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Pseudomonas Exotoxin A Prevents β-Adrenoceptor-Induced Up-regulation of G_i Protein α-Subunits and Adenylyl Cyclase Desensitization in Rat Heart Muscle Cells

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SUMMARY

Exposure of rat heart muscle cells to noradrenaline (1 μM) for 48 hr led to a decrease in the number of β_1-adrenoceptors of 50% and a concomitant decrease in adenylyl cyclase stimulation by isoprenaline and forskolin of about 60 and 30%, respectively. In addition, the levels of two inhibitory guanine nucleotide-binding protein (G_i protein) α-subunits ($G_{i\alpha 40}$ and $G_{i\alpha 41}$) were increased in membranes of noradrenaline-treated cells. Evidence is presented that noradrenaline induces this increase by activation of β-adrenoceptors. First, the noradrenaline action was mimicked by the β-adrenoceptor agonist isoprenaline. Second, β-adrenoceptor blockade by timolol but not α-adrenoceptor blockade by prazosin prevented the noradrenaline-induced up-regulation of $G_{i\alpha}$ proteins. Furthermore, timolol but not prazosin abolished the noradrenaline-induced down-regulation of β_1-adrenoceptors and the decreases in receptor-dependent (isoprenaline) and -independent (forskolin) adenylyl cyclase stimulation. The specific protein synthesis inhibitor *Pseudomonas* exotoxin A was used to study whether the noradrenaline-induced up-regulation of G_i α-subunits depends on increased synthesis of these proteins. This toxin inhibits peptide chain elongation by ADP-ribosylating elongation factor 2. Treatment of rat heart muscle cells with *Pseudomonas* exotoxin A (1 ng/ml) completely prevented the noradrenaline-induced increase in $G_{i\alpha}$ proteins, measured by both pertussis toxin-catalyzed ADP-ribosylation and immunoblotting with anti-$G_{i\alpha}$ antibodies. Most importantly, *Pseudomonas* exotoxin A also completely prevented the noradrenaline-induced decrease in forskolin-stimulated adenylyl cyclase activity. Furthermore, the noradrenaline-induced decrease in isoprenaline-stimulated adenylyl cyclase activity was significantly attenuated by the toxin, although the down-regulation of β_1-adrenoceptors caused by noradrenaline treatment was not affected. The data presented suggest that prolonged activation of β-adrenoceptors in rat heart muscle cells, in addition to causing a receptor down-regulation, induces the synthesis of $G_{i\alpha}$ proteins, which then apparently mediate a decreased adenylyl cyclase responsiveness. The data, additionally, suggest that the synthesis of $G_{i\alpha}$ proteins is under control of the activity of the adenylyl cyclase system and that altered levels of these proteins may play a major role in long term regulation of signal transduction by this enzyme.

Long term exposure of cultured rat heart muscle cells to noradrenaline causes a down-regulation of β-adrenoceptors and a decrease in β-adrenoceptor-dependent and -independent cyclic AMP accumulation (1). As recently reported, similar changes are observed when adenylyl cyclase activities are studied in membranes of noradrenaline-treated rat heart muscle cells (2). Most interestingly, the noradrenaline-induced decrease in adenylyl cyclase responsiveness was pertussis toxin sensitive and was accompanied by a marked increase in plasma membrane G_i protein α-subunits. Functional alterations similar to those observed in rat heart muscle cells were reported to occur in membrane preparations of severely failing human hearts. In addition to a down-regulation of β-adrenoceptors, adenylyl cyclase stimulation by β-adrenoceptor agonists and receptor-independent adenylyl cyclase stimulators is reduced in hearts of these patients (3, 4). Furthermore, in hearts from patients with dilated cardiomyopathy, an increased level of $G_{i\alpha}$ proteins was reported (4–6). These similarities suggested that the increase in G_i α-subunits observed in severely failing human hearts may also be due to a prolonged exposure of the hearts to increased noradrenaline concentrations, which are known to be elevated in these patients (7).

Cultured rat heart muscle cells contain both α_1- and β_1-adrenoceptors, with the number of α_1-adrenoceptors exceeding

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ABBREVIATIONS: G_i, inhibitory guanine nucleotide-binding protein associated with adenylyl cyclase; G protein, guanine nucleotide-binding protein; G_s, stimulatory guanine nucleotide-binding protein associated with adenylyl cyclase; G_t, transducin, a G protein found in retinal rod outer segments; G_o a guanine nucleotide-binding protein of unknown function in brain; SDS, sodium dodecyl sulfate; CGP 12177, (4-3-*tert*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid.

that of β_1-adrenoceptors by a factor of 2 to 3 (8). Thus, it was necessary to establish, first, by activation of which type of adrenoceptors noradrenaline induces the increase in $G_{i\alpha}$ proteins and, second, whether the same type of adrenoceptors is also responsible for the decrease in receptor-independent adenylyl cyclase stimulation after noradrenaline treatment. Furthermore, we wanted to know whether the increase in $G_{i\alpha}$ proteins observed in plasma membranes of cells treated with noradrenaline is due to increased synthesis of the proteins or, for example, to a translocation from intracellular compartments into the plasma membrane. Finally, it was of major interest to study whether blockade of the up-regulation of $G_{i\alpha}$ proteins would also prevent the noradrenaline-induced decrease in adenylyl cyclase responsiveness. We report here that both β-adrenoceptor blockade and specific protein synthesis inhibition by *Pseudomonas* exotoxin A prevent the noradrenaline-induced increase in G_i α-subunits and the noradrenaline-induced decrease in receptor-independent adenylyl cyclase stimulation.

Experimental Procedures

Materials. ATP, GTP, cyclic AMP, creatine kinase, and iron-saturated transferrin were from Boehringer Mannheim (Mannheim, FRG). Creatine phosphate, dexamethasone, gelatin (G 0510), bovine insulin, 3-isobutyl-1-methylxanthine, isoprenaline, (−)-timolol, noradrenaline, and molecular weight markers (SDS-6H) were from Sigma (Deisenhofen, FRG). Trypsin, bovine serum albumin, Coomassie brilliant blue R 250, and reagents for SDS-polyacrylamide gel electrophoresis were from Serva (Heidelberg, FRG). Collagenase (125–250 units/mg, CLS II; Worthington), fetal calf serum, horse serum, and CMRL 1415 ATM medium were from Biochrom (Berlin, FRG). Nitrocellulose membranes (BA 85) were from Schleicher and Schüll (Dassel, FRG). Peroxidase-conjugated goat anti-rabbit IgG was from Kirkegaard and Perry (Gaithersburg, MD). Centricon 10 microconcentrators were from Amicon (Witten, FRG). Pertussis toxin was purified as described by Gierschik *et al.* (9). (−)-[^{3}H]CGP 12177 (44 Ci/mmol) and L-[4,5-^{3}H] leucine (146 Ci/mmol) were purchased from Amersham Buchler (Braunschweig, FRG). [α-^{32}P]ATP was obtained from New England Nuclear (Dreieich, FRG). [^{32}P]NAD was prepared from [α-^{32}P]ATP as described by Cassel and Pfeuffer (10). *Pseudomonas* exotoxin A was obtained from Swiss Serum and Vaccine Institute (Bern, Switzerland) and was dissolved in 100 mM Tris·HCl, pH 8.1, for a stock concentration of 100 μg/ml. Forskolin was kindly donated by Dr. H. Metzger, Hoechst AG (Frankfurt, FRG) and was dissolved in absolute ethanol for a stock concentration of 10 mM. Prazosin was a gift from Pfizer (Karlsruhe, FRG). The C-terminal decapeptide of α_t was kindly supplied by Prof. U. Weber (Tübingen, FRG). All other reagents were from commercial sources and of the highest purity available.

Cell culture technique and preparation of cytoplasmic fractions or membranes. Preparation and culturing of rat heart muscle cells have been described in detail previously (11). In brief, the procedures are as follows: preparation of hearts from 30–100 1- to 5-day-old neonatal rats under sterile conditions; disaggregation of heart tissue at 37° with a trypsin (0.12%)/collagenase (0.03%) salt solution (Ca^{2+}- and Mg^{2+}-free); and seeding of the cells (1–2 × 10^5 cells/cm^2) in Nunclon plastic flasks (Nunclon Plastics, Roskilde, Denmark) in CMRL 1415 ATM medium supplemented with 10% fetal calf serum, 10% horse serum, and 0.02 mg/ml gentamycin. Serum-containing medium was replaced after 24 hr of culture by a serum-free culture medium (CMRL 1415 ATM medium containing 2 μM insulin, 0.1 μM dexamethasone, 0.4 μM iron-saturated transferrin, and 0.4 μM bovine serum albumin) supplemented with 1 mM ascorbic acid and 10 mM HEPES, pH 7.4. The cells were cultured for 48 or 60 hr in the presence of the indicated additions, with daily medium changes. Noradrenaline decomposed by less than 20% within 24 h, as tested by high pressure liquid chromatography with fluorimetric detection. After the culture period, cell-bound noradrenaline was removed by washing the cells three times with 5 ml of ice-cold buffer A [20 mM Tris·HCl, pH 8.0, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (0.6 M stock solution in dimethylsulfoxide)]. Thereafter, the cells were removed from the plastic culture flasks by scraping and were homogenized in ice-cold buffer A with a glass homogenizer (Braun, Melsungen, FRG) (10 strokes). The homogenates were centrifuged at 40,000 × g for 30 min at 4°. For preparation of cytoplasmic fractions, the supernatants were centrifuged three times in Centricon 10 microconcentrators at 5000 × g for 1 hr at 4°, then diluted in buffer A, frozen in liquid nitrogen, and stored at −70°. To obtain crude membrane preparations, the pellets were resuspended with a syringe needle in buffer A and diluted to a concentration of 3–6 mg/ml. For preparation of purified membranes, the suspensions of crude membranes were centrifuged through a discontinuous sucrose density gradient, consisting of 25% and 30% sucrose (w/w), at 100,000 × g for 2 hr and the membranes at the buffer/25% sucrose interface were collected (2). Crude and purified membranes were snap frozen in liquid nitrogen and stored at −70°. Protein concentrations were determined as described by Bradford (12), using bovine IgG as a standard.

[^{3}H]CGP 12177 binding assay. Binding of the antagonist [^{3}H] CGP 12177 to the different membrane preparations was performed in a reaction mixture (400 μl) containing 50 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, and 2.5 nM [^{3}H]CGP 12177 (2.5 × 10^4 cpm/tube). The incubation was started by addition of the crude membranes (200–400 μg/tube) and was carried out for 45 min at 37°. Under these conditions, binding equilibrium was obtained. The reaction was terminated by the addition of ice-cold incubation buffer, followed by rapid filtration through Whatman GF/C glass fiber filters and subsequent washing of the filters with 15 ml of incubation buffer. Nonspecific binding was determined in the presence of 10 μM timolol and amounted to 20–30% of total binding at 2.5 nM [^{3}H]CGP 12177. The assays were performed in triplicate and were repeated at least twice, with similar results. Representative experiments are shown with the individual experimental error.

Adenylyl cyclase assay. The adenylyl cyclase activity of crude heart muscle cell membranes was determined in a reaction mixture containing 50 μM [α-^{32}P]ATP (0.2 μCi/tube), 5 mM $MgCl_2$. 0.1 mM EGTA, 1 mM dithiothreitol, 0.1 mM cyclic AMP, 1 mM 3-isobutylmethylxanthine, 5 mM creatine phosphate, 0.4 mg/ml creatine kinase, and the additions indicated, in 50 mM triethanolamine·HCl, pH 7.4, in a total volume of 100 μl. After thermal preequilibration of the reaction mixture, including the adenylyl cyclase stimulators, for 5 min at 30°, the reaction was started by addition of the membranes (100 μg) and was allowed to proceed for 10 min at 30°. Termination of the reaction and isolation of the cyclic AMP formed were carried out as described (13). Adenylyl cyclase activity was measured in triplicate, and each experiment shown herein was repeated at least twice using different membrane preparations, with similar results being obtained. Representative experiments are shown with the individual experimental error.

Pertussis toxin-catalyzed ADP-ribosylation. Pertussis toxin was preactivated in 100 mM Tris·HCl, pH 8.0, with 50 mM dithiothreitol for 1 hr at room temperature. The activated toxin, at a final concentration of 29 μg/ml was then added to a reaction mixture (final volume, 50 μl) containing 30–45 μg of purified membrane protein, 100 mM Tris·HCl, pH 8.0, 25 mM dithiothreitol, 2 mM ATP, and 50 nM [^{32}P]NAD (1 μCi/tube). The reaction mixture was incubated for 1 hr at 37° and the reaction was terminated by the addition of SDS sample buffer (14) and subsequent heating for 5 min at 95°. SDS-polyacrylamide gel electrophoresis and autoradiography were performed as described (15). Where indicated, the resolving gel was supplemented with 4 M deionized urea. Quantitation of [^{32}P]ADP-ribose incorporation was performed by cutting the bands out from the dried gel, as previously described (16). Densitometric determination of the intensity of the radiolabeling was performed using a LKB Bromma 2202 Ultroscan

laser densitometer (München, FRG) with a Hewlett Packard 3390 A integrator. Similar results were obtained with both methods. Staining of the gels with Coomassie blue was carried out as described (15).

***Pseudomonas* exotoxin A-catalyzed ADP-ribosylation.** *Pseudomonas* exotoxin A-mediated radiolabeling of rat heart muscle cell cytoplasmic fractions was performed by activating toxin in 300 mM dithiothreitol for 30 min at room temperature (17). The activated toxin (15 μg/ml) or an equivalent amount of buffer for "no toxin" control was then added to a reaction mixture containing 80 μg of cytoplasmic fractions, 20 mM Tris·HCl, pH 8.1, 1 mM EDTA, and 50 nM [^{32}P]NAD (1 μCi/tube). The reaction mixture was incubated for 1 hr at 37°, and the reaction was terminated by the addition of SDS sample buffer and heating for 5 min at 95°.

Immunoblotting. After SDS-polyacrylamide gel electrophoresis, proteins were transferred from the gels to nitrocellulose membranes, with a constant current (125 mA), for 12 hr in a Bio-Rad transblot apparatus (15). Thereafter, the membranes were incubated in 20 mM Tris·HCl, pH 7.5, 500 mM NaCl, with 3% gelatin to block nonspecific protein binding. The membranes were then incubated for 24 hr at room temperature in the same buffer containing 1% gelatin and the various rabbit antisera. After being washed, the membranes were incubated in 20 mM Tris·HCl, pH 7.5, 500 mM NaCl, with 1% gelatin and a second antibody (1 μg of peroxidase-conjugated goat anti-rabbit IgG/ml), for 2 hr at room temperature. After repeated washings, the papers were stained in 16.6 mM Tris·HCl, pH 7.5, 415 mM NaCl, 16% (v/v) methanol, 0.015% H_2O_2, 0.5 mg/ml 4-chloro-1-naphtol, for 10 min at room temperature. Antiserum DS/4 was generated by immunizing rabbits with the C-terminal decapeptide of α_t coupled to keyhole limpet hemocyanin, as described (18).

Measurement of protein synthesis. To label cell proteins, the rat heart muscle cells were incubated for 4 hr at 37° with [^{3}H]leucine (0.2 μCi/ml). Thereafter, the cells were washed three times with ice-cold Tyrode solution. Protein was precipitated with ice-cold 10% (w/v) trichloroacetic acid. The precipitated material was dissolved in 0.2 M NaOH before scintillation counting.

Statistical calculations. Statistical significances were calculated according to the one-sided Student *t* test for unpaired observations, with $p \leq 0.05$ taken as significant.

Results

Effects of α- and β-adrenoceptor activation on the level of G_i protein α-subunits. We have recently shown that treatment of rat heart muscle cells for 3 days with 1 μM noradrenaline induces an increase in the level of plasma membrane G_i α-subunits and a concomitant decrease in β-adrenoceptor-dependent and -independent adenylyl cyclase stimulation (2). To determine whether the noradrenaline-induced increase in the level of $G_{i\alpha}$ was due to the α- or β-adrenoceptor stimulatory effect of noradrenaline, the influence of α- and β-adrenoceptor blockade on the noradrenaline-induced increase in pertussis toxin-catalyzed ADP-ribosylation was examined (Fig. 1). The noradrenaline (1 μM, 48 hr)-induced increase in pertussis toxin-catalyzed [^{32}P]ADP-ribosylation into 40-kDa proteins was virtually not affected by the α-adrenoceptor antagonist prazosin (1 μM). In contrast, the concomitant presence of the β-adrenoceptor antagonist timolol (1 μM) completely prevented the agonist action. Quantitation of the radioactivity incorporated into the 40-kDa bands revealed an approximately 70% increase in membranes of noradrenaline- and noradrenaline- plus prazosin-treated cells versus membranes of control cells, whereas in membranes of noradrenaline- plus timolol-treated cells no difference from control membranes was observed. Both timolol and prazosin, added without noradrenaline, had no effect by themselves (data not shown).

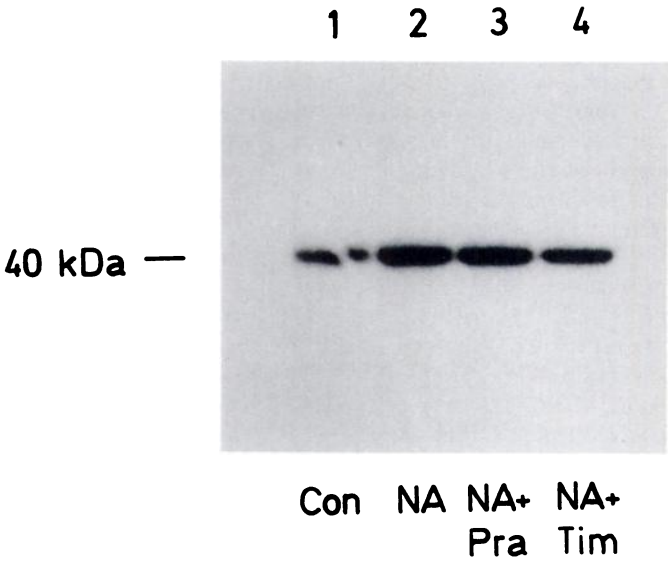

Fig. 1. Influence of α- and β-adrenoceptor activation on pertussis toxin-catalyzed ADP-ribosylation in rat heart muscle cell membranes. Heart muscle cells were incubated for 48 hr without additions (*Con*), with 1 μM noradrenaline (*NA*), with 1 μM noradrenaline plus 1 μM prazosin (*NA+Pra*), or with 1 μM noradrenaline plus 1 μM timolol (*NA+Tim*). The membranes were purified and subjected to pertussis toxin-catalyzed [^{32}P]ADP-ribosylation and SDS-polyacrylamide gel electrophoresis, as described in Experimental Procedures. Each lane contained 45 μg of membrane protein. An autoradiogram of the dried gel is shown. The position of the pertussis toxin substrates (40 kDa) is indicated on the *left*.

As recently reported, the noradrenaline-induced increase in pertussis toxin-catalyzed radiolabeling in membranes of rat heart muscle cells involves two distinct $G_{i\alpha}$ proteins, a major 40-kDa and a minor 41-kDa protein (2). Therefore, it was studied whether treatment of the cells with the β-adrenoceptor agonist isoprenaline (1 μM) for 48 hr would also induce an increase in the level of both $G_{i\alpha}$ proteins (Fig. 2). Quantitation of the labeled 40-kDa bands revealed that, similar to noradrenaline, exposure of the cells to isoprenaline leads to an increase in pertussis toxin-catalyzed [^{32}P]ADP-ribosylation by about 120%. When SDS-polyacrylamide gel electrophoresis of the pertussis toxin-ADP-ribosylated membranes was performed in the presence of 4 M urea, an increase in the labeling of both the 40-kDa and the 41-kDa pertussis toxin substrate was observed in membranes of isoprenaline-treated rat heart muscle cells. These data, thus, indicate that the noradrenaline-induced increase in $G_{i\alpha}$ proteins in rat heart muscle cell membranes is β-adrenoceptor mediated.

Effects of α- and β-adrenoceptor activation on desensitization of adenylyl cyclase stimulation. In the absence of noradrenaline, in the culture medium, neither α-adrenoceptor blockade by 1 μM prazosin nor β-adrenoceptor blockade by 1 μM timolol had an effect on subsequently measured adenylyl cyclase stimulation by 100 μM isoprenaline or 100 μM forskolin. Furthermore, [^{3}H]CGP 12177 binding measured in membranes of rat heart muscle cells treated with only prazosin or timolol was not altered (data not shown). In contrast, exposure of rat heart muscle cells to noradrenaline (1 μM) for 48 hr led to a decrease in isoprenaline (100 μM)-stimulated adenylyl cyclase activity in rat heart muscle cell membranes by 59 ± 5% (mean ± SD, three experiments). In addition, receptor-independent adenylyl cyclase stimulation by forskolin (100 μM) was diminished in membranes of noradrenaline-treated cells by 26 ± 1%.

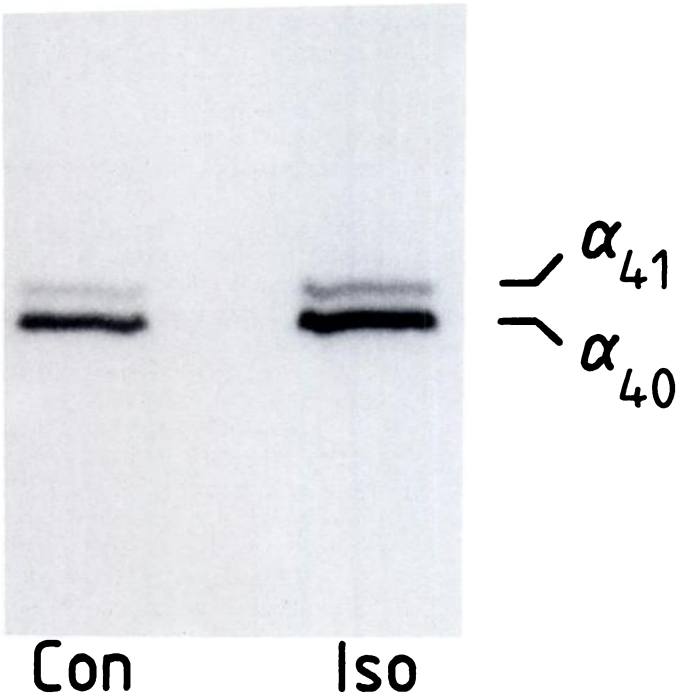

Fig. 2. Effect of isoprenaline on pertussis toxin-catalyzed ADP-ribosylation of two G proteins in rat heart muscle cells. After incubation of rat heart muscle cells in the absence (*Con*) or presence of 1 μM isoprenaline (*Iso*) for 48 h, purified membranes were prepared. The membranes (30 μg each) were subjected to pertussis toxin-catalyzed [^{32}P]ADP-ribosylation. For SDS-polyacrylamide gel electrophoresis, the resolving gel was supplemented with 4 M urea. An autoradiogram of the dried gel is shown. The positions of the two pertussis toxin substrates (α_{40}, α_{41}) are indicated on the *right*.

Specific binding of the β-adrenoceptor antagonist (−)-[^{3}H]CGP 12177 to rat heart cell membranes was reduced in membranes of noradrenaline-treated cells by about 50% (Table 1). This decrease in [^{3}H]CGP 12177 binding was due to a decrease in binding capacity without a change in binding affinity (1, 19). β-Adrenoceptor blockade by 1 μM timolol completely abolished the noradrenaline-induced decrease in [^{3}H]CGP 12177 binding. Most important, the concomitant presence of timolol also abolished the noradrenaline-induced decrease in isoprenaline (100 μM)- and forskolin (100 μM)-stimulated adenylyl cyclase activities. In contrast, α-adrenoceptor blockade by 1 μM prazosin did not prevent either the noradrenaline-mediated decrease in [^{3}H]CGP 12177-binding or the decrease in isoprenaline- and forskolin-stimulated adenylyl cyclase activities. These data indicate that the decrease in [^{3}H]CGP 12177 binding and the decrease in β-adrenoceptor-dependent and -independent adenylyl cyclase stimulation observed in membranes of noradrenaline-treated cells were due to the β-adrenoceptor stimulatory effect of the catecholamine.

***Pseudomonas* exotoxin A-catalyzed ADP-ribosylation of elongation factor 2.** *Pseudomonas* exotoxin A inhibits protein synthesis by ADP-ribosylating elongation factor 2 and, thus, preventing peptide chain elongation (20). In cytoplasmic preparations of rat heart muscle cells, *Pseudomonas* exotoxin A catalyzed the ADP-ribosylation of an approximately 100-kDa protein, presumably elongation factor 2 (Fig. 3). No other protein was specifically labeled in the presence of *Pseudomonas* exotoxin A either in cytoplasmic fractions or in membranes of rat heart muscle cells. Incubation of intact rat heart muscle cells for 60 hr with 1 ng/ml *Pseudomonas* exotoxin A led to a marked (≈ 70%) decrease in subsequent [^{32}P]ADP-ribosylation of rat heart cell cytosol using *Pseudomonas* exotoxin A and [^{32}P]NAD (Fig. 3). [^{3}H]Leucine incorporation into cellular proteins was decreased by approximately 30% by this *Pseudomonas* exotoxin A (1 ng/ml) treatment (data not shown). Total cellular protein content and cell viability, measured as spontaneous contractions, were not altered. Furthermore, the Coomassie blue-stained gels from membranes of rat heart muscle cells treated without or with 1 ng/ml *Pseudomonas* exotoxin A for 60 hr exhibited virtually no difference in the distribution and density of protein bands. However, when cells were exposed to higher *Pseudomonas* exotoxin A concentrations (10 ng/ml), the subsequently performed *Pseudomonas* exotoxin A-catalyzed [^{32}P]ADP-ribosylation was almost prevented. [^{3}H]Leucine incorporation into cellular proteins was decreased by more than 50%, and marked alterations in protein patterns in Coomassie blue-stained gels from these membranes were observed (data not shown).

Effect of *Pseudomonas* exotoxin A on the level of $G_{i\alpha}$ proteins. To determine the effects of protein synthesis inhibition by *Pseudomonas* exotoxin A on the level of $G_{i\alpha}$ proteins in rat heart muscle cells, the cells were first preincubated for 12 hr in the absence or presence of *Pseudomonas* exotoxin A (1 ng/ml). Thereafter, both cell types were treated without or with noradrenaline (1 μM) for an additional 48-hr period, with *Pseudomonas* exotoxin A (1 ng/ml) still being present. The influence of *Pseudomonas* exotoxin A treatment on the noradrenaline-induced increase in G_i α-subunits was first studied by measuring pertussis toxin-catalyzed ADP-ribosylation in purified membranes. In the experiment shown in Fig. 4, expo-

TABLE 1

Influence of α- and β-adrenoceptor activation on adenylyl cyclase activity and [^{3}H]CGP 12177 binding in rat heart muscle cell membranes

Rat heart muscle cells were incubated for 48 hr in the absence or presence of 1 μM noradrenaline, 1 μM noradrenaline plus 1 μM timolol, or 1 μM noradrenaline plus 1 μM prazosin, and crude membranes were prepared. Adenylyl cyclase activities were determined in a reaction mixture containing 100 μM GTP in the presence of either 100 μM isoprenaline or 100 μM forskolin. Specific [^{3}H]CGP 12177 binding was determined as described in Experimental Procedures. The values are given as mean of triplicates ± standard deviation.

	Control	Noradrenaline	Noradrenaline + timolol	Noradrenaline + prazosin
Adenylyl cyclase activity (pmol of cAMP × mg of protein^{-1} × 10 min^{-1})				
Isoprenaline	110.2 ± 3.3	50.3 ± 6.9	108.5 ± 4.1*	51.0 ± 4.3
Forskolin	459.5 ± 35.7	331.2 ± 39.7	480.3 ± 53.0*	323.3 ± 13.7
[^{3}H]CGP 12177 bound (fmol × mg of protein^{-1})	22.4 ± 0.8	11.5 ± 1.8	23.2 ± 1.1*	10.8 ± 0.2

* The values are significantly higher than the corresponding values of the noradrenaline-treated group, $p \leq 0.05$.

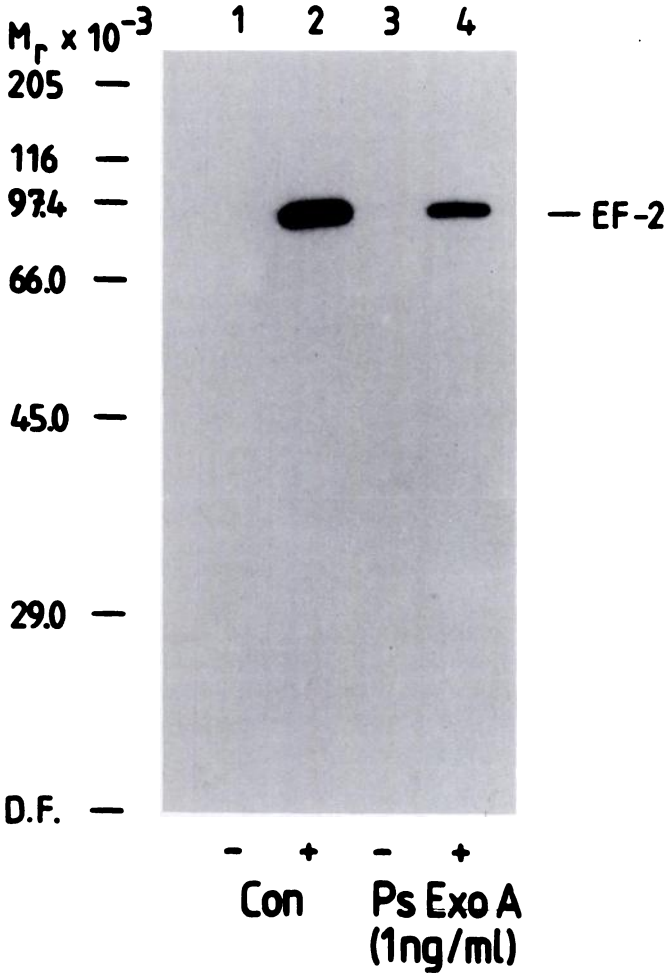

Fig. 3. Influence of *Pseudomonas* exotoxin A pretreatment on *Pseudomonas* exotoxin A-catalyzed ADP-ribosylation in rat heart cell cytoplasmic fractions. After incubation of rat heart muscle cells for 60 hr in the absence (*Con*) or presence of 1 ng/ml *Pseudomonas* exotoxin A (*PsExoA*), cytoplasmic fractions were prepared. *Pseudomonas* exotoxin A-catalyzed [^{32}P]ADP-ribosylation and SDS-polyacrylamide gel electrophoresis were carried out as described in Experimental Procedures. − and +, The absence and presence of *Pseudomonas* exotoxin A, respectively. Each lane contained 80 μg of cytoplasmic proteins. The positions of molecular weight marker proteins and of elongation factor II (*EF-2*) are indicated. *D.F.*, dye front.

sure of rat heart muscle cells to noradrenaline (1 μM) led to an increase in pertussis toxin-catalyzed ADP-ribosylation of 200%. In the absence of noradrenaline, *Pseudomonas* exotoxin A had a small inhibitory effect (maximally 30%) on radiolabeling by pertussis toxin. However, the protein synthesis-inhibitory toxin completely abolished the noradrenaline-induced increase in pertussis toxin-catalyzed ADP-ribosylation.

To confirm the evidence that the effect of *Pseudomonas* exotoxin A on pertussis toxin-catalyzed radiolabeling was due to alterations in the level of G_i protein α-subunits, immunoblotting of purified rat heart cell membranes with the rabbit antiserum DS/4 was performed (Fig. 5). This antiserum is reactive against the α-subunits of G_t, G_{i1}, G_{i2}, and G_{i3}, whereas the serum is only marginally reactive against the α-subunit of G_o. Because no 39-kDa protein was labeled by pertussis toxin in purified rat heart cell membranes, immunoblotting of these membranes with this antiserum is a quantitative assay for $G_{i\alpha}$ proteins (2). As reported before, the intensity of the $G_{i\alpha}$ band was clearly increased in membranes of noradrenaline-treated cells. This increase in immunoreactivity caused by noradrenaline exposure was completely abolished when the cells were additionally treated with *Pseudomonas* exotoxin A.

Because the noradrenaline-induced increase in pertussis toxin-catalyzed radiolabeling involves two distinct pertussis toxin substrates ($G_{i\alpha40}$, $G_{i\alpha41}$), the effect of *Pseudomonas* exotoxin A treatment on the increase of these two G_i protein α-subunits was determined. As shown in Fig. 6, exposure of the cells to 1 ng/ml *Pseudomonas* exotoxin A completely abolished the noradrenaline-induced increase in the level of $G_{i\alpha40}$ as well as of $G_{i\alpha41}$.

Effect of *Pseudomonas* exotoxin A on desensitization of adenylyl cyclase stimulation. Because *Pseudomonas* exotoxin A abolished the noradrenaline-induced increase in G_i α-subunits, it was of major interest to determine whether this treatment had also functional consequences with regard to noradrenaline-induced desensitization (Table 2). In the absence of noradrenaline, *Pseudomonas* exotoxin A treatment had virtually no effect on adenylyl cyclase stimulation by isoprenaline (100 μM) or forskolin (100 μM) in rat heart cell membranes. Furthermore, the binding of [^{3}H]CGP 12177 to β-adrenoceptors was also not altered by the toxin. However, *Pseudomonas* exotoxin A completely abolished the noradrenaline-induced decrease in adenylyl cyclase stimulation by forskolin (100 μM). Furthermore, the noradrenaline-mediated decrease in isoprenaline-stimulated adenylyl cyclase activity, although still present, was also significantly reduced by *Pseudomonas* exotoxin A treatment, from about 60 to 35%. On the other hand, the noradrenaline-induced decrease in (−)-[^{3}H]CGP 12177 binding was not affected by the toxin.

Discussion

We have recently reported that, in addition to a down-regulation of β_1-adrenoceptors, long term exposure of cultured rat heart muscle cells to noradrenaline leads to a decrease in β-adrenoceptor-mediated and receptor-independent adenylyl cyclase stimulation and to a concomitant increase in the level of two distinct G_i protein α-subunits ($G_{i\alpha40}$ and $G_{i\alpha41}$) (2). We now show that the noradrenaline-induced decrease in β_1-adrenoceptors and the decrease in isoprenaline- and forskolin-stimulated adenylyl cyclase activities were abolished by the β-adrenoceptor antagonist timolol but not by the α-adrenoceptor antagonist prazosin. Furthermore, β-adrenoceptor but not α-adrenoceptor blockade prevented the noradrenaline-induced increase in G_i α-subunits. Finally, an apparent increase in the level of the two distinct pertussis toxin substrates was also induced by treatment of the cells with the β-adrenoceptor agonist isoprenaline. These results indicate that prolonged agonist occupancy of β-adrenoceptors not only induced the down-regulation of β_1-adrenoceptors and the decrease in β-adrenoceptor-stimulated adenylyl cyclase activity but was also responsible for the decrease in forskolin-stimulated adenylyl cyclase activity and the concomitant increase in G_i α-subunits. In contrast, noradrenaline stimulation of α-adrenoceptors, which are present in rat heart muscle cells at an even higher concentration than β-adrenoceptors (8), had apparently no effect on the parameters studied, either on the number of β_1-adrenoceptors, on the receptor-dependent and -independent adenylyl cyclase stimulation, nor on the level of G_i α-subunits.

The specific protein synthesis inhibitor *Pseudomonas* exo-

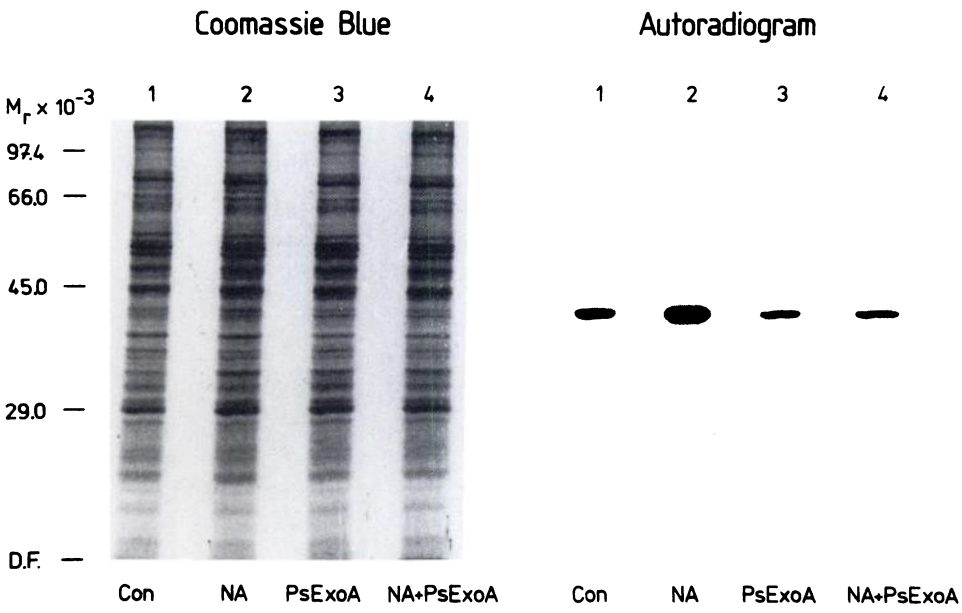

Fig. 4. Effect of *Pseudomonas* exotoxin A on the noradrenaline-induced increase in pertussis toxin-catalyzed ADP-ribosylation in rat heart muscle cell membranes. Heart muscle cells were pretreated in control medium for 12 hr and then incubated in control medium without (*Con*) or with 1 μM noradrenaline (*NA*) for 48 hr or were pretreated in the presence of 1 ng/ml *Pseudomonas* exotoxin A for 12 hr and then incubated in the presence of 1 ng/ml *Pseudomonas* exotoxin A without (*PsExoA*) or with 1 μM noradrenaline (*NA+PsExoA*) for 48 hr. Purified membranes were prepared and subjected to pertussis toxin-catalyzed [^{32}P]ADP-ribosylation. SDS-polyacrylamide gel electrophoresis and Coomassie blue staining were performed as described in Experimental Procedures. Each lane contained 44 μg of membrane protein. The Coomassie blue stain and the autoradiogram of the same gel are shown. The positions of molecular weight marker proteins are indicated.

toxin A was used to study whether the β-adrenoceptor-mediated desensitization and increase in G_i α-subunits were dependent on protein synthesis. Treatment of the cells with 1 ng/ml *Pseudomonas* exotoxin A for 60 hr led to a marked reduction of subsequent *Pseudomonas* exotoxin A-catalyzed [^{32}P]ADP-ribosylation, thus suggesting an ADP-ribosylation or a decrease in the level of elongation factor 2 by the toxin pretreatment. Under this condition, protein synthesis, estimated by [^{3}H] leucine incorporation into cellular proteins, was inhibited by about 30%. However, total cellular protein level, β_1-adrenoceptor number, and isoprenaline- or forskolin-stimulated adenylyl cyclase activities were not affected by this toxin treatment. In contrast, the noradrenaline-induced increase in pertussis toxin-catalyzed radiolabeling was completely prevented by *Pseudomonas* exotoxin A. In the absence of noradrenaline, *Pseudomonas* exotoxin A had only a small inhibitory effect on the pertussis toxin substrates. Immunoblotting with anti-$G_{i\alpha}$ antibodies confirmed the suggestion that the *Pseudomonas* exotoxin A-induced blockade of the β-adrenoceptor-mediated increase in pertussis toxin substrates was due to an effect of *Pseudomonas* exotoxin A on the level of $G_{i\alpha}$ proteins and not on the accessibility of the G_i proteins to pertussis toxin. Furthermore, *Pseudomonas* exotoxin A prevented the noradrenaline-induced increase in the level of the two distinct G_i α-subunits, $G_{i\alpha 40}$ and $G_{i\alpha 41}$. It is suggested from these data that the noradrenaline-mediated increase in the level of the G_i α-subunits depends on *de novo* synthesis of these proteins, and is not due, for example, to a translocation of these proteins into the plasma membrane compartment. Recently, mRNAs for $G_{i\alpha 2}$ and $G_{i\alpha 3}$, but not for $G_{i\alpha 1}$, were found in the neonatal and adult rat heart (21). The major 40-kDa pertussis toxin substrate present in rat heart muscle cell membranes appears to be of the $G_{i\alpha 2}$-subtype, because it comigrates with the major pertussis toxin substrate present in HL-60 cells, which was shown to be of the G_{i2}-subtype (22). The minor 41-kDa pertussis toxin substrate is, therefore, suggested to be the α-subunit of G_{i3}, although final proof for these assumptions remains to be provided.

Concomitantly with the inhibition of the noradrenaline-induced increase in $G_{i\alpha}$, the *Pseudomonas* toxin treatment abolished the noradrenaline-mediated decrease in forskolin-stimulated adenylyl cyclase activity. These results corroborate the evidence that the β-adrenoceptor-induced desensitization of receptor-independent adenylyl cyclase stimulation in rat heart muscle cells is due to an up-regulation of inhibitory G protein α-subunits. Three distinct G_i proteins are known so far (23). However, it is unclear at present which one of these proteins is the "real" adenylyl cyclase-inhibitory G protein. The data showing that two distinct $G_{i\alpha}$ proteins are up-regulated by β-adrenoceptor activation in rat heart muscle cells may even suggest that both $G_{i\alpha 40}$ and $G_{i\alpha 41}$ are involved in the decreased adenylyl cyclase activity.

Under the conditions used, *Pseudomonas* exotoxin A had no effect on the noradrenaline-induced down-regulation of β_1-adrenoceptors. These findings suggest that, in contrast to the increase in the level of G_i α-subunits, the decrease in the number of β_1-adrenoceptors does not depend on protein synthesis, at least not with the same sensitivity. Most interestingly, although the β_1-adrenoceptor down-regulation induced by noradrenaline was not prevented by *Pseudomonas* exotoxin A, the noradrenaline-induced decrease in β-adrenoceptor-mediated adenylyl cyclase stimulation was significantly attenuated by the toxin. These results suggest that the β-adrenoceptor-mediated increase in $G_{i\alpha}$ also contributes by 30–40% to the decrease in β-adrenoceptor-mediated adenylyl cyclase stimulation.

It has recently been reported that diphtheria toxin, which like *Pseudomonas* exotoxin A inhibits protein synthesis by ADP-ribosylating elongation factor 2, attenuates the isoprenaline-induced desensitization of adenylyl cyclase stimulation in A431 human epidermoid carcinoma cells (24). Moreover, in these cells cycloheximide, another less specific protein synthesis inhibitor, completely abolished the dibutyryl cyclic AMP-induced refractoriness to β-adrenoceptor stimulation. The authors proposed that the inhibition of protein synthesis prevents the synthesis of an unknown "rapidly turning over refractoriness" protein that is induced in response to catecholamine-stimulated production of cyclic AMP and that inhibits adenylyl cyclase. It may be speculated that, in accordance with the

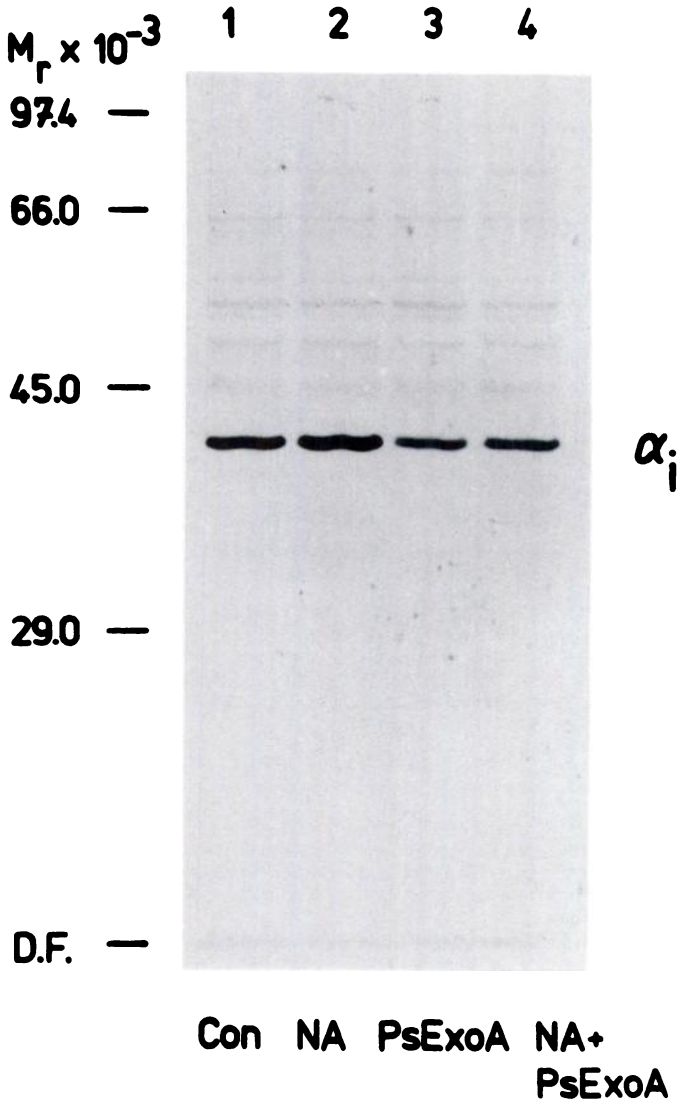

Fig. 5. Effect of *Pseudomonas* exotoxin A on the noradrenaline-induced increase in the level of $G_{i\alpha}$ in rat heart muscle cell membranes. Purified membranes were prepared after culture of heart muscle cells without additions (*Con*), with 1 μM noradrenaline (*NA*), with 1 ng/ml *Pseudomonas* exotoxin A (*PsExoA*), or with 1 μM noradrenaline plus 1 ng/ml *Pseudomonas* exotoxin A (*NA+PsExoA*), as described in the legend to Fig. 4. Membranes (each lane, 44 μg of membrane protein) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with an antiserum reactive against the α-subunits of G_t and G_i. The positions of molecular weight marker proteins and of the immunoreactive α-subunits of G_i (α_i) are indicated.

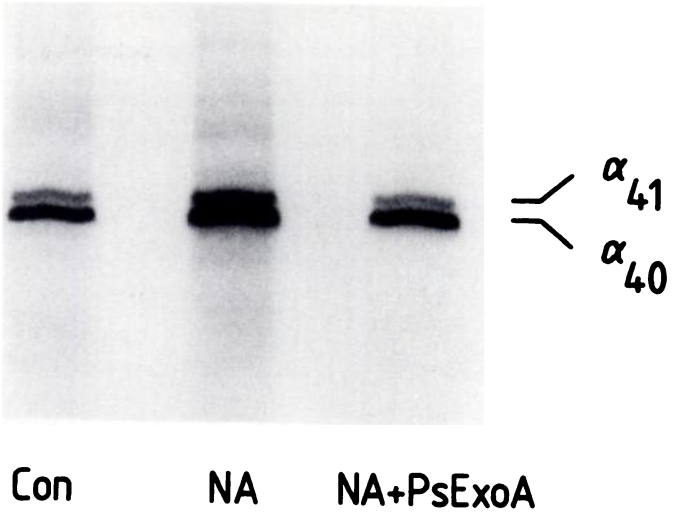

Fig. 6. Influence of *Pseudomonas* exotoxin A on the noradrenaline-induced increase in pertussis toxin-catalyzed ADP-ribosylation of two G proteins in rat heart muscle cell membranes. Purified membranes were prepared after culture of rat heart muscle cells without any additions (*Con*), with 1 μM noradrenaline (*NA*) or with 1 μM noradrenaline plus 1 ng/ml *Pseudomonas* exotoxin A (*NA+PsExoA*), as described in the legend to Fig. 4. The membranes were subjected to pertussis toxin-catalyzed [^{32}P]ADP-ribosylation. Each lane contained 44 μg of membrane protein. For SDS-polyacrylamide gel electrophoresis, the resolving gel was supplemented with 4 M urea. An autoradiogram of the dried gel is shown. The positions of the two distinct pertussis toxin substrates (α_{40}, α_{41}) are indicated on the *right*.

results presented herein, these "rapidly turning over refractoriness" proteins are the α-subunits of G_i. The up-regulation of these proteins induced by noradrenaline treatment was completely prevented by the *Pseudomonas* exotoxin A treatment, even though total cellular protein content was not altered and, moreover, other components of the adenylyl cyclase system, like the β-adrenoceptors, the G_s protein, and the adenylyl cyclase itself, were apparently not affected. It is interesting to note here that the promoter region of the human gene of the α-subunit of G_{i2} has recently been reported to contain possible DNA binding domains for AP-2 (25), a factor that may mediate the transcriptional effects of cyclic AMP (26). Thus, alterations of $G_{i\alpha}$ protein levels, possibly induced by alterations in intracellular cyclic AMP concentrations, may play a major regulatory role in long term regulation of adenylyl cyclase activity.

In membrane preparations from hearts of patients with dilated cardiomyopathy, both a down-regulation of β_1-adrenoceptors and an apparent increase in the level of G_i α-subunits has recently been reported (4–6). Results obtained from noradrenaline exposure of rat heart muscle cells suggest that the increase in $G_{i\alpha}$ and the desensitization to receptor-independent adenylyl cyclase stimulation observed in severely failing human hearts may be due to prolonged exposure of these hearts to rather high concentrations of noradrenaline (1, 2). The results of the present study now lead to the suggestion that the apparent increase in G_i protein α-subunits in these human hearts may be induced by the β-adrenoceptor stimulatory effect of the increased noradrenaline concentrations found in patients with heart failure. Furthermore, if prolonged β-adrenoceptor agonist exposure is the mechanism of the increase in the level of $G_{i\alpha}$ in hearts of patients with dilated cardiomyopathy, this increase may also be due to new synthesis of $G_{i\alpha}$ proteins.

In *Pseudomonas aeruginosa* septicemia in humans, *Pseudomonas* exotoxin A has been identified as an important pathogenetic factor (27). However, at present, it is unknown whether this toxin has any importance in the pathogenesis of the decreased cardiac responsiveness to inotropic stimulation in *Pseudomonas* septicemia. The results of the present study suggest that, if relevant concentrations of *Pseudomonas* exotoxin A would be present in the heart in *Pseudomonas* septicemia, the toxin would lead to a partial attenuation of catecholamine-induced desensitization and, therefore, possibly to an increased catecholamine cardiotoxicity.

Acknowledgments

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TABLE 2

Influence of *Pseudomonas* exotoxin A on the noradrenaline-induced decrease in adenylyl cyclase stimulation and [^{3}H]CGP 12177 binding in rat heart muscle cell membranes

Rat heart muscle cells were incubated in the absence or presence of 1 μM noradrenaline, 1 ng/ml *Pseudomonas* exotoxin A, or 1 μM noradrenaline plus 1 ng/ml *Pseudomonas* exotoxin A, as described in the legend to Fig. 4, and crude membranes were prepared. Adenylyl cyclase activities were determined in the presence of either 100 μM isoprenaline or 100 μM forskolin. Specific [^{3}H]CGP 12177-binding was determined as described in Experimental Procedures. The values are given as mean of triplicates ± standard deviation.

	Control	Noradrenaline	*Pseudomonas* exotoxin A	Noradrenaline + *Pseudomonas* exotoxin A
Adenylyl cyclase activity (pmol cAMP × mg of protein^{-1} × 10 min^{-1})				
Isoprenaline	83.5 ± 5.7	34.2 ± 3.7	82.1 ± 4.5	53.0 ± 2.7*
Forskolin	297.0 ± 29.3	230.1 ± 34.7	322.2 ± 15.7	294.2 ± 36.4*
[^{3}H]CGP 12177 bound (fmol × mg of protein^{-1})	19.2 ± 1.2	10.5 ± 1.3	19.4 ± 3.7	12.3 ± 1.3

* The values are significantly higher than the corresponding values of the noradrenaline-treated group, $p \leq 0.05$.

References

1. Reithmann, C., and K. Werdan. Noradrenaline-induced desensitization in cultured heart cells as a model for the defects of adenylate cyclase system in severe heart failure. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **339**:138–144 (1989).
2. Reithmann, C., P. Gierschik, D. Sidiropoulos, K. Werdan, and K. H. Jakobs. Mechanism of noradrenaline-induced heterologous desensitization of adenylate cyclase stimulation in rat heart muscle cells: increase in the level of inhibitory G-protein α-subunits. *Eur. J. Pharmacol.* **172**:211–221 (1989).
3. Bristow, M. R., R. Ginsburg, W. Minobe, R. S. Cubiccioti, W. S. Sageman, K. Lurie, M. E. Billingham, D. C. Harrison, and E. B. Stinson. Decreased catecholamine sensitivity and β-adrenergic receptor density in failing human hearts. *N. Engl. J. Med.* **307**:205–211 (1982).
4. Feldman, A. M., A. E. Cates, W. B. Veazey, R. E. Hershberger, M. R. Bristow, K. L. Baughman, W. A. Baumgartner, and C. Van Dop. Increase of the 40,000 mol. wt. pertussis toxin substrate (G-protein) in the failing human heart. *J. Clin. Invest.* **82**:189–197 (1988).
5. Neumann, J., H. Scholz, V. Döring, W. Schmitz, L. Von Meyerinck, and P. Kalmar. Increase in myocardial G_i-proteins in heart failure. *Lancet* **2**:936–937 (1988).
6. Böhm, M., P. Schnabel, and E. Erdmann. Increase of a 40 kDa pertussis toxin substrate accompanies reduced cAMP formation and inotropic responsiveness in the failing human heart in dilated but not in ischemic cardiomyopathy. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **339**(suppl):R52 (1989).
7. Cohn, J. N., T. B. Levine, M. T. Olivari, V. Garber, D. Lura, G. S. Francis, A. B. Simon, and R. Rector. Plasma noradrenaline as a guide to prognosis in patients with congestive heart failure. *N. Engl. J. Med.* **311**:819–823 (1984).
8. Kupfer, L. E., J. P. Bilezikian, and R. B. Robinson. Regulation of *alpha* and *beta* adrenergic receptors by triiodothyronine in cultured rat myocardial cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **334**:275–281 (1986).
9. Gierschik, P., D. Sidiropoulos, M. Steißlinger, and K. H. Jakobs. Na^+ regulation of formyl peptide receptor-mediated signal transduction in HL 60 cells: evidence that the cation prevents unoccupied receptors from activating the G-protein. *Eur. J. Pharmacol.* **172**:481–492 (1989).
10. Cassel, D., and T. Pfeuffer. Mechanism of cholera toxin action: covalent modification of the guanyl nucleotide binding protein of the adenylate cyclase system. *Proc. Natl. Acad. Sci. USA* **75**:2669–2673 (1978).
11. Werdan, K., B. Wagenknecht, B. Zwißler, L. Brown, W. Krawietz, and E. Erdmann. Cardiac glycoside receptors in cultured heart cells. II. Characterization of a high affinity and a low affinity binding site in heart muscle cells from neonatal rats. *Biochem. Pharmacol.* **33**:1873–1886 (1984).
12. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1986).
13. Jakobs, K. H., W. Saur, and G. Schultz. Reduction of adenylate cyclase activity in lysates of human platelets by the α-adrenergic component of epinephrine. J. Cyclic Nucleotide Res. **2**:381–392 (1976).
14. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
15. Gierschik, P., D. Sidiropoulos, A. Spiegel, and K. H. Jakobs. Purification and immunochemical characterization of the major pertussis-toxin-sensitive guanine-nucleotide binding protein of bovine-neutrophil membranes. *Eur. J. Biochem.* **165**:185–194 (1987).
16. Gierschik, P., J. Falloon, G. Milligan, M. Pines, J. I. Gallin, and A. Spiegel. Immunochemical evidence of a novel pertussis toxin substrate in human neutrophils. *J. Biol. Chem.* **261**:8058–8062 (1986).
17. Eide, B., P. Gierschik, and A. Spiegel. Immunochemical detection of guanine nucleotide binding proteins mono-ADP-ribosylated by bacterial toxins. *Biochemistry* **25**:6711–6715 (1986).
18. Goldsmith, P., P. Gierschik, G. Milligan, C. G. Unson, R. Vinitsky, H. L. Malech, and A. M. Spiegel. Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophil and brain. *J. Biol. Chem.* **262**:14683–14688 (1987).
19. Karliner, J. S., and P. C. Simpson. β-Adrenoceptor and adenylate cyclase regulation in cardiac myocyte growth. *Basic Res. Cardiol.* **83**:655–663 (1988).
20. Iglewski, B. H., and D. Kabat. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. *Proc. Natl. Acad. Sci. USA* **72**:2284–2288 (1975).
21. Luetje, C. W., K. M. Tietje, J. L. Christian, and N. M. Nathanson. Differential tissue expression and developmental regulation of guanine nucleotide binding regulatory proteins and their messenger RNAs in rat heart. *J. Biol. Chem.* **263**:13357–13365 (1988).
22. Murphy, P. M., B. Eide, P. Goldsmith, M. Brann, P. Gierschik, A. Spiegel, and H. L. Malech. Detection of multiple forms of $G_{i\alpha}$ in HL 60 cells. *FEBS Lett.* **221**:81–86 (1987).
23. Lochrie, M. A., and M. I. Simon. G protein multiplicity in eukaryotic signal transduction systems. *Biochemistry* **27**:4957–4965 (1988).
24. De Bernardi, M., and G. Brooker. Diphtheria toxin prevents catecholamine desensitization of A431 human epidermoid carcinoma cells. *Proc. Natl. Acad. Sci. USA* **84**:2246–2250 (1987).
25. Weinstein, L. S., A. M. Spiegel, and A. D. Carter. Cloning and characterization of the human gene for the α-subunit of G_{is}, a GTP-binding signal transduction protein. *FEBS Lett.* **232**:333–340 (1988).
26. Imagawa, M., R. Chiu, and M. Karin. Transcription factor AP-2 mediates induction of two different signal-transduction pathways: protein kinase C and cAMP. *Cell* **51**:251–260 (1987).
27. Pollack, M., and L. S. Young. Protective activity of antibodies to exotoxin A and lipopolysaccharide at the onset of *Pseudomonas aeruginosa* septicemia in man. *J. Clin. Invest.* **63**:276–286 (1979).

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