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The effect of Gi-protein inactivation on basal, and β_1- and β_2AR-stimulated contraction of myocytes from transgenic mice overexpressing the β_2-adrenoceptor

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1 The atria and ventricles of transgenic mice (TGβ_2) with cardiac overexpression of the human β_2-adrenoceptor (β_2AR) were initially reported to show maximum contractility in the absence of β-AR stimulation. However, we have previously observed a different phenotype in these mice, with myocytes showing normal contractility but reduced βAR responses. We have investigated the roles of cyclic AMP and Gi in basal and βAR function in these myocytes.

2 ICI 118,551 at inverse agonist concentrations decreased contraction by 32%. However, the cyclic AMP antagonist Rp-cAMPS had no effect on contraction in TGβ_2 myocytes, indicating that there was no tonic influence of raised cyclic AMP. These findings cannot be explained by the proposed model for inverse agonism, where the activated receptor (R*) raises cyclic AMP levels and so increases contraction in the absence of agonist.

3 After pertussis toxin (PTX) pretreatment to produce inactivation of Gi, the basal contraction in 1 mM Ca^{2+} was increased in TGβ_2 mice ($7.82 \pm 0.47\%$, $n=23$) compared to LM mice ($3.60 \pm 0.59\%$, $n=11$) ($P<0.001$). The contraction amplitude of myocytes to the maximal concentration of isoprenaline was also increased significantly by PTX in TGβ_2 mice ($9.40 \pm 1.22\%$, $n=8$) and was no longer reduced compared to LM mice ($8.93 \pm 1.50\%$, $n=11$). Both β_1- and β_2AR subtypes were affected both by the original desensitization and by the resensitization with PTX.

4 PTX treatment has therefore restored the original phenotype, with high basal contractility and little further effect of isoprenaline. We suggest that both β-AR desensitization and lack of increased basal contraction in ventricular myocytes from our colony of TGβ_2 mice were due to increased activity of PTX-sensitive G-proteins.

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Abbreviations: β_2AR, β_2-adrenoceptor; Gi, inhibitory G-protein; ISO, isoprenaline; LM, littermate; PTX, Pertussis toxin; TG, transgenic

Introduction

β-Adrenoceptors (βARs) are integral membrane proteins mediating the effects of catecholamines on heart rate and force. β_1 and β_2 subtypes increase contractile force through an action on adenylyl cyclase, coupled via the stimulatory guanine nucleotide binding protein (Gs) (Kaumann & Molenaar, 1997). The β_1AR subtype is the predominant influence on ventricular contraction, but the β_2ARs assume greater importance in man, especially during the development of heart failure (del Monte *et al*., 1993; Harding *et al*., 1994). The β_1AR-selective β-blockers now being used for heart failure (as well as angina and hypertension) can upregulate the β_2AR subtype in atria (Hall *et al*., 1990). Recent evidence suggests that a polymorphism in the β_2AR subtype has a strong prognostic effect for mortality in patients with cardiomyopathies (Liggett *et al*., 1998).

Because of this interest in the β_2AR subtype, a transgenic mouse was created with a large (200 fold) overexpression of the human β_2AR (TGβ_2) (Milano *et al*., 1994). The TGβ_2 phenotype itself initially showed high basal contractility, with little further stimulation by isoprenaline. Certain βAR antagonists, termed inverse agonists (Bond *et al*., 1995), produced negative inotropic effects and reduced the high basal contractility in isolated atria. Neutral antagonists blocked this negative inotropic effect. This led to the suggestion that a proportion of the β_2ARs were spontaneously active in the absence of agonists: in the overexpressing mice this proportion was sufficient to increase contraction to levels equivalent to those produced by maximum isoprenaline stimulation.

However, later studies confirmed the lack of response to isoprenaline but did not detect the raised basal contractility (Heubach *et al*., 1999). It was suggested that an adaptation had occurred to oppose the effect of the spontaneously active β_2ARs. β_2ARs in a cell line have been shown to couple to both Gs and Gi (the inhibitory guanine nucleotide-binding protein), with phosphorylation by protein kinase A (PKA) switching the β_2AR towards Gi (Daaka *et al*., 1997). It would be predicted that PKA activity would be high in TGβ_2 myocardium, predisposing to Gi coupling. Additionally, Gi levels are reported to be increased in the TGβ_2 mouse (Nagaraja *et al*., 1997). Activation of Gi by muscarinic or adenosine A_1 receptors is known to suppress the effects of simultaneous stimulation through Gs. It was therefore hypothesized that excess Gi coupling was decreasing the observed effects of the overexpressed β_2AR in the hearts of TGβ_2 mice.

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In this investigation we have confirmed that basal contractility is not increased in the myocytes from TGβ_2 mice at either low or high Ca^{2+} levels, and that cyclic AMP does not support contraction in the absence of βAR stimulation. By using isolated ventricular myocytes we have excluded the possibility that endogenously released catecholamines might be modulating contractility. We show that pertussis toxin treatment, which inactivates Gi, restores responses to βAR stimulation in myocytes from TGβ_2 ventricle. In this we agree with another study, which has been published while the experiments reported here were in progress (Xiao *et al*., 1999). We further show here that both overexpressed β_2AR and the native β_1AR are involved in the spontaneous down-regulation and pertussis toxin-induced recovery of response. Additionally, we demonstrate that pertussis toxin treatment increases basal contraction of TGβ_2 myocytes towards levels seen during agonist stimulation, thus completely restoring the original phenotype.

Methods

Confirmation of transgenic status

All studies complied with the United Kingdom Home Office Regulation Governing the Care and Use of Laboratory Animals. TGβ_2 mice and non-transgenic littermate mice had a mixed genetic background and their age ranged from 8 weeks to 12 months. Animals were derived from transgenic/transgenic or littermate/littermate pairings. The genetic status of the offspring was checked prior to experimentation. Mouse genomic DNA was extracted from tail clips by the Genera Purgene system with DNeasy Tissue Kit (Qiagen Ltd., U.K.). TGβ_2 mice were identified by Southern blot analysis with an 850 bp SV-40 DNA probe (Milano *et al*., 1994). The method did not distinguish between homo-and heterozygous genotype for the human β_2AR transgene.

Isolation of ventricular myocytes

Ventricular myocytes were isolated by enzymatic dissociation as previously described (Heubach *et al*., 1999). Mice were killed by cervical dislocation and the heart quickly removed and placed into ice-cold Krebs-Henseleit (KH) solution (composition in mM: NaCl 119, KCl 4.7, $MgSO_4$ 0.94, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.5, $CaCl_2$ 1), previously bubbled with 95% O_2/5% CO_2 to bring the pH to 7.4. The heart was then mounted on a needle canal (23G 1 1/4″) attached to a Langendorff perfusion apparatus and equilibrated with KH solution containing 1 mM Ca^{2+} for 5 min at 37°C. The perfusate was then changed to a low calcium (LC) medium of the following composition (mM): NaCl 120, KCl 5.4, $MgSO_4$ 5, pyruvate 5, glucose 20, taurine 20, HEPES 10, nitrilotriacetic acid 5, containing 12–15 μM, calcium as measured with a calcium electrode, also at 37°C and bubbled with 100% O_2 at pH 6.95, for 5 min. This was followed by 1 min perfusion with the same low calcium solution (with nitrilotriacetic acid omitted) at pH 7.4 with 200 μM calcium and 2 I.U. (Sigma P-8038) protease type XXIV added. This enzyme solution was then switched to one containing no added protease but 1.0 mg ml^{-1} (Worthington 4376 CLS-2) collagenase and 0.6 mg ml^{-1} (Sigma H-3506) hyaluronidase for a further 10 min, after which the ventricles were chopped and incubated in the same enzyme solution for 2×10 min. The medium was shaken gently throughout this incubation at 35°C, and kept under an atmosphere of 100% O_2, the dispersed cells were then strained through gauze of mesh size 300 μm and supernatant was centrifuged at $40 \times g$ for 1 min at room temperature. Cells were washed and resuspended in KH solution at room temperature until used.

Pertussis toxin treatment

Myocytes were incubated with pertussis toxin (PTX) (1.5 μg ml^{-1}) at 35°C for at least 3 h. PTX-treated myocytes were compared with cells that had been kept at 35°C in the absence of PTX for an equal time. After PTX treatment, both PTX-treated and non-treated cells were kept at room temperature until the time of experiments.

Measurement of cell contraction

The contraction of single ventricular myocyte was recorded as previously described (Harding *et al*., 1988). Myocytes were placed into a Perspex cell bath on the stage of an inverted microscope (Nikon or Zeiss IM), and superfused continuously with KH solution (32°C) equilibrated with 95% O_2/5% CO_2 at 1.8 to 2 ml min^{-1}. Cells were stimulated at a basal rate of 1 Hz by bipolar pulses through platinum electrodes placed along the bath. Unloaded cell shortening was measured with a video-camera/length detection system, with spatial resolution 1 to 512 and time resolution 10 ms. Cells were allowed to stabilize for at least 10 min at 1 mM Ca^{2+}, before they were challenged with increasing concentrations of Ca^{2+} or cumulative concentrations of ISO ($3 \times 10^{-11} \sim 10^{-6}$) until either a maximum response was reached, or the myocyte developed arrhythmias.

For the studies with Rp-cAMPS or inverse agonist, myocytes were contracting in near-physiological (2 mM) or high Ca^{2+} (4 mM) Rp-cAMPS (100 μM final concentration) was added to the reservoir and allowed to recirculate for 40 min. For the antagonist studies myocytes were preincubated with either 50 nM ICI 118,551 (selective β_2AR antagonist) or CGP 20712A (selective β_1AR antagonist) for 20–30 min as previously described (del Monte *et al*., 1993): at these concentrations there was no effect of the antagonist on basal contraction. When inverse agonist effects of ICI 118,551 were required, the concentration used was 1 or 3 μM, which was the maximum used in the original report (Milano *et al*., 1994). Inverse agonist effects were maximal after 5 min exposure and reversed with approximately the same time course.

Radioligand receptor binding assay

Myocytes were suspended in 25 mM Tris-HCl buffer (pH 7.4) containing 0.32 mM sucrose, and ventricles (trimmed free of connective tissue, great vessels and valves) were suspended in buffer containing (mM): Tris-HCl 50, EGTA 5, EDTA 5, $MgCl_2$ 4, ascorbic acid 1, phenylmethylsulphonylfluoride (PMSF) 0.5 (pH 7.4). Both were homogenized at 4°C with a Polytron homogenizer, then myocytes were further disrupted by repeated passage through a sterile 21G 1.5 needle. The homogenate was then centrifuged at low speed (myocytes; $1000 \times g$, ventricle; $175 \times g$) for 10 min at 4°C to remove unhomogenized debris, and the supernatant centrifuged at $40{,}000 \times g$ for 20 min (myocytes) or $50{,}000 \times g$ for 15 min (ventricle) at 4°C. Aliquots of membrane were incubated with [^{125}I]-iodocyanopindolol (ICYP) (specific activity: 2000 Ci $mmol^{-1}$) in the absence and presence of 200 μM ISO (to define non-specific binding). Incubation was carried out at 37°C for 120 min in duplicate or triplicate. The

incubation was terminated by rapid vacuum filtration through glass-fibre filters using a cell harvester (Brandel). Filters were rapidly washed with 3×5 ml ice-cold assay buffer and counted in a Gamma counter (Packard Instruments, Downers Grove, IL, U.S.A) at an efficiency of 80%. Protein concentration was determined by the method of Bradford (Bradford, 1976) with IgG used as the standard. The experiments were analysed for one binding site by non-linear curve fitting with the equation:

$$Beq = (B_{max} * [ICYP]/(K_D + [ICYP])$$

where Beq is the amount of ICYP binding at equilibrium, B_{max} is the maximum density of β-ARs and K_D is the equilibrium dissociation constant of ICYP.

Immunoblotting techniques

Immunoblotting techniques were performed as previously described (Gierschik *et al*., 1985). The polyclonal antiserum (MB1) was raised in rabbits against the terminal decapeptide of retinal transducin α (KENLKDCGLF) coupled to keyhole limpet haemocyanin as described (Goldsmith *et al*., 1987). The antiserum recognized Giα-1 and Giα-2. Blots were stained with an HRP-labelled goat anti IgG antiserum. The immunoreactive bands were visualized with the ECL detection system (Amersham).

Statistical analyses

Numbers of myocytes and numbers of hearts are given in parentheses. Where only one number is given this indicates the number of hearts, with only one cell from each heart being used for that experimental protocol. Results were expressed as the means $\pm$ standard error of the mean (s.e.mean). Between group comparison was made by unpaired Student's *t*-test and paired *t*-test was applied for within group comparison. Agonist (ISO) EC_{50} values were calculated by nonlinear regression analysis using the HYPMIC program. A value of $P < 0.05$ was considered to be statistically significant.

Drugs

Pertussis toxin and (−)-ISO were obtained from RBI (Natick, MA, U.S.A.). Other drugs were obtained from Sigma (Pool, Dorset, U.K.).

Results

β-AR binding and Giα expression

Radioligand binding studies confirmed the increase in β-AR number in the transgenic mice, which are directly descended from the TG4 mice used in the initial report (Milano *et al*., 1994). B_{max} values in whole ventricle were 5120 ± 580 fmol mg protein^{-1} for TGβ_2 mice ($n = 10$) compared with 13.1 ± 0.7 fmol mg^{-1} for LM controls ($n = 7$), an approximately 400 fold overexpression. K_D values were 6.1 ± 0.9 pM for TGβ_2 and 9.0 ± 1.4 pM for LM. In isolated myocyte preparations the overexpression was even more pronounced, with TGβ_2 B_{max} at 8158 ± 3467 ($n = 7$) and LM having 11.8 ± 2.3 fmol·mg protein^{-1} ($n = 10$). Expression of Giα-1 and 2 was tested at two different ages by Western blotting of membranes. Levels were increased significantly by 60 days (Figure 1), and the alteration was maintained up to 120 days.

Concentration-response curves to the non-selective β-AR agonist (−)-isoprenaline in ventricular myocytes

Single ventricular myocytes isolated from TGβ_2 mice or LM mice were superfused with normal KH buffer solution containing 1.0 mM Ca^{2+}. The concentration-response curve of myocytes to isoprenaline (ISO) with changes in contraction amplitude expressed as percentage of the maximum response is shown in Figure 2. There was no significant difference in the EC_{50} value (concentration for half-maximal response) between TGβ_2 mice (1.92 ± 0.37 nM, $n = 6$; mean $\pm$ s.e.mean) and LM mice (2.69 ± 0.31 nM, $n = 19$).

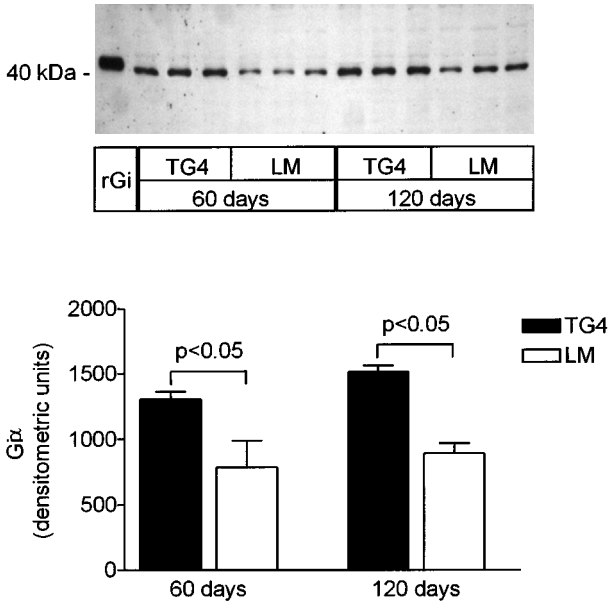

Figure 1 Gia expression in left ventricular myocardium from 60 and 120-day-old LM and TG4 mice. The upper panel shows a representative Western blot. For comparison, immunoreactive signal of recombinant Giα-2 and Giα-3 (rGi) is shown. The bar graph represents the densitometric quantification ($n = 5$ for each group).

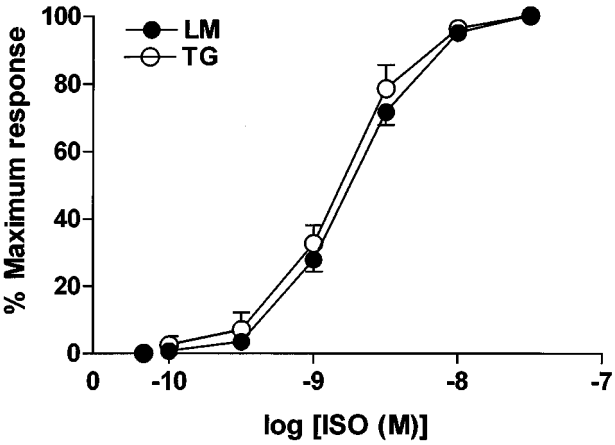

Figure 2 Comparison of the normalized concentration-response curves to (−)-isoprenaline (ISO) in myocytes from TGβ_2 (open circles, $n = 6$) and LM (solid circles, $n = 19$) mice. The range of concentrations of ISO was from 3×10^{-11} to 3×10^{-8}. The concentration–response curves were not significantly different between TGβ_2 and LM mice.

Basal, ISO- and calcium-stimulated contraction of ventricular myocytes

Figure 3 shows that baseline contraction amplitude in 1 mM Ca^{2+} was not different between TGβ_2 ($3.54\pm0.25\%$, $n=36$ cells/23 hearts) and LM mice ($3.63\pm0.28\%$, $n=41$ cells/35 hearts). Although ISO could directly enhance contraction amplitude in ventricular myocytes from TGβ_2 mice or LM mice, the ISO-induced maximum contraction amplitude was decreased in TGβ_2 mice ($5.65\pm0.34\%$, $n=36$ cells/23 hearts) relative to LM mice ($9.09\pm0.52\%$, $n=41$ cells/35 hearts) ($P<0.001$). The decreased positive inotropic response indicates that there is a functional βAR desensitization in TGβ_2 mice overexpressing the β_2AR. Contraction of myocytes in maximum Ca^{2+} (4–6 mM) was not different between TGβ_2 mice ($11.9\pm1.1\%$, $n=16$ cells/11 hearts) and LM mice ($13.1\pm0.5\%$, $n=23$ cells/18 hearts). This suggests that the contractile function of myocytes mediated by Ca^{2+} was not affected in TGβ_2 mice.

Inverse agonism in TGβ_2 myocytes: a comparison with a cyclic AMP antagonist

The lack of a tonic enhancement in contraction contradicts the original hypothesis that the overexpressed β_2ARs are stimulating cyclic AMP and so increasing contractility in the absence of agonist (Milano *et al*., 1994). However, it is possible that mechanisms distal to the cyclic AMP/protein kinase A interaction are decreasing contraction in a compensatory manner (e.g. changes in myofilament sensitivity). TGβ_2 myocytes were therefore challenged with an inverse agonist concentration of ICI 118,551 (1 μM) to discover whether tonic stimulation through the β_2AR was occurring. The amplitude of contraction was decreased significantly (Figure 4), although the magnitude of the decrease (32%) was less than the 80% in the original report (Bond *et al*., 1995). All decreases were fully reversible on washout of ICI 118,551, or on addition of 10 μM ISO in the presence of the β_1AR antagonist CGP20712A (300 nM) (Basal; $9.63\pm1.23\%$, +ICI; $6.09\pm0.68\%$, +ICI+ISO/CGP; $12.5\pm1.3\%$, $n=7$ cells/four hearts). The experiments shown in Figure 4 were performed in 4 mM Ca^{2+}. Similar results were obtained in 2 mM Ca^{2+} (basal; $9.34\pm0.60\%$, +ICI; $6.57\pm0.40\%$ shortening, $P<0.001$, $n=14$ cells/eight hearts). A small (<10%) but significant decrease in amplitude was also seen in control myocytes at 4 mM Ca^{2+}.

If the action of inverse agonists is to decrease tonic production of cyclic AMP, then a similar effect should be obtained using Rp-cAMPS, an inactive analogue of cyclic AMP which competes for binding at protein kinase A (PKA). However, no significant decrease in contraction in TGβ_2 myocytes was observed using 100 μM Rp-cAMPS (Figure 4). This concentration is sufficient to inhibit responses to maximal concentrations of ISO in guinea-pig or human myocytes (Money-Kyrle *et al*., 1998), or in myocytes from LM controls (ISO; $5.89\pm0.64\%$, ISO+Rp-cAMPS; $3.29\pm0.65\%$, $P<0.01$, basal; 3.31 ± 1.29, $n=4$ myocytes/hearts).

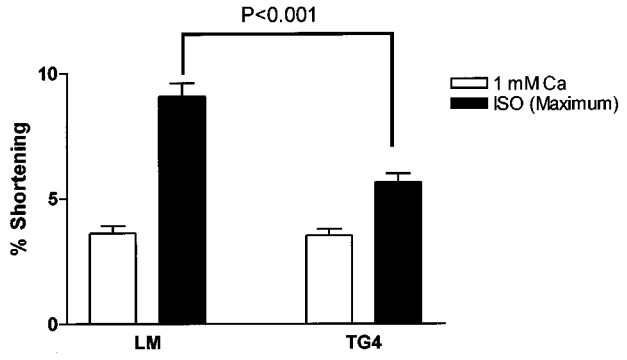

Figure 3 ISO (3×10^{-8}–10^{-6}) stimulated maximum contraction amplitude in ventricular myocytes of TGβ_2 mice (36 cells/23 hearts) and LM mice (41 cells/35 hearts). Unfilled columns: baseline, 1 mM Ca^{2+}; solid columns: baseline plus maximal concentration of ISO.

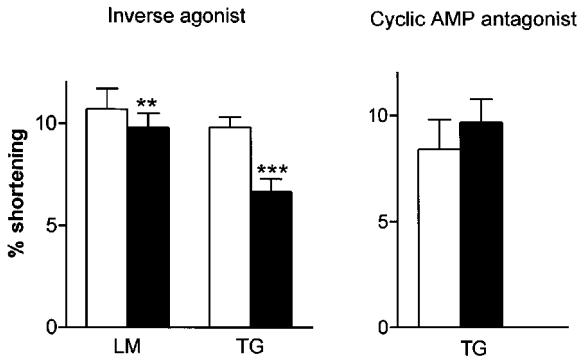

Figure 4 Left panel: effect on contraction amplitude of 5–10 min exposure to the inverse agonist ICI 118,551 (1 μM) in myocytes from LM ($n=7$ cells/six hearts) and TG ($n=10$ cells/eight hearts) rats. All effects were reversible. Right panel: effect on contraction amplitude of 40 min exposure to 100 μM Rp-cAMPS in myocytes from TG rats ($n=9$ cells/seven hearts). Open bars, control conditions; solid bars, presence of inverse agonist/Rp-cAMPS. $^{**}P<0.01$, $^{***}P<0.001$ compared to control, paired *t*-test.

Enhancement of basal and ISO-stimulated contraction amplitude of myocytes from TGβ_2 mice following pertussis toxin (PTX) treatment

The effectiveness of PTX to inactivate Gi was confirmed by loss of the anti-adrenergic effect of carbachol. Before PTX treatment 1 μM carbachol reduced contraction stimulated by 10 nM ISO from 6.99 ± 0.88 to $3.80\pm0.68\%$ shortening (basal $3.29\pm0.65\%$) ($P<0.02$, $n=7$ myocyte, four hearts). After PTX treatment the contraction amplitude in ISO was unchanged by carbachol (ISO alone; $7.43\pm1.32\%$, ISO+carbachol; $7.37\pm1.24\%$, basal; $3.71\pm0.32\%$, $n=6$ myocytes, four hearts).

After PTX pretreatment for inactivation of inhibitory G-protein (Gi), basal contractility of TGβ_2 heart cells in 1 mM Ca^{2+} was increased more than 2 fold ($P<0.001$). The basal contractility of LM heart cells was not changed by PTX treatment (Figure 5). Maximum contraction amplitude to ISO was also increased significantly in TGβ_2 myocytes ($9.40\pm1.22\%$, $n=8$ cells/six hearts) relative to that in non PTX-treated TGβ_2 myocytes ($5.79\pm0.40\%$, $n=29$ cells/18 hearts) and was not different from that of LM mice before ($8.93\pm1.50\%$, $n=11$ cells/eight hearts) or after ($9.89\pm0.73\%$, $n=19$ cells/12 hearts) PTX treatment. This indicates that PTX treatment has restored the original phenotype. After PTX pretreatment the contraction of myocytes to maximum Ca^{2+} was not different in TGβ_2 mice or LM mice (LM: $12.4\pm1.3\%$ shortening, $n=11$ cells/nine hearts, TGβ_2: $11.9\pm0.9\%$, $n=8$ cells/seven hearts).

Stimulation through β_1- and β_2AR subtypes

Concentration-response curves to ISO were constructed on myocytes in the presence and absence of the specific

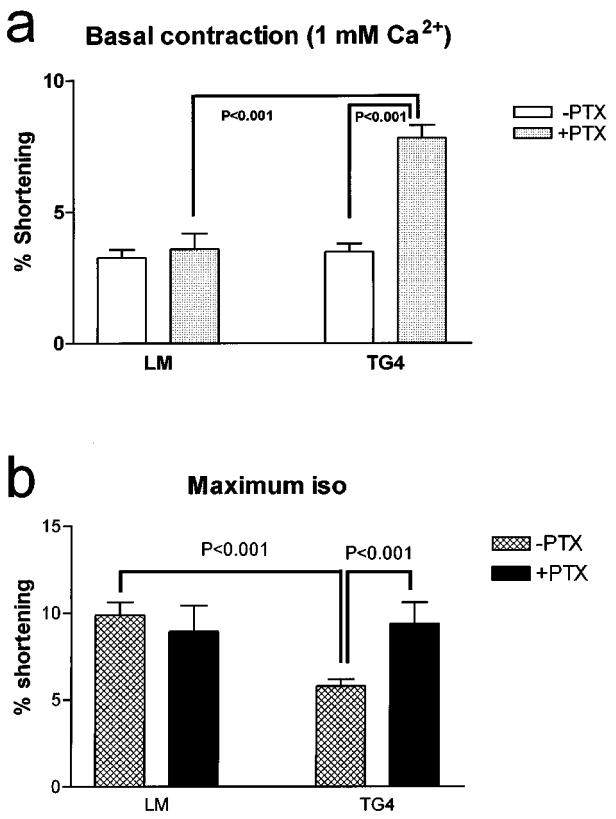

Figure 5 Effect of pertussis toxin on (a) basal (LM 11 cells/eight hearts, TGβ_2 23 cells/18 hearts) and (b) maximum ISO-stimulated (LM 11 cells/eight hearts, TGβ_2 8 cells/six hearts) contraction amplitude of myocytes.

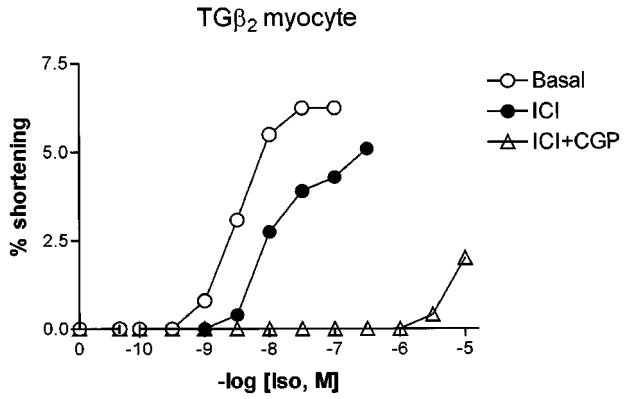

Figure 6 Sequential concentration-response curves to isoprenaline in a single myocyte from a TGβ_2 mouse. The first curve was constructed in the absence of antagonists, the second after 30 min exposure to ICI 118,551 (50 nM) and the third after 30 min exposure to both ICI 118,551 and 300 nM CGP 20712A. The antagonists remained in contact with the cell throughout the subsequent exposure to isoprenaline.

antagonists ICI 118,551 (β_2AR) and CGP 20712A (β_1AR). ICI 118,551 at the concentration used (50 nM) would be predicted to produce a 2 log unit shift in a purely β_2AR-mediated effect, while 300 nM CGP 20712A would shift a pure β_1AR effect by >3 log units (del Monte *et al*., 1993). Smaller shifts would indicate mixed responses. In myocytes from LM animals, the initial EC_{50} (concentration to produce half-maximal response) was 3.22 ± 0.83 nM ($n=12$). Addition of 50 nM ICI 118,551 produced little shift, increasing the EC_{50} to only 4.48 ± 1.72 nM ($n=7$): subsequent addition of CGP 20712A as well as ICI 118,551 to four of these cells gave a 2–3 log unit shift. Addition of CGP 20712A alone increased the EC_{50} to 39.8 ± 2.9 nM ($n=5$, $P<0.05$). This indicates that the response of ventricular myocytes to ISO in LM mice is largely β_1AR-mediated. Because repeated concentration-response curves are more difficult in mouse myocytes than in other species, due to their high rate of spontaneous arrhythmia, we also performed the experiments by using a maximal concentration of ISO and adding the antagonists when a steady increase in contraction had been reached. In LM myocytes, addition of 50 nM ICI 118,551 increased (rather than decreased) contraction by $3.4\pm6.2\%$ ($n=3$, NSD), confirming the lack of β_2AR contribution.

Fewer TGβ_2 myocytes than LM could be used for experiments involving repeated concentration-response curves, because of the decreased effect of ISO. When these could be performed, results indicated a mixed β_1- and β_2AR response. Initial addition of ICI 118,551 increased the EC_{50} from 2.73 ± 1.34 to 8.70 ± 2.89 nM, with an average log shift of 0.80 ± 0.21 units ($n=5$, $P<0.05$). This indicates a significant effect mediated through β_2ARs. However, some β_1AR contribution was still evident first, because the shift was less than predicted for a pure β_2AR-mediated response, and second, because addition of CGP 20712A also produced a shift either before ($n=3$, 0.25–3.4 log units) or in the presence of ICI 118,551 ($n=3$, 0.1–2.95 log units). Addition of antagonist during exposure to a single concentration of ISO gave a similar result, with five of seven myocytes (six hearts) decreasing contraction ($>10\%$ change) in response to ICI 118,551 and six of eight myocytes (seven hearts) decreasing contraction with CGP 20712A. An example of a complete concentration-response curve experiment is given in Figure 6 and several traces showing sequential addition of antagonists are displayed in Figure 7.

Following pertussis toxin treatment in TGβ_2 myocytes, the mixed β_1- and β_2AR response to ISO was still evident, with ICI 118,551 decreasing the response in four of five myocytes and CGP 20712A decreasing it in three of four cells (two hearts). Although this does not give as accurate a measure of the β_1:β_2AR ratio as full concentration-response curves, the result suggests that pertussis toxin treatment up-regulates both β_1- and β_2AR responses.

Discussion

This study presents strong evidence for the role of increased Gi in the spontaneous deviation of the TGβ_2 mice from the original phenotype. Initially, it had been reported that ventricular contraction in TGβ_2 mice was maximally activated in the absence of βAR agonist stimulation, and this was thought to be due to the presence of tonically active β_2ARs. Adenylate cyclase activity was increased, and the consequent rise in cyclic AMP was the suggested mechanism for the raised contractility. In contrast, we have shown that basal contraction in ventricular myocytes from TGβ_2 mice was not supported tonically by cyclic AMP, since Rp-cAMPS did not decrease contraction amplitude. Nor was contraction in basal (1 mM) or high (4–8 mM) Ca^{2+} different between LM and TGβ_2 myocytes. We have shown for the first time that treatment with pertussis toxin, to inactivate Gi, increased basal contraction until it reached levels similar to those seen with high ISO in LM mice.

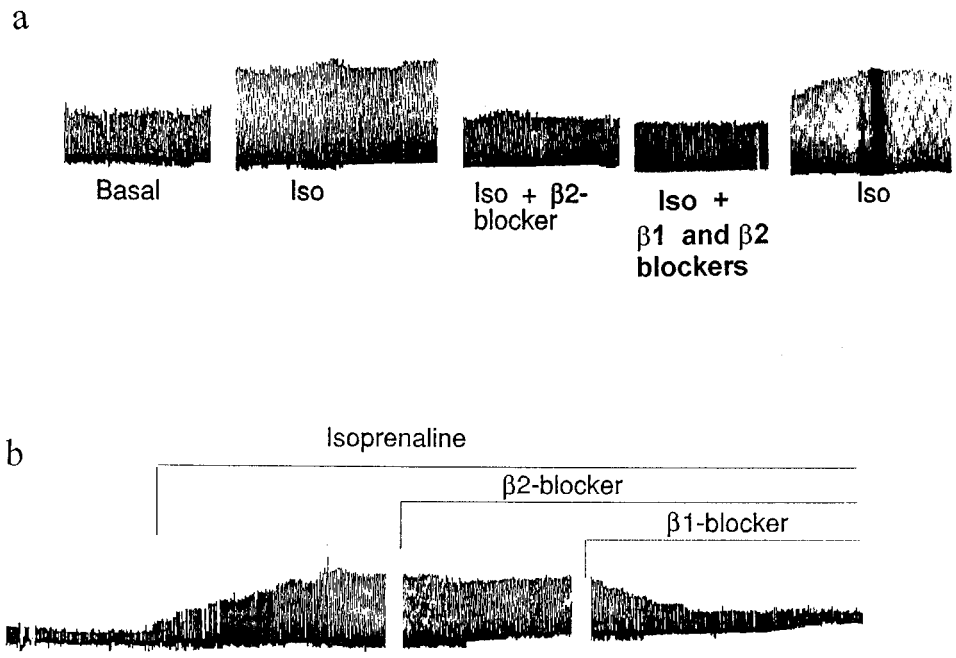

Figure 7 Examples traces of sequential additions of antagonists (β_1AR = 300 nM CGP20712A, β_2AR-blocker = 50 nM ICI 118,551) in the presence of isoprenaline (10^{-7} M). The effect of isoprenaline in cell (a) is predominantly mediated by β_2ARs, since ICI 118,551 abolishes the response with little further effect of CGP 20712A. The effect in cell (b) is mediated predominantly by β_1ARs.

Inactivation of Gi also restored the response to ISO in ventricular myocytes from TGβ_2 mice. These data agree well with those of Xiao *et al.* (1999) who were the first to show the functional relevance of raised Gi in the TGβ_2 mice. We have investigated the contribution of the β_1- and β_2AR subtypes to ISO-stimulated contraction in LM and TGβ_2 mice under the various conditions. More of the response to ISO was mediated by β_2AR in TGβ_2 mice than in LM, which are almost entirely under β_1AR control. However, there was still a significant contribution from the β_1AR even in TGβ_2 myocytes, with the concentration-response curve shifted less than predicted by ICI 118,551 and additionally shifted by CGP 20712A. This indicates that despite the extreme excess of β_2AR, the contribution of β_1AR was still detectable, and implies that the efficiency of the overexpressed receptors was lower than that of the native ones.

In a proportion of myocytes, responses to ISO were lost completely (previously reported: Heubach *et al.*, 1999) which indicates that both β_1- and β_2AR had been down-regulated by the increase in Gi. After pertussis toxin treatment the ISO-stimulated responses were again mediated by both receptor subtypes. This argues against an exclusive relationship between the β_2AR subtype and Gi, as has been suggested (Xiao *et al.*, 1999). We have found that β_1ARs in guinea-pig myocytes also show PTX-sensitive changes in contractility during desensitization, again indicating an interaction of Gi with this subtype (Ranu *et al.*, 2000).

Taking into account both our studies and those of Xiao *et al.* (1999) it appears that there is a gradual modification of the initial phenotype of the TGβ_2 mouse, in which basal activity is maximal due to a large excess of the activated form of the β_2AR (R*) and cannot be increased further by ISO. Gi is up-regulated, probably in response to the continual activation of the adenylyl cyclase pathway: there is a site for the cyclic AMP-activated transcription factor AP-2 on the promoter region of the Giα-2 gene (Imagawa *et al.*, 1987). Additionally, the high cyclic AMP levels encourage the switch of β_2AR-coupling from Gs to Gi, by PKA-dependent phosphorylation of the receptor (Daaka *et al.*, 1997). Increased coupling through Gi suppresses the effect of R*, decreasing first the basal activation and finally the response to ISO through β_2AR. The high levels of Gi also suppress in addition the response to ISO through the β_1AR. Treatment with pertussis toxin reverses this sufficiently to allow the recovery of the βAR responses, as in the work of Xiao *et al.* (1999) and can also restore the activation of basal activity (present study).

Several intriguing questions remain. First, why does this change occur? We have shown that it is not age of the mouse *per se* that produces the change in phenotype (Heubach *et al.*, 1999), which implies that it must be due to some genetic drift. It has recently been shown that overexpression of the β_2AR above a certain level (60 fold) results in development of a cardiomyopathy, and that this is more rapidly fatal as the receptor level increases (Liggett *et al.*, 2000). We have paired transgenic to transgenic for mating, and the hearts now have a 400 fold or greater excess of the β_2AR compared to 200 fold in the original report. It may be we have a higher mortality rate due to cardiomyopathy, and that animals which survive to the time of experiment are those in which Gi has up-regulated in a protective fashion. Second, why is the alteration seen in ventricle but not in right atria, which retain the original increased chronotropic activity (Heubach *et al.*, 1999)? Third, what exactly is inverse agonism? We observe a 32% decrease in contraction amplitude in these myocytes with 1 μM ICI 118,551 (an inverse agonist concentration) despite the observations that basal contraction was not higher than that in LM and that the cyclic AMP antagonist Rp-cAMPS did not decrease contraction. This is not consistent with the hypothesis that inverse agonism represent a reduction in the tonic stimulation of adenylyl cyclase by R* (Milano *et al.*, 1994). This model is worth continuing investigation, because of the strong parallels between spontaneous desensitization due to up-regulation of Gi in TGβ_2 mice and the natural history of human heart failure.

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References

BOND, R.A., LEFF, P., JOHNSON, T.D., MILANO, C.A., ROCKMAN, H.A., McMINN, T.R., APPARSUNDARAM, S., HYEK, M.F., KENAKIN, T.P., ALLEN, L.F. & LEFKOWITZ, R.J. (1995). Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta 2-adrenoceptor. *Nature,* **374,** 272–276.

BRADFORD, M.M. (1976). A rapid a sensitive method for the quantification of microgram quantities of protein utilising the principle of protein-dye binding. *Anal. Biochem.,* **72,** 248–254.

DAAKA, Y., LUTTRELL, L.M. & LEFKOWITZ, R.J. (1997). Switching of the coupling of the beta2-adrenergic receptor to different G proteins by protein kinase A. *Nature,* **390,** 88–91.

DEL MONTE, F., KAUMANN, A.J., POOLE-WILSON, P.A., WYNNE, D.G. & HARDING, S.E. (1993). Coexistence of functioning β1- and β2-adrenoceptors in single myocytes from human ventricle. *Circulation,* **88,** 854–863.

GIERSCHIK, P., CODINA, J., SIMONS, C., BIRNBAUMER, L. & SPIEGEL, A. (1985). Antisera against a guanine nucleotide binding protein from retina cross-react with the β-subunit of the adenylyl cyclase-associated guanine nucleotide binding protein, Ns and Ni. *Proc. Natl. Acad. Sci. U.S.A.,* **82,** 727–731.

GOLDSMITH, P., GIERSCHIK, P., MILLIGAN, G., UNSON, C.G., VINITSKY, R., MALECH, H.I. & SPIEGEL, A.M. (1987). Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophils and brain. *J. Biol. Chem.,* **262,** 14683–14688.

HALL, J.A., KAUMANN, A.J. & BROWN, M.J. (1990). Selective beta 1-adrenoceptor blockade enhances positive inotropic responses to endogenous catecholamines mediated through beta 2- adrenoceptors in human atrial myocardium. *Circ. Res.,* **66,** 1610–1623.

HARDING, S.E., BROWN, L.A., WYNNE, D.G., DAVIES, C.H. & POOLE-WILSON, P.A. (1994). Mechanisms of beta-adrenoceptor desensitisation in the failing human heart. *Cardiovasc. Res.,* **28,** 1451–1460.

HARDING, S.E., JONES, S.M. & POOLE-WILSON, P.A. (1988). Contractile responses of myocytes isolated from human atria to isoprenaline, calcium, forskolin and histamine. *J. Physiol.,* **406,** 219P (Abstract).

HEUBACH, J.F., TREBEB, I., WETTWER, E., HIMMEL, H.M., MICHEL, M.C., KAUMANN, A.J., KOCH, W.J., HARDING, S.E. & RAVENS, U. (1999). L-type calcium current and contractility in ventricular myocytes from mice overexpressing the cardiac β_2-adrenoceptor. *Cardiovasc. Res.,* **42,** 173–182.

IMAGAWA, M., CHIU, R. & KARIN, M. (1987). Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell,* **51,** 251–260.

KAUMANN, A.J. & MOLENAAR, P. (1997). Modulation of human cardiac function through 4 beta-adrenoceptor populations. *Naunyn Schmiedebergs Arch. Pharmacol.,* **355,** 667–681.

LIGGETT, S.B., TEPE, N.M., LORENZ, J.N., CANNING, A.M., JANTZ, T.D., MITARAI, S., YATANI, A. & DORN II, G.W. (2000). Early and delayed consequences of beta(2)-adrenergic receptor overexpression in mouse hearts: critical role for expression level. *Circulation,* **101,** 1707–1714.

LIGGETT, S.B., WAGONER, L.E., CRAFT, L.L., HORNUNG, R.W., HOIT, B.D. & McINTOSH, T.C. (1998). The Ile164 β_2-adrenergic receptor polymorphism adversely affects the outcome of congestive heart failure. *J. Clin. Invest.,* **102,** 1534–1539.

MILANO, C.A., ALLEN, L.F., ROCKMAN, H.A., DOLBER, P.C., McMINN, T.R., CHIEN, K.R., JOHNSON, T.D., BOND, R.A. & LEFKOWITZ, R.J. (1994). Enhanced myocardial function in transgenic mice overexpressing the β2-adrenergic receptor. *Science,* **264,** 582–586.

MONEY-KYRLE, A.R., DAVIES, C.H. & HARDING, S.E. (1998). The role of cyclic AMP in the frequency-dependent changes of contraction of guinea-pig cardiomyocytes. *Cardiovasc. Res.,* **37,** 532–540.

NAGARAJA, S., IYER, S., EICHBERG, J. & BOND, R.A. (1997). Cardiac β_2-adrenoceptor overexpression in transgenic mice results in G protein alterations. *Pharmacologist,* **39,** 40 (Abstract).

RANU, H.K., MAK, J.C., BARNES, P.J. & HARDING, S.E. (2000). Gi-dependent suppression of β_1-adrenoceptor effects in ventricular myocytes from norepinephrine-treated guinea-pigs. *Am. J. Physiol.,* **278,** H1807–H1814.

XIAO, R.P., AVDONIN, P., ZHOU, Y.Y., CHENG, H., AKHTER, S.A., ESCHENHAGEN, T., LEFKOWITZ, R.J., KOCH, W.J. & LAKATTA, E.G. (1999). Coupling of β_2-adrenoceptor to G_1 proteins and its physiological relevance in murine cardiac myocytes. *Circ. Res.,* **84,** 43–52.

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