width the first term predominates, greatly simplifying the analysis.
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15. The steady-state variance is expected to differ as the channel approaches equilibrium at different potentials, since its value depends on the rate and amount of charge movement of the transitions between states populated at each potential. The maximum value occurs when forward and backward rates are equal. Unfortunately, absolute values of steady-state gating variance are not readily obtained because of the difficulty of determining background noise at different potentials. The background noise is dependent on the patch characteristics, and frequently it increases with membrane polarization and the lifetime of the patch, the most probable cause being seal instability.
16. Obtaining the clean traces required for successful noise analysis occurred less frequently at very negative potentials such as −110 mV, where seal instability often produced flickering "chatter" noise, particularly at the beginning of the return pulse. Thus, we cannot with certainty discount the presence of a rising phase of the OFF variance lasting less than 0.4 ms.
17. The transition from the open state to the last closed state has been found to be relatively voltage-independent [W. N. Zagotta and R. W. Aldrich, *J. Gen. Physiol.* **95**, 29 (1990); (*26*)].
18. The classic application of nonstationary noise analysis has been to estimate the single-channel current from macroscopic ionic currents [F. J. Sigworth, *J. Physiol.* **307**, 97 (1980)]. Applied to ionic currents, it is sufficient that a single level of conductance exists in order to produce a parabolic mean-variance curve. A parabolic variance-mean curve arises if the single-channel variance has the form $var = \phi \cdot i - i^2$, where ϕ is the elementary event and is constant with time. Assuming noninteraction between channels, the relation for N channels (Var versus I) becomes $Var = \phi \cdot I - I^2/N$, which has a peak at $(N\phi/2, N\phi^2/2)$. For ionic currents, the elementary event ϕ is the single-channel current, and the peak of the parabola corresponds to an open probability of 0.5. In the case of gating currents, the requirements to apply the formula $Var = \phi \cdot I - I^2/N$ are more stringent (*6*). Activation must consist of uncorrelated transitions, implying that the channel is made up of independent two-state gating subunits that undergo irreversible transitions. Another requirement is favorable bandwidth [$(B >> \alpha)$, where α is the unimolecular rate constant governing a single subunit]. With these conditions, the elementary event has the value $\phi = 2Bq$, thus providing an estimate of the single-transition gating charge q. Gating current fluctuations cannot reach the peak of the parabola without violating the conditions of the two-state model, as it corresponds to $\alpha = B$.
19. The following linear eight-state model was developed by F. Bezanilla, E. Perozo, and E. Stefani (*26*) to account for the kinetics and voltage dependence of macroscopic gating currents in the range of −150 to 40 mV

$$C_O \underset{\beta_0}{\overset{\alpha_0}{\rightleftharpoons}} C_1 \underset{\beta_1}{\overset{\alpha_1}{\rightleftharpoons}} C_{11} \underset{\beta_1}{\overset{\alpha_1}{\rightleftharpoons}} C_{12} \underset{\beta_1}{\overset{\alpha_1}{\rightleftharpoons}} C_2 \underset{\beta_2}{\overset{\alpha_2}{\rightleftharpoons}} C_3 \underset{\beta_3}{\overset{\alpha_3}{\rightleftharpoons}} C_4 \underset{\beta_4}{\overset{\alpha_4}{\rightleftharpoons}} O$$

The equivalent of stage I in this work may include transitions between states C_0 through C_2, which has equivalent charges of 1.81 e_0 for α_0, β_0 and 1.24 e_0 for α_1, β_1. The large charge of stage II may comprise the cluster of transitions from C_2 through O with equivalent charges of 0.89 e_0 for α_2, β_2, 3.5 e_0 ($=q_{max}$) for α_3, β_3, and 0.35 e_0 for α_4, β_4.
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Enhanced Myocardial Function in Transgenic Mice Overexpressing the β_2-Adrenergic Receptor

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Transgenic mice were created with cardiac-specific overexpression of the β_2-adrenergic receptor. This resulted in increased basal myocardial adenylyl cyclase activity, enhanced atrial contractility, and increased left ventricular function in vivo; these parameters at baseline in the transgenic animals were equal to those observed in control animals maximally stimulated with isoproterenol. These results illustrate a useful approach for studying the effect of gene expression on cardiac contractility. Because chronic heart failure in humans is accompanied by a reduction in the number of myocardial β-adrenergic receptors and in inotropic responsiveness, these results suggest a potential gene therapy approach to this disease state.

Receptors coupled to heterotrimeric guanine nucleotide–binding proteins (G proteins), such as the adrenergic receptors, increase cellular concentrations of second messengers like adenosine 3′,5′-monophosphate (cAMP) and diacylglycerol, thereby regulating cellular function. The binding of the sympathetic neurotransmitter norepinephrine or the adrenal medullary hormone epinephrine to β-adrenergic receptors (β-ARs) in the heart stimulates adenylyl cyclase, raises the concentration of cAMP, and increases cardiac contractility. Increased secretion of catecholamines and stimulation of myocardial β-ARs are critical for augmenting cardiac function during stress.

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In chronic congestive heart failure, an illness affecting more than 4 million Americans, there is impairment of the myocardial β-AR system. Failing human ventricular myocardium contains 50% fewer β-ARs and shows parallel decreases in agonist-stimulated adenylyl cyclase activity and even greater decreases in agonist-mediated inotropy (*1*). Nonreceptor-mediated stimulation of adenylyl cyclase with NaF, however, appears to be intact in the failing myocardium, and contractile responses to other agents, such as calcium or histamine, are also not impaired in the diseased myocardium (*1*). This supports the concept that impaired inotropic responsiveness in heart failure results from focal receptor defects. Therapeutic interventions involving the administration of agonists to stimulate β-ARs have an inherently limited efficacy, given the reduction in receptor targets in the diseased myocardium.

The development of transgenic technology, and emerging techniques for in vivo gene transfer (*2*), suggest a strategy for improving cardiac function by overexpressing the β-AR in the myocardium. Although transgenic mice have been reported that express, for example, the c-*myc* protooncogene or SV-40 T antigen affecting cardiac growth (*3*), to date few experiments have documented the ability of a transgene to significantly improve myocardial function (*4*).

The transgene construct used in these experiments contained the murine alpha myosin heavy chain (α-MHC) promoter ligated to the coding sequence for the human β_2-AR (*5*). This promoter effects a pattern of transgene expression similar to that of the endogenous α-MHC, which is the predominant heavy chain isoform in adult murine atria and ventricles. This isoform is normally not expressed in smooth or skeletal muscle, nor is it expressed in the ventricular chamber during development. Thus, potentially lethal developmental effects of β-AR activation are avoided. Under normal conditions, β_1-ARs compose the majority of total myocardial β-ARs, which can undergo down-regulation as described above. Therefore, we used the β_2-AR for this study, which does not appear to undergo down-regulation in clinical heart failure (*1*, *6*). In addition, the β_2-AR couples to adenylyl cyclase with greater efficacy than the β_1-AR (*7*). Furthermore, expression of myocardial β_1-ARs in transgenic mice has resulted in only moderate expression without changes in contractility (*8*). The transgene construct was terminated with a portion of the SV-40 intron, which was included as 3′ untranslated sequence to improve transcription and increase stability of the mRNA transcript.

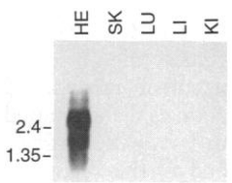


Fig. 1. Northern analysis. Total RNA was extracted from five tissues [heart (HE), quadriceps skeletal muscle (SK), lung (LU), liver (LI), and kidney (KI)] from transgenic mice by the RNAzol method (*17*), fractionated on a 1.2% agarose–formaldehyde gel (30 μg per lane), and transferred to nitrocellulose. After 6 hours of prehybridization, hybridization was carried out overnight with a random primer, [α-^{32}P]deoxycytidine triphosphate–labeled SV-40 intron DNA probe (1×10^6 to 2×10^6 cpm per milliliter of a 50% formamide solution). The membrane was then washed twice in 0.1× saline sodium citrate at 65°C for 30 min and exposed to film at −70°C for 36 hours.

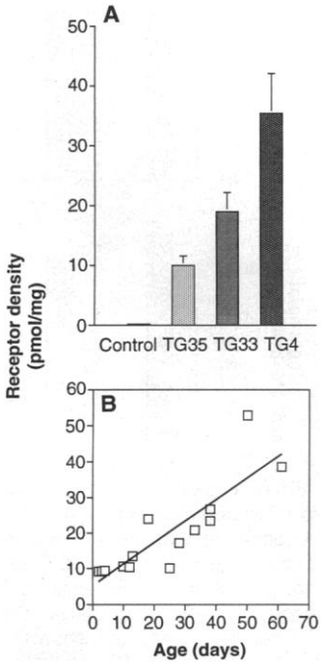


Fig. 2. Transgene expression by radioligand binding. (**A**) Ligand binding assays were performed on membranes (*18*) prepared from the hearts of 18 animals approximately 2 months of age [control (nontransgenic), $n = 5$; TG35, $n = 3$; TG33, $n = 6$; and TG4, $n = 4$]. The control value was 0.18 ± 0.04 pmol/mg. (**B**) Estimated B_{max} of whole heart membrane preparations from each of 14 TG4 animals plotted against the animal's age. Ages ranged from 2 to 61 days, and β_2-AR density ranged from 9.2 to 52.8 pmol/mg.

This transgene construct was microinjected into the pronuclei of one-cell mouse embryos, which were then surgically reimplanted into pseudopregnant female mice (*9*). Offspring were screened by Southern (DNA) blot analysis with a probe to the SV-40 sequences, and three founders containing the transgene were identified. Grossly, these founders demonstrated no phenotypic changes, and three lines with demonstrated transmission of the transgene were established (TG4, TG33, and TG35). Neonatal mortality in the transgenics did not differ from that in the nontransgenic animals, and there were no adult deaths. Second generation adult animals, approximately 2 to 4 months of age, were used for biochemical and physiologic studies. These animals had similar heart to body weight ratios relative to their littermate controls, and there was no evidence of developmental defects, myocyte necrosis, or fibrosis.

Transgene expression assessed by Northern analysis on total RNA from various tissues revealed intense cardiac expression, with no detectable expression elsewhere (Fig. 1). Transgene expression was quantitated by ligand binding assays (Fig. 2A), which demonstrated overexpression in the three transgenic lines of approximately 55 times (TG35) to 195 times (TG4) the amount of β-AR in control tissue. This high expression was present in all three lines and therefore cannot be attributed to the site of genomic integration.

In the transgenic myocardium, the amount of total myocardial β-AR increased in a linear manner in the transgenics during the first 2 months of life (Fig. 2B); this paralleled α-MHC expression, which at birth is confined to the atria and then progressively increases in the ventricles during the first several weeks of life. The high amounts of receptor expression and linear increase during the first 2 months may also result from activation of the α-MHC promoter by the increased intracellular concentrations of cAMP (a consequence of β-AR activation); β-AR blockade in vivo reduces expression of α-MHC (*10*).

Polyclonal antibodies to the human β_2-AR (*11*) allowed for in situ demonstration of receptor expression in the adult mouse heart (Fig. 3). Consistent with the pattern of α-MHC expression, strong labeling was present throughout the atria and ventricles, with all myocytes being labeled. Coronary vasculature was not labeled. Myocyte labeling was confined predominantly to the sarcolemma, including the intercalated disks, with some fainter intracellular labeling, which may represent transverse tubules. This pattern of expression confirmed that the receptor protein undergoes the appropriate posttranslational modifications and processing necessary for membrane insertion.

To assess whether the myocardium of the transgenic animals overexpressing β_2-ARs had enhanced adenylyl cyclase activity, we incubated myocardial membranes with [α-^{32}P]adenosine triphosphate (ATP) and measured the rate of cAMP formation. Basal and isoproterenol-stimulated rates

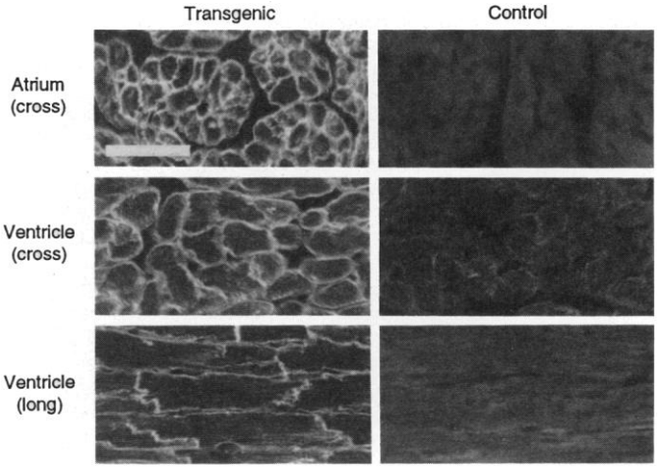


Fig. 3. In situ demonstration of transgene expression. Immunohistochemical labeling (*19*) of the human β_2-AR in TG4 (transgenic) and control myocardial frozen sections was done with rabbit antiserum to the COOH-terminus of the human β_2-AR (*11*). Atrium (cross), cross-sectioned atria; ventricle (cross), cross-sectioned ventricle; ventricle (long), longitudinal sectioned ventricle; size bar, 50 μm.

were measured in two transgenic lines (TG4 and TG33) and compared to that of controls. Both basal and isoproterenol-stimulated cyclase activity were increased in the transgenic animals relative to controls. These increases were most pronounced in TG4 (Fig. 4), consistent with the high amount of receptor expression in this line. Increases in basal cyclase activity were statistically significant in both TG4 (Fig. 4) and TG33 (*12*) relative to control values. In TG4 animals, the basal adenylyl cyclase activity was as high as the maximal isoproterenol-stimulated activity in the controls (Fig. 4). This large increase in basal cyclase is consistent with emerging concepts of receptor action: A small fraction of the total receptor pool is thought to exist in the activated conformation even in the absence of agonist (*13*). With the very high amount of receptor present in the transgenics, this small fraction of receptors in the activated conformation is sufficient to activate adenylyl cyclase. Isoproterenol sensitivity was also enhanced in TG4 animals, with the median effective concentration (EC_{50}) for isoproterenol (2.2×10^{-8} M) being an order of magnitude lower than that for control animals (1.6×10^{-7} M) (Fig. 4).

To directly assess the effect of β_2-AR overexpression on myocardial inotropy, we determined isometric tension development in isolated atria. Basal isometric tension development was increased for both the right and left atria in TG4 animals relative to controls (Fig. 5, A and B). In the left atria, basal tension was approximately three times that of control atria. Baseline isometric tension in the transgenics was not different from the maximal isoproterenol-stimulated tension in control atria. Initial studies were conducted at the intrinsic beating rates, which were significantly greater in atria from TG4 animals (Fig. 5C). Because increased rate might contribute to the increased tension measured in the transgenic myocardium, the relation of rate to developed tension was assessed. From a rate of 240 to 360 beats per minute, a negative force-rate relation was demonstrated (*12*). Thus, measurements of isometric tension at the intrinsic atrial rate may actually underestimate the degree of increased inotropy present in the transgenic atria. Isometric tension was also increased in TG33 transgenic atria at a rate which did not differ from that of controls (*12*). All atria studied were weighed, and no significant differences were noted between transgenic and control tissue weights.

TG4 atria showed no further inotropic response to doses of isoproterenol that maximally stimulated isometric tension in controls (Fig. 5, A and B). Thus, it appears that even in the absence of agonist, sufficient receptors were present in the activated state to stimulate adenylyl cyclase and effect a maximal inotropic response. Isoproterenol did increase adenylyl cyclase activity in the transgenic animals, but increases in inotropy were not generated, presumably because downstream effectors beyond the cyclase became limiting (such as calcium channel opening and contractile protein adenosine triphosphatase activity). Although baseline inotropy was equally enhanced in TG4 and TG33, basal cyclase activation was considerably greater in the line that expressed more receptor (TG4). This suggests that small increases in basal cyclase activity may be sufficient to induce a maximal inotropic response. In addition, there may be β-AR–mediated pathways for increasing myocardial inotropy independent of cAMP formation. Indeed, myocardial calcium channels that are coupled directly to stimulatory G proteins may contribute to the β-AR–mediated inotropic response (*14*).

Recent technological advances have enabled cardiac catheterization and hemodynamic measurements in anesthetized mice (*15*). Using such techniques, we assessed functional changes in the left ventricle in vivo in TG4 mice; heart rate (HR), continuous left ventricular (LV) pressures, and aortic pressures were recorded at baseline and after progressive doses of isoproterenol. At baseline, LV end diastolic pressures and mean aortic pressures (Fig. 6C) were similar between TG4 animals and controls. The maximum first derivative of the LV pressure (LV dP/dt_{max}) (Fig. 6A) and HR (Fig. 6B) was, however, significantly increased in the TG4 animals; baseline LV dP/dt_{max} was 70% greater than the control value. LV relaxation at baseline was also significantly enhanced in the TG4 animals as compared with controls; peak negative dP/dt was -5328 ± 1371 in TG4 animals and -3313 ± 546 mm Hg per second in controls. With the administration of isoproterenol, there was no significant change (relative to baseline) in LV dP/dt_{max} or HR for the transgenics (Fig. 6, A and B), confirming a maximally activated β-AR system. Control animals demonstrated progressive increases in LV dP/dt_{max} after isoproterenol infusion and at the highest doses generated a LV dP/dt_{max} that was not statistically different

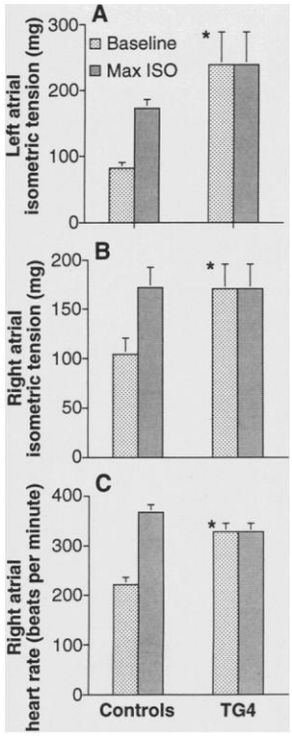
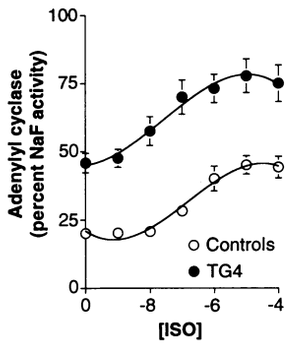


Fig. 5. Baseline and maximally stimulated responses in isolated atria. Atria were suspended under optimal resting tension in modified Kreb's bicarbonate solution at 30°C (*21*). Left atria were paced at the rate exhibited by the spontaneously beating right atria of the same animal. Three responses are shown: (**A**) left atrial developed tension; (**B**) right atrial developed tension; and (**C**) right atrial heart rate. Baseline responses were obtained before maximally stimulating the response with isoproterenol (1×10^{-8} M). Data shown are the mean values ± SEM for control animals (n = 7 or 8) and TG4 animals (n = 5 or 6). In all three responses, the baseline values for the TG4 animals were greater than the baseline in the control animals (*, $P < 0.05$, Student's t test), but not different from either control isoproterenol-stimulated values or from TG4 isoproterenol-stimulated values.

Fig. 4. Adenylyl cyclase activity of membranes (*18*) prepared from TG4 and control hearts was determined (*13*) under basal conditions and in the presence of isoproterenol (1×10^{-9} to 1×10^{-4} M) or NaF (10 mM). Membranes (5 to 20 μg of protein per 50 μl) were incubated for 10 min at 37°C, and [α-^{32}P]ATP was isolated and quantitated (*20*). Basal and isoproterenol-stimulated cyclase activities were normalized to the percent of the activity achieved with NaF. Each point represents the mean ± SEM of seven independent experiments performed in duplicate. Both basal and maximal isoproterenol-stimulated values for TG4 were significantly greater than control values (Student's t test, $P < 0.01$). Mean NaF-stimulated cyclase activity (±SD) for the transgenic membranes (445.5 ± 165.5 pmol of cAMP per minute per milligram of protein) was not significantly different from that of the controls (606.4 ± 207.8) (Student's t test, $P > 0.05$).



from that in TG4 animals (Fig. 6A). Baseline HR was also significantly increased in the TG4 animals and remained greater than that of controls throughout the isoproterenol infusion (Fig. 6B). At the highest dose of isoproterenol, a significant decrease in the mean aortic pressure was seen in the TG4 animals relative to controls (Fig. 6C), which probably resulted from peripheral β-AR–stimulated vasodilation uncompensated by further increases in the cardiac output (since the inotropic state of the TG4 LV was already maximal). These experiments confirmed in vivo that the transgenic left ventricle, which serves the critical function of supporting the systemic circulation, had been converted to a maximal inotropic state in TG4 animals.

These data demonstrate that overexpression of the β_2-AR results in a significant population of spontaneously isomerized receptors, present in the active conformation in the absence of agonist, which can therefore activate adenylyl cyclase and maintain a physiologic response. Thus, the biochemical and physiologic phenotype observed with overexpression of the wild-type receptor parallels the properties of mutant β- and α-ARs, which have been shown to be constitutively active (*13, 16*).

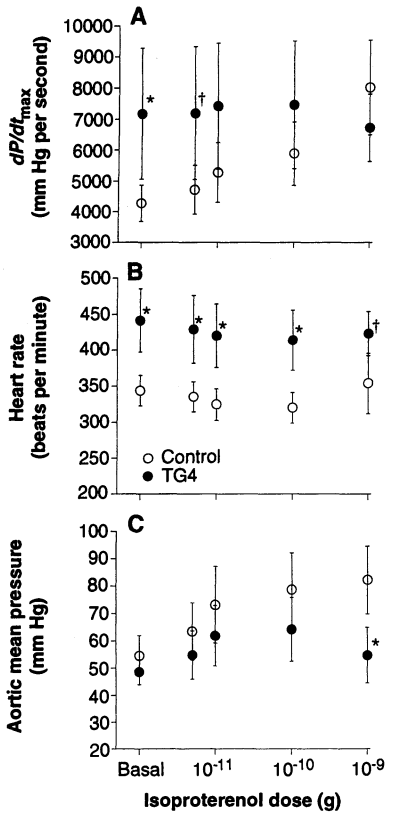


Fig. 6. In vivo assessment of left ventricular function. Seven TG4 (transgenic) animals and seven controls were anesthetized and aortic and left ventricular catheters placed (*15*). Three measured parameters are shown at baseline and after doses of isoproterenol: (**A**) LV dP/dt_{max}; (**B**) heart rate; and (**C**) mean aortic pressure. Data are reported as means ± SD and were analyzed with a two-way repeated measures analysis of variance with post hoc tests based on *t* test with Bonferroni correction for five comparisons (*, $P < 0.005$; †, $P < 0.05$).

Several lines of evidence support the concept that the phenotype observed in the TG4 animals is not due to the action of endogenous catecholamines on the large pool of transgenic myocardial β_2-ARs. First, neutral antagonists, such as alprenolol, which completely displace endogenous catecholamines from the receptors, did not significantly reduce the increased basal adenylyl cyclase activity, atrial contractility, or baseline LV dP/dt_{max} (*12*). However, an antagonist with negative efficacy (which shifts the receptor from the active to inactive conformation), such as ICI-118551, was able to markedly reduce these increased basal responses in transgenic animals (*12*). In addition, the relative elevation of basal isometric tension development measured in these animals persisted after depletion of greater than 99% of endogenous catecholamines by reserpine treatment (*12*). Finally, the predominant endogenous myocardial catecholamine, norepinephrine, has markedly reduced efficacy for the transgenic β_2-AR relative to the endogenous β_1-AR (*12*).

This study demonstrates the potential of genetic engineering as a modality for enhancing myocardial and ventricular function and highlights the use of transgenic mice for studying the functional consequences of altered myocardial gene expression in vivo. Given the well-characterized deficiencies in the myocardial β-AR system in chronic heart failure, these findings have clinical relevance. As methods for in vivo gene transfer develop, such an approach may prove to be useful in this or other disease states where the consequences of augmenting receptor function in a single or limited number of tissues would be beneficial.

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18. We prepared crude myocardial membranes by homogenizing whole hearts in ice-cold lysis buffer [5 mM tris-HCl (pH 7.4), 5 mM EDTA, leupeptin (10 μg/ml), and aprotinin (20 μg/ml)]. Nuclei were sedimented at 500*g* for 15 min. The supernatant was filtered through cheese cloth, and membranes were sedimented at 40,000*g* for 15 min. Membranes were washed once in binding buffer [75 mM tris-HCl (pH 7.4), 12.5 mM $MgCl_2$, and 2 mM EDTA] before resuspension. Ligand binding assays were done in duplicate on membranes in 500 μl of binding buffer with saturating concentrations of the β-AR ligand, [^{125}I]cyanopindolol (approximately 500 pM; specific activity, 2200 Ci/mmol), and nonspecific binding was determined in the presence of alprenolol (20 μM). Assays were done at 37°C for 60 min and then filtered over glass fiber filters, which were washed and counted in a gamma counter. Specific binding (estimated B_{max}) was normalized to membrane protein [M. M. Bradford, *Anal. Biochem.* **72**, 248 (1976)] and reported as the mean picomoles of receptor per milligram of membrane protein ± SEM.

19. For direct immunofluorescence micrographs, 7-μm frozen sections were rinsed three times for 3 min in phosphate-buffered saline (PBS) and 3 min in PBS with 0.05% Triton X-100 (Triton-PBS). Sections were then blocked with serum diluent (10% goat serum in PBS with 0.1% bovine serum albumin and 0.1% sodium azide) and rinsed for 15 min in Triton-PBS before overnight incubation at 4°C in primary antibody (*11*) (1:500 dilution in serum diluent). The sections were then washed four times for 10 min in Triton-PBS at room temperature and incubated for 1 hour in fluorescein isothiocyanate–conjugated goat antibody to rabbit immunoglobulin G (1:50 dilution in serum diluent). After five 3-min rinses in PBS, the sections were mounted with the use of NaI (25 g liter^{-1}) in 1:1 PBS:glycerol and photographed.
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21. Mice were anesthetized with pentobarbital (50 mg/kg) and the hearts rapidly excised. Atria were dissected and placed in carbogenated modified Kreb's bicarbonate solution [25 mM $NaHCO_3$ (pH 7.4), 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.2 mM KH_2PO_4, 1.75 mM $CaCl_2 \cdot 2H_2O$, 10 mM glucose, 0.1 mM NaS_2O_5, 0.03 mM EDTA], supplemented with ascorbic acid (1.1×10^{-4} M), cocaine (1×10^{-5} M), corticosterone (4×10^{-5} M), and phentolamine (3×10^{-6} M). Left atria were paced at 3 to 7 Hz, with a pulse duration of 3 ms and voltage set at threshold plus 20%.
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