

Differential regulation of inotropy and lusitropy in overexpressed Gs α myocytes through cAMP and Ca²⁺ channel pathways

Song-Jung Kim, ... , Charles J. Homcy, Stephen F. Vatner

J Clin Invest. 1999;103(7):1089-1097. <https://doi.org/10.1172/JCI4848>.

Article

We investigated the mechanisms responsible for altered contractile and relaxation function in overexpressed Gs α myocytes. Although baseline contractile function (percent contraction) in Gs α mice was similar to that of wild-type (WT) mice, left ventricular myocyte contraction, fura-2 Ca²⁺ transients, and Ca²⁺ channel currents (I_{Ca}) were greater in Gs α mice in response to 10⁻⁸ M isoproterenol (ISO) compared with WT mice. The late phase of relaxation of the isolated myocytes and fura-2 Ca²⁺ transients was accelerated at baseline in Gs α but did not increase further with ISO. *In vivo* measurements using echocardiography also demonstrated enhanced relaxation at baseline in Gs α mice. Forskolin and CaCl₂ increased contraction similarly in WT and Gs α mice. Rp-cAMP, an inhibitor of protein kinase, blocked the increases in contractile response and Ca²⁺ currents to ISO in WT and to forskolin in both WT and Gs α . It also blocked the accelerated relaxation in Gs α at baseline but not the contractile response to ISO in Gs α myocytes. Baseline measurements of cAMP and phospholamban phosphorylation were enhanced in Gs α compared with WT. These data indicate that overexpression of Gs α accelerates relaxation at end diastolic but does not affect baseline systolic function in isolated myocytes. However, the enhanced responses to sympathetic stimulation partly reflect increased Ca²⁺ channel activity; *i.e* the cellular mechanisms mediating these effects appear to involve a cAMP-independent as well [...]

Find the latest version:

<https://jci.me/4848/pdf>



Differential regulation of inotropy and lusitropy in overexpressed Gs α myocytes through cAMP and Ca²⁺ channel pathways

Song-Jung Kim,¹ Atsuko Yatani,¹ Dorothy E. Vatner,¹ Satoshi Yamamoto,¹ Yoshihiro Ishikawa,¹ Thomas E. Wagner,² Richard P. Shannon,¹ Young-Kwon Kim,¹ Gen Takagi,¹ Kuniya Asai,¹ Charles J. Homcy,³ and Stephen F. Vatner¹

¹Cardiovascular and Pulmonary Research Institute, Allegheny University of the Health Sciences, Pittsburgh, Pennsylvania 15212, USA

²Department of Molecular Medicine, Clemson University, and the Greenville Hospital System/Clemson University Biomedical Cooperative, Greenville, South Carolina 29605, USA

³COR Therapeutics Inc., South San Francisco, California 94080, USA

Address correspondence to: Stephen F. Vatner, Cardiovascular Research Institute, Allegheny University of the Health Sciences, 15th floor, South Tower, 320 East North Avenue, Pittsburgh, Pennsylvania 15212, USA. Phone: (412) 359-8255; Fax: (412) 359-8257; E-mail: svatner@pgh.ugh.edu

Received for publication August 7, 1998, and accepted in revised form February 23, 1999.

We investigated the mechanisms responsible for altered contractile and relaxation function in overexpressed Gs α myocytes. Although baseline contractile function (percent contraction) in Gs α mice was similar to that of wild-type (WT) mice, left ventricular myocyte contraction, fura-2 Ca²⁺ transients, and Ca²⁺ channel currents (I_{Ca}) were greater in Gs α mice in response to 10⁻⁸ M isoproterenol (ISO) compared with WT mice. The late phase of relaxation of the isolated myocytes and fura-2 Ca²⁺ transients was accelerated at baseline in Gs α but did not increase further with ISO. *In vivo* measurements using echocardiography also demonstrated enhanced relaxation at baseline in Gs α mice. Forskolin and CaCl₂ increased contraction similarly in WT and Gs α mice. Rp-cAMP, an inhibitor of protein kinase, blocked the increases in contractile response and Ca²⁺ currents to ISO in WT and to forskolin in both WT and Gs α . It also blocked the accelerated relaxation in Gs α at baseline but not the contractile response to ISO in Gs α myocytes. Baseline measurements of cAMP and phospholamban phosphorylation were enhanced in Gs α compared with WT. These data indicate that overexpression of Gs α accelerates relaxation at end diastolic but does not affect baseline systolic function in isolated myocytes. However, the enhanced responses to sympathetic stimulation partly reflect increased Ca²⁺ channel activity; *i.e.* the cellular mechanisms mediating these effects appear to involve a cAMP-independent as well as a cAMP-dependent pathway.

J. Clin. Invest. 103:1089–1097 (1999).

Introduction

The sympathetic nervous system plays a major role in the regulation of cardiovascular function under various stress conditions, such as hypotension, exercise, and the fight-or-flight reaction, by releasing catecholamines, which, in turn, activate the β -adrenergic receptor-Gs-adenylyl cyclase (β AR-Gs-AC) pathway. To understand the physiological and pathological mechanisms of this cascade when the stimulation is chronic, murine models have been created by altering expression of components of the β AR signaling pathway (1–9). These models all demonstrated enhanced efficacy of the β AR-Gs-AC signaling pathway (1–9). However, the chronic augmentation of β AR signaling in the model with overexpression of cardiac Gs α leads to myocyte hypertrophy and cardiomyopathy as these animals age (2, 4, 5), which differs from what has been reported in the other models (8, 9). Another controversial issue is whether Gs α can alter L-type Ca²⁺ channel activity in a cAMP-PKA-independent manner. Prior studies exist supporting such a mechanism *in vitro* (10–13), although there is another

that contradicts this position (14). The current model of overexpressed Gs α presents a unique opportunity to reconcile this controversy. To address these mechanistic questions, it is necessary to study the contractile function of isolated myocytes, which has not been done previously in the model of overexpressed Gs α (1–7). Furthermore, all prior studies have concentrated on inotropic function *in vivo* (2–5), neglecting another important controlling mechanism, lusitropic function. The isolated myocyte technique lends itself to examination of that aspect of myocardial function as well. Finally, it was important to conduct the present investigation in adult as opposed to neonatal (7) myocytes, because (a) it is possible to examine contractile and relaxation function as well as Ca²⁺ channel activity in adult cells, and (b) it is well known that β AR-Gs-AC regulation differs markedly in neonatal animals (15–17).

The first goal of this study was to investigate the regulation of isolated left ventricular (LV) myocyte contractility by the β AR-Gs-AC pathway and the extent to which myocyte contractile function is altered by overexpression

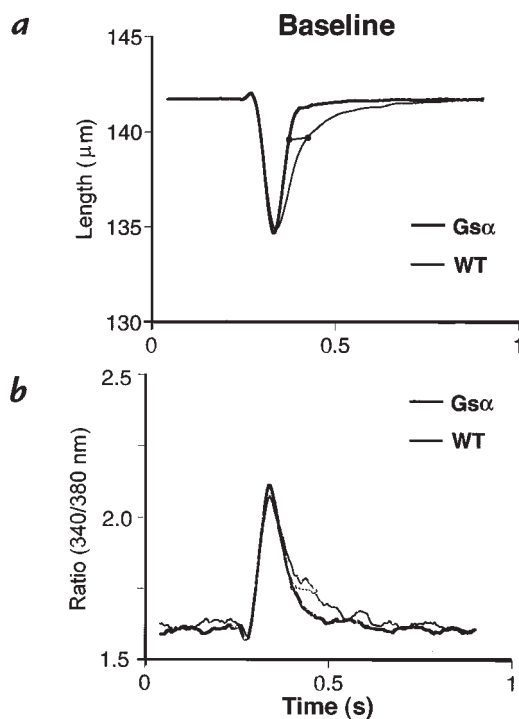


Figure 1
Superimposed representative length (a) and Ca²⁺ transient (b) recordings from a WT myocyte (thin line) and Gs α -overexpressed myocyte (thick line). Neither myocyte contractile function nor systolic fura-2 signaling was affected in overexpressed Gs α myocytes. However, the recovery in late diastole was faster in Gs α , as noted by the time to 70% recovery of relaxation (circles on tracings). WT, wild-type.

of cardiac Gs α , as this approach will provide an assessment of intrinsic contractile function independent of the extracellular matrix and hemodynamic and neurohormonal effects. As part of this goal, it was important to determine whether lusitropic function was affected in a fashion parallel to inotropic function in the isolated myocytes. Once it was determined that lusitropic function was altered at baseline both in isolated myocytes and *in vivo*, the mechanism was investigated by measuring baseline cAMP and phospholamban phosphorylation. The next goal of this study was to determine whether the mechanism for the enhanced effects in response to β AR stimulation involved an action on the Ca²⁺ channel, potentially independent of cAMP. This was accomplished using a dual approach. First, Ca²⁺ channel function was assessed directly using patch-clamp techniques. Second, the effects of β AR stimulation were examined after the cAMP pathway was blocked with Rp-cAMP, an inhibitor of protein kinase A (PKA), which should abolish the enhanced inotropic effects induced by β AR stimulation of AC via Gs.

Methods

Preparation of LV myocytes. Cardiac myocytes were prepared from WT and Gs α -overexpressed (Gs α) mice 15 \pm 2 weeks old, as described previously (18). In brief, the heart was rapidly excised and submerged in Ca²⁺-free Tyrode's solution containing (in mmol/l): 140 NaCl, 5.4 KCl, 1 MgCl₂, 0.33 NaH₂PO₄, 10 glu-

cose, and 5 HEPES (pH 7.4). The aorta was cannulated with a blunt-tip needle (20 gauge) on a perfusion apparatus. The heart was perfused for 3 min with Tyrode's solution and then perfused for 18 min with Tyrode's solution with 2% FCS (Sigma Chemical Co., St. Louis, Missouri, USA) and 75 U/ml each of collagenase 1 and 2 (Worthington Biochemical, Freehold, New Jersey, USA) at 37°C. All solutions were continuously bubbled with 95% O₂ and 5% CO₂ at 37°C. Isolated myocytes were stored in Tyrode's solution with 2% FCS and 10% BSA (Fraction V; Sigma Chemical Co.) before the experiment. Myocytes were stored at room temperature, and mechanical studies were completed within 3 h after isolation to minimize deterioration due to prolonged storage of cells. Whether the mice were transgenic was blinded until the results of the studies were completed.

Measurement of contractile and relaxation function. Myocytes were transferred to a warmed (37°C) and continuously perfused cell chamber located on an inverted microscope stage (Nikon Inc., Melville, New York, USA). The chamber was perfused with physiological buffer containing (in mmol/l): 120 NaCl, 2.6 KCl, 1.2 MgCl₂, 1.2 KH₂PO₄, 11 glucose, 5 HEPES, 25 NaHCO₃, 2 taurine, 1 pyruvate, and 1 CaCl₂. Myocyte contraction was induced at 1 Hz by platinum field electrodes that were placed in the cell chamber and attached to a stimulator (S48; Grass Instrument Co., Quincy, Massachusetts, USA). Cell images were continuously monitored through a \times 40 objective lens (Nikon Inc.) and transmitted to a charge-coupled device (CCD) video camera (TM-640; Pulnix, Mountain View, California, USA). The output from the CCD camera was displayed on a video monitor (PVM-135; Sony, New York, New York, USA). Myocyte length was measured using a video motion edge detector (VED103; Crescent Electronics, Sandy, Utah, USA), and the data were acquired at 240 images per second. Myocyte dimensions were calibrated

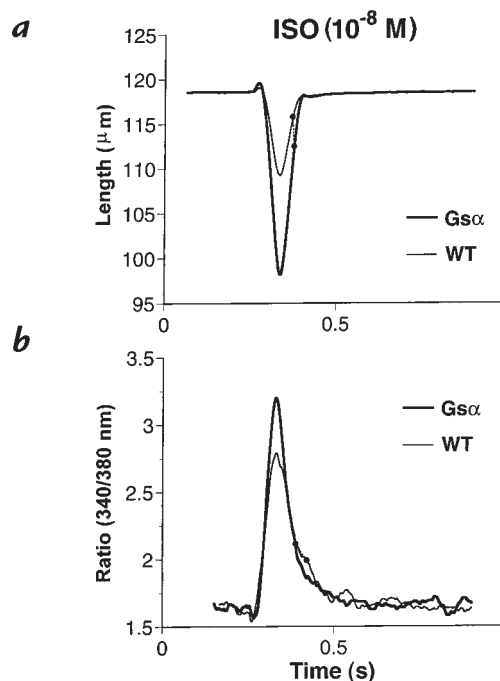


Figure 2
Superimposed representative contraction (a) and Ca²⁺ transient (b) recordings in response to ISO (10⁻⁸ M) in WT (thin line) and Gs α (thick line). In response to ISO, the enhanced contractile function was associated with an increased Ca²⁺ transient in Gs α . Note that differences in late diastolic recovery were less apparent after ISO (compare with Figure 1), as the time to 70% recovery of relaxation was no longer accelerated in Gs α . ISO, isoproterenol.

Table 1Contractile and relaxation function in response to ISO (10^{-8} M) in myocytes from WT and $Gs\alpha$ mice

	Baseline	ISO	Baseline	ISO
Myocyte contractile and relaxation				
Diastolic length (μm)	139 \pm 6	136 \pm 5	138 \pm 4	141 \pm 6
Systolic length (μm)	130 \pm 6	125 \pm 5	130 \pm 4	121 \pm 5
Contraction (%)	6.2 \pm 0.8	8.4 \pm 1.1 ^B	6.1 \pm 0.3	14.2 \pm 0.9 ^{A,B}
-dL/dt _{max} ($\mu\text{m/s}$)	-369 \pm 56	-532 \pm 84 ^B	-376 \pm 28	-885 \pm 114 ^{A,B}
+dL/dt _{max} ($\mu\text{m/s}$)	317 \pm 48	465 \pm 55 ^B	341 \pm 38	808 \pm 90 ^{A,B}
TR 70% (ms)	57 \pm 7	43 \pm 2 ^B	46 \pm 7 ^A	47 \pm 6
<i>n</i>	6	6	6	6
Myocyte Ca ²⁺ transient				
Baseline (340/380 nm)	1.56 \pm 0.04	1.61 \pm 0.05	1.66 \pm 0.09	1.62 \pm 0.04
Amplitude (340/380 nm)	0.51 \pm 0.06	0.67 \pm 0.04 ^B	0.55 \pm 0.09	0.87 \pm 0.08 ^{A,B}
TRC 70% (ms)	139 \pm 17	112 \pm 11 ^B	94 \pm 9 ^A	82 \pm 5 ^A
<i>n</i>	5	5	4	4

-dL/dt_{max}, the maximum rate of contraction. +dL/dt_{max}, the maximum rate of relaxation. TR 70%, the time for 70% recovery of relaxation; TRC 70%, the time for 70% recovery of Ca²⁺ transient. ISO, isoproterenol. WT, wild-type. Values are means \pm SE. ^A*P* < 0.05 vs. respective WT. ^B*P* < 0.05 vs. respective baseline.

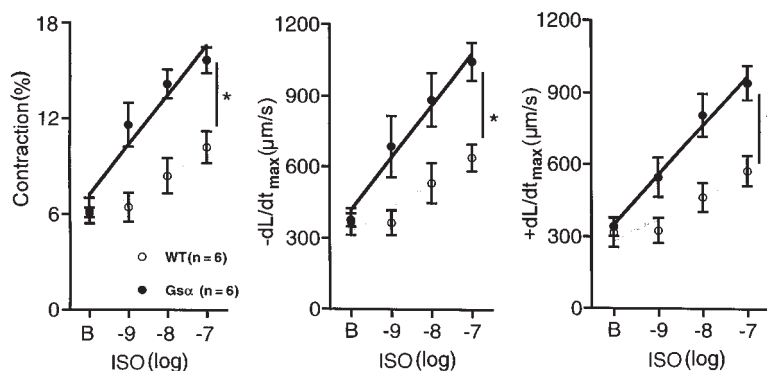
with a hemocytometer grid placed on the microscope stage. Sarcomere length was measured in isolated myocytes using light microscopy. There was no difference in sarcomere length (WT: 1.88 \pm 0.08 μm , *n* = 3; $Gs\alpha$: 1.90 \pm 0.09 μm , *n* = 3).

Myocyte contractile and relaxation function in WT and $Gs\alpha$ mice was assessed (a) with isoproterenol (ISO; 10^{-9} to 10^{-7} M) to determine the extent to which β AR function is altered (10 myocytes from 6 WT, and 9 myocytes from 6 $Gs\alpha$); (b) with forskolin (10^{-8} to 10^{-6} M), which directly activates AC, to determine whether intrinsic AC activity was also enhanced (10 myocytes from 6 WT, and 11 myocytes from 6 $Gs\alpha$); (c) with Rp-cAMP (200 μM), which blocks PKA, to determine whether the enhanced contractile and relaxation function in response to ISO (7 myocytes from 5 WT, and 10 myocytes from 5 $Gs\alpha$) and forskolin (10 myocytes from 4 WT, and 11 myocytes from 4 $Gs\alpha$) in $Gs\alpha$ mice is solely due to an enhanced β AR-Gs-AC signaling pathway or whether a cAMP-independent mechanism is also operative (7 myocytes from 5 WT, and 10 myocytes from 5 $Gs\alpha$); and (d) with CaCl₂ (2 and 3 mM) to determine whether the inotropic and lusitropic responses of $Gs\alpha$ myocytes to non- β AR-mediated stimulation were altered (9 myocytes from 6 WT, and 11 myocytes from 6 $Gs\alpha$). Myocytes were preincubated with Rp-cAMP for at least 30 min before the experiment. A study by Gjertsen *et al.* (19) demonstrated that Rp-cAMP is a nonhydrolyzable,

membrane-permeable, and selective antagonist of cAMP. Rp-cAMP, per se, did not affect contractile function in isolated myocytes and failed to inhibit the contractile responses to cAMP-independent mechanisms, *e.g.*, Ca²⁺ (20, 21).

Measurement of myocyte Ca²⁺ transients. Myocytes were loaded with 3.8 mM of fura-2 (Sigma Chemical Co.), dissolved in DMSO, and maintained at room temperature (25°C) for 30 min in Tyrode's solution with 10% BSA (Fraction V; Sigma Chemical Co.), *i.e.*, without Ca²⁺. After loading, cells were washed with Tyrode's solution for 30 min and placed in the myocyte perfusion chamber on the microscope, as already described here. The myocytes were excited by ultraviolet light (wavelengths 340 and 380 nm, alternately), and the fura emission wavelength (510 nm) was synchronously monitored by the Photocan dual-beam spectrofluorophotometer (Photon Technology International, Monmouth Junction, New Jersey, USA). Intracellular-free Ca²⁺ was measured as the fluorescence ratio (340/380 nm), and the measurement of the fluorescent signal was averaged from an area within a single cell (22). Loaded myocytes were stimulated at 1 Hz, and measurements from an individual myocyte were taken before and during ISO stimulation. These experiments were conducted in 32 myocytes from 5 WT mice, and 26 myocytes from 4 $Gs\alpha$ mice.

Measurement of L-type Ca²⁺ channel current. Whole-cell currents were recorded using patch-clamp techniques in WT (*n* = 5) and

**Figure 3**

Enhanced contractile (percent contraction, -dL/dt) and relaxation (+dL/dt) function in response to ISO in $Gs\alpha$ compared with WT, and the upward shift of the dose-response curves. **P* < 0.05 vs. respective WT.

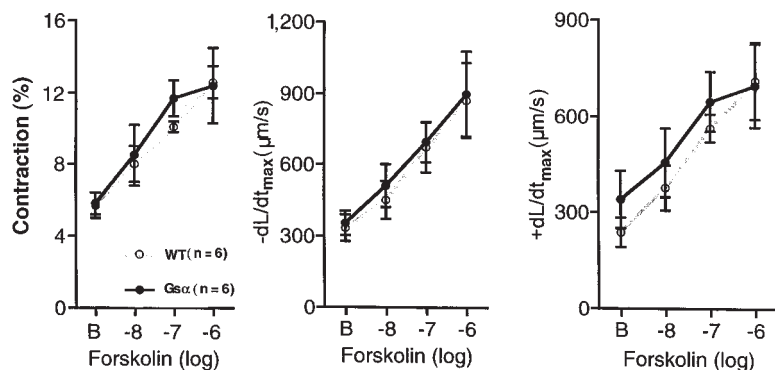


Figure 4
Contraction and relaxation in response to forskolin (10^{-8} to 10^{-6} M). Forskolin elicited similar increases in contractile and relaxation function in $Gs\alpha$ and WT.

$Gs\alpha$ ($n = 5$) mice as described previously (23). The patch pipettes had a resistance of 2 M Ω or less. The experimental chamber (0.2 ml) was placed on a microscope stage, and the external solution changes were made using a modified Y-tube technique (24). The external solution contained (in mmol/l): 2 CaCl₂, 1 MgCl₂, 135 tetraethyl ammonium chloride, 5, 4-aminopyridine, 10 glucose, and 10 HEPES (pH 7.3). The pipette solution was (in mmol/l): 100 Cs aspartate, 20 CsCl, 1 MgCl₂, 2 MgATP, 0.5 GTP, 5 EGTA or 10 BAPTA, and 5 HEPES (pH 7.3). These external and internal solutions provided isolation of Ca²⁺ channel currents (I_{Ca}) from other membrane currents, such as Na⁺ and K⁺ channel currents, and also from Ca²⁺ flux through the Na⁺/Ca²⁺ exchanger (25). In the initial characterization of basic I_{Ca} kinetics, cells were dialyzed with 5 mM EGTA, as we have shown previously that Ca²⁺-dependent inactivation properties can be reliably measured under these experimental conditions (23, 26). To determine responses to β AR stimulation, myocytes were dialyzed with the faster Ca²⁺ chelator BAPTA to minimize Ca²⁺-dependent inactivation and subsequent negative regulation of Ca²⁺ channels (26).

Membrane capacitance was measured using voltage ramps of 0.8 V/s from a holding potential of -50 mV. No difference in myocyte size, evaluated by cell capacitance, was observed (WT: 113.7 \pm 3.1 pF, $n = 40$; $Gs\alpha$: 115.0 \pm 3.0 pF, $n = 53$). Myocyte Ca²⁺ channel function in WT and $Gs\alpha$ mice was assessed with ISO (10^{-9} to 10^{-6} M), forskolin (5×10^{-6} M), and Rp-cAMP (100 μ M). All experiments were done at room temperature.

Determination of cAMP levels. Whole hearts from both $Gs\alpha$ ($n = 7$) and WT ($n = 8$) were prepared by rapid freezing with liquid nitrogen. Tissue cAMP levels were determined by a double-antibody RIA that uses a prereacted antibody complex with the use of a specific assay kit (cAMP[¹²⁵I] RIA kit; Du Pont NEN Research Products, Boston, Massachusetts, USA) (27).

Western blot analysis for phospholamban phosphorylation. Cardiac myocytes were isolated from both $Gs\alpha$ and WT mice. Proteins were separated by SDS-PAGE on a 15% polyacrylamide gel for

phospholamban. For immunological detection of phospholamban phosphorylation, blots were probed as described previously (28) with polyclonal antibodies raised against a phospholamban peptide phosphorylated at Ser¹⁶ (1:10,000) (PhosphoProtein Research, West Yorkshire, United Kingdom).

Echocardiography. Echocardiography was performed using ultrasonography (Apogee X-200; Interspec Inc., Ambler, Pennsylvania, USA) as described previously (2). In brief, a dynamically focused 9-MHz annular array transducer was applied from below, using the saline bag as a standoff. M-mode measurements of LV internal dimension (LVID) were sampled from more than three beats and averaged. End diastole (d) was measured at the time of the apparent maximal LV diastolic dimension, and end systole (s) was measured at the time of the most anterior systolic excursion of the posterior wall. LV ejection fraction (LVEF), as an index of systolic function, was calculated by the cubed method: $LVEF = [(LVIDd)^3 - (LVIDs)^3]/LVIDd^3$.

Diastolic function was assessed using the E/A ratio, with E representing early diastolic flow and A representing late diastolic flow after atrial systole (29).

Data analysis. The camera images at 240 samples per second were converted to length measurements by the video edge detector and were analyzed by the data acquisition system. A combination of five-point median smoothing with three-point linear smoothing was performed to have minimal effect on the data; the median filtering rid the wave form of any noise spikes, and the linear filtering approximated the transitions between samples of the length of signal. This results in a slight underestimation of the true dL/dt values, but it has little effect on the relaxation calculations. Also, a minimum of three beats were analyzed to avoid underestimation during the peak contraction. Shortening was calculated by the length differences from diastole to systole. Measurements from the calcium transients were used to evaluate the peak change in the calcium ratio from baseline values. As already explained here, three beats were averaged

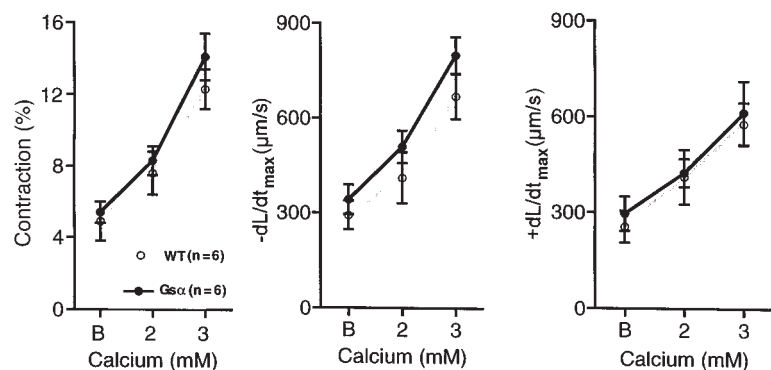


Figure 5
Contraction and relaxation in response to CaCl₂ (2 and 3 mM). CaCl₂ elicited similar increases in contractile and relaxation function in $Gs\alpha$ and WT.

Table 2Contractile and relaxation function in response to ISO (10^{-8} M) following Rp-cAMP (200 μ m) in myocytes from WT and Gs α mice

	WT			Gs α		
	Baseline		ISO Rp-cAMP	Baseline		ISO Rp-cAMP
	Pre	Post		Pre	Post	
Diastolic length (μ m)	139 \pm 4	146 \pm 11	142 \pm 9	144 \pm 4	147 \pm 9	152 \pm 11
Systolic length (μ m)	136 \pm 4	138 \pm 10	134 \pm 7	135 \pm 4	139 \pm 7	134 \pm 9
Contraction (%)	6.0 \pm 0.5	5.5 \pm 0.7	5.8 \pm 0.5	5.9 \pm 0.4	5.6 \pm 1.2	12.1 \pm 1.2 ^{A,B}
-dL/dt _{max} (μ m/s)	-387 \pm 45	-350 \pm 84	-358 \pm 95	-352 \pm 25	-350 \pm 95	-855 \pm 138 ^{A,B}
+dL/dt _{max} (μ m/s)	290 \pm 36	256 \pm 80	272 \pm 76	306 \pm 32	263 \pm 79	663 \pm 85 ^{A,B}
TR 70% (ms)	63 \pm 4	62 \pm 14	56 \pm 10	47 \pm 3 ^A	57 \pm 4 ^B	50 \pm 5
n	15	5	5	15	5	5

-dL/dt_{max}, the maximum rate of contraction. +dL/dt_{max}, the maximum rate of relaxation. TR 70%, the time for 70% recovery of relaxation. ^A $P < 0.05$ vs. respective WT. ^B $P < 0.05$ vs. respective pre-baseline.

on a temporal basis by aligning the leading edge of each waveform at 50% of peak level, after smoothing the data with a 21-point Savitsky-Goulet polynomial routine (PTI Software, Monmouth Junction, New Jersey, USA)

All myocyte data for contraction, relaxation, and fura-2 signaling were averaged to obtain one data point from each animal. These data are expressed as mean \pm SE. Comparison of the data between WT and Gs α were performed by Student's *t* test for grouped comparisons, with differences considered significant at $P < 0.05$. Dose-response relationships were compared between WT and Gs α using regression analysis.

Results

Baseline contractile and relaxation function. Figure 1 shows representative contraction/relaxation and Ca²⁺ transient recordings at baseline in WT and Gs α . Although peak amplitude of contraction and Ca²⁺ transients were not different from WT myocytes, the late relaxation phase in Gs α myocytes was significantly shorter; this was associated with accelerated Ca²⁺ uptake. As summarized in Table 1, indices of systolic function (percent contraction and -dL/dt) were similar in Gs α and WT, as was one index of diastolic function, the rate of relaxation (+dL/dt). However, as noted in Figure 1, the late phase of relaxation was accelerated in Gs α : the time for 70% recovery of relaxation was less ($P < 0.05$) in Gs α (46 \pm 7 ms) compared with WT (57 \pm 7 ms) (Table 1). Similar data were observed for the Ca²⁺ transients. The amplitude of systolic Ca²⁺ uptake function was similar at baseline for WT myocytes (0.51 \pm 0.06) and Gs α myocytes (0.55 \pm 0.09), whereas the late recovery was significantly faster in Gs α -overexpressed myocytes. For example, the time to 70% recovery of the Ca²⁺ transient was less ($P < 0.05$) in Gs α myocytes (94 \pm 9 ms) compared with myocytes from WT mice (139 \pm 17 ms).

To determine whether results of contractile and relaxation function obtained in isolated myocytes are similar to what is observed *in vivo*, systolic and diastolic function was also assessed by echocardiography. Consistent with the *in vitro* data, we found a significant ($P < 0.05$) increase in relaxation, using the E/A ratio as an index of diastolic

function (Gs α : 1.29 \pm 0.04; WT: 1.19 \pm 0.02). However, ejection fraction as an index of LV systolic function *in vivo* was not different at baseline in the Gs α and WT mice (Gs α : 71 \pm 2%, $n = 3$; WT: 73 \pm 2%, $n = 3$).

Contraction and relaxation responses to isoproterenol, forskolin, and CaCl₂. Figure 2 shows representative contraction/relaxation and Ca²⁺ transient recordings in response to isoproterenol (ISO) in Gs α and WT mice. Peak systolic contraction and fura-2 signaling were enhanced in Gs α after ISO. The differences in late diastolic recovery between Gs α and WT mice were no longer apparent, because this part of relaxation was accelerated by ISO in WT but not in Gs α mice. Figure 3 compares dose-response data for contractile function (percent contraction, -dL/dt_{max}) and relaxation function (+dL/dt_{max}) in Gs α and WT, whereas Table 1 compares the data at one dose. In response to ISO, myocyte contractile indices and rate of relaxation (+dL/dt_{max}) were increased more ($P < 0.05$) in Gs α than in WT myocytes, and the dose-response curve to ISO was shifted (Figure 3). The slopes of the dose responses for both contractile and relaxation indexes were significantly greater in Gs α myocytes ($P < 0.05$) compared with WT myocytes. For example, at ISO 10^{-8} M, percent contraction in Gs α myocytes was increased ($P < 0.05$) (14.2 \pm 0.9 vs. 8.4 \pm 1.1%) and the maximum rate of contraction (-dL/dt_{max}) was increased ($P < 0.05$) (-885 \pm 114 vs. -532 \pm 84 μ m/s), compared with myocytes from WT. The maximum rate of relaxation (+dL/dt_{max}) in Gs α myocytes was also increased ($P < 0.05$) compared with WT myocytes (808 \pm 90 vs. 465 \pm 55 μ m/s) (Table 1). Of note, the enhanced contractile responses to ISO were associated with increased Ca²⁺ transients measured by fura-2 (Figure 2). The amplitude of the Ca²⁺ signal in Gs α myocytes was significantly increased (from 0.55 \pm 0.09 to 0.87 \pm 0.08; $P < 0.05$) compared with WT controls (from 0.51 \pm 0.06 to 0.67 \pm 0.04; $P < 0.05$). ISO reduced the time to 70% recovery of relaxation in WT (from 57 \pm 7 to 43 \pm 2 ms) but did not further reduce the time to 70% recovery of relaxation in Gs α (from 46 \pm 7 to 47 \pm 6 ms). Similarly, the time for 70% decay of the Ca²⁺ transient was

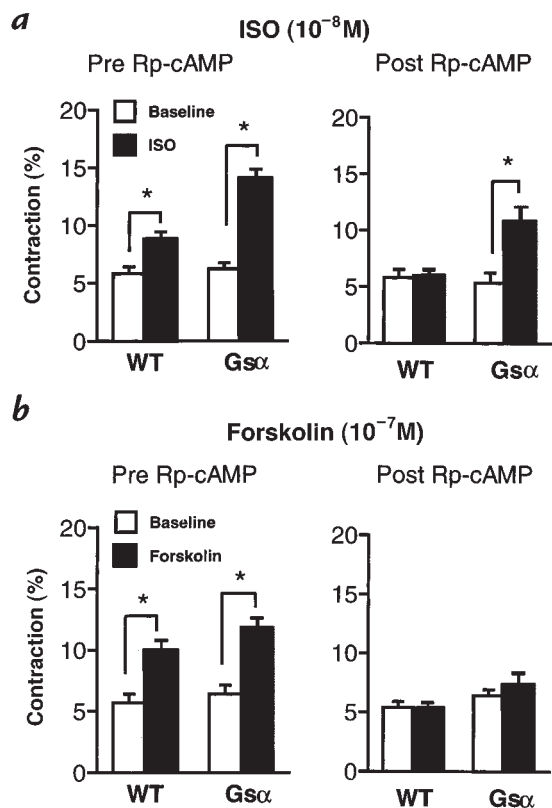


Figure 6 Contractile function in response to ISO (**a**) and forskolin (**b**) in the presence of Rp-cAMP. Rp-cAMP blocked the increase in percent contraction in response to ISO in WT and to forskolin in both WT and Gs α , but not the response to ISO in Gs α myocytes. * $P < 0.05$ vs. respective baseline.

reduced ($P < 0.05$) with ISO in WT myocytes (from 139 ± 17 to 112 ± 11 ms) but not in Gs α myocytes (from 94 ± 9 to 82 ± 5 ms.). To determine whether the enhanced ISO responses observed in Gs α myocytes were a consequence of an action proximal to AC activation, the effects of forskolin on contractile function were measured. Forskolin elicited similar increases in contractile and relaxation function in Gs α and WT mice (Figure 4). Furthermore, non- β AR-mediated inotropic stimulation by CaCl₂ was not altered in Gs α myocytes (Figure 5).

Contractile and relaxation function in the presence of Rp-cAMP. As shown in Table 2 and Figure 6, Rp-cAMP completely blocked LV contractile and relaxation function in response to ISO in WT myocytes but not in Gs α myocytes. Interestingly, after Rp-cAMP, the differences in the time to 70% recovery of the relaxation between WT and Gs α myocytes at baseline were abolished (WT: 62 ± 14 ms; Gs α : 57 ± 4 ms) because Rp-cAMP prolonged the time for 70% recovery in Gs α myocytes (from 47 ± 3 to 57 ± 4 ms) ($P < 0.005$). However, increases in contractile function in both Gs α and WT myocytes in response to forskolin (10^{-7} M) were completely abolished in the presence of Rp-cAMP (Figure 6). Thus, the increased contractile function in Gs α myocytes in response to ISO is not simply a result of enhanced AC activity but, rather, may involve a cAMP-independent mechanism. We exam-

ined the L-type Ca²⁺ channel to determine whether its regulation was similarly altered in Gs α myocytes.

Ca²⁺ channel function in response to ISO and the effects of Rp-cAMP. In an attempt to characterize more fully the mechanisms for the enhanced inotropy in Gs α myocytes, Ca²⁺ channel activity was measured in myocytes from Gs α ($n = 5$) and WT ($n = 5$) mice. Although the current-voltage (I-V) relationships in Gs α myocytes are similar to those in WT myocytes, the Ca²⁺ channel current (I_{Ca}) density was significantly less ($P < 0.01$) (Figure 7). However, the response to dihydropyridines was not altered. For example, 0.1 μ M Bay K 8644 (a dihydropyridine agonist) increased I_{Ca} and also shifted the I-V relationship to negative potentials (Gs α : 2.1 ± 0.3 -fold and 12.3 ± 1.4 mV, $n = 8$; WT: 2.3 ± 0.1 -fold and 14.4 ± 0.7 mV, $n = 17$). Similarly, a dihydropyridine antagonist, nifedipine (1 μ M), reduced I_{Ca} amplitude to $9.59 \pm 0.03\%$ of baseline in Gs α ($n = 5$), similar to that observed in WT ($11.20 \pm 0.02\%$ of baseline, $n = 5$). Interestingly, the maximal I_{Ca} response to ISO in Gs α was significantly higher compared with WT myocytes (Gs α : 3.1 ± 0.2 -fold, $n = 16$; WT: 2.3 ± 0.1 -fold, $n = 42$; $P < 0.001$) (Figure 8). However, there was no difference in the effects of forskolin (5 μ M) (Gs α : 2.0 ± 0.1 -fold, $n = 14$; WT: 2.1 ± 0.1 -fold, $n = 6$), indicating that the enhanced responsiveness reflects signaling at the level of Gs α . To support this hypothesis, the effects of ISO were reexamined in the presence of Rp-cAMP (100 μ M). Consistent with the enhanced response to ISO as measured by myocyte contraction, I_{Ca} response to ISO in the presence of Rp-cAMP was significantly higher in Gs α ($P < 0.001$) compared with WT myocytes (Figure 9).

Determination of cAMP concentration and Western blot analysis. The concentration of cAMP in the heart was significantly higher ($P < 0.05$) in Gs α mice (1.21 ± 0.08

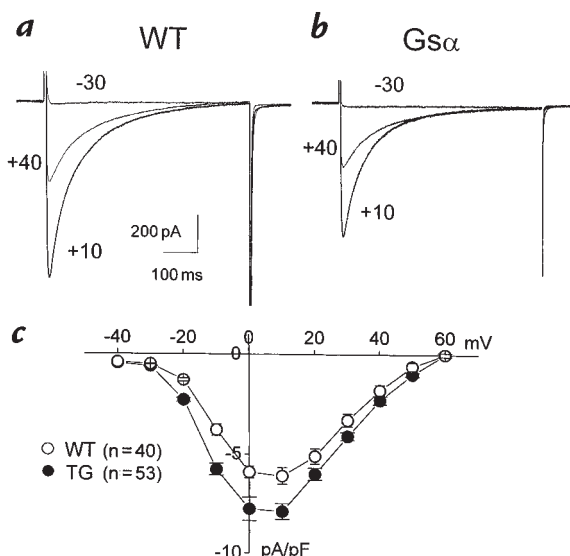


Figure 7 Representative I_{Ca} recordings in WT (**a**) and Gs α (**b**) mice. Currents were elicited from a holding potential of -50 mV to the indicated test potentials. (**c**) A comparison of the current-voltage relationships in WT and Gs α mice. I_{Ca} was normalized to the cell capacitance to give current densities (pA/pF). Average peak I_{Ca} densities for WT and Gs α mice were 8.4 ± 0.5 (pA/pF) and 6.6 ± 0.3 (pA/pF), respectively. Numbers correspond to number of cells. I_{Ca}, Ca²⁺ channel currents.

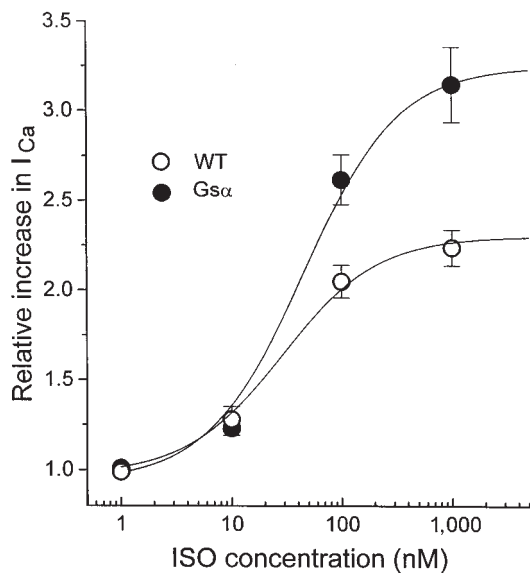


Figure 8 Concentration-dependent effects of ISO on I_{Ca} in WT and $Gs\alpha$ mice. The increase of current amplitude relative to baseline was plotted against ISO concentration. The increase in I_{Ca} amplitude in $Gs\alpha$ myocytes in response to ISO was significantly higher than in WT myocytes ($P < 0.005$). Data are mean \pm SE from 16–42 cells.

pmol/mg tissue, $n = 7$) compared with WT mice (0.94 ± 0.08 pmol/mg tissue, $n = 8$). Furthermore, phospholamban phosphorylation (Ser¹⁶) was higher at baseline in myocytes from $Gs\alpha$ mice (Figure 10).

Discussion

Activation of the sympathetic nervous system plays a major role in maintaining cardiovascular homeostasis by increasing inotropy, chronotropy, and lusitropy. These changes are mediated by activation of the β AR signaling pathway, leading to PKA activation and phosphorylation of intracellular proteins. A key target of PKA is the sarcolemmal L-type Ca^{2+} channel, which, when phosphorylated, enhances Ca^{2+} entry into the cell (30). Although this signaling pathway is clearly important in the acute and subacute maintenance of cardiovascular homeostasis under conditions of stress, the extent to which chronic stimulation of this pathway is beneficial or deleterious remains controversial (2). To understand the physiological and pathological mechanisms of this cascade with chronic stimulation, a murine model was created by overexpressing myocardial $Gs\alpha$, a component of the β AR signaling pathway (1–7). Recent studies in our laboratory on this model demonstrated that cardiac $Gs\alpha$ overexpression enhances inotropic and chronotropic responses to endogenous sympathetic stimulation in younger animals, but as the animals age, a cardiomyopathy develops (2, 5). These studies of cardiac function were carried out using echocardiography in anesthetized mice. These *in vivo* techniques, however, are limited. The intrinsic regulation of LV myocyte inotropy by the β AR- Gs -AC pathway and the extent to which myocyte contraction and relaxation is altered by overexpression of $Gs\alpha$, independent of the extracellular matrix and hemodynamic and

neurohormonal effects, cannot be directly assessed. Furthermore, the prior *in vivo* studies of cardiac function in mice with overexpressed cardiac $Gs\alpha$ did not measure the effects on relaxation (2, 5).

In the current investigation, we have, to our knowledge characterized for the first time both contractile and relaxation function in isolated myocytes from mice with overexpressed cardiac $Gs\alpha$. Although baseline contractile function was not altered in $Gs\alpha$ myocytes, consistent with previous *in vivo* observations in this model (2), myocyte contractile function was augmented in response to ISO and was associated with increased Ca^{2+} transients, assessed by fura loading, and with Ca^{2+} channel activity, assessed with patch-clamp measurements. Because forskolin, which stimulates cAMP distal to the β AR, elicited similar increases in contractile and relaxation function and Ca^{2+} channel activity in both $Gs\alpha$ and WT mice, and, further, because forskolin's action was blocked by Rp-cAMP, it can be concluded that altered AC catalytic activity was not the responsible mechanism. The results with $CaCl_2$ treatment, which increases inotropy independent of cAMP, and the lack of any observed differences between WT and $Gs\alpha$ myocytes in their contractile responses, also support the position that augmented inotropic responses to β AR stimulation with ISO in $Gs\alpha$ myocytes is a consequence of enhanced signaling via the β AR pathway rather than an alteration in Ca^{2+} handling or Ca^{2+} sensitivity at the subcellular-myofilament level.

We therefore attempted to determine whether the enhanced signal mediated by β AR stimulation in $Gs\alpha$ myocytes was due solely to increased cAMP production.

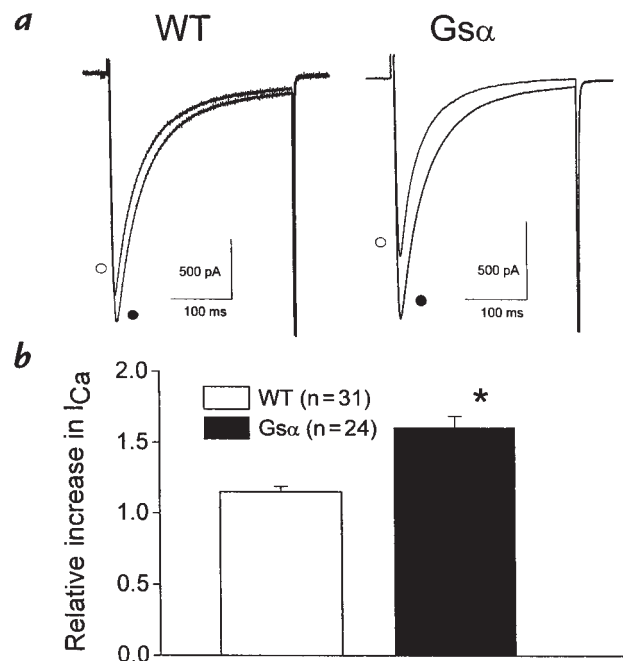


Figure 9 Change to I_{Ca} response in the presence of Rp-cAMP. (a) Current traces were recorded in WT and $Gs\alpha$ myocytes from a holding potential of -50 mV to 0 mV and were superimposed before (open circles) and after ISO (filled circles). (b) Mean increase of I_{Ca} elicited by ISO assessed in the presence of Rp-cAMP, ISO, increased I_{Ca} in $Gs\alpha$ myocytes, but not in WT. Numbers correspond to number of cells. * $P < 0.001$ vs. respective WT.

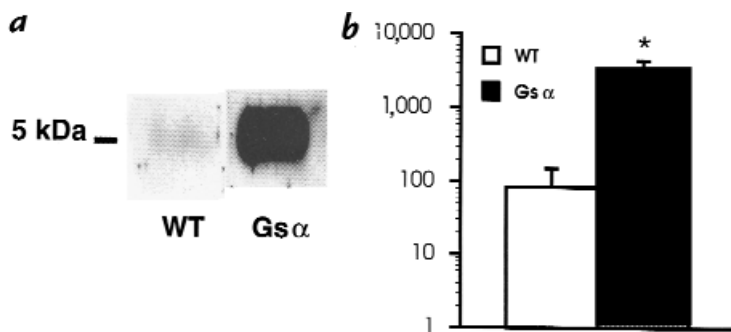


Figure 10
(a) Western blots of baseline phospholamban phosphorylation at Ser¹⁶ in isolated cardiac myocytes from WT and Gs α mice. **(b)** Baseline phospholamban phosphorylation at Ser¹⁶ in Gs α myocytes ($n = 3$) is increased compared with WT ($n = 3$). Y axis is in arbitrary units.

To accomplish this, the effects of ISO stimulation were also examined by PKA blockade with Rp-cAMP. In WT myocytes, ISO no longer elicited an increase in systolic contraction after Rp-cAMP, indicating that essentially the entire response to ISO was cAMP-mediated. As already noted, the Gs α myocytes did not respond to forskolin with increased contraction after Rp-cAMP. In contrast, the Gs α myocytes still responded to ISO with enhanced contraction in the presence of Rp-cAMP. These experiments suggested that overexpressed Gs α permitted ISO to exert a positive inotropic effect independent of cAMP, *e.g.*, potentially by an action directly or indirectly on the Ca²⁺ channel. In support of this hypothesis, measurement of I_{Ca} demonstrated a significantly increased response to ISO in the presence of Rp-cAMP in Gs α myocytes but not in WT myocytes. Interestingly, a recent study by Muntz *et al.* (6) demonstrated that the Gs α protein was localized in the T-tubules and intercalated disks in the Gs α myocytes. These and other data indicate that colocalization of the various components of both the β AR signaling unit (β AR-Gs-AC) and its targets, particularly those regulating Ca²⁺ handling, allows efficient and rapid activation of all components necessary to enhance contractility in response to β AR stimulation. Although Hartzell *et al.* (14) demonstrated that inotropic response to β AR stimulation was exclusively due to a cAMP-dependent pathway, other studies have identified another pathway by which β AR agonists can increase Ca²⁺ currents via a cAMP-independent pathway (10–13). The data in the present manuscript support the latter point of view (10–13) and thus may help to resolve this controversy.

The results of Ca²⁺ currents in this investigation differ in certain ways from a recent study by Lader *et al.* (7), which also used our Gs α myocytes but those that were dialyzed with the protein kinase inhibitor PKI and found to still exhibit an increase in I_{Ca} compared with nondialyzed Gs α myocytes. The study by Lader *et al.* found enhanced I_{Ca} at baseline in Gs α myocytes, which appears to be inconsistent with the current investigation. There is one important difference in the two studies: Lader *et al.* studied neonatal, cultured myocytes, whereas the current study used freshly prepared adult myocytes. Our preliminary data suggest that Gs α overexpression is enhanced in neonatal transgenic hearts compared with that in adult transgenic hearts (Vatner, D., unpublished data). Similarly, in neonatal myocytes from transgenic mice with β_2 -AR overexpression, the baseline Ca²⁺ currents were also higher (31), but Ca²⁺ channel activity in

adult β_2 -AR-overexpressed myocytes is reduced compared with WT myocytes (32). Thus, the discrepancy between Ca²⁺ channel activity in neonatal and adult myocytes appears consistent for both β_2 -AR overexpression and Gs α overexpression and points to an important limitation in extrapolating from neonatal or fetal to adult physiological regulation. This is particularly relevant to β AR-Gs-AC regulation, which is well recognized to be different in neonatal animals (15–17).

As already noted, prior studies in the Gs α model did not examine diastolic function. In the current investigation, echocardiographic assessment demonstrated enhanced relaxation *in vivo*, without an alteration in systolic contraction in overexpressed Gs α . The recovery of the late phase of diastole for both the myocyte length and fura signal was accelerated in the overexpressed Gs α myocytes at baseline. This suggests an interesting possibility that Ca²⁺ reuptake during late diastole is regulated by Gs α even at baseline. In further support of this possibility, after Rp-cAMP, the differences in recovery during late diastole were no longer different in Gs α and WT myocytes. The cellular basis for the accelerated relaxation in Gs α myocytes is not clear. It is possible that there is an enhanced SR Ca²⁺ uptake due to an increase in phosphorylation of phospholamban (33) in Gs α myocytes. Indeed, both increased cAMP levels at baseline and enhanced phospholamban phosphorylation were observed in the present study. It is also possible that other Ca²⁺ regulatory proteins are altered in this transgenic model, independent from, but potentially in response to, the actual genetic perturbation.

In conclusion, overexpression of Gs α resulted in more rapid relaxation at end diastole, but it does not affect baseline systolic function in isolated myocytes. Improved baseline diastolic function, independent from systolic function, was also observed *in vivo*. Both inotropic and lusitropic responses to β AR stimulation are enhanced in Gs α myocytes. The enhanced inotropic response to β AR stimulation partly reflects increased Ca²⁺ channel activity, and the cellular mechanisms mediating effects on both systolic and diastolic function appear to involve both a cAMP-independent as well as a cAMP-dependent pathway.

1. Gaudin, C., *et al.* 1995. Overexpression of Gs α protein in the hearts of transgenic mice. *J. Clin. Invest.* **95**:1676–1683.
2. Iwase, M., *et al.* 1996. Adverse effects of chronic endogenous sympathetic drive induced by cardiac Gs α overexpression. *Circ. Res.* **78**:517–524.
3. Uechi, M., *et al.* 1998. Depressed heart rate variability and arterial barore-

- flex in conscious transgenic mice with overexpression of cardiac Gs α . *Circ. Res.* **82**:416–423.
4. Vatner, D.E., et al. 1998. Overexpression of myocardial Gs α prevents full expression of catecholamine desensitization despite increased β -adrenergic receptor kinase. *J. Clin. Invest.* **101**:1916–1922.
 5. Iwase, M., et al. 1997. Cardiomyopathy induced by cardiac Gs α overexpression. *Am. J. Physiol.* **272**:H585–H589.
 6. Muntz, K.H., et al. 1997. Localisation of cardiac Gs α in transgenic mice overexpressing Gs α . *J. Mol. Cell. Cardiol.* **29**:1649–1653.
 7. Lader, A.S., et al. 1998. Cardiac Gs α overexpression enhances L-type calcium channels through an adenylyl cyclase independent pathway. *Proc. Natl. Acad. Sci. USA.* **95**:9669–9674.
 8. Milano, C.A., et al. 1994. Enhanced myocardial function in transgenic mice overexpressing the β_2 -adrenergic receptor. *Science.* **264**:582–586.
 9. Koch, W.J., et al. 1995. Cardiac function in mice overexpressing the β -adrenergic receptor kinase or a β ARK inhibitor. *Science.* **268**:1350–1353.
 10. Pelzer, S., et al. 1990. Membrane-delimited stimulation of heart cell calcium current by β -adrenergic signal-transducing G $_s$ protein. *Am. J. Physiol.* **259**:H264–H267.
 11. Yatani, A., et al. 1987. A G protein directly regulates mammalian cardiac calcium channels. *Science.* **238**:1288–1292.
 12. Yatani, A., and Brown, A.M. 1989. Rapid β -adrenergic modulation of cardiac calcium channel currents by a fast G protein pathway. *Science.* **245**:71–74.
 13. Cavalié, A., Allen, T.J.A., and Trautwein, W. 1991. Role of the GTP-binding protein G $_s$ in the β -adrenergic modulation of cardiac Ca channels. *Pflugers Arch.* **419**:433–443.
 14. Hartzell, H.C., Méry, P.-F., Fischmeister, R. and Szabo, G. 1991. Sympathetic regulation of cardiac calcium current is due exclusively to cAMP-dependent phosphorylation. *Nature.* **351**:573–576.
 15. Rockson, S.G., et al. 1981. Cellular mechanisms of impaired adrenergic responsiveness in neonatal dogs. *J. Clin. Invest.* **67**:319–327.
 16. Cros, G.H., Chanez, P.O., Michel, A., Boucard, M. and Serrano, J.-J. 1988. Post-natal evolution of rat cardiac beta-adrenoceptors. *Life Sci.* **43**:699–706.
 17. Zeiders, J.L., Seidler, F.J., and Slotkin, T.A. 1997. Ontogeny of regulatory mechanisms for β -adrenoceptor control of rat cardiac adenylyl cyclase: targeting of G-proteins and the cyclase catalytic subunit. *J. Mol. Cell. Cardiol.* **29**:603–615.
 18. Kim, S.-J., et al. 1999. An α -cardiac myosin heavy chain gene mutation impairs contraction and relaxation function of cardiac myocytes. *Am. J. Physiol.* In press.
 19. Gjertsen, B.T., et al. 1995. Novel (Rp)-cAMPs analogs as tools for inhibition of cAMP-kinase in cell culture. *J. Biol. Chem.* **270**:20599–20607.
 20. Bell, D., and McDermott, B.J. 1994. Use of the cyclic AMP antagonist, Rp-cAMPS, to distinguish between cyclic AMP-dependent and cyclic AMP-independent contractile responses in rat ventricular cardiomyocytes. *J. Mol. Cell. Cardiol.* **26**:1439–1448.
 21. Zhou, Y.-Y., et al. 1997. Localized cAMP-dependent signaling mediates β_2 -adrenergic modulation of cardiac excitation-contraction coupling. *Am. J. Physiol.* **273**:H1611–H1618.
 22. Williford, D.J., Sharma, V.K., Korth, M., and Shen, S. 1990. Spatial heterogeneity of intracellular Ca $^{2+}$ concentration in nonbeating guinea pig ventricular myocytes. *Circ. Res.* **66**:234–241.
 23. Masaki, H., Sato, Y., Luo, W., Kranias, E.G., and Yatani, A. 1997. Phospholamban deficiency alters inactivation kinetics of L-type Ca $^{2+}$ channels in mouse ventricular myocytes. *Am. J. Physiol.* **272**:H606–H612.
 24. Yamamoto, S., Kuntzweiler, T.A., Wallick, E.T., Sperelakis, N., and Yatani, A. 1996. Amino acid substitutions in the Na $^+$, K $^+$ -ATPase α 1 subunit alter the cation regulation of pump current expressed in HeLa cells. *J. Physiol. (Lond.)* **495**:467–485.
 25. Weir, W.G. 1990. Cytoplasmic [Ca $^{2+}$] in mammalian ventricle: dynamic control by cellular processes. *Annu. Rev. Physiol.* **52**:467–485.
 26. Sako, H., Green, S., Kranias, E.G., and Yatano, A. 1997. Modulation of Ca $^{2+}$ channel currents by isoproterenol studied in transgenic ventricular myocytes with altered SR Ca $^{2+}$ content. *Am. J. Physiol.* **273**:C1666–C1672.
 27. Frandsen, E.K., and Krishna, G. 1976. A simple ultrasensitive method for the assay of cyclic AMP and cyclic GMP in tissues. *Life Sci.* **18**:529–541.
 28. Luo, W., et al. 1998. Transgenic approaches to define the functional role of dual site phospholamban phosphorylation. *J. Biol. Chem.* **273**:4734–4739.
 29. Feigenbaum, H. 1994. *Echocardiography*. Lea & Febiger. Philadelphia, PA. 151–155.
 30. Bers, D.M. 1991. Excitation-contraction coupling and cardiac contractile force. In *Developments in cardiovascular medicine*. Kluwer Academic Publishers Boston, MA. 49–69.
 31. Health, B., An, R.H., Koch, W., Lefkowitz, R.J., and Kass, R.S. 1998. Targeting of cAMP-dependent increases in L-type Ca $^{2+}$ (I_{Ca}) over delayed K $^+$ channel (I_K) activity by overexpression of the β_2 -adrenergic receptor (β_2 AR) in the developing mouse heart. *Biophys. J.* **74**:A35. (Abstr.)
 32. Yatani, A., Szigeti, G.P., Liggett, S., and Dorn, G.W., II. 1999. Cardiac specific overexpression of β_2 -adrenergic receptors is associated with decreased Ca $^{2+}$ current density and increased myocyte size. *Biophys. J.* **76**:A369. (Abstr.)
 33. Li, L., Chu, X., Kranias, E.G., and Bers, D.M. 1998. Phosphorylation of phospholamban is more crucial than troponin I phosphorylation in PKA mediated acceleration of relaxation in cardiac myocytes. *Biophys. J.* **74**:A248. (Abstr.)