Cardiac Hypertrophy to Cardiac Failure. In heart failure, however, it is still unresolved which factors predispose or accelerate the progression from cardiac hypertrophy to cardiac failure. In heart failure, several neurohormonal mechanisms are activated, among which are the circulating and tissue renin-angiotensin systems (RAS) including the myocardium (M. Paul, unpublished data, 1994). These animals develop cardiac hypertrophy and fulminant hypertension. Recently, transgenic animals became available that overexpress mouse renin at particularly high concentrations in tissues, including the myocardium (M. Paul, unpublished data, 1994). These animals develop cardiac hypertrophy and fulminant hypertension. In the present study, we addressed the question of whether similar alterations of sympathetic neuroeffector mechanisms occur in hypertrophic myocardium of transgenic rats that exhibit one important pathophysiological alteration that similarly occurs in heart failure, namely, activation of tissue renin activity in several tissues including the heart.

Methods

Transgenic Animals

Transgenic rats [TG(mREN2)27] were housed and bred in the animal laboratory of the Max Delbrück Zentrum in Berlin. At the age of 5 weeks, rats were transferred to the animal laboratory of the Klinikum Großhadern, University of Munich (Germany). Sprague-Dawley (SD) control rats were obtained from the Laboratorium für Versuchstierkunde in Hannover (Germany). These were the animals into which the transgene was originally introduced. Animals were housed according to animal care guidelines of the University of Munich. Only male rats were used. The animals were held on a standard laboratory animal diet (Altromin) and tap water ad libitum. They were exposed to a 12-hour light/dark cycle at 20° to 22°C. Experiments were performed when rats were 12 to 14 weeks old, at which time hypertension is fully established [ie, TG (mREN2)27, 230±8 mm Hg versus SD, 115±12 mm Hg]. Rats were killed by a blow on the head, and the hearts were rapidly removed. In TG(mREN2)27 compared with SD rats, absolute and relative heart weights increased significantly [TG (mREN2)27, 1.82±0.03 g, 4.92±0.11 mg/g, respectively; SD, 1.42±0.03 g, 3.78±0.08 mg/g; \( P < 0.001 \)]. TG(mREN2)27 hearts exhibited concentric hypertrophy but no dilatation or any signs of heart failure such as excessive scarring. No signs of venous congestion were observed in any other organ.
Adenylyl Cyclase Determination

Particulate washed membrane fractions (10 000g sediment) were prepared from homogenates of rat hearts. Adenylyl cyclase activity was determined in a reaction mixture containing 50 μmol/L Tris-HCl, 100 μmol/L EGTA, 1 mmol/L 3-isobutyl-l-methylxanthine, 5 mmol/L creatine phosphate, 0.4 mg/mL creatine kinase, and 0.1 mmol/L cyclic AMP (cAMP) at pH 7.4 in a final volume of 100 μL. The mixture was preincubated for 5 minutes at 37°C. The incubation time was 20 minutes at the same temperature. Reactions were stopped by the addition of 500 μL of 120 mmol/L zinc acetate. Next, the zinc acetate was neutralized by 600 μL NaHCO₃ (144 mmol/L). After centrifugation for 5 minutes at 10 000g, 0.8 mL of the supernatant was applied on neutral alumina columns equilibrated with 0.1 mmol/L Tris-HCl, pH 7.5. The effluent was collected and [32P]-cAMP determined by measurement of radioactivity in a liquid scintillation spectrometer (LKB Wallac 1272 Clingamma).

Membrane Preparation for Receptor and G Protein Determinations

Myocardial tissue was chilled in 30 mL ice-cold homogenization buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, pH 7.4). Connective tissue was trimmed away and myocardial tissue minced with scissors; membranes were prepared with a motor-driven glass-polytetrafluoroethylene homogenizer for 1 minute. Then the membrane preparation was homogenized by hand for 1 minute with a glass-bar homogenizer. The homogenate was spun at 450g (Beckman JA 20 rotor) for 10 minutes. The supernatant was filtered through two layers of cheesecloth, diluted with an equal volume of ice-cold 1 mol/L KC1, and stored on ice for 10 minutes. This suspension was centrifuged at 100 000g for 45 minutes. The final pellet was resuspended in 50 vol incubation buffer (50 mmol/L Tris-HCl, 10 mmol/L MgCl₂, pH 7.4) and homogenized for 1 minute with a glass-bar homogenizer. This suspension was recentrifuged at 100 000g for 45 minutes. The final pellet was resuspended in incubation buffer (50 vol) and stored at -70°C. Storage did not alter the results.

Radioligand Binding Studies

Assays were performed in a total volume of 250 μL incubation buffer (for composition, see above). Incubation was carried out at 37°C for 60 minutes. These conditions allowed complete equilibrium of the receptors with the radioligand. The reaction was terminated by rapid vacuum filtration through Whatman GF/C filters; filters were immediately washed three times with 6 mL ice-cold incubation buffer. All experiments were performed in triplicate. Myocardial β-adrenergic receptors were studied using [125I]-cyanopindolol ([125I]-Cyp). β-Adrenergic receptor subtype studies were used using the β₁-selective and β₂-selective antagonists CGP 207,12A and ICI 118,551, respectively, as described previously. Specific activity was 2000 Ci/mmol. (−)Propranolol (3 μmol/L) was used to determine nonspecific binding.

Pertussis Toxin-Induced [32P]-ADP Ribosylation

[32P]ADP ribosylation of Gαₛ by pertussis toxin was performed for 12 hours at 4°C in a volume of 50 μL containing 100 mmol/L Tris-HCl (pH 8.0) at 20°C, 25 mmol/L dithiothreitol, 2 mmol/L ATP, 1 mmol/L GTP, 50 mmol/L NAD (800 Ci/mmol), and 20 μg/mL pertussis toxin that had been activated by incubation with 50 mmol/L dithiothreitol for 1 hour at 20°C before the labeling reaction as described earlier.17,18 Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10% wt/vol) acrylamide, 16 cm total gel length). Gels were stained with Coo massie blue and dried before autoradiography was performed.

Immunoblotting Techniques

Immunoblotting techniques were performed as described before.19 The polyclonal antiserum MB 1 was raised in rabbits against the C-terminal decapetide of retinal transducin a (KENLKDCGLF) coupled to keyhole limpet hemocyanine as described by Goldsmith et al.19 The antiserum recognized Gₛ₁ and Gₛ₂, but not Gₛ₃ and Gₛ₄ (not shown). Blots were stained with an alkaline phosphatase-labeled goat anti-IgG antiserum.

S49 Lymphoma cye" Cells

S49 lymphoma cye" cells were grown in suspension culture in RPMI 1640 medium supplemented with 10% (vol/vol) fetal serum (culture volume ≤100 mL) or 10% (vol/vol) horse serum (culture volume ≥100 mL), NaHCO₃ (44 mmol/L), glucose (5.5 mmol/L), l-glutamine (5 mmol/L), nonessential amino acids, sodium pyruvate (1 mmol/L), penicillin (50 U/mL), and streptomycin (50 μg/mL) in a humidified atmosphere of 90% air and 10% CO₂. Cell density was maintained at approximately 1X10⁶ cells per milliliter. Cells (1X10⁸ to 2X10⁸ cells in 10 to 20 L medium) were harvested by centrifugation in a Beckman JA-10 rotor at 1000g for 20 minutes at 4°C. The pellets were resuspended in 50 mL triethanolamine-HCl (10 mmol/L; pH 7.4 at 20°C). The final pellet was resuspended in 100 to 130 mL lysis buffer containing sucrose (0.25 mol/L), Tris-HCl (20 mmol/L; pH 7.5 at 20°C), MgCl₂ (1.5 mmol/L), ATP (1 mmol/L), benzamidine (3 mmol/L), leupeptin (1 μmol/L), phenylmethylsulfonyl fluoride (1 mmol/L), and soybean trypsin inhibitor (2 μg/mL). Cells were homogenized by nitrogen cavitation. The cavitate was centrifuged in a JA-20 rotor at 1500g for 45 seconds at 4°C to remove unbroken cells and nuclei and was filtered through two layers of cheesecloth. A crude membrane fraction was isolated from the resulting supernatant by centrifugation in a JA-20 rotor at 5000g for 20 minutes 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), phenylmethylsulfonyl fluoride (1 mmol/L), and soybean trypsin inhibitor (2 μg/mL), and stored at -80°C. The membrane protein yield was approximately 100 mg per 10⁶ cells.

Reconstitution of Myocardial Gₛ₁ Into S49 cye" Membranes

Reconstitution assays were performed according to Sternweis et al.20

Contraction Studies

Experiments were performed on electrically driven (1 Hz) rat papillary muscles. Papillary muscles of uniform size (diameter, <1.0 mm; length, 3 to 6 mm) were dissected in aerated bathing solution (for composition, see below) at room temperature from the left ventricles of TG(mREN2)27 and control rats. The preparations were attached to a bipolar platinum stimulating electrode and suspended individually in 75-mL glass tissue chambers for recording of isometric contractions. The bathing solution was a modified Tyrode's solution containing (mmol/L) NaCl 119.8, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1.05, NaH₂PO₄ 0.42, NaHCO₃ 22.6, Na₂EDTA 0.05, ascorbic acid 0.28, and glucose 5.0. It was continuously gassed with 95% O₂/5% CO₂ and maintained at 35°C (pH 7.4). Contraction force was measured with an inductive force transducer (W. Flecck) attached to a Helfige Helco Scriptron or Gould recorder. Each muscle was stretched to the length at which contraction force was maximal. The resting force (approximately 5 mN) was kept constant throughout the experiment. Preparations were electrically paced at 1 Hz with rectangular pulses of 5 milliseconds duration (Grass stimulator SD 9). The voltage was approximately 20% greater than threshold. All preparations were allowed to equilibrate in drug-free bathing solution until complete mechanical stabilization. Positive inotropic response
to isoproterenol and calcium were determined with cumulative concentration-response curves.

**Norepinephrine and Neuropeptide Y Determinations**

For norepinephrine measurements, tissue samples were homogenized with a polytron in 0.1 mol/L Tris-HCl (pH 7.4). After centrifugation (10 000g, 30 minutes), norepinephrine was extracted with alumina and determined by high-performance liquid chromatography with electrochemical detection as described by Beschi et al. Neuropeptide Y was determined with a commercially available radioimmunoassay (Amersham-Buchler).

**Miscellaneous**

Protein was determined according to Lowry et al using bovine serum albumin as standard. SDS-PAGE was performed as described by Lammli. 5'-Nucleotidase activity was analyzed with the method of Dixon and Purdom. Materials

Forskolin was donated by Dr Jürgen Metzger (Hoechst AG, Frankfurt, Germany). GTP, guanylylimidodiphosphate [Gpp(NH)p], ATP, creatine phosphate, and creatine kinase were purchased from Boehringer-Mannheim and isobutylmethylxanthine from EGA-Chemie. The ligand 2CI-P-Cyp was from Amersham-Buchler, dithiothreitol from Serva, and pertussis toxin from Llist Biological Laboratories. CGP 207.12A [2-hydroxy-5-(2-hydroxy-3-(4-(1-methyl-4-trifluoromethyl)-3-isopropyl-aminoethoxy)-benzamido-hydrochlo-

FIG 1. Line graphs show concentration-response curves for effects of isoproterenol (top, isoprenaline on figure), guanylylimidodiphosphate [Gpp(NH)p] (middle), and forskolin (bottom) (all 0.01 to 100 μmol/L) on adenylyl cyclase activity of left ventricular membranes from transgenic rats (TG(mREN2)27) and age-matched Sprague-Dawley rats (SP) as controls. Basal adenylyl cyclase activities were: top, 1008±234 in TG(mREN2)27 (n=9) and 1144±135 in SP (n=10); middle, 114.8±27.3 in T

**Statistics**

Data are mean±SEM. Statistical significance was estimated with Student's t test for unpaired observations and ANOVA according to Wallenstein et al. A probability value less than .05 was considered significant. K₅ values were determined graphically in each individual experiment.

**Results**

**Adenylyl Cyclase Activity**

Fig 1 (top) shows the ability of the β-adrenergic receptor agonist isoproterenol to increase adenylyl cyclase. The effect of isoproterenol was significantly reduced in TG(mREN2)27 compared with SD rats. The maximal effect of isoproterenol was smaller by approximately 60% in TG(mREN2)27 than in SD rats. To determine whether the reduced stimulation of adenylyl cyclase is due to alterations in the levels of β-adrenergic receptors or of G protein, we investigated the effects of the metabolically stable GTP derivative Gpp(NH)p. As with isoproterenol, the effect of Gpp(NH)p was significantly reduced starting at 1 mmol/L in TG(mREN2)27 compared with SD rats (Fig 1, middle). To further study whether the observed changes are due to an impairment of the activity of the catalytic subunit of the adenylyl cyclase, we investigated the effect of forskolin, which directly activates the catalyst. The effect of forskolin was only slightly reduced in TG(mREN2)27 rats at 1 μmol/L forskolin, whereas at 0.01 to 1 μmol/L or 10 to 100 μmol/L, a small but nonsignificant reduction was present. The data did not differ when related to any protein or 5'-nucleotidase activity (Table). However, in some cell systems, the effects of forskolin depend on Gₛ. Therefore, basal and forskolin-stimulated adenylyl cyclase activities were measured alone and in the presence of MnCl₂, which has been observed to uncouple the catalyst from the influence of G proteins. At 5 mmol/L MnCl₂, Gpp(NH)p did not stimulate basal or forskolin-activated adenylyl cyclase activities, indicating that under this condition, the catalyst stimulation was independent of G proteins (data not shown). Fig 2 (right) shows the effects of MnCl₂ on basal and forskolin-stimulated adenylyl cyclase activities. The presence of MnCl₂ did not completely antagonize the reduction of the effects of forskolin or forskolin plus Gpp(NH)p. Thus, the activity of the catalyst in TG(mREN2)27 rats appears to be mildly depressed. However, it is noteworthy that the reduction after stimulation of adenylyl cyclase by isoproterenol or Gpp(NH)p was much greater than after stimulation with forskolin.
**β- and β2-Adrenergic Receptors and Maximal Stimulatory Effects on Adenylyl Cyclase Activity in Myocardial Membranes of Transgenic and Sprague-Dawley Rats**

<table>
<thead>
<tr>
<th>Membrane Protein, mg</th>
<th>5'-Nucleotidase, (nmol/L P)/mg·min</th>
<th>Membrane Protein, mg</th>
<th>5'-Nucleotidase, (nmol/L P)/mg·min</th>
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</thead>
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<tr>
<td><strong>β-Adrenergic receptors</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>β-AR</td>
<td>36.7±2.8* (n=10)</td>
<td>1.04±0.11 (n=7)</td>
<td>26.7±1.2 (n=10)</td>
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<tr>
<td>β1-AR</td>
<td>22.0±2.2* (n=9)</td>
<td>0.67±0.09 (n=7)</td>
<td>14.9±1.9 (n=9)</td>
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<tr>
<td>β2-AR</td>
<td>14.5±2.1 (n=10)</td>
<td>0.39±0.04 (n=8)</td>
<td>13.6±2.4 (n=9)</td>
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<tr>
<td>Adenylyl cyclase activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoproterenol (100 μmol/L)</td>
<td>1415±162* (n=6)</td>
<td>17.4±0.9* (n=5)</td>
<td>609±101 (n=7)</td>
</tr>
<tr>
<td>Gpp(NH)p (30 μmol/L)</td>
<td>2123±147* (n=4)</td>
<td>25.4±3.1* (n=7)</td>
<td>1397±169 (n=8)</td>
</tr>
<tr>
<td>Forskolin (100 μmol/L)</td>
<td>2834±321 (n=8)</td>
<td>32.7±3.9 (n=7)</td>
<td>2497±238 (n=6)</td>
</tr>
<tr>
<td><strong>TG(mREN2)27</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane Protein, mg</td>
<td>32.3±3.8</td>
<td>0.59±0.21</td>
<td>26.7±3.1</td>
</tr>
<tr>
<td>5'-Nucleotidase, (nmol/L P)/mg·min</td>
<td>28.0±3.2</td>
<td>0.73±0.18</td>
<td>22.3±2.8</td>
</tr>
</tbody>
</table>

*Significant differences vs TG(mREN2)27.

**β-Adrenergic Receptors**

To investigate whether a decrease in the number of β-adrenergic receptors plays a role in the reduction of the isoproterenol-stimulated adenylyl cyclase activity in TG(mREN2)27 rats, we studied the β-adrenergic receptors and subtypes with β1-selective antagonists CGP 207.12A and ICI 118.551, TG(mREN2)27 rats, we studied the β-adrenergic receptors and subtypes with β1-selective antagonists CGP 207.12A and ICI 118.551, respectively. Fig 3 (top) summarizes the data for radio-ligand saturation experiments. The density of β-adrenergic receptors was significantly lower in TG(mREN2)27 compared with SD rats when related to milligrams of membrane protein. However, when binding data were related to 5'-nucleotidase activity, a small difference was present, but this was not significant (Fig 3, top, right). The antagonist affinity as judged from the K<sub>i</sub> values for β1-Cyp binding did not differ between the two groups (SD, 46.4 [42.8 to 52.2] pmol/L, n=10; TG (mREN2)27, 46.8 [38.2 to 57.4] pmol/L, n=10). Fig 3 (middle) shows the data for the number of β1-adrenergic receptors. A significant decrease of β1-adrenergic receptors by 32% or 28% was observed when data were related to milligrams of protein or 5'-nucleotidase activity, respectively. There was no difference in the number of β1-adrenergic receptors between TG(mREN2)27 and SD rats when related to membrane protein or 5'-nucleotidase activity (Table). Thus, a reduction in the number of β1-adrenergic receptors was observed in myocardial membranes, suggesting that this alteration could be involved in the reduced effects of isoproterenol on adenylyl cyclase.

**Stimulatory G Protein α-Subunits**

Because the effects of Gpp(NH)p on adenylyl cyclase activity were also reduced in TG(mREN2)27 rats, it is likely that postreceptor events such as alterations of G protein function or content could play a role. To study the function of stimulatory G protein α-subunits, we performed reconstitution experiments using S49 cell membranes, which genetically lack G<sub>α</sub>. G<sub>α</sub> was solubilized from myocardial membranes and reconstituted with S49 cell membranes. Reconstitution of G<sub>α</sub> into S49 cell membranes increased basal adenylyl cyclase activity by 105% to 115% (Fig 4). There was no difference in basal adenylyl cyclase activity between the membranes with reconstituted G<sub>α</sub> from SD or TG(mREN2)27 rats (Fig 4, top). The adenylyl cyclase activity after stimulation with isoproterenol (Fig 4, middle) and Gpp(NH)p (Fig 4, bottom) also did not differ between the two groups. Taken together, these data suggest that an alteration of G<sub>α</sub> function does not play a role in cardiac adenylyl cyclase desensitization of TG(mREN2)27 rats.

Fig 2. Bar graphs show effect of manganese chloride (MnCl<sub>2</sub>, 5 mmol/L) on basal adenylyl cyclase activity and adenylyl cyclase activity stimulated by forskolin (10 μmol/L) or forskolin plus guanylylimidodiphosphate [Gpp(NH)p, 100 μmol/L] in left ventricular membranes from transgenic rats [TG(mREN2)27, hatched columns] and age-matched Sprague-Dawley rats (open columns) as controls. Adenylyl cyclase was studied in the absence of MgCl<sub>2</sub>. Each column gives the data for four to six independent experiments performed in triplicate. Adenylyl cyclase was significantly reduced after application of forskolin+Gpp(NH)p and forskolin+MnCl<sub>2</sub> or forskolin+Gpp(NH)p+MnCl<sub>2</sub>.
Inhibitory G Protein α-Subunits

Inhibitory G\(\alpha\)_subunits were investigated by pertussis toxin–catalyzed \(^{32}\)P\(\text{ADP}\) ribosylation and immunologically with an antisera (MB 1) raised against the C-terminus of retinal transducin \(\alpha\) (KENLKDCGLF). A representative autoradiograph (Fig 5, left) for \(^{32}\)P\(\text{ADP}\) ribosylation of myocardial membranes shows that the incorporation of radioactivity into G\(\alpha\) (approximately 40 kD) was more pronounced in myocardial membranes from TG(mREN2)27 than from SD rats. Correspondingly, more immunoreactive G\(\alpha\) was observed on Western blots in TG(mREN2)27 rats (Fig 5, right). Competition of immunostaining with the synthetic peptide KENLKDCGLF, against which the antisera MB 1 was raised, showed that the antisera MB 1 specifically recognized a 40-kD protein in myocardial membranes, which comigrates with purified G\(\alpha\)/G\(\beta\) from bovine brain (not shown). Fig 6 summarizes the data for \(^{32}\)P\(\text{ADP}\) ribosylation with pertussis toxin. In myocardial membranes from TG(mREN2)27 rats, pertussis toxin substrate increased by 75% to 95%. Similar results were obtained when the amount of pertussis toxin substrate was related to milligrams of membrane protein or 5'-nucleotidase activity as myocardial membrane marker (right ordinate). Each column gives the data of six to eight independent experiments done in triplicate. \(^{32}\)P\(\text{ADP}\) indicates \(^{32}\)P-cyanopindolol.

Fig 4. Bar graphs show basal adenylyl cyclase activity (top) and adenylyl cyclase activity stimulated by isoproterenol (middle, isoprenaline in figure) and guanylylimidodiphosphate \([\text{Gpp (NH)}_\text{p}}, \text{bottom}]) in S49 cyc– membranes reconstituted with G\(\alpha\) solubilized from left ventricular membranes of transgenic rats [TG(mREN2)27] and age-matched Sprague-Dawley rats (SP) as controls. Each column gives the data of six to eight independent experiments done in triplicate.

To this end, the question had to remain open of whether the increased G\(\alpha\) expression is of functional nature in TG(mREN2)27 compared with SD rats and thus is likely to play a role in the depressed basal and guanine nucleotide–stimulated adenylyl cyclase activities.

Fig 5. Representative autoradiography (left) and immunological detection (Western blot, right) of G\(\alpha\) (approximately 40 kD) in left ventricular membranes from transgenic rats [TG (mREN2)27] and age-matched Sprague-Dawley rats (SPDR) as controls. \(^{32}\)P\(\text{ADP}\) ribosylation by pertussis toxin plus \(^{32}\)P\(\text{NAD}\) (left) was performed as described in "Methods." Immunological detection (right) was performed with an antisera raised against the C-terminus of retinal transducin \(\alpha\). Membranes were separated by sodium dodecyl sulfate–polyacrylamide (10%) gel electrophoresis before autoradiography or electrophoretic transfer of proteins. G\(\alpha\)/G\(\beta\) (0.5 μg) purified from bovine brain was investigated in parallel as standard. Each lane contained 25 μg protein for \(^{32}\)P\(\text{ADP}\) ribosylation and Western blotting.

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Fig 6. Bar graphs show incorporation of radioactivity by pertussis toxin–catalyzed $[^{32}P]$ADP ribosylation into approximately 40-kD protein of left ventricular membranes from transgenic rats [TG(mREN2)27] and age-matched Sprague-Dawley rats (SP) as controls. Data are given in equivalents of radioactivity incorporations into G$_{i}$G$_{o}$ standard from bovine brain and were related to 5'-nucleotidase activity as myocardial membrane marker (left ordinate) or milligram of membrane protein (right ordinate).

relevance for the reduced adenylyl cyclase activity. To address this issue, we studied adenylyl cyclase in myocardial membranes that were treated with pertussis toxin plus NAD to inactivate G$_{i}$. Fig 8 shows a representative autoradiograph of $[^{32}P]$ADP ribosylation by pertussis toxin in membranes treated with pertussis toxin plus NAD and control membranes treated with NAD and heat-inactivated pertussis toxin. $[^{32}P]$ADP ribosylation of G$_{i}$ was markedly depressed by membrane treatment with pertussis toxin plus NAD. This observation suggests that under this experimental condition, the major portion of G$_{i}$ proteins in the membranes treated with active pertussis toxin was ADP-ribosylated. Fig 9 shows adenylyl cyclase activity in control membranes and membranes with ADP-ribosylated G$_{i}$. As shown in the previously described experiments, basal and Gpp(NH)p-stimulated adenylyl cyclase activities were depressed in control membranes from TG(mREN2)27 rats. This depressed adenylyl cyclase activity was restored when membranes were treated with intact pertussis toxin plus NAD. The latter finding suggests that the increase in G$_{i}$ protein expression is of functional relevance for the desensitized cardiac adenylyl cyclase activity in TG(mREN2)27 rats.

Contraction Force

To study whether the desensitization of adenylyl cyclase activity caused by the apparent reduction of $\beta$-adrenergic receptors and the increase of G$_{i}$ proteins has functional relevance for inotropy in hypertrophied myocardium from TG(mREN2)27 rats, we studied the positive inotropic effects of the $\beta$-adrenergic receptor agonist isoproterenol and Ca$^{2+}$ on papillary muscle strips from TG(mREN2)27 and control SD rats. Fig 10 summarizes the data. Compared with SD rats (Fig 10A), the positive inotropic effect of isoproterenol was markedly reduced in TG(mREN2)27 rats (Fig 10B). In contrast, the positive inotropic effect after an increase in the extracellular Ca$^{2+}$ concentration did not differ between the two groups. This held true for the potency, as judged
Fig 10. Line graphs show concentration-response curves for effects of isoproterenol (isoprenaline on figure, 0.001 to 10 μmol/L) and calcium (1.8 to 15 mmol/L) on isolated, electrically driven papillary muscles from transgenic rats [TG(mREN2)27] and age-matched Sprague-Dawley rats (SPDR) as controls. Predrug values of force of contraction were 3.9±0.3 mN (n=18) in SPDR (A) and 4.1±0.4 mN (n=16) in TG(mREN2)27 (B).

from the EC50 values, as well as for the efficacy, as judged from the maximal effects (not shown).

Myocardial Norepinephrine and Neuropeptide Y Concentrations

An increased release of norepinephrine and the coreleased neuropeptide from sympathetic cardiac nerves have been suggested to lead to a depletion of cardiac norepinephrine and neuropeptide Y stores in failing heart muscle. Thus, cardiac norepinephrine concentrations were taken as an indirect measure of cardiac catecholamine turnover in this study. Fig 11 shows the myocardial levels of norepinephrine and neuropeptide Y in samples from TG(mREN2)27 and SD rats. In the myocardium from transgenic rats, myocardial norepinephrine and neuropeptide Y concentrations were markedly reduced by 70% and 92%, respectively. Taken together, these findings suggest that an increased cardiac sympathetic nerve activity could result in depleted cardiac norepinephrine and neuropeptide Y stores and could play a role in heterologous desensitization in TG(mREN2)27 rats.

Discussion

Long-standing hypertension with consecutive myocardial hypertrophy has been observed to be associated with a high incidence of heart failure. The hypertrophy process is regarded as an adaptive mechanism when an increased workload is imposed on the myocardium. However, when and why the hypertrophied heart begins to fail remains unresolved. In heart failure, the renin-angiotensin-aldosterone system is activated in response to a reduced cardiac output. Genetic linkage studies in human hypertensive subjects and laboratory animals have shown that a genetically determined variation of the RAS contributes to the increase in blood pressure in these conditions. Recently, a transgenic animal model was developed, which harbors the mouse Ren-2 gene. This gene is derived from some mouse strains, which express it highly in certain tissues and translate it into an unglycosylated protein. Transgenic animals develop fulminant hypertension. The model is characterized by an activation of the tissue RAS and a depressed plasma and kidney renin activity. As control, non-inbred SD rats were used. The controls were of the same strain from which the transgenic strain was generated. The apparent advantage of this model is the monogenic pathogenesis of hypertension, which allows the investigation of phenotypic changes in various organs caused by a genetic perturbation of one system, such as the RAS. This approach was also useful to overcome problems inherent in the choice of the appropriate controls in genetic models of spontaneous hypertension.

In heart failure in humans, a stimulation of the sympathetic nervous system occurs besides an activation of the RAS. Between both systems a close interrelation exists. Angiotensin II is known to facilitate norepinephrine release by presynaptic angiotensin II receptors. Furthermore, it stimulates the adrenergic system at central sites and activates sympathetic ganglion cells in the adrenal medulla. Although important pathophysiological differences between human heart failure and any rat model with local activation of the RAS would still remain, one experimental approach used to mimic the activation of the local RAS is the genetic overexpression of renin in various tissues, as occurs in TG(mREN2)27 rats. From these observations it appeared likely that the TG(mREN2)27 transgenic animals could also exhibit changes of neurohormonal adrenergic activation and postsynaptic neuroeffector mechanisms.

In TG(mREN2)27 rats, myocardial concentrations of norepinephrine and neuropeptide Y, which is coreleased from sympathetic nerve endings, were significantly reduced. Similar findings were reported in myocardial samples from patients with end-stage heart failure. Bristow et al reported a reduction of norepinephrine and neuropeptide Y levels by 90%. Since in severe heart failure norepinephrine is released from the myocardium, it has been suggested that the enhanced stimulation of cardiac sympathetic nerves produces a depletion of cardiac norepinephrine stores in the presence of elevated plasma norepinephrine concentrations. The similarity between TG(mREN2)27 animals and the failing...
human heart raises the question of whether similar changes of postsynaptic neuroeffector mechanisms occur.

On the cellular level, stimulation of sympathetic nerves results in the release of norepinephrine, which stimulates cardiac β- and β-adrenergic receptors, which couple through a heterotrimeric stimulatory G protein (αsβγ) to the catalytic subunit of the adenylyl cyclase, which forms cAMP from ATP. The increase of the cellular cAMP concentration activates a cAMP-dependent protein kinase and thereby initiates the increase in the cytosolic Ca\(^{2+}\) and the positive inotropic response.\(^{26}\) The adenylyl cyclase activity is also under the control of inhibitory G proteins and receptors, namely, A\(_1\)-adenosine receptors and m-cholinergic receptors, which produce antiadrenergic effects on contraction force.

In heart failure caused by dilated cardiomyopathy, the density of cardiac β-adrenergic receptors has been reported to be reduced, whereas the density of β\(_2\)-adrenergic receptors was unchanged.\(^{9,27}\) Similar observations were made in TG(mREN2)27 animals, in which the number of β\(_2\)-adrenergic receptors was reduced and the β\(_2\)-adrenergic receptor density unchanged. This finding is compatible with an increased stimulation of cardiac sympathetic nerves, because the released norepinephrine has an approximately 30 times higher potency to activate β\(_2\)-adrenergic receptors than to bind to β\(_1\)-adrenergic receptors.\(^{28}\) This effect is a true reduction of receptor molecules and cannot be explained by a different yield of membrane proteins, because similar results were obtained when the receptor density was related to 5'-nucleotidase activity as myocardial membrane marker. Consistent with the reduction of β-adrenergic receptors, the stimulation of adenylyl cyclase by the β-adrenergic receptor agonist isoproterenol was reduced. However, a reduced number of β-adrenergic receptors is not a general finding in models for hypertensive cardiac hypertrophy. In renal hypertensive rats,\(^{17,29}\) deoxycorticosteroid-treated rats, and Dahl salt-sensitive hypertensive rats,\(^{40,41}\) a desensitization of adenylyl cyclase in the absence of β-adrenergic receptor downregulation is well documented. Thus, data on receptor changes have to be interpreted with great caution and cannot be uncritically transferred from one model of hypertensive cardiac hypertrophy to another. In several models of secondary\(^{12}\) or genetic hypertension with cardiac hypertrophy\(^{25,26}\) as well as in human heart failure,\(^{11,12}\) a defect of the adenylyl cyclase complex distal to β-adrenergic receptor change has been characterized. In TG(mREN2)27 rats, the effects of the diterpen derivative forskolin, which directly stimulates the catalytic of the adenylyl cyclase,\(^{26}\) were studied. The effects of forskolin were slightly reduced in TG(mREN2)27 compared with SD rats. However, the effects of forskolin could depend on G\(_s\), in several tissues. Thus, the effects were studied in the presence of MnCl\(_2\), which has been reported to uncouple the catalyst from the influences of G proteins.\(^{27,28}\) Also under these conditions a slight but significant reduction in the effects of forskolin was observed, indicating that the catalytic activity is reduced in TG(mREN2)27 rats. Similar results were observed in deoxycorticosteroid-treated rats, in which a desensitization of adenylyl cyclase was observed that was mainly due to a depressed activity of the catalyst but independent of β-adrenergic receptor changes.\(^{17}\) In this respect, it is interesting that in TG(mREN2)27 rats, the transgene is highly expressed in the adrenals.\(^{44}\) The increased basal production of angiotensin II is a well-known stimulus for steroid secretion and production in the adrenals.\(^{45}\) Thus, one could speculate that increased steroid production could be involved not only in the elevation of blood pressure in TG(mREN2)27 rats but also in the depressed activity of the catalyst in the myocardium. Although all other components of the β-adrenergic receptor/G protein-regulated adenylyl cyclase system are similarly reduced in TG(mREN2)27 rats, even though the pathophysiology might be entirely different, this observation is different from what has been observed in human heart failure. In the latter condition, the effects of forskolin were only reduced when GTP was present in the medium,\(^{42}\) not when the effects of forskolin were studied in the absence of guanine nucleotides\(^{41}\) or when MnCl\(_2\)\(^{12}\) was added. Thus, in the human failing myocardium, the activity of the catalyst appeared to be unchanged.

A further localization of adenylyl cyclase defects in TG(mREN2)27 rats was suggested by the observed reduced effect of the metabolically stable guanine nucleotide derivative Gpp(NH)p. Since the effects of Gpp(NH)p were more reduced than those of forskolin, an additional alteration had to be suspected on the G protein level. This observation could be explained by a reduced activity of G\(_s\), on adenylyl cyclase activity. To address potential alterations of G\(_s\) function, G\(_s\) solubilized from TG(mREN2)27 and SD myocardial membranes were reconstituted with S49 ccr\(^+\) cell membranes. No differences were observed between the two groups. This is similar to the data obtained in failing human myocardium in which no decrease of G\(_s\) activity, as judged from reconstitution experiments,\(^{12}\) cholera toxin labeling of G\(_s\),\(^{12,46}\) or G\(_s\) mRNA levels,\(^{47,48}\) was observed.

The second possible mechanism for receptor-independent adenylyl cyclase desensitization was an increased amount of G\(_s\) proteins. In fact, pertussis toxin substrates increased markedly in myocardial membranes of TG (mREN2)27 compared with SD rats. However, the labeling of G\(_s\) at a C-terminal cysteine residue can be influenced by the substrate quality of G\(_s\), the amount of β\(\gamma\)-subunits and guanine nucleotides, and preexisting covalent modifications of the C-terminus.\(^{49}\) Therefore, an immunochemical technique to quantify G\(_s\) proteins was applied by using a polyclonal antiserum (MB 1) raised against the C-terminus of retinal transducin \(\alpha\) (KENLKDCGLF). With the use of immunoblots, a similar increase of G\(_s\) was detected. These findings suggest that the increase in pertussis toxin substrates indeed relates to a true increase in G\(_s\) protein expression. Similar results were obtained in various models of hypertensive cardiac hypertrophy. In spontaneously hypertensive rats, increases in pertussis toxin substrates,\(^{43,50}\) immunodetectable G\(_s\),\(^{43,50}\) and G\(_s\) mRNA levels,\(^{50,51}\) were observed. In renal hypertensive rats, rats with reduced renal mass,\(^{17}\) and Dahl salt-sensitive rats on a high sodium chloride diet,\(^{40}\) an increase in G\(_s\) expression was observed to accompany heterologous adenylyl cyclase desensitization. In addition, pertussis toxin substrates,\(^{11,13}\) immunochemical G\(_s\),\(^{11}\) and G\(_s\) mRNA were observed to be increased in patients with end-stage heart failure. Information on the causal relation between the increase in G\(_s\) and reduced adenylyl
cycase activity was provided by experiments in pertussis toxin–treated cardiac membranes. This experimental procedure was performed to inactivate Gβγ. As reported before for human myocardial membranes,12 the reduced basal and Gpp(NH)p-stimulated adenyl cyclase activities were completely antagonized by pertussis toxin treatment in TG(mREN2)27 rats. Thus, Gα is causally involved in heterologous adenyl cyclase desensitization in this model.

The mechanisms of increased Gβγ are not completely clear, although there is evidence that a prolonged stimulation of β-adrenergic receptors with a consequent increase in the cellular cAMP concentration could play a crucial role. In rats treated with isoproterenol, increases in pertussis toxin substrates52 and Gβγ mRNA levels53 were observed, whereas Gα, mRNA levels remained unchanged.54 In nuclear run-on assays, Müller et al54 showed that the increase in Gγ mRNA is caused by an increase in transcriptional activity after isoproterenol treatment in rats. Similarly, norepinephrine upregulated Gα protein expression in cultivated neonatal rat cardiomyocytes.55 This increase in Gα and the heterologous adenyl cyclase desensitization were sensitive to β-adrenergic receptor antagonists but not to α-adrenergic receptor blockers.55 Direct evidence for an involvement of cAMP was provided in S49 cell lines, which genetically lack Gβγ and the Gβγ-dependent protein kinase, respectively. In these models, β-adrenergic receptor stimulation did not induce any heterologous desensitization.56 These findings suggest that the induction of heterologous adenyl cyclase desensitization and the increased expression of Gβγ involve an intact receptor-cAMP-protein phosphorylation cascade. Taken together, the depleted norepinephrine stores, the β-selective adrenergic receptor downregulation, and the increased expression of Gα lead to the suggestion that the neuroeffector defects might be caused by an increased sympathetic nerve activity in TG(mREN2)27 rats.

If adenyl cyclase desensitization were relevant for myocardial contraction, one would expect that the positive inotropic responses to β-adrenergic receptor agonists would be reduced. This question was studied in isolated papillary muscle strips. The positive inotropic effects of isoproterenol were reduced in TG(mREN2)27 compared with SD rats. A reduction of the positive inotropic responsiveness after β-adrenergic stimulation was also observed in spontaneously hypertensive57 and renal hypertensive57 rats, suggesting that β-adrenergic subsensitivity in these models has a functional relevance for the regulation of contractile force. Again, in the failing human heart, the decline of β-adrenergic receptors and Gα proteins is closely related to a reduced responsiveness of the failing human heart to β-adrenergic receptor agonists and cAMP-phosphodiesterase inhibitors.58,59

In summary, in TG(mREN2)27 rats, a heterologous adenyl cyclase desensitization was observed that is caused by a downregulation of β-adrenergic receptor, an increased expression of Gα, and a mild dysfunction of the catalyst, whereas β2-adrenergic receptors and Gα, were unchanged. Because norepinephrine and neuropeptide Y stores are depleted in TG(mREN2)27 rats, an activation of cardiac sympathetic nerves could play a causal role in inducing the neuroeffector changes. The results show that in pathological situations in which the tissue RAS is activated, accompanying changes of the sympathetic nervous system are likely and emphasize the close interrelation between these two systems in cardiovascular disorders. Since the observed alterations of neuroeffector mechanisms are very similar to those observed in heart failure, one might speculate that these defects could already occur in hypertensive cardiac hypertrophy and could play an important pathophysiological role in the development of contractile dysfunction and progression to heart failure in later stages of the syndrome.

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Beta-adrenergic neuroeffector mechanisms in cardiac hypertrophy of renin transgenic rats.

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