional consequence was a complete inhibition of high affinity agonist binding in both conditions. To examine the covalent modification of the Giα C-terminus by more rigorous criteria, we investigated the immunochemical properties of Giα in membranes following pertussis toxin plus NAD or NEM treatment using antisera raised against the C-terminus of retinal transducin α. Figure 3 shows immunoblots of membranes treated with pertussis toxin plus NAD and of control membranes using the antiserum DS 4. Interestingly, pertussis toxin plus NAD (3 mmol/L) treatment increased the immunoreactivity of Giα in all membranes studied. In addition, pertussis toxin treatment reduced the electrophoretic mobility of

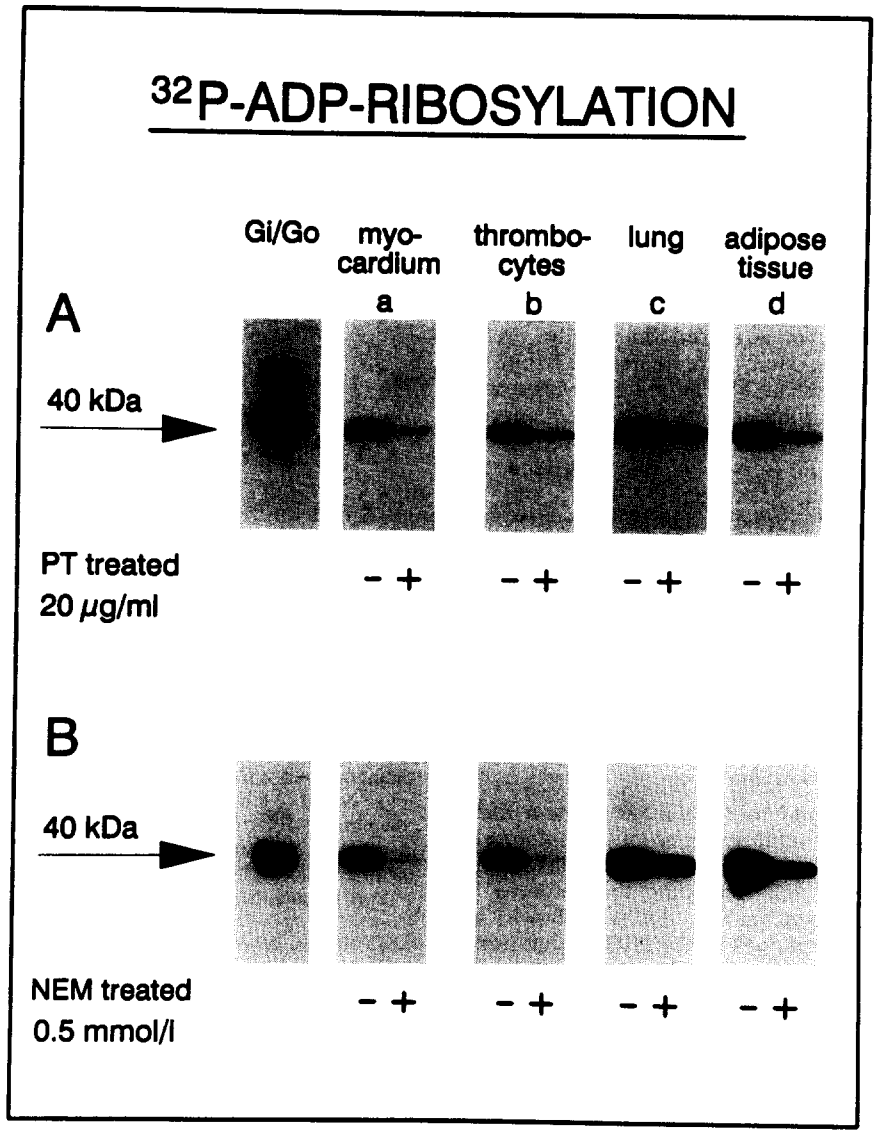

Fig. 2. [^{32}P]ADP-ribosylation of G-protein α-subunits ($\approx$40 kDa) in membranes from human heart (a), thrombocytes (b), lung (c) and adipose tissue (d) following treatment with pertussis toxin plus NAD (3 mmol/L) (A, pertussis toxin) and NEM (B) with the respective control conditions. Membranes were treated with pertussis toxin plus NAD or NEM as described in the Materials and Methods. Samples (25 µg) were [^{32}P]ADP-ribosylated with pertussis toxin and [^{32}P]NAD and were separated by SDS–PAGE before autoradiography. Gi/Go α-subunits isolated from bovine brain [^{32}P]ADP-ribosylated with pertussis toxin and [^{32}P]NAD are shown as standard.

Giα proteins on SDS–PAGE. In some pertussis toxin-treated membranes, a portion of Giα comigrated with the non-ADP-ribosylated form of Giα. This observation provides evidence that, especially in lung and adipose tissue, pertussis toxin treatment did not produce complete ADP-ribosylation of Giα (Fig. 2). Interestingly, there was no Giα in native membranes which comigrated with the pertussis toxin-modified form. In all of the non-treated tissues and cell types, Giα immunoreactivity was confined to a single immunoreactive polypeptide with a higher electrophoretic mobility. These results indicate that the G-proteins present in these membranes are unlikely to be modified by endogenous ADP-ribosylation in native plasma membranes.

Hoshino *et al.* [13] recently demonstrated that treatment with NEM resulted in alkylation of a C-terminal cysteine residue which is the site of pertussis toxin-catalysed ADP-ribosylation on these G-protein α-subunits. To study the question of whether an increase of immunoreactivity and an alteration of protein mobility is a general phenomenon of C-terminal covalent modifications or specific for ADP-ribosylation of the C-terminus, we investigated membranes treated with the sulfhydryl group-alkylating agent NEM. As shown

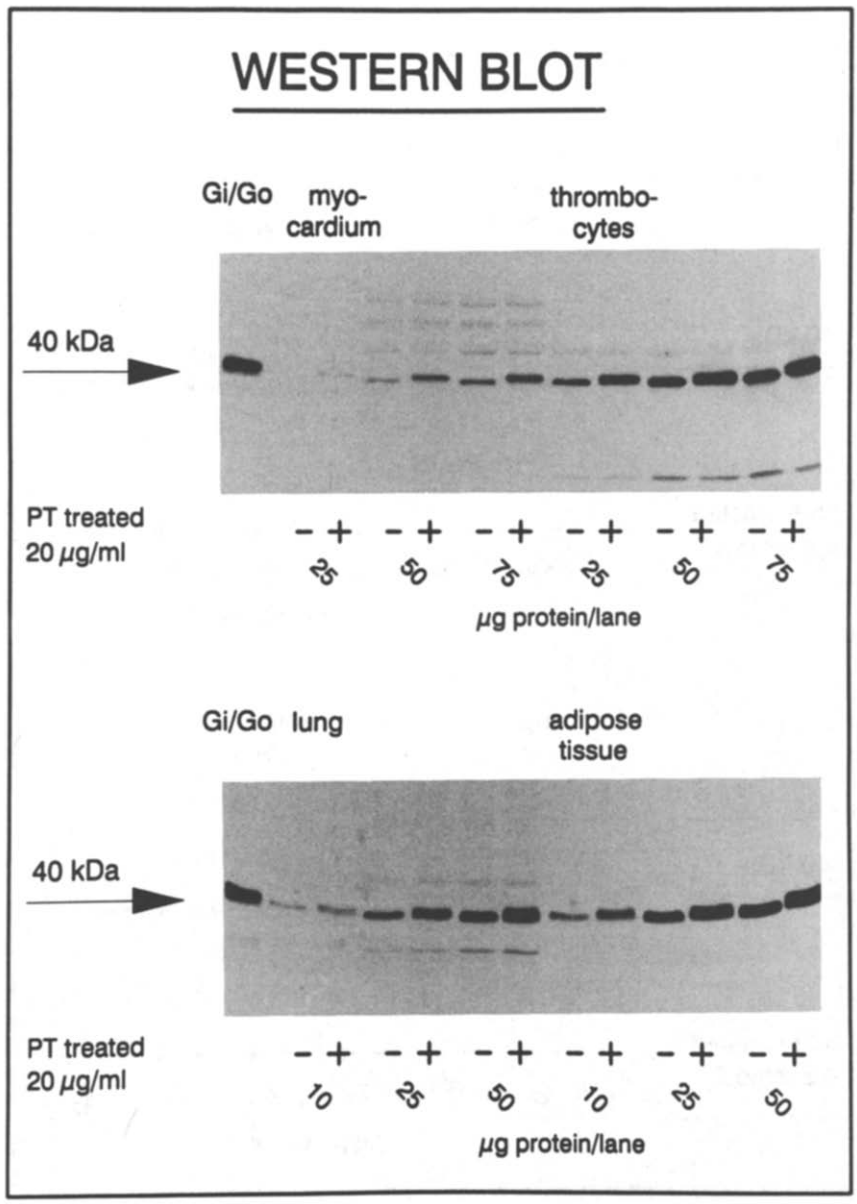

Fig. 3. Immunochemical detection (western blots) of Giα in pertussis toxin- plus NAD- (3 mmol/L) (pertussis toxin) treated membranes from human heart (upper panel, left), thrombocytes (upper panel, right), lung (lower panel, left) and adipose tissue (lower panel, right). Membranes were treated with pertussis toxin plus NAD as described in Materials and Methods. Samples (10–75 µg/lane as indicated) were separated on a 10% SDS–PAGE before electrophoretic transfer to nitrocellulose membranes. Gi/Go α-subunits (1.2 µg) isolated from bovine brain are shown as standard.

in Fig. 4, NEM slightly decreased immunoreactivity of Giα in all tissues studied. There was no effect of NEM treatment on electrophoretic mobility on SDS–PAGE.

In order to study whether the increase by pertussis toxin treatment and the decrease by NEM treatment is a general phenomenon or just a peculiarity of antiserum DS4, four additional antisera raised against the same decapeptide KENLKDCGLF designated DS1, DS2, DS3 and AS were studied for comparison. Figure 5 shows that with all antisera, a consistent increase of immunoreactivity was observed following pertussis toxin treatment. In summary, pertussis toxin treatment enhanced immunoreactivity and reduced electrophoretic mobility of Giα on SDS–PAGE. This appears to be a general phenomenon for antisera raised against synthetic peptides corresponding to the C-terminus.

Next, we set out to determine whether the "heavy" form of Giα observed on immunoblots of membrane proteins treated with pertussis toxin and NAD corresponds to ADP-ribosylated Giα. To this end,

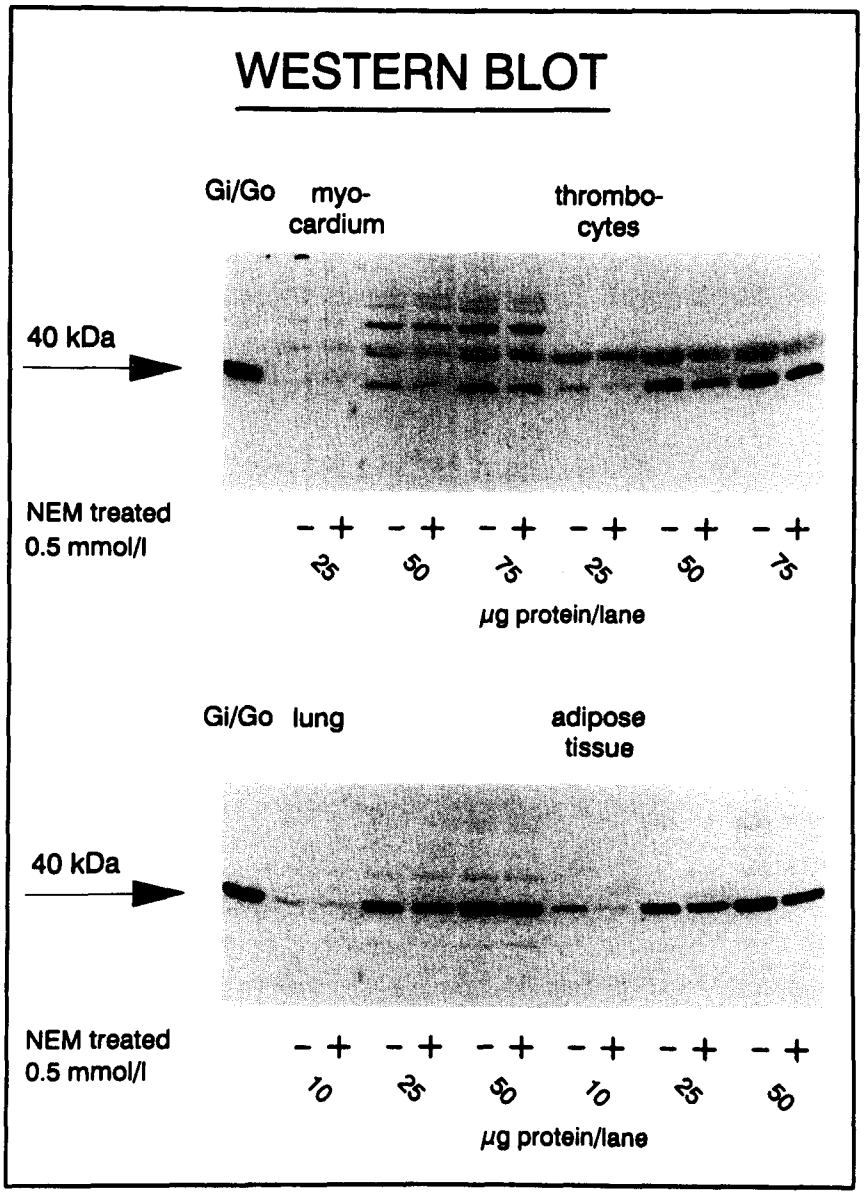

Fig. 4. Immunochemical detection (western blot) of Giα in NEM-treated membranes from human heart (upper panel, left), thrombocytes (upper panel, right), lung (lower panel, left) and adipose tissue (lower panel, right). Membranes were treated with NEM as described in Materials and Methods. Samples (10–75 µg/lane as indicated) were separated on a 10% SDS–PAGE before electrophoretic transfer to nitrocellulose membranes. Gi/Go α-subunits (1.2 µg) isolated from bovine brain are shown as standard.

we examined whether NAD was required for the pertussis toxin-dependent change in the electrophoretical and immunochemical properties of Giα. Much to our surprise, both the increase in immunoreactivity and the decrease in electrophoretic mobility were observed even when the pretreatment was performed in the absence of NAD and addition of NAD did not lead to a further alteration of these two parameters (Fig. 6, left panel). Thus, NAD was either not required for these effects of pertussis toxin or was already present as a contaminant in the myocardial membrane preparation. To discriminate between these two possibilities, we determined the effect of pretreating the membranes with pertussis toxin and increasing concentrations of unlabeled NAD on the ability of Giα to serve as a substrate for [^{32}P]ADP-ribosylation in the second reaction (Fig. 6, right panel). The results show that the [^{32}P]ADP-ribosylation was inhibited concentration dependently by NAD present in the pre-incubation. Half-maximal and maximal ($\geq$95%) inhibition were observed at $\approx$1 mmol/L and 3 mmol/L NAD, respectively. In additional experiments (Fig. 6, right panel, lane 2 vs 3), we found that this inhibition

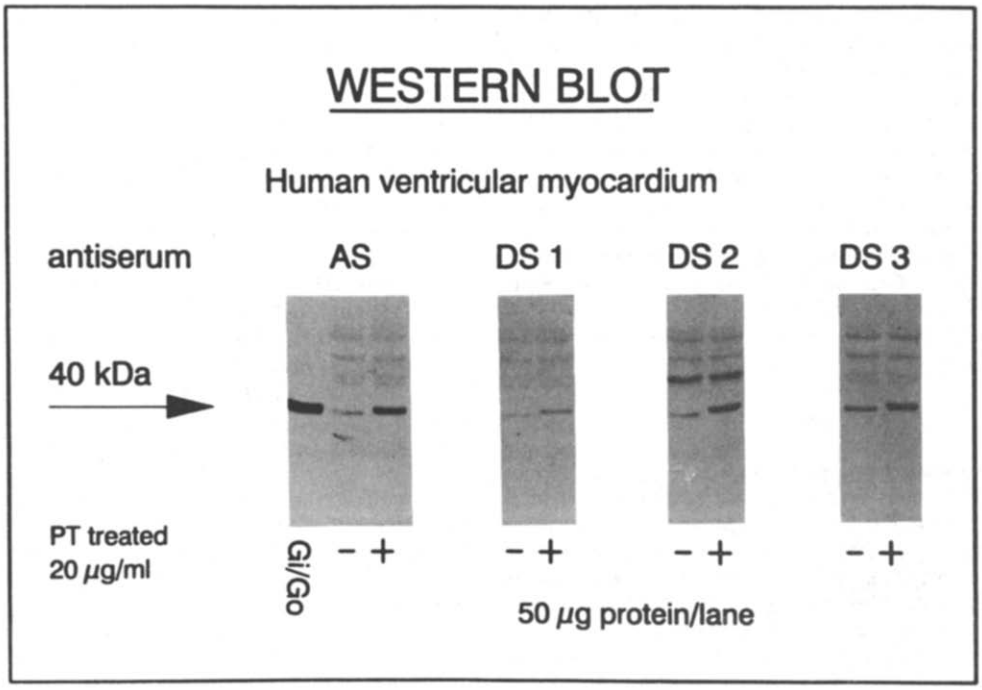

Fig. 5. Immunochemical detection (western blot) of Giα in pertussis toxin- plus NAD- (3 mmol/L) (pertussis toxin) treated human myocardial membranes by antisera raised in different rabbits and at different institutions against the C-terminus of Giα (KENLKDCGLF). Samples (50 μg) were pertussis toxin- plus NAD-treated as described in Materials and Methods and separated on a 8% SDS–PAGE before electrophoretic transfer to nitrocellulose membranes.

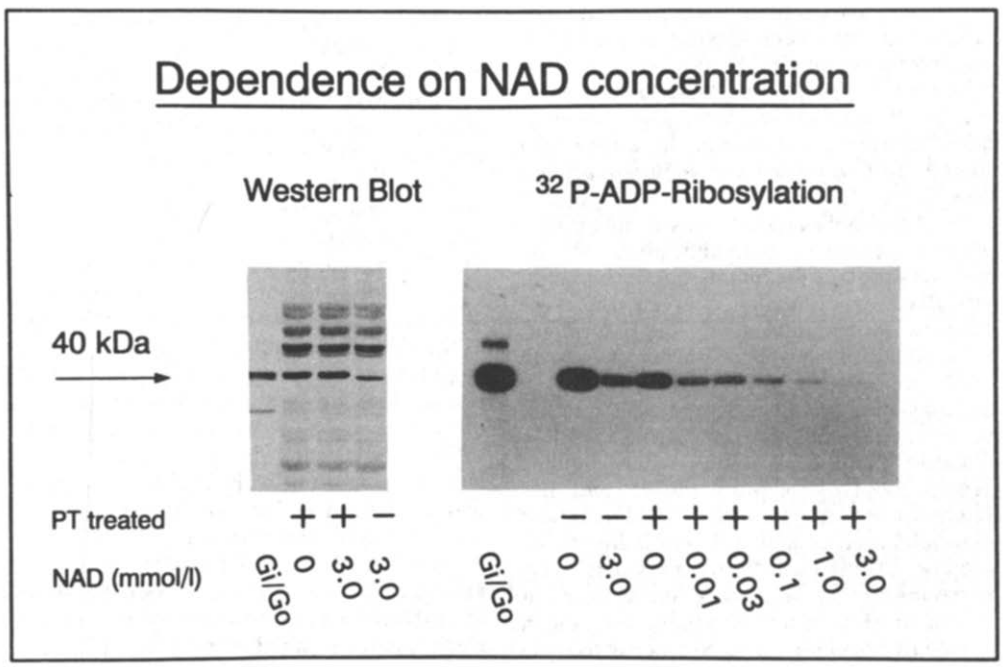

Fig. 6. Western blot (left panel) and [^{32}P]ADP-ribosylation by pertussis toxin and [^{32}P]NAD (right panel) of human myocardial membranes treated with pertussis toxin plus NAD at different concentrations (0–3 mmol/L). Membranes were pertussis toxin- plus NAD- (0–3 mmol/L) treated as described in Materials and Methods. Following washing and treatment with pertussis toxin and [^{32}P]NAD, samples (25 μg) were separated on a 8% SDS–PAGE before autoradiography. Membranes (75 μg) were electrophoretically transferred to nitrocellulose sheets for western blotting. Gi/Go α-subunits (1.2 μg) isolated from bovine brain are shown as standard.

could be due to a carry-over of unlabeled NAD^+ into the [^{32}P]ADP-ribosylation reaction. These experiments also revealed that pretreating the membranes with pertussis toxin in the absence of unlabeled NAD led to only a small decrease in [^{32}P]-ADP-ribosylation in the second reaction (Fig. 6, right panel lane 1 vs 3). Finally, we examined the effect of the ADP-ribosylation product nicotinamide on the ability of pertussis toxin to increase the immunoreactivity and decrease the electrophoretic mobility of Giα in the absence of exogeneous NAD. Nicotinamide (50 mmol/L) almost completely abolished these two effects of pertussis toxin (not shown).

DISCUSSION

Heterotrimeric G-proteins occur ubiquitiously in eukaryotic cells and transduce receptor-generated signals to effectors such as adenylate cyclase, ionic channels of phospholipase C [14]. The quantification of Giα proteins with pertussis toxin labeling is fraught with several technical and biological uncertainties. The accessibility of the C-terminus of Giα for pertussis toxin was observed to limit the [^{32}P]ADP-ribosylation reaction [15]. Improvement of the substrate quality of Giα has been observed to strongly depend on the detergent used [16]. In addition, a variable amount of endogenous ADP-ribosylation depending on the activity of an endogenous ADP-ribosyltransferase [17] and an endogenous ADP-ribosyltransferase inhibition [18] may exert potentially important influences on the quantification of G-protein α-subunits with this technique. To overcome these problems, immunochemical methods have been applied. Recently, we observed differences in the quantification of Giα from various human tissues by pertussis toxin labeling and by a novel radioimmunoassay [7]. However, data on the influence of covalent modification of the C-terminus of the Giα-proteins on immunoreactivity are sparse.

The aim of the present study was to investigate the influence of covalent modifications of the C-termini of G-protein α-subunits on the immunoreactivity. Two experimental interventions were used which can potentially modify the C-terminus of Giα, i.e. ADP-ribosylation with pertussis toxin plus NAD and alkylation by NEM. NEM is a sulfhydryl alkylating agent which is known to abolish receptor-dependent inhibition of adenylate cyclase [19, 20] and to inhibit high affinity agonist binding to G-protein-coupled receptors (20–25 and this study). Both pertussis toxin as well as NEM treatment resulted in inhibition of high affinity binding to *m*-cholinoceptors. Thus, in both instances it is likely that the modification took place at the C-terminus of the α-subunit which is involved in the coupling of receptors to effectors [14]. Since both pertussis toxin and NEM treatment of the membranes resulted in a marked depression of [^{32}P]ADP-ribosylation of Giα with pertussis toxin, this finding suggests that the cysteine of the C-terminus itself is involved or its substrate quality for the ADP-ribosyltransferase of pertussis toxin is altered [2].

In contrast to the finding on agonist binding and pertussis toxin labeling, pertussis toxin and NEM treatment resulted in the opposite effects on immunoreactivity of Giα. NEM reduced antiserum binding, whereas pertussis toxin increased immunostaining of Giα on western blots. This effect was observed with antisera from different animals DS1–DS4, AS). From these experiments, it is likely that alkylation produces steric inhibition of antiserum binding to the C-terminus, while modifications by pertussis toxin, e.g. ADP-ribosylation, induce conformational changes which might facilitate antiserum binding to Giα. Interestingly, Klinker and Seifert [26] observed opposite effects of lipopeptide stimulation on high affinity GTPase activity in HL 60 membranes. Thus, the different modifications could not only produce differences in immunoreactivity but also in functional responses. Experiments with hybrid peptides indicated that the C-terminal amino acids phenylalanine (Giα_1, Giα_2, transducin α) and tyrosine (Goα, Giα_3) play an important role in the antigenicity of G-protein α-subunits [27]. Thus, one could hypothesize that conformational changes of the C-terminus could produce the increase of immunoreactivity following ADP-ribosylation by pertussis toxin. Interestingly, pretreatment of membranes with pertussis toxin without NAD resulted in a similar increase of immunoreactivity and a similar decrease of electrophoretic mobility as treatment with pertussis toxin plus NAD. Thus, pertussis toxin could have produced covalent modifications in the absence of the substrate NAD distinct from ADP-ribosylation of the C-terminus. A second explanation could be the presence of endogenous NAD in the membranes allowing pertussis toxin to ADP-ribosylate Giα in amounts sufficient to increase immunoreactivity on western blots. To test these hypotheses, we studied the immunoreactivity of Giα in the presence of nicotinamide which is able to inhibit pertussis toxin-catalysed ADP-ribosylation [28]. The increase in immunoreactivity and the shift in electrophoretic mobility by pertussis toxin both in the presence and absence of NAD were abolished by nicotinamide. This finding is in accordance with Roerig *et al.* [29] and in favor of the previous notion that the effects of pertussis toxin are due to the ADP-ribosylation of the C-terminus rather than due to other covalent modifications by pertussis toxin. Since [^{32}P]ADP-ribosylation by pertussis toxin was similar in membranes treated without pertussis toxin and without NAD and in membranes treated with pertussis toxin, but without NAD it appears likely that only a minor portion of the total Giα pool present in a given membrane is [^{32}P]ADP-ribosylated by pertussis toxin, at least with the methodology used here. Ribeiro-Neto and Rodbell [30] observed an increase of immunoreactivity and a decrease of electrophoretic mobility of Giα in bovine brain membranes in the absence of ADP-ribosylation substrate NAD. However, in experiments with 50 mmol/L nicotinamide to further inhibit the ADP-ribosylating activity of pertussis toxin possibly activated by endogenous NAD no evidence for other covalent modifications as ADP-ribosylation was detected. These presented experiments are in favor

of the concept that pertussis toxin is able to covalently modify Giα only by its ADP-ribosylating activity.

These findings are principally important for the quantification of Giα-proteins. For example, any increase of Giα measured with this type of antisera (DS4) could be due to the presence of increased levels of endogeneously modified or ADP-ribosylated Giα. Thus, the knowledge of the presence of the ADP-ribosylated or otherwise modified forms of Giα is crucial. One approach was presented by Eide *et al.* [31] who raised an antiserum in rabbits against ADP-ribose coupled to keyhole limpet hemocyanine. This antiserum recognized the ADP-ribosylated but not the native form of Giα. In this study, we compared the electrophoretic mobility of the ADP-ribosylated and the unmodified Giα on SDS–PAGE. It is known that pertussis toxin treatment reduces the electrophoretic mobility of Giα. Experiments without or with low concentrations of NAD [29 and this study] or NADase [29] or nicotinamide [29 and this study] showed that reduced mobility of Giα is due to ADP-ribosylation of Giα. The comparison of the native and the ADP-ribosylated membranes from various human tissues showed that native membranes did not contain a form of Giα comigrating with exogenously ADP-ribosylated Giα. This indicates that in human heart, fat, lung and thrombocyte membranes no endogenously ADP-ribosylated form of Giα occurs. The comparison of immunostained material of native and pertussis toxin-treated membranes represents an important tool to detect endogenously ADP-ribosylated or pertussis toxin-modified Giα, because it can be separated from the native form by SDS–PAGE.

It is concluded that treatment of membranes with pertussis toxin of NEM results in the opposite effects on immunoreactivity of Giα although both experimental conditions antagonized high affinity agonist binding and [^{32}P]ADP-ribosylation with pertussis toxin. These findings could be explained by covalent modifications by both experimental interventions of the C-terminus of the G-protein α-subunit. In pertussis toxin-treated membranes, the immunoreactivity of Giα was increased while the electrophoretic mobility was impaired. Experiments with nicotinamide showed that these changes are due to the ADP-ribosylation of Giα by pertussis toxin. Comigration of immunoreactive material of Giα from native membranes with the slowly migrating pertussis toxin ADP-ribosylated Giα could provide a valuable tool to detect endogenous modifications of G-protein α-subunits. In membranes from human heart, thrombocytes, lung and adipose tissue no endogenously modified (i.e. ADP-ribosylated) Giα was detected.

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