Failure of [^{32}P]ADP-ribosylation by pertussis toxin to determine $G_i\alpha$ content in membranes from various human tissues

Improved radioimmunological quantification using the ^{125}I-labelled *C*-terminal decapeptide of retinal transducin

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The quantitative determination of pertussis-toxin-sensitive guanine-nucleotide-binding proteins (G-proteins) in cell membranes is still a problem. Pertussis-toxin-catalysed [^{32}P]ADP-ribosylation strongly relies on the substrate quality of the α-subunits and is influenced by the concentration of nucleotides, $\beta\gamma$-subunits, the physicochemical properties of the membranes influencing the availability of $G_i\alpha$ for pertussis toxin, and covalent modification of $G_i\alpha$. Quantification of immunoreactive material on Western blots can be only imprecisely performed by two-dimensional densitometry. In order to generate a method for quantification of pertussis-toxin-sensitive G-proteins in membranes we have developed a fast and sensitive radioimmunoassay. The *C*-terminal decapeptide of retinal transducin α (KENLKDCGLF) was ^{125}I-labelled and used as tracer. Polyclonal antiserum (DS 4) was raised against this peptide. $G_i\alpha$ proteins were determined by competition of solubilized membranes for ^{125}I-KENLKDCGLF binding to DS 4 using dilutions of retinal transducin α as standard. The interassay variation was less than 10%, with a sensitivity of 2.5 μg/ml. The density of $G_i\alpha$ was highest in human adipose tissue, followed by HL60 cells, lung, mononuclear leucocytes, thrombocytes and left ventricular myocardium. A striking difference was observed between the density of $G_i\alpha$ and the amount of incorporation of [^{32}P]ADP-ribose into the 40 kDa membrane proteins by pertussis toxin in the same samples. This is also demonstrated by comparison of the weak [^{32}P]ATP-ribosylation of pertussis toxin substrates with the density of immunoreactive $G_i\alpha$ on Western blots in tissues such as lung. This study shows that the $G_i\alpha$ content can be exactly determined by a sensitive and fast radioimmunoassay using iodinated synthetic peptide homologues of $G_i\alpha$ proteins. Radioimmunological quantification of $G_i\alpha$ might be able to detect the 'true' $G_i\alpha$ content of membranes without being hampered by influences on the [^{32}P]ADP-ribosylation reaction. It is concluded that this newly developed method may become an important tool for studying expression of $G_i\alpha$ proteins in a variety of tissues or cell types, and for precisely quantifying the changes caused by pathological conditions.

INTRODUCTION

Heterotrimeric guanine-nucleotide-binding proteins (G-proteins) play a pivotal role in the hormonal regulation of cellular effectors such as adenylate cyclase, phospholipase C and ion channels (Gilman, 1987). G-protein subtypes are classified by the distinct structures of their α-subunits. The α-subunits of one family of G-proteins ($G_i1\alpha$, $G_i2\alpha$, $G_i3\alpha$, $G_0\alpha$ and retinal transducin α) with molecular masses of approx. 39–42 kDa can be covalently modified by a pertussis-toxin-induced ADP-ribosylation reaction (Gilman, 1987; Milligan, 1988; Birnbaumer & Brown, 1990). In the presence of [^{32}P]NAD$^+$, it is possible to tag $G_i\alpha$ and to identify these proteins on SDS/PAGE after electrophoretic separation of the membrane proteins. This technique has frequently been used to quantify and classify $G_i\alpha$ in membranes of various organs (Reisine, 1990). A more direct approach to assessing $G_i\alpha$ is electrophoretic transfer and Western blotting using antibodies raised against synthetic peptides (Pines *et al.*, 1985; Goldsmith *et al.*, 1987) or native G-protein (Gierschik *et al.*, 1985). In general, quantification is performed by densitometric evaluation of autoradiograms or densitometry of Western blots. Both methods suffer from technical and biological uncertainties. The ADP-ribosylation reaction may be dependent on a number of influences, such as the concentration of nucleotides (Ribeiro-Neto *et al.*, 1987*a*), the varying accessibility of the ADP-ribosylation substrates to pertussis toxin (Ribeiro-Neto *et al.*, 1987*a*,*b*) in the membranes, the level of $\beta\gamma$-subunits present in the membrane (Neer *et al.*, 1984; Ribeiro-Neto *et al.*, 1985), the unknown amount of endogenously ADP-ribosylated $G_i\alpha$ (Tanuma *et al.*, 1988), and the uncertain activity of endogenous NADase enzyme activity which metabolizes the substrate of the ADP-ribosylation reaction (Longabaugh *et al.*, 1986). The immunoblotting technique is time-consuming and hampered by a lack of precision when attempts are made to quantify immunoreactivity on Western blots by densitometry. Herein we describe a fast and sensitive radioimmunoassay for the quantification of $G_i\alpha$ in cell membranes. Pertussis toxin substrates and the amount of $G_i\alpha$ were measured in membranes from various human tissues. The data obtained with this method demonstrate a marked difference between the immunodetectable $G_i\alpha$ levels and the intensity of pertussis toxin labelling in membranes from various human cells and tissues.

Abbreviations used: G-protein, guanine-nucleotide-binding protein; $G_i\alpha$, α-subunit of the inhibitory G-protein; $G_s\alpha$, α-subunit of the stimulatory G-protein; $G_0\alpha$, α-subunit of a G-protein of unknown function in brain; PMSF, phenylmethanesulphonyl fluoride.

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EXPERIMENTAL

Materials

Bolton–Hunter reagent for peptide iodination (specific radioactivity 2000 Ci/mmol) was purchased from Amersham–Buchler (Braunschweig, Germany). The *C*-terminal decapeptide of transducin α was kindly provided by Professor U. Weber (University of Tübingen, Germany). ATP and GTP were from Boehringer, (Mannheim, Germany). Immunoprecipitin (staphylococcal Protein A adsorbant) suspension was from Gibco/BRL (Eggenstein, Germany). Sodium deoxycholate, gelatin type B, Tween 20, 4-chloronaphthol, Triton X-100 and bovine serum albumin were purchased from Sigma (Deisenhofen, Germany). Reagents for SDS/PAGE (acrylamide, *NN'*-methylenebisacrylamide, *NNN'N'*-tetramethyletylenediamine, ammonium hydrogen sulphate 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.) or purified as described (Feldman *et al.*, 1988). Antiserum DS 4 was raised against the *C*-terminal decapeptide of bovine retinal transducin. The second antibody was affinity-purified goat anti-rabbit IgG (peroxidase-labelled), from Kirkegaard & Perry (Gaithersburg, MD, U.S.A.). Transfer nitrocellulose membranes were from Schleicher and Schüll (Dassel, Germany). Sep-Pak C_{18} cartridges were obtained from Waters (Königstein, 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 cells and tissues

Human leukaemia (HL60) cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (culture volume < 100 ml) or 10% (v/v) horse serum (culture volume > 100 ml), $NaHCO_3$ (44 mmol/l), glucose (5.5 mmol/l), L-glutamine (5 mmol/l), non-essential amino acids, sodium pyruvate (1 mmol/l), penicillin (50 units/ml) and streptomycin (50 μg/ml) in a humidified atmosphere of 90% air and 10% CO_2 (Collins *et al.*, 1977). The cell density was maintained at approx. 1×10^6 cells/ml. Cells were induced to differentiate into mature myeloid forms by cultivation in the presence of 1.25% (v/v) dimethyl sulphoxide for 5 days.

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 mononuclear leucocytes were isolated from 30 ml of peripheral venous blood anticoagulated with heparin according to Böyum (1968). After dilution (1:1) with phosphate-buffered saline (NaCl, 138 mmol/l; Na_2HPO_4, 8 mmol/l; KH_2PO_4, 1.5 mmol/l; KCl, 1.4 mmol/l), samples of 20 ml were carefully layered on the Ficoll–Metrizoate solution. After centrifugation at 1000 *g* for 30 min, the coat of mononuclear leucocytes was washed four times (each at 400 *g*, 12 min). Human myocardial tissue was obtained during cardiac transplantation in 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. Approx. 10–20 g (wet weight) of peripheral tumour-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 °C until membrane preparation was carried out.

Membrane preparations

Differentiated HL60 cells [$(1–2) \times 10^{10}$ cells in 10–20 litres of medium] were harvested by centrifugation in a Beckman type JA-10 rotor at 1000 *g* for 20 min at 4 °C. The pellets were resuspended in 50 ml of triethanolamine/HCl (10 mmol/l) (pH 7.4 at 20 °C)/NaCl (140 mmol/l). Cells were resedimented by centrifugation in a Beckman type JA-20 rotor at 1500 *g* for 20 min at 4 °C. The final pellet was resuspended in 100–150 ml of lysis buffer containing sucrose (0.25 mol/l), Tris/HCl (20 mmol/l) (pH 7.5 at 20 °C), $MgCl_2$ (1.5 mmol/l), ATP (1 mmol/l) benzamidine (3 mmol/l), leupeptin (1 μmol/l), PMSF (1 mmol/l), and soybean trypsin inhibitor (2 μg/ml). Cells were homogenized by nitrogen cavitation as described by Gierschik *et al.* (1987). The cavitate was centrifuged in a JA-20 rotor at 1500 *g* for 45 s at 4 °C to remove unbroken cells and nuclei and filtered through two layer of cheesecloth. A crude membrane fraction was isolated from the resulting supernatant by centrifugation in a JA-20 rotor at 5000 *g* for 20 min at 4 °C. The membranes were washed three times with a buffer containing Tris/HCl (20 mmol/l) (pH 7.5 at 20 °C), EDTA (1 mmol/l), dithiothreitol (1 mmol/l), benzamidine (3 mmol/l), PMSF (1 mmol/l), leupeptin (10 μmol/l) and soybean trypsin inhibitor (2 μg/ml, resuspended to 10 mg of protein/ml with this buffer and stored at −80 °C. The yield of membrane protein was approx. 100 mg/10^{10} cells.

For preparation of membranes of human thrombocytes and platelets, cells were suspended in Tris/HCl (10 mmol/l)/EDTA (1 mmol/l)/dithiothreitol (1 mmol/l) containing sucrose (0.25 mmol/l) and disrupted with an Ultra-Turrax (Jahnke and Kunkel, Staufenbreisgau, Germany), and then homogenized with a Teflon–glass homogenizer. This suspension was centrifuged at 4000 *g*, and the pellet was resuspended and washed three times 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 motor-driven 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 mol/l) and stirred on ice for 15 min. This suspension was centrifuged at 100000 *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 100000 *g* for 45 min. The final pellet was resuspended in buffer (50 vol.) and was stored at −80 °C. Storage did not alter the results.

For radioimmunological quantification of $G_i\alpha$, the solubilization of membranes was performed in three-detergent buffer consisting of Tris/HCl (100 mmol/l), $MgCl_2$ (5 mmol/l), KCl (100 mmol/l), 1% Triton X-100, 0.5% SDS and 1% sodium deoxycholate at pH 8.15. Membranes were suspended in detergent buffer at a protein concentration of 10 μg/μl and were continuously stirred on ice for 2 h. Afterwards, tubes were centrifuged at 30000 *g* for 30 min. Under these conditions, $G_i\alpha$-proteins were completely solubilized (results not shown).

Immunoblotting

Immunoblotting techniques were performed according to

Gierschik *et al.* (1985). The retinal transducin holoprotein (G_t) and α-subunits of transducin were purified from bovine rod outer segments as described elsewhere (Pines *et al.*, 1985; Gierschik *et al.*, 1985). The polyclonal antiserum DS 4 was raised in rabbits against the *C*-terminal decapeptide of retinal transducin (KENLKDCGLF) coupled to keyhole-limpet haemocyanin, as described by Goldsmith *et al.* (1987). After electrophoretic separation, proteins were transferred from the SDS/polyacrylamide gel (10%, 16 cm length) to nitrocellulose (125 mA, 12 h, Bio-Rad Transblot apparatus). Under these conditions, one immunoreactive $G_i\alpha$ band was detected. The sheets were immersed in 100 ml of 3% gelatin in TBS buffer (Tris/HCl, 20 mmol/l; NaCl, 500 mmol/l; pH 7.5) and shaken for 1 h at room temperature. Then they were incubated in the first antibody solution (DS 4) containing 200 μl of antiserum in 50 ml of 3% gelatin in TBS (24 h, 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-labelled goat anti-rabbit IgG in 60 ml of 1% gelatin in TBS) for 1 h. After repeated washings with 0.05% Tween in TBS, the sheets were transferred to 100 ml of Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in Tris/HCl (0.1 mol/l) containing NaCl (100 mmol/l) and $MgCl_2$ (5 mmol/l) at pH 8.5, in 20 ml of methanol and 60 μl of 30% H_2O_2. Colour 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, $G_i1\alpha$ and $G_i2\alpha$, and weakly reactive against $G_i3\alpha$ and $G_o\alpha$.

[^{32}P]ADP-ribosylation by pertussis toxin

[^{32}P]ADP-ribosylation of $G_i\alpha$ by pertussis toxin was performed for 1 h at 37 °C in a volume of 50 μl containing Tris/HCl (100 mmol/l) (pH 8.0 at 20 °C), 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 h at 20 °C prior to the labelling 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.

Immunoprecipitation of [^{32}P]ADP-ribosylated $G_i\alpha$

Immunoprecipitation of $G_i\alpha$ that had been [^{32}P]ADP-ribosylated with pertussis toxin was performed as described by Colman (1984). In brief, [^{32}P]ADP-ribosylated HL60 membranes (50 μg/sample) were pelleted by centrifugation at 10000 *g* for 10 min at 4 °C in an Eppendorf microcentrifuge, then washed once with and resuspended in buffer containing Tris/HCl (20 mmol/l), pH 7.5, dithiothreitol (1 mmol/l) and EDTA (1 mmol/l) (60 μl/sample). Samples were supplemented with immunoprecipitation buffer (240 μl) containing Tris/HCl (100 mmol/l), pH 7.5, KCl (100 mmol/l), $MgCl_2$ (5 mmol/l), 1% (v/v) Triton X-100, 0.5% (w/v) SDS, 1% (w/v) sodium deoxycholate and PMSF (1 mmol/l), vortex-mixed vigorously and then incubated for 60 min at 4 °C with occasional vortex-mixing. The antiserum was diluted in immunoprecipitation buffer and added to each sample in a volume of 50 μl. In peptide competition experiments, samples were supplemented with 5 μl of undiluted serum and 30 μl of peptide diluted in immunoprecipitation buffer, as specified in the Figure legends. Following an overnight incubation at 4 °C, samples were supplemented with 100 μl of *Staphylococcus aureus* Protein A suspension (Immunoprecipitin), vortex-mixed and incubated for 2 h at 4 °C. The immunoprecipitates were collected by centrifugation and washed with 3 × 500 μl of immunoprecipitation buffer. The final pellet was resuspended in 80 μl of SDS/PAGE sample buffer, boiled for 5 min and subjected to SDS/PAGE and autoradiography.

Iodination of the peptide KENLKDCGLF

The *C*-terminal synthetic peptide was iodinated by conjugation to the ^{125}I-labelled acylating Bolton–Hunter reagent (Bolton & Hunter, 1973). In brief, 5 μg of peptide in 10 μl of borate buffer (0.2 mol/l; pH 8.5) was added to the Bolton–Hunter reagent, which had been evaporated under a gentle stream of nitrogen. The reaction was carried out for 15 min on ice with occasional vortex-mixing. In order to quench the reaction, the sample was supplemented with 0.5 ml of glycine (0.2 mol/l) in borate (0.1 mol/l) buffer, pH 8.5, and incubated for 5 min on ice.

The iodinated peptide was purified by chromatography on a Sep-Pak C_{18} cartridge. In brief, the iodination mixture [1 ml diluted in 0.1% (v/v) trifluoroacetic acid in water] was applied to the cartridge, which had been equilibrated previously with 5 ml of 0.1% (v/v) trifluoroacetic acid in water. Following a wash with 5 ml of 0.1% (v/v) trifluoroacetic acid in water, the peptide was eluted from the matrix by using a step gradient (10 steps, 1 ml each), starting with 0.1% (v/v) trifluoroacetic acid in water and ending with 0.1% (v/v) trifluoroacetic acid in acetonitrile. Three peaks of radioactive materials were obtained. The first peak was eluted immediately at 0.1% trifluoroacetic acid in water [0% (v/v) acetonitrile] and contained about 80% of the radioactivity. As this peak was also observed when the peptide was omitted from the labelling reaction, this material most likely corresponds to Bolton–Hunter reagent conjugated to glycine and/or unreacted hydrolysis product. The two other peaks, each containing about 10% of the radioactivity, were peptide-specific and were centred at about 20% (v/v) and 40% (v/v) acetonitrile respectively. The material present in the third peak bound more specifically to the antiserum DS 4 than did material found in the second peak. The fractions of this peak containing the highest amounts of radioactivity (6.8×10^6 c.p.m./μl) were combined and stored at −80 °C.

Radioimmunoassay

The assay was performed in a final volume of 80 μl in 3.5 ml polypropylene tubes, comprising 20 μl of the radiolabelled peptide diluted to 10000 c.p.m./tube in sodium phosphate buffer (50 mmol/l) containing 0.25% gelatin at pH 7.5, 20 μl of antiserum and 40 μl of solubilized membranes, transducin α in sodium phosphate buffer (50 mmol/l) at pH 7.5, or solubilization buffer instead of membranes. Standard curves were prepared by using 0.25–25 μg of retinal transducin α/ml, prepared as reported previously (Pines *et al.*, 1985; Goldsmith *et al.*, 1987). The use of antiserum DS 4 at a final dilution of 1:100 resulted in binding of about 20% of the total radioactivity. The assay was performed at 4 °C for 18 h. For precipitation of immune complexes, samples were supplemented with 30 μl of staphylococcal Protein A suspension and incubated for 30 min on ice. The tubes were centrifuged at 10000 *g* for 10 min. The sediment was washed twice with sodium phosphate buffer (50 mmol/l) containing 0.25% gelatin at pH 7.5, and the adsorption of radioactivity to the final pellet was determined.

Miscellaneous

Protein was determined according to Lowry *et al.* (1951) using bovine serum albumin as standard. SDS/PAGE was performed as described by Laemmli (1970).

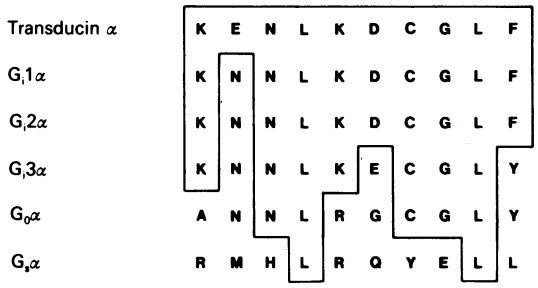

Fig. 1. Comparison of the amino acid sequences of the *C*-terminal decapeptides of pertussis-toxin-sensitive G-protein α-subunits

The *C*-terminus of $G_s\alpha$ is shown for comparison. Residues identical to those of transducin α are enclosed in the framed area.

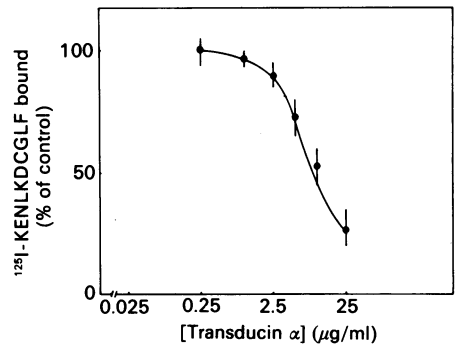

Fig. 2. Standard curve of transducin α competition with ^{125}I-KENLKDCGLF binding

Data points give the means ± S.E.M. of eight separate assays. The experiments were performed as described in the Experimental section.

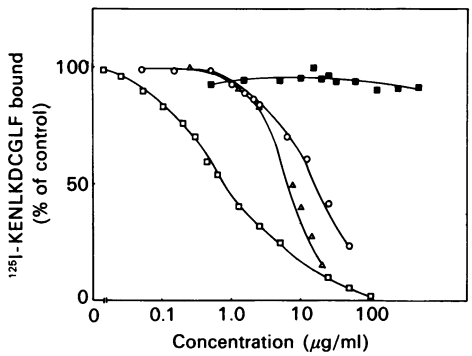

Fig. 3. Competition by KENLKDCGLF (□) retinal transducin α (△), RMHLRQYELL (■, *C*-terminus of $G_s\alpha$) and G_i/G_o from bovine brain (○) for ^{125}I-KENLKDCGLF binding to DS 4 antiserum

Incubation conditions were as described in the Experimental section.

RESULTS

Selectivity and sensitivity of the radioimmunoassay

In order to improve the quantitative determination of $G_i\alpha$, a sensitive radioimmunoassay was developed. The synthetic peptide KENLKDCGLF, corresponding to the *C*-terminus of retinal transducin α (Fig. 1), was iodinated according to Bolton & Hunter (1973). Consistent with the amino acid sequences of the *C*-termini of $G_i\alpha$ subtypes (Fig. 1), the polyclonal antiserum DS 4, which was raised against the *C*-terminus of transducin α, was strongly reactive against transducin α, $G_i1\alpha$ and $G_i2\alpha$, and weakly reactive against $G_i3\alpha$ and $G_o\alpha$ (results not shown). A typical standard curve of eight assays using retinal transducin α is depicted in Fig. 2. The sensitivity of the radioimmunoassay was about 2.5 μg/ml (100 ng/tube). Half-maximal displacement was observed at a mean concentration of 10 μg/ml (0.4 μg/tube). The interassay variation was less than 10%. The unlabelled synthetic peptide displaced ^{125}I-KENLKDCGLF from the antibody as did transducin α or holo G_i/G_o from bovine brain (Fig. 3). The synthetic decapeptide RMHLRQYELL, which corresponds to the *C*-terminus of $G_s\alpha$ and shares only 20% sequence identity with the *C*-termini of transducin α or the $G_i\alpha$ subtypes (Fig. 1), did not displace binding of the tracer to DS 4 (Fig. 3). In order to study whether the synthetic peptide competes with $G_i\alpha$ for binding to antiserum DS 4 after covalent modification by pertussis-toxin-catalysed [^{32}P]ADP-ribosylation, immunoprecipitation experiments were performed. KENLKDCGLF displaced DS 4 binding to the 40 kDa pertussis toxin substrate in a concentration-dependent manner (Fig. 4*a*). At a peptide concentration of 90 μg/ml, binding of [^{32}P]ADP-ribosylated $G_i\alpha$ to DS 4 was completely abolished. This corresponds well with the total displacement of ^{125}I-KENLKDCGLF binding to antiserum DS 4 by 100 μg of synthetic peptide/ml (Fig. 3). Moreover, antiserum DS 4 is shown to bind in a concentration-dependent manner to ADP-ribosylated $G_i\alpha$ of HL60 cells (Fig. 4*b*).

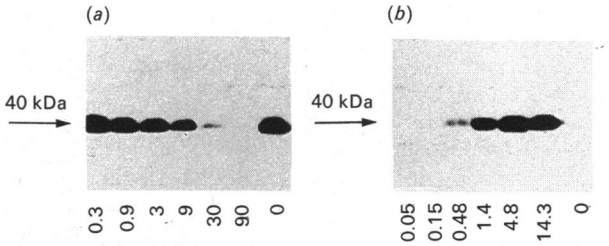

Fig. 4. Inhibition of immunoprecipitation by the synthetic peptide KENLKDCGLF (*a*) and concentration-dependent immunoprecipitation by the antiserum DS 4 (*b*) of pertussis-toxin-[^{32}P]ADP-ribosylated $G_i\alpha$ from membranes of HL60 cells

[^{32}P]ADP-ribosylation was performed as described in the Experimental section. [^{32}P]ADP-ribosylated membranes were incubated overnight with 5 μl of undiluted DS 4 antiserum and 30 μl of peptide, with dilutions giving final concentrations of 0.3–90 μg of peptide/ml or antiserum dilutions giving final concentrations of 0.05–14.3 μg of DS 4 antiserum/ml in a total volume of 275 μl. Concentrations of peptide (*a*) and antibody (*b*) are indicated. Afterwards, membrane proteins were separated on SDS/PAGE (10% gels) before autoradiography.

Dilutions of membranes from various human tissues were capable of completely antagonizing binding of ^{125}I-KENLKDCGLF to the antiserum DS 4. Comparison of the density of $G_i\alpha$ proteins in a variety of organs showed that the density in membranes of human adipose tissue is about 6 times greater than in human myocardium and about 3 times greater than in human peripheral lung. The data are summarized in Table 1.

Pertussis-toxin-catalysed [^{32}P]ADP-ribosylation of $G_i\alpha$ content

[^{32}P]ADP-ribose incorporation into 40 kDa $G_i\alpha$ proteins has previously been used as a measure of the $G_i\alpha$ content of membranes. In order to study whether incorporation of [^{32}P]ribose into 40 kDa pertussis toxin substrates corresponds to the radioimmunological quantification of $G_i\alpha$ proteins, pertussis-toxin-catalysed [^{32}P]ADP-ribosylation of 40 kDa membrane pro-

Table 1. $G_i\alpha$ and pertussis toxin substrates in membranes from various human cells and tissues

$G_i\alpha$ content is given in μg of transducin α equivalents/mg of protein, and pertussis toxin substrates are given in c.p.m. of [^{32}P]ADP-ribose incorporated into a 40 kDa membrane protein/50 μg of protein. Values are means ± S.E.M. for numbers of experiments given in parentheses.

Cells or tissue	$G_i\alpha$ (μg/mg)	Pertussis toxin substrates (c.p.m./50 μg)
HL60 cells (1)	13.6	12145
Thrombocytes (7)	5.2±0.6	4090±377
Mononuclear leucocytes (7)	7.5±0.8	5994±1086
Left ventricular myocardium (5)	4.8±0.3	5229±938
Lung (6)	10.5±0.9	1389±108
Adipose tissue (7)	25.4±1.5	64895±1919

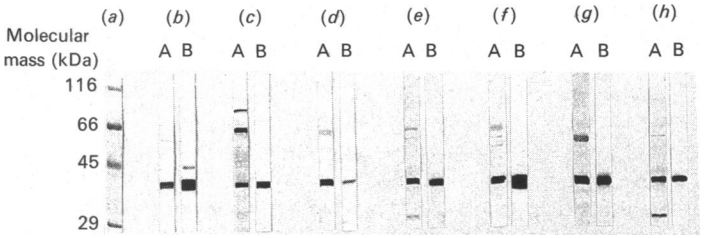

Fig. 5. Immunochemical detection (Western blot, lane A) of $G_i\alpha$ and pertussis-toxin-catalysed incorporation of [^{32}P]ADP-ribose into 40 kDa membrane proteins (lane B) in samples from various human cells and tissues

Membranes were prepared and separated by SDS/PAGE (10% gels) before transfer to nitrocellulose sheets and immunoblotting with the antiserum DS 4 reactive against the α-subunits of G_i and retinal transducin. Each lane contained 150 μg of membrane protein. For pertussis toxin labelling, membranes were incubated for 60 min at 30 °C with activated pertussis toxin and [^{32}P]NAD^+ as substrate and subjected to SDS/PAGE and autoradiography for 18 h as described in the Experimental section. Each lane contained 50 μg of membrane protein. Molecular mass marker proteins are shown for comparison (*a*). Note that the intensity of staining of immunoreactive material at 40 kDa in different cells and tissues differs from the intensity of autoradiographic 40 kDa bands of pertussis-toxin-induced [^{32}P]ADP-ribosylation of the very same samples. (*b*) G_i/G_0 standards, (*c*) heart, (*d*) lung, (*e*) mononuclear leucocytes, (*f*) adipose tissue, (*g*) HL60 cells, (*h*) thrombocytes.

teins was performed in membranes of the same samples. As the ability of pertussis toxin to ADP-ribosylate $G_i\alpha$ was strongly inhibited by the detergent buffer, the [^{32}P]ADP-ribosylation reaction was performed with membranes rather than with detergent extracts. The autoradiograms shown in Fig. 5 demonstrate a marked difference in intensity of labelling between the groups. The labelling was very weak in the lung, but strong in adipose tissue. To investigate whether the different intensities of pertussis-toxin-induced [^{32}P]ADP-ribose labelling are due to a variable content of $G_i\alpha$ in the membranes of the different human cells and tissues, portions of the same samples were subjected to SDS/PAGE and immunoblotting with DS 4, and these are shown for comparison (Fig. 5). These experiments show that the differences in the immunoreactive $G_i\alpha$ contents were much smaller than those of the pertussis toxin substrates. The most pronounced differences between immunoreactive $G_i\alpha$ and pertussis toxin substrates were obtained in human lung and human mononuclear leucocyte membranes, in which pertussis toxin labelling was weak but immunoreactive $G_i\alpha$ content was similar to that in the other tissues and cells studied. These experiments show that pertussis-toxin-induced [^{32}P]ADP-ribosylation does not reflect the amount of immunodetectable $G_i\alpha$.

DISCUSSION

Many neurotransmitters and drugs mediate their effects on cellular effectors by interfering with pertussis-toxin-sensitive heterotrimeric G_i-proteins (Gilman, 1987; Milligan, 1988; Birnbaumer & Brown, 1990). In various conditions, such as pharmacological interventions, pathological conditions or under the influence of hormones or the autonomic nervous system, the response of the target tissues is altered due to changes in the levels of $G_i\alpha$. In adipocytes $G_i\alpha$ is down-regulated on stimulation of the cells with the A_1 adenosine receptor agonist (−)-N^6-phenylisopropyladenosine (Green *et al.*, 1990). In membranes of adipose tissue the amount of $G_i\alpha$ is decreased in obese (*ob/ob*) mice (Begin-Heick, 1990) and in hypothyroid and obese patients (Ohisalo & Milligan, 1989). In diabetes, there is a decline in $G_i\alpha$ expression and in $G_i\alpha$-mediated effects on hepatocyte adenylate cyclase activity (Gawler *et al.*, 1987). On the other hand, there is an increase in the expression of $G_i\alpha$ in rat cardiomyocytes exposed to noradrenaline (Reithmann *et al.*, 1990) and in canine myocardium following parasympathectomy (Hodges *et al.*, 1989). In human dilated cardiomyopathy, a 35–40% increase in $G_i\alpha$ has also been observed (Feldman *et al.*, 1988; Neumann *et al.*, 1988; Böhm *et al.*, 1989). In these studies, $G_i\alpha$ expression was studied using pertussis-toxin-catalysed [^{32}P]ATP-ribosylation or immunoblotting techniques, with subsequent two-dimensional densitometry of 40 kDa bands of autoradiograms or on Western blots. The uncertainties of the quantification of $G_i\alpha$-protein expression by the aforementioned techniques prompted us to develop an accurate, fast and sensitive radioimmunoassay for the determination of $G_i\alpha$ in solubilized membranes.

The antiserum used in our study was raised against the peptide KENLKDCGLF, corresponding to the *C*-terminus of retinal transducin α. Consistent with the identity of this peptide with the *C*-termini of other pertussis toxin substrates (Fig. 1), it was strongly reactive against $G_i1\alpha$ $G_i2\alpha$ and transducin α, but only weakly reactive against $G_i3\alpha$ and G_0. The sensitivity and specifity of a radioimmunoassay is dependent on the affinity and cross-reactivity of the major antibodies of the polyclonal antiserum with the tracer and the major epitopes of the $G_i\alpha$-proteins in the solubilized membranes. The competition by KENLKDCGLF for immunoprecipitation of $G_i\alpha$ from HL60 cell membranes demonstrates that the antibodies of the polyclonal antiserum DS 4 cross-react with KENLKDCGLF and with the immunogenic epitopes of $G_i\alpha$ in HL60 membranes. Thus binding of ^{125}I-KENLKDCGLF was inhibited in a concentration-dependent manner by the unlabelled peptide itself and by detergent extracts of membranes from HL60 cells and from other human cells and tissues. In contrast, the *C*-terminal decapeptide of $G_s\alpha$, RMHLRQYELL, which possesses only two identical amino acid residues to transducin α or $G_i\alpha$ *C*-termini, failed to decrease the binding of the tracer to the antiserum. Immunoprecipitation of pertussis-toxin-catalysed [^{32}P]ADP-ribosylated $G_i\alpha$ was dependent on the DS 4 antiserum concentration. In addition, KENLKDCGLF inhibited immunoprecipitation of the ADP-ribosylated form of $G_i\alpha$ concentration-dependently. These experiments provide the information that DS 4 binds to $G_i\alpha$ even when the *C*-terminus is covalently modified by pertussis-toxin-catalysed ADP-ribosylation. The binding to ADP-ribosylated $G_i\alpha$ can be antagonized by the unchanged KENLKDCGLF *C*-

terminus. The competition curve of G_o/G_i isolated from bovine brain for ^{125}I-KENLKDCGLF binding was flat and revealed two components of binding to DS 4. It is not unreasonable to hypothesize that this reflects the lower affinity of the antiserum DS 4 for $G_o\alpha$ compared with $G_i\alpha$ in the mixture of these G-protein standards.

The limitation of this assay is the lack of specifity of the antiserum to recognize different subtypes of $G_i\alpha$ proteins. DS 4 is more reactive against $G_i1\alpha$, $G_i2\alpha$ and retinal transducin α than against $G_i3\alpha$ and $G_o\alpha$. Therefore the quantitative measurement of $G_i\alpha$ (as judged by transducin α equivalents) might underestimate the $G_i\alpha$ content in tissues expressing a high amount of $G_i3\alpha$ or $G_o\alpha$. Hence the application of the assay at present is to obtain reliable quantification of changes of $G_i\alpha$ expression following pharmacological or hormonal influences in one given tissue. Further studies with more specific antisera will show whether it is possible to quantify $G_i\alpha$ protein subtypes specifically by immunochemical methods.

In all samples of lung tissue the ratio of the 40 kDa pertussis toxin substrate to immunoreactive $G_i\alpha$ was lower than in the other cells and tissues. This indicates that a certain amount of $G_i\alpha$ proteins that can be detected by radioimmunological quantification are not ADP-ribosylated. Hence there is a discrepancy between the apparent amount of $G_i\alpha$, measured by pertussis-toxin-catalysed ADP-ribosylation, and the true amount of the proteins, as detected by radioimmunological methods. This suggestion is supported by the observation of similar amounts of immunoreactive $G_i\alpha$ on Western blots of membranes from human thrombocytes, mononuclear leucocytes, HL60 cells and human lung tissue, despite marked differences in the intensity of [^{32}P]ADP labelling of pertussis toxin substrates. The question arises as to which factors might be involved in the impaired pertussis-toxin-catalysed ADP-ribosylation. Covalent modification of $G_i\alpha$-proteins by the mono-ADP-ribosyltransferase activity of pertussis toxin is dependent on a number of cofactors. ATP enhances ADP-ribosylation of $G_i\alpha$ (Ribeiro-Neto *et al.*, 1987*a*; Codina *et al.*, 1990), most likely by direct activation of pertussis toxin (Lim *et al.*, 1985) via a facilitated dissociation of the toxin subunits (Burns & Manclark, 1986). In contrast, guanine nucleotides influence the substrate quality of $G_i\alpha$ for pertussis-toxin-catalysed ADP-ribosylation (Ribeiro-Neto *et al.*, 1987*a*; Codina *et al.*, 1990). ADP-ribosylation is facilitated most effectively by GTP, GDP and guanosine 5'-[β-thio]diphosphate and least by non-hydrolysable GTP analogues (Ribeiro-Neto *et al.*, 1987*a*). Since ADP-ribosylation is further augmented by β-subunits (Neer *et al.*, 1984; Ribeiro-Neto *et al.*, 1985), it was suggested that the GDP-liganded $\alpha\beta\gamma$-complex is the most susceptible substrate for the pertussis toxin ADP-ribosyltransferase (Tsai *et al.*, 1984; Mattera *et al.*, 1986). Hence the amounts of nucleotides and $\beta\gamma$-subunits, as well as the accessibility of $G_i\alpha$ for these cofactors that influence the activation state of the G-protein and pertussis toxin, could explain the impaired [^{32}P]ADP-ribosylation in some of the human cells and tissues studied. Moreover, covalent endogenous modification of the cysteine residue four residues from the *C*-terminus, which is the acceptor of pertussis-toxin-catalysed ADP-ribosylation (Van Meurs *et al.*, 1987), could impair exogenous [^{32}P]ADP-ribosylation. Recently, ADP-ribose linked to cysteine has been detected as a novel ADP-ribosyl protein linkage in liver plasma membranes (Jacobson *et al.*, 1990). These findings are in accordance with the isolation of an endogenous ADP-ribosyltransferase in erythrocytes which is capable of ADP-ribosylating $G_i\alpha$ *in vitro* (Tanuma *et al.*, 1988). The observation of weak exogenous [^{32}P]ADP-ribosylation of human thrombocytes (the present study) raises the interesting possibility that $G_i\alpha$ is ADP-ribosylated by an endogenous ADP-ribosyltransferase, which had been reported to be cytosolic (Molina y Vedia *et al.*, 1988) and to be activated by nitric oxide (Brüne & Lapetina, 1990) in human thrombocytes. In addition, endogenous ADP-ribosylation has been suggested to be regulated by endogenous activators or inhibitors of ADP-ribosyltransferase activity. Accordingly, the presence of an endogenous inhibitor of pertussis-toxin-catalysed ADP-ribosylation has been demonstrated in membrane extracts of rat liver (Hara-Yokoyama & Furuyama, 1988). If endogenous ADP-ribosylation plays a physiological role by post-translation modification of G_i function, one would suggest that other mechanisms should be able to reverse this covalent modification of $G_i\alpha$. Consistently with this, an ADP-ribosyl protein hydrolase C activity which cleaves mono-ADP-ribosyl linkages from the cysteine residues of $G_i\alpha$ has been observed in cytosolic fractions of human erythrocytes (Tanuma & Endo, 1990). Moreover, there is evidence that proteins which possess a CaaX-motif (where C is cysteine, a is an aliphatic amino acid and X is any amino acid) in their *C*-terminal domain (proteins such as ras and $G_i\alpha$) are post-translationally modified by this ether-linked prenyl group (Glomset *et al.*, 1990). Whether this modification of the ADP-ribose acceptor is occurring in $G_i\alpha$ of the studied cells and tissues is unclear and requires further investigation. Ribeiro-Neto *et al.* (1987*b*) reported that the non-ionic detergent Lubrol-PX augmented pertussis-toxin-catalysed ADP-ribosylation of $G_i\alpha$ in thyroid membranes by a factor of 25–30. Since not only the [^{32}P]ADP-ribosylation of $G_i\alpha$ in membranes but also that of isolated G-proteins was enhanced (Ribeiro-Neto *et al.*, 1987*a*), it was suggested that the physicochemical properties of both the membrane and the G-protein are altered, giving a better accessibility of $G_i\alpha$ to pertussis toxin or other cofactors. The importance of physicochemical factors is substantiated by the findings of Hodges *et al.* (1989), who reported an increase in immunoreactive $G_i\alpha$ on immunoblots after cardiac parasympathectomy in dog myocardial membranes. The increase in immunoreactive $G_i\alpha$ was only detected by pertussis-toxin-catalysed ADP-ribosylation in cholate extracts rather than in native membranes. These data indicate that physicochemical membrane properties might obscure changes in pertussis toxin labelling of $G_i\alpha$. In addition, incubation with thyrotropin of bovine thyroid slices decreased the pertussis-toxin-catalysed [^{32}P]ADP-ribosylation of $G_i\alpha$ in native membranes but not in Lubrol PX-treated membranes, indicating that the accessibility of $G_i\alpha$ for pertussis toxin and other cofactors can be hormonally modified (Ribeiro-Neto *et al.*, 1987*b*).

Taken together, the altered effects of cofactors on pertussis-toxin-catalysed ADP-ribosylation, covalent modification of the ADP-ribose acceptor of the *C*-terminus by endogenous ADP-ribosylation or prenylation, and physicochemical properties of the membrane environment and/or the G-protein itself provide interesting explanations for the difference between apparent $G_i\alpha$, as studied by [^{32}P]ADP-ribosylation with pertussis toxin, and true $G_i\alpha$ content, as investigated by the novel radioimmunochemical technique. From the present experiments it is concluded that radioimmunoassays using ^{125}I-labelled decapeptides, derived from $G_i\alpha$ proteins, as tracers might be a useful tool to study G-protein expression and changes thereof in pathological conditions or following experimental manipulations.

This work was performed with financial support from the Deutsche Forschungsgemeinschaft. We thank Miss Elisabeth Ronft for her expert technical assistance and D. Sidiropoulos for his help in generating antiserum DS 4. We thank Dr. B. Reichart (Department of Cardiac Surgery, Klinikum Grosshadern), Dr. L. Sunder-Plassmann (Department of Surgery, Klinikum Grosshadern), Dr. L. Sunder-Plassmann (Department of Surgery, Klinikum Grosshadern) and Dr. A. Feller (Klinikum Rechts der Isar, Department of Plastic Surgery) for their help in providing human tissues.

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Received 17 December 1990/4 March 1991; accepted 7 March 1991