Cardioprotection specific for the G protein G_{i2} in chronic adrenergic signaling through β_2-adrenoceptors

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Two subtypes of β-adrenoceptors, β_1 and β_2, mediate cardiac catecholamine effects. These two types differ qualitatively, e.g., regarding G protein coupling and calcium channel stimulation. Transgenic mice overexpressing human β_2-adrenoceptors survive high-expression levels, unlike mice overexpressing β_1-adrenoceptors. We examined the role of inhibitory G_i proteins, known to be activated by β_2- but not β_1-adrenoceptors, on the chronic effects of human β_2-adrenoreceptor overexpression in transgenic mice. These mice were crossbred with mice where $G\alpha_{i2}$, a functionally important cardiac G_i α-subunit, was inactivated by targeted gene deletion. Survival of β_2-adrenoreceptor transgenic mice was reduced by heterozygous inactivation of $G\alpha_{i2}$. Homozygous knockout/β_2-adrenoreceptor transgenic mice died within 4 days after birth. Heterozygous knockout/β_2-adrenoreceptor transgenic mice developed more pronounced cardiac hypertrophy and earlier heart failure compared with β_2-adrenoreceptor transgenic mice. Single calcium-channel activity was strongly suppressed in heterozygous knockout/β_2-adrenoreceptor transgenic mice. In cardiomyocytes from these mice, pertussis toxin treatment *in vitro* fully restored channel activity and enhanced channel activity in cells from homozygous $G\alpha_{i2}$ knockout animals. Cardiac $G\alpha_{i3}$ protein was increased in all $G\alpha_{i2}$ knockout mouse strains. Our results demonstrate that $G\alpha_{i2}$ takes an essential protective part in chronic signaling of overexpressed β_2-adrenoceptors, leading to prolonged survival and delayed cardiac pathology. However, reduction of calcium-channel activity by β_2-adrenoreceptor overexpression is due to a different pertussis-toxin-sensitive pathway, most likely by $G\alpha_{i3}$. This result indicates that subtype-specific signaling of β_2-adrenoreceptor functionally bifurcates at the level of G_i, leading to different effects depending on the $G\alpha$ isoform.

L-type calcium channel | single channel recording | mouse genetics | survival curve | pertussis toxin

Cardiac stimulatory catecholamine effects are mediated by both β_1- and β_2-adrenergic receptors (β-adrenoceptors), mainly through cAMP-dependent protein kinase A-catalyzed phosphorylation of cardiac proteins involved in calcium homeostasis, such as phospholamban and the L-type calcium channel. Evidently, this pathway is compromised in heart failure. Because of the clear-cut evidence that β_2-adrenoceptors mediate acute functional effects in human cardiomyocyte *in vitro* (1, 2) and *in vivo* (3), a rationale for β_2-adrenoreceptor gene therapy exists (4–6). Whether and where β_2-adrenoreceptor stimulation or inhibition, by pharmacological or genetic means, will have its place in heart failure therapy is an open question (7–9).

Inherent in the clinical discussion is recent molecular insight into differences between cardiac β_1- and β_2-adrenoreceptor stimulation at the signal-transduction level (10, 11). In rat cardiomyocytes, a β_2-agonist, zinterol, was shown to increase calcium current in a manner qualitatively distinct from β_1-adrenoreceptor stimulation (12), consistent with single-channel effects reported later (13). Xiao *et al.* (14) also showed that β_2- but not β_1-effects were potentiated by inactivation of $G_{i/o}$ protein by using pertussis toxin (PTX), and β_2-adrenoreceptor coupling to $G\alpha_{i2}$ and $G\alpha_{i3}$ could be directly demonstrated in mice (15). G_i coupling then may lead to activation of protein phosphatases (16), or activate, by means of c-Src and Ras, the mitogen-activated protein kinase pathway (17, 18). In addition, β_2- but not β_1-adrenoreceptor activation modulates sodium/proton exchanger in a G protein-independent manner (19). The distinct effect of β_1-adrenoreceptor vs. β_2-adrenoreceptor stimulation on cardiac apoptosis (20) also likely reflects (antiapoptotic) G_i signaling through β_2-adrenoreceptor activation (21, 22). Which of the abundant cardiac G_i proteins, i.e., $G\alpha_{i2}$ or $G\alpha_{i3}$, figures more prominently in these effects has hitherto not been addressed.

The most striking difference between β_1-adrenoreceptor and β_2-adrenoreceptor signaling in a pathophysiological sense comes from studies using a transgenic approach: mice overexpressing human β_2-adrenoceptors in the heart from early life (by α-MHC promotor) display increased contractility (23, 24) and calcium currents (25) at a young age. These effects vanish during adulthood (26, 27). β_2-Adrenoreceptor transgenic mice appear to have normal life expectancy (24) if expression levels are moderately high, i.e., <4,000–5,000 fmol of receptors per mg of membrane protein (28). In sharp contrast, mice containing a β_1-adrenoreceptor construct (at ≈600 fmol/mg) develop severe hypertrophy and die of heart failure (29).

It is tempting to hypothesize that β_2-adrenoreceptor stimulation, besides its known cardiac stimulatory effects, exerts some sort of protection against hypertrophy and failure, possibly by parallel chronic G_i signaling. Testing this idea could shed some light on the question whether the well known up-regulation of G_i found in human heart failure (e.g., ref. 30) is maladaptive or beneficial (31, 32).

PTX, an inhibitor of $G_{i/o}$ signaling, has been a valuable tool to dissect G_i-dependent components of β_2-adrenoreceptor signaling. This compound, because of its systemic toxicity, is ill-suited to study the phenomena associated with chronic β_2-adrenoreceptor overexpression and signaling *in vivo*. Therefore, we used a genetic approach to determine the role of G_i in chronic effects of β_2-adrenoreceptor signaling. To this end, we crossed mice with targeted deletion of $G\alpha_{i2}$ (33, 34) with mice overexpressing the human β_2-adrenoreceptor (23). We report here that the three most relevant endpoints, mortality, cardiac hypertrophy, and calcium-channel activity, are affected in heterozygous and homozygous $G\alpha_{i2}$ knockout/β_2-adrenoreceptor transgenic mice. We conclude that $G\alpha_{i2}$ figures crucially in the prevention of

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Abbreviation: PTX, pertussis toxin.

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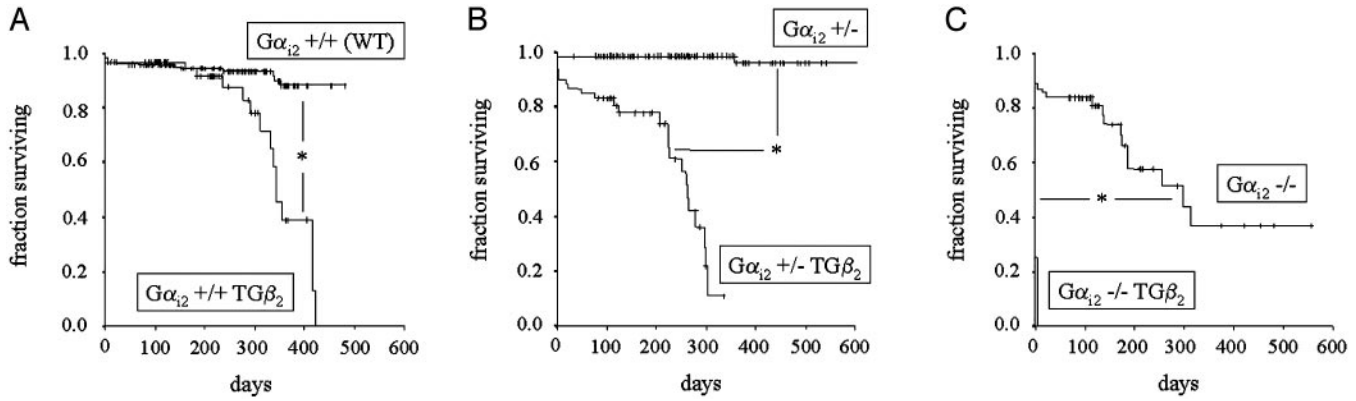

Fig. 1. Kaplan–Meier survival curves of knockout and transgenic mice. Vertical bars indicate a censoring event (usually representing killing of an animal for experiment). (*A*) WT (Gα_{i2} +/+) and β_2-adrenoreceptor transgenic (TG β_2) mice. (*B*) Heterozygous Gα_{i2} knockout mice without (Gα_{i2} +/−) or with (Gα_{i2} +/− TG β_2) cardiac overexpression of β_2-adrenoceptors. (*C*) Homozygous Gα_{i2} knockout mice without (Gα_{i2} −/−) or with (Gα_{i2} −/− TG β_2) cardiac overexpression of β_2-adrenoceptors. *, Significant difference (log-rank test) among the two curves depicted, respectively.

hypertrophy and in survival, but not in the calcium-channel-suppressing function of chronic β_2-adrenoreceptor signaling.

Materials and Methods

Animals. Mice with cardiac-specific heterozygous overexpression of human β_2-adrenoceptors (TG β_2, ref. 23) were fully back-crossed (>5 generations) into the C57/Bl6 strain and bred with their respective nontransgenic littermates. Littermates served as the WT (or Gα_{i2} +/+) controls. Gα_{i2} knockout animals (Gα_{i2} −/−, ref. 33) were fully back-crossed (>5 generations) into the C57/Bl6 strain. Offspring were generated by breeding heterozygous and homozygous knockout animals. Hybrid mice (Gα_{i2} +/− TG β_2 and Gα_{i2} −/− TG β_2) were generated by using three breeding schemes: mating of Gα_{i2} −/− × Gα_{i2} +/+ TG β_2 yielded offspring at approximately Mendelian distribution (Gα_{i2} +/− TG β_2, $n = 21$; Gα_{i2} +/−, $n = 26$). Mating of G$_i\alpha_2$ −/− × G$_i\alpha_2$ +/− TG β_2 yielded less than expected Gα_{i2} −/− TG β_2 ($n = 2$), whereas the other genotypes occurred at the expected frequencies (Gα_{i2} +/− TG β_2, $n = 8$; Gα_{i2} +/−, $n = 9$; Gα_{i2} −/−, $n = 6$). In 23 breeding protocols using Gα_{i2} +/− TG β_2 × Gα_{i2}+/−, the following genotypes were obtained at approximate Mendelian frequency: Gα_{i2} +/+, $n = 26$; Gα_{i2}+/−, $n = 28$; Gα_{i2} −/−, $n = 16$; Gα_{i2} +/+ TG β_2, $n = 17$; Gα_{i2} +/− TG β_2, $n = 30$; again except for Gα_{i2} −/− TG β_2, ($n = 6$), which fell below expectation by half (4.9% instead of 12.5%).

Genotyping. A tail-clip analysis was performed at 3–4 weeks of age. After preparation of genomic DNA, a PCR was run. To genotype Gα_{i2} (GenBank accession no. NM_008138), we used the following primer pairs: wild type (+), forward, 5′-GAT CAT CCA TGA AGA TGG CTA CTC AGA AG-3′; reverse, 5′-CCC CTC TCA CTC TTG ATT TCC TAC TGA CAC-3′. Knockout (−), forward, 5′-CAG GAT CAT CCA TGA AGA TGG CTA C-3′; reverse, 5′-GCA CTC AAA CCG AGG ACT TAC AGA AC-3′.

Both reactions were run over 35 cycles (saturation). Amplified sequences were 805 bp for the WT allele and 509 bp for the targeting construct.

A similar strategy was used to identify the presence of the human β_2-adrenoreceptor transgene (accession no.Y00106) in mice (23). The following primers, human β_2-adrenoreceptor, forward, 5′-ACA TTG TGC ATG TGA TCC-3′; reverse, 5′-ATT CCT CCC TTG TGA ATC-3′, when used over 35 PCR cycles, yielded a strong signal of the expected size (337 bp) in native human myocardial tissue and in transgenic animals. However, a weak signal could also be seen with endogenous murine β_2-adrenoceptors, which are 82% homologous at the cDNA level. To eliminate these wrong positive results, PCR products were extracted, precipitated, washed, and subjected to digestion with *Eco*RV, which recognizes a restriction site unique to the human gene, leading to specific fragments (219 and 118 bp in length).

Isolation of Cardiac Myocytes. Single ventricular myocytes were isolated from the hearts of 3- to 9-month-old mice by enzymatic dissociation by using the method described (27). In brief, hearts were perfused with a collagenase solution (Worthington type I and II, 75 units·liter^{-1}) in a Langendorff setup and subsequently cut into small chunks. Myocytes were harvested by pouring the suspension through cheesecloth.

Single-Channel Recording. Single-channel recordings were performed by using the cell-attached configuration of the patch-clamp method as described (35). Cells were placed in disposable Petri dishes containing 3 ml of a high-potassium depolarizing solution [25 mM KCl/120 mM potassium glutamate/2 mM $MgCl_2{\cdot}6H_2O$/10 mM Hepes/2 mM EGTA/10 mM dextrose/1 mM $CaCl_2$/1 mM Na_2-ATP (pH 7.3) with KOH]. The patch pipettes (borosilicate glass, 6–8 MΩ) were filled with the pipette solution (70 mM $BaCl_2{\cdot}2H_2O$/110 mM sucrose/10 mM Hepes, with pH adjusted to 7.4 with tetraethylammonium hydroxide). Ba^{2+} currents were elicited by voltage steps (150 ms at 1.66 Hz) from −100 mV to +20 mV (≥180 sweeps per experiment). Data were sampled at 10 kHz and filtered at 2 kHz (−3 dB, four-pole Bessel) by using an Axopatch 200 A amplifier (Axon Instruments, Foster City, CA). PCLAMP software (CLAMPEX 5.5.1,

Table 1. Body weight and heart weight of transgenic animals

Animals	Body weight, g	Heart weight, g	Heart/body weight ratio, %	Animals, *n*
WT	29.7 ± 0.8	0.164 ± 0.007	0.55 ± 0.02	10
Gα_{i2} +/−	32.9 ± 1.3	0.154 ± 0.005	0.47 ± 0.02	10
Gα_{i2} +/+ TGβ_2	34.1 ± 4.2	0.221 ± 0.039	0.65 ± 0.12	6
Gα_{i2} +/− TGβ_2	25.3 ± 0.9†	0.291 ± 0.012*	1.15 ± 0.07*	10

Body and heart weights were determined after killing or spontaneous death of 7- to 10-month-old animals of different genotypes. Mean ± SEM of *n* animals per group. Gα_{i2} +/− TGβ_2 mice revealed. *, Significant ($P < 0.05$) differences versus all other genotypes; †, significant ($P < 0.05$) differences versus Gα_{i2} +/− and Gα_{i2} +/+ TGβ_2 (ANOVA and posttests with Bonferroni correction, $P < 0.05$).

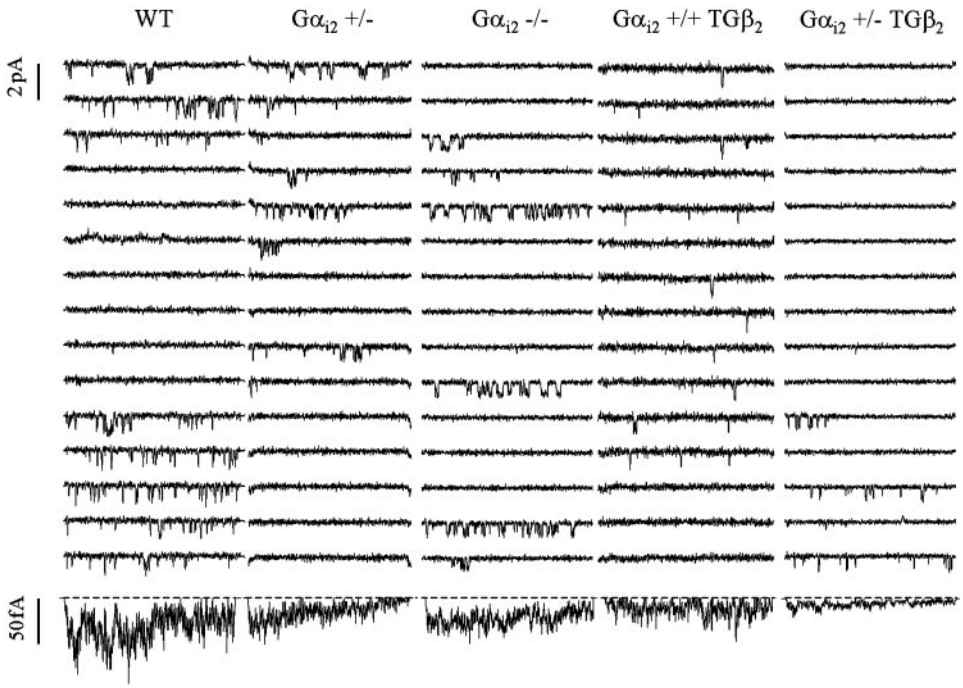

Fig. 2. Comparison of gating properties (70 mM Ba^{2+}) observed for the different genotypes (for abbreviations, see Fig. 1). Fifteen consecutive single-channel sweeps (150-ms pulses to +20 mV; holding potential, −100 mV) are shown together with the ensemble average current of the whole experiment (bottom traces, from left to right, 180, 180, 240, 180, and 360 sweeps). Note that the single-channel activity is markedly lower in the Gα_{i2} +/− TGβ_2 cell (compare with Table 2).

FETCHAN, and PSTAT 6) was used for data acquisition and analysis (Axon Instruments).

Data Analysis and Statistics. Mostly, experiments with one single channel (i.e., no stacked openings above unitary amplitude level) were analyzed in this study. Linear leak and capacity currents (averaged nonactive sweeps) were digitally subtracted. Openings and closures were identified by the half-height criterion. The fraction of active sweeps within a channel-containing patch (availability), the open probability within active sweeps (open probability), and the peak value of single-channel ensemble average currents (I_{peak}) were determined as described (35). In double-channel patches, these parameters were corrected for the number of channels as described (36). Time constants of open- and closed-time histograms were estimated by the maximum-likelihood method (37). For multiple comparisons among groups, ANOVA followed by Bonferroni-corrected posttest was done. For simple comparisons (e.g., PTX effects), an unpaired Student's two-tailed t test was used. Kaplan–Meier survival curves were statistically compared by log-rank test. Throughout, a level of $P < 0.05$ was considered significant. Values are given as mean ± SEM.

SDS/PAGE and Western Blot Analysis. For isolation of cardiac membrane protein fractions, mouse hearts where frozen in liquid nitrogen and homogenized in a buffer containing 50 mM

Table 2. Calcium-channel gating under basal conditions

Conditions	WT	Gα$_{i2}$ +/−	Gα$_{i2}$ −/−	Gα$_{i2}$ +/+ TGβ$_2$	Gα$_{i2}$ +/− TGβ$_2$
I_{peak}, fA	49.2 ± 13.1	25.2 ± 6.3	26.4 ± 5.2	32.3 ± 8.8	20.8 ± 10.4
Availability, %	41.5 ± 5.3	34.7 ± 3.4	42.4 ± 5.5	47.7 ± 7.7	21.1 ± 4.1†
Open probability, %	8.94 ± 1.99	7.00 ± 2.43	5.88 ± 1.11	4.75 ± 1.19	4.57 ± 2.25
Mean open time, ms	0.45 ± 0.03	0.44 ± 0.05	0.37 ± 0.05	0.46 ± 0.07	0.35 ± 0.06
τ_{open}, ms	0.42 ± 0.04	0.43 ± 0.06	0.32 ± 0.05	0.46 ± 0.07	0.29 ± 0.06
Mean closed time, ms	4.27 ± 0.99	3.81 ± 0.92	3.99 ± 0.6	7.53 ± 1.89	8.57 ± 2.96
$\tau_{closed1}$, ms	0.49 ± 0.07	0.54 ± 0.07	0.64 ± 0.09	1.04 ± 0.29	0.45 ± 0.08†
Proportion	0.66 ± 0.04	0.74 ± 0.04	0.75 ± 0.03	0.55 ± 0.07*	0.72 ± 0.04
$\tau_{closed2}$, ms	12.0 ± 2.5	13.5 ± 2.6	14.0 ± 1.3	16.5 ± 2.6	23.3 ± 9.6
Proportion	0.34 ± 0.04	0.26 ± 0.04	0.25 ± 0.03	0.45 ± 0.07*	0.28 ± 0.04
n	19	16	15	12	13
n_o	13	11	11	9	12

Measurements were carried out at a test potential of +20 mV (holding potential, −100 mV) by using 70 mM Ba^{2+} as charge carrier. Mean ± SEM of n experiments are given. Peak ensemble average current (I_{peak}), availability (fraction of active sweeps within a channel containing patch), and open probability were corrected for the number of channels in multichannel patches. Closed times were analyzed for one-channel patches only (n_o). *,Significant versus Gα$_{i2}$ +/− and Gα$_{i2}$ −/− (ANOVA followed by posttests with Bonferroni correction, $P < 0.05$); †,Significant versus Gα$_{i2}$ +/+ TGβ$_2$ (ANOVA followed by posttests with Bonferroni correction, $P < 0.05$).

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Table 3. Effect of PTX on calcium-channel gating

Conditions	WT	$G\alpha_{i2}$ +/−	$G\alpha_{i2}$ −/−	$G\alpha_{i2}$ +/+ TGβ_2	$G\alpha_{i2}$ +/− TGβ_2
I_{peak}, f*A*	60.3 ± 26.0	56.7 ± 12.2*	68.8 ± 15.6*	44.2 ± 11.0	93.3 ± 31.8*
Availability, %	58.9 ± 10.0	47.8 ± 8.4	53.4 ± 6.2	53.2 ± 5.3	68.7 ± 7.7*
Open probability, %	10.5 ± 2.56	16.0 ± 4.0	13.3 ± 4.2	7.2 ± 1.4	17.3 ± 9.0
Mean open time, ms	0.32 ± 0.06	0.49 ± 0.08	0.42 ± 0.07	0.65 ± 0.06	0.48 ± 0.11
τ_{open}, ms	0.32 ± 0.08	0.50 ± 0.09	0.43 ± 0.14	0.61 ± 0.08	0.44 ± 0.08
Mean closed time, ms	3.67 ± 1.04	2.16 ± 0.74	2.05 ± 0.77	3.38 ± 0.83	2.00 ± 0.65
$\tau_{closed1}$, ms	0.47 ± 0.03	0.31 ± 0.07	0.34 ± 0.08	0.44 ± 0.07	0.24 ± 0.05
Closed1 fraction, %	70 ± 3	76 ± 4	78 ± 4	52 ± 6	74 ± 6
$\tau_{closed2}$, ms	11.2 ± 2.4	6.94 ± 2.72	7.04 ± 2.57*	9.51 ± 2.64	5.69 ± 3.21
n	6†	6	4	6†	3
n_o	6	5	4	4	3

Measurements were carried out after a 3-h incubation with PTX (1.5 μg/ml, 37°C) at a test potential of +20 mV (holding potential, −100 mV), using 70 mM Ba^{2+} as charge carrier. Mean ± SEM of *n* experiments are given. The peak value of ensemble average currents (I_{peak}), availability (fraction of active sweeps within a channel containing patch), and open probability were corrected for the number of channels in multichannel patches. Closed times were analyzed for one-channel patches only (n_o). *,Significant versus baseline values (Table 2) of the respective genotype (*t* test, $P < 0.05$).

†Includes data from unpublished experiments ($n = 3$ for WT and $n = 4$ for $G\alpha_{i2}$ +/+ TGβ_2) performed during our previous study (27).

Tris·HCl and 1 mM phenylmethylsulfonyl fluoride, pH 7.4. The homogenized tissue was centrifuged (15 min, 3,400 × *g*), and the resulting supernatant was recentrifuged (40 min, 100,000 × *g*). The resulting pellets were solubilized in sample buffer [50 mM Tris·HCl (pH 6.8)/2% (wt/vol) SDS/20% (vol/vol) glycerol/5 mM DTT], shaken for 30 min, and subjected to SDS/PAGE on a 12.5% acrylamide running gel and a 4% stacking gel. For protein analysis an equal amount of 15 μg per homogenate was loaded. SDS/PAGE was blotted to poly(vinylidene difluoride) membranes (Bio-Rad) by using a semidry Western blot system. $G\alpha_s$, $G\alpha_{i2}$, and $G\alpha_{i3}$ proteins were detected by specific antisera (Sigma-Aldrich product nos. 65090, 64090, and 64915, respectively; dilution, 1:2,000). Protein bands were analyzed densitometrically.

Results and Discussion

Generation and Survival of Transgenic Mice. In all breeding schemes used to generate knockout ($G\alpha_{i2}$ +/−, $G\alpha_{i2}$ −/−), transgenic (TG β_2), or hybrid, double-mutant ($G\alpha_{i2}$ +/− TG β_2) mice, viable offspring were obtained at roughly Mendelian rates (see *Materials and Methods*). Notably, $G\alpha_{i2}$ −/− TG β_2 were observed at less than half of the expected Mendelian distribution. At birth, these animals appeared smaller and weaker than their littermates.

Survival was monitored for all genotypes for at least 1 year (Fig. 1). WT and $G\alpha_{i2}$ +/− did not reveal mortality or any clinical signs of disease during this period. (Fig. 1*A* and *B*). Mean survival was 445 ± 8 days (WT, $n = 227$) and 642 ± 9 days ($G\alpha_{i2}$ +/−, $n = 191$), respectively. In contrast, $G\alpha_{i2}$ −/− developed gastrointestinal symptoms (diarrhea, rectal prolapse, anal bleeding), loss of body weight (significant vs. $G\alpha_{i2}$+/− and $G\alpha_{i2}$ +/+ in male animals) and fatigue after a few months. Survival time (307 ± 40 days, $n = 59$) was significantly shorter than with heterozygous $G\alpha_{i2}$ +/−. These findings qualitatively corroborate the original description of this mouse line (33).

TG β_2 mice, in agreement with Liggett *et al.* (28), developed fatigue, dyspnea, and cyanosis of mucous membranes when approaching 1 year of age. The survival (mean, 340 ± 17 days,

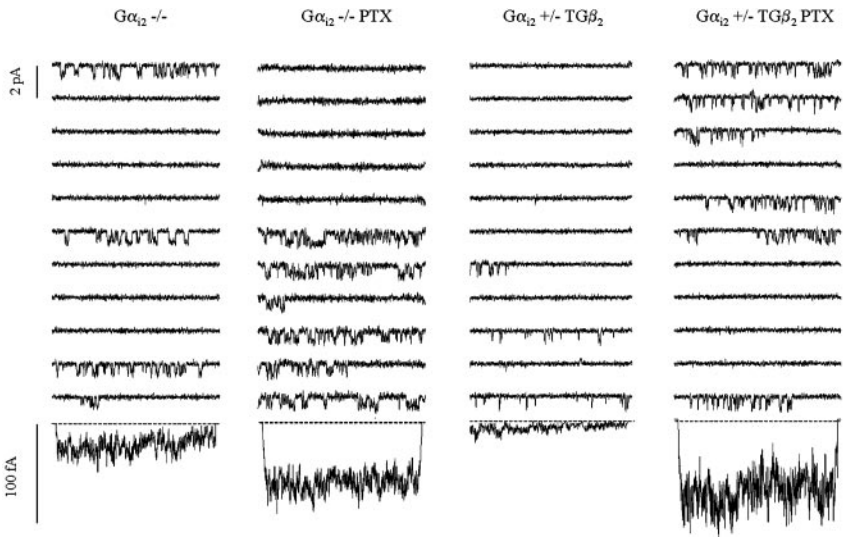

Fig. 3. PTX (3-h incubation with 1.5 μg/ml, 37°C) elevates single-channel activity in $G\alpha_{i2}$ −/− cells (*Left*) and restores unitary events and average currents toward WT levels in $G\alpha_{i2}$ +/− TGβ_2 (*Right*). Note that control and PTX data are from different cells, respectively. Eleven consecutive single-channel sweeps (compare with Fig. 2) are shown together with the average current of the whole ensemble (bottom traces, from left to right, 240, 360, 360, and 240 sweeps).

$n = 61$) curve (Fig. 1*A*) fell steeply in this age range, and most animals had died by 1 year of age (significant vs. WT). Heterozygous hybrid mice ($G\alpha_{i2}$ +/− TG β_2) showed a similar clinical phenotype, but at earlier ages (Fig. 1*B*). Survival was significantly shortened (mean, 223 ± 15 days, $n = 59$), compared with both $G\alpha_{i2}$ +/− and nonhybrid TG β_2 mice. At necropsy, these animals ($G\alpha_{i2}$ +/− TG β_2) revealed lung and liver congestion, ascites, and an enlarged and dilated heart, often with thrombotic material in the atria. Only in a few cases (3 of 24), death occurred without prior clinical signs, and, in all these cases, massive thrombi were found in the atrial cavities. Heart weight of heterozygous hybrid mice was enhanced at the time of death, compared with the other genotypes (killed at a similar age range of 7–10 months). Together with a slightly reduced body weight, this gave rise to a highly significant increase of heart/body weight ratio (Table 1) in $G\alpha_{i2}$ +/− TG β_2, indicating cardiac hypertrophy. At the same age, heart/body weight ratio was slightly but not significantly enhanced in simple transgenic TG β_2 mice, in agreement with their clinical phenotype, developing heart failure and death at a higher age (Fig. 1).

All homozygous hybrid mice ($G\alpha_{i2}$ −/− TG β_2, $n = 8$) died between day 1 and 4 after birth (Fig. 1*C*). Necropsy revealed a dilated heart in the two cases examined macroscopically. Unfortunately, autolysis at the time of necropsy prevented meaningful histological examination. All these findings are in line with our hypothesis that protective effects (prolonged survival, suppression of cardiac hypertrophy and failure) are mediated by $G\alpha_{i2}$ in mice overexpressing the β_2-adrenoreceptor.

Calcium-Channel Gating Under Basal Conditions. Single channels were examined by using the cell-attached configuration, with the same protocol (holding potential, −100 mV; test potential, +20 mV; 70 mM $BaCl_2$) as described (27). All genotypes except for the homozygous hybrid mice ($G\alpha_{i2}$ −/− TG β_2) could be tested at adult age. The apparent channel density (i.e., the probability to find at least one channel in a technically stable patch) amounted to 18–21% in all genotypes, suggesting that our single-channel data are predictive for whole-cell current levels, which were not measured in this study. The majority of patches analyzed contained only one active channel (see Tables 2 and 3). Channel activity (Fig. 2 and Table 2) was similar to our report (27) regarding WT and TG β_2 mice, although the reduction in single-channel activity in TG β_2 turned out to be nonsignificant in this study. This outcome may be due to comparably large scatter (we used a wider age range, but age-dependent changes were not obvious), or perhaps because we now used monoallelic transgene carriers throughout (see *Materials and Methods*). Channel activity was not significantly different between WT and $G\alpha_{i2}$ −/− mice, confirming earlier studies at the level of whole-cell currents (38) or cardiac contractility (39). In striking contrast, a clear-cut reduction in single-channel activity and ensemble average currents (Fig. 2 and Table 2) was observed with heterozygous hybrid $G\alpha_{i2}$ +/− TG β_2 mice. Both a reduced availability (fraction of active sweeps within a channel-containing patch) and a lower open probability due to altered closed time distribution contributed to this effect. The phenomenon is qualitatively similar to, but more pronounced than our observations with TG β_2 mice (27). Therefore, loss of one allele of $G\alpha_{i2}$ did not ameliorate but rather aggravated the suppression of calcium-channel activity induced by β_2-adrenoreceptor overexpression, in contrast to our initial hypothesis. An explanation would be that other G proteins, such as $G\alpha_{i3}$, mediate the suppressive effects of β_2-adrenoceptors on the L-type calcium channel. To test this idea, myocytes of all available genotypes were pretreated with PTX (1.5 μg/ml, 3 h, 37°C), and single-channel activity was examined afterward.

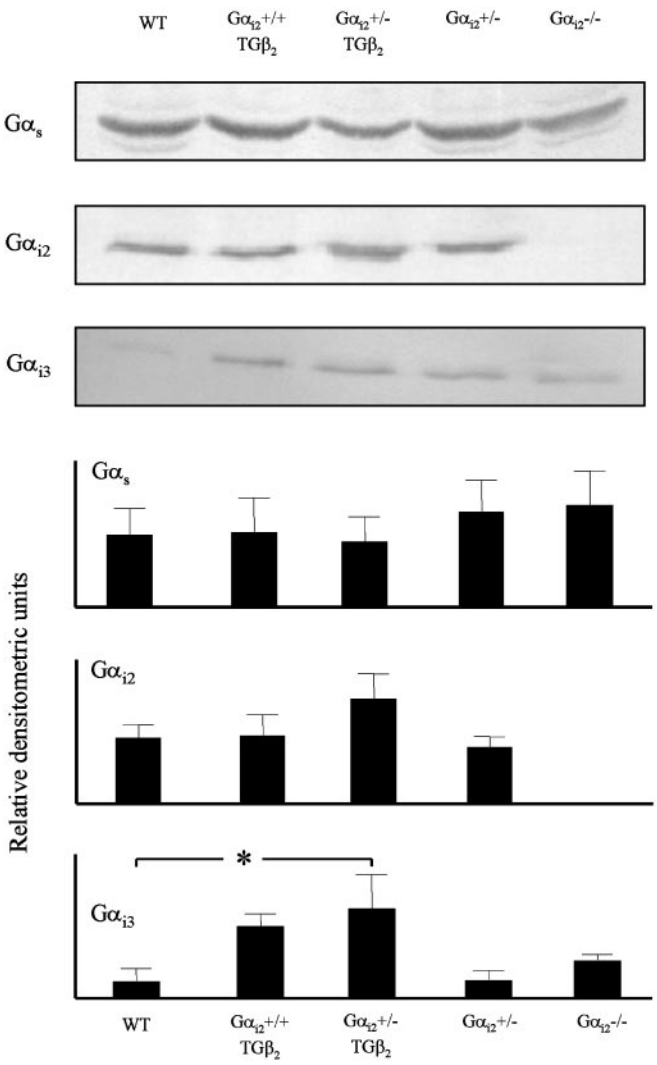

Fig. 4. G protein expression in murine cardiac membrane homogenates depending on genotype (for abbreviations, see Fig. 1). G_s levels are unaltered, whereas $G\alpha_{i2}$ is not detectable in $G\alpha_{i2}$ −/− and tends to be enhanced in $G\alpha_{i2}$ +/− TGβ_2. $G\alpha_{i3}$ appears somewhat up-regulated in $G\alpha_{i2}$ −/− and is markedly increased in TGβ_2 and $G\alpha_{i2}$ +/− TGβ_2 mouse heart. Statistics (mean ± SEM) are derived from $n = 5$–7 samples per genotype for G_s, $G\alpha_{i2}$, and $G\alpha_{i3}$. *, Significant difference (ANOVA and posttests with Bonferroni correction, $P < 0.05$).

Effects of PTX on Single-Channel Gating. PTX pretreatment had no effect on WT ($G\alpha_{i2}$ +/+) channels (Table 3), as reported (27). However, in channels from homozygous mice lacking $G\alpha_{i2}$ ($G\alpha_{i2}$ −/−), PTX significantly elevated single-channel activity (Fig. 3 and Table 3), and normal channel activity was fully restored in channels from heterozygous hybrid mice ($G\alpha_{i2}$ +/− TG β_2), as depicted in Fig. 3 and statistically verified in Table 3. Even in heterozygous knockout mice ($G\alpha_{i2}$ +/−), a significant elevation (compared with baseline, Table 2) of channel activity was observed after PTX pretreatment. The changes in ensemble average current were mostly due to an elevation of open probability and a shortening of closed times, exactly opposing the phenomena induced by β_2-adrenoreceptor overexpression. This finding argues in favor of a role for a PTX-sensitive G protein other than $G\alpha_{i2}$ to regulate calcium-channel activity. $G\alpha_{i3}$ is a likely candidate, given its abundance in heart and stimulation by β_2-adrenoreceptor activation (15). Therefore, levels of G proteins were measured by Western blot analysis.

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Expression Levels of G Proteins. Protein levels of $G\alpha_s$, $G\alpha_{i2}$, and $G\alpha_{i3}$ were determined as depicted in Fig. 4. G_s levels were not significantly different among genotypes. $G\alpha_{i2}$ was undetectable in homozygous mice ($G\alpha_{i2}$ $-/-$) as expected. Levels seemed slightly enhanced in heterozygous hybrid mice ($G\alpha_{i2}$ $+/-$ TG β_2), confirming earlier findings in adult TG β_2 mice (40). $G\alpha_{i3}$ levels appeared to be increased in homozygous knockouts ($G\alpha_{i2}$ $-/-$), in line with observations of Rudolph *et al.* (34) in other tissues and cells. More strikingly, a severalfold increase was observed in β_2-adrenoreceptor transgenic hearts and in hybrid mutant mice ($G\alpha_{i2}$ $+/-$ TG β_2, significant vs. WT). Taken together, those genotypes reveal an increased expression of $G\alpha_{i3}$, where reduction in single-channel activity was described in this (Table 2) and the previous study (27), and where PTX raises basal channel activity (Table 3). Up-regulation of $G\alpha_{i3}$ probably represents compensation for lack of $G\alpha_{i2}$ (function) (34, 41). $G\alpha_{i3}$ is the most likely candidate to exert suppression of calcium-channel activity, although a role of other PTX substrates ($G\alpha_{i1}$ or $G\alpha_o$) cannot be safely excluded at present. Distinct roles of $G\alpha_{i2}$ and $G\alpha_{i3}$ have already been described for muscarinic regulation of cardiac ventricular calcium currents, which fully depends on $G\alpha_{i2}$ but not $G\alpha_{i3}$ (38, 42).

In conclusion, our data strongly suggest that cardiac hypertrophy and failure due to β_2-adrenoreceptor overexpression are compensated by $G\alpha_{i2}$ activation, but single-channel regulation of L-type calcium channels follows a different pathway. We propose that calcium-channel regulation is rather mediated by $G\alpha_{i3}$, and this G protein is indeed up-regulated. Therefore, the chronic β_2-adrenoreceptor stimulation drives G_i protein isoforms to several distinct, subtype-specific signaling cascades, the details of which merit further investigation.

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