Differential regulation of inotropy and lusitropy in overexpressed Gsα myocytes through cAMP and Ca^{2+} channel pathways

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We investigated the mechanisms responsible for altered contractile and relaxation function in overexpressed Gsα myocytes. Although baseline contractile function (percent contraction) in Gsα mice was similar to that of wild-type (WT) mice, left ventricular myocyte contraction, fura-2 Ca^{2+}transients, and Ca^{2+} channel currents (I_{ca}) were greater in Gsα 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 Ca^{2+} transients was accelerated at baseline in Gsα but did not increase further with ISO. *In vivo* measurements using echocardiography also demonstrated enhanced relaxation at baseline in Gsα mice. Forskolin and $CaCl_2$ increased contraction similarly in WT and Gsα mice. Rp-cAMP, an inhibitor of protein kinase, blocked the increases in contractile response and Ca^{2+} currents to ISO in WT and to forskolin in both WT and Gsα. It also blocked the accelerated relaxation in Gsα at baseline but not the contractile response to ISO in Gsα myocytes. Baseline measurements of cAMP and phospholambation phosphorylation were enhanced in Gsα compared with WT. These data indicate that overexpression of Gsα 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 Ca^{2+} channel activity; *i.e* the cellular mechanisms mediating these effects appear to involve a cAMP-independent as well as a cAMP-dependent pathway.

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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 (βAR-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 βAR signaling pathway (1–9). These models all demonstrated enhanced efficacy of the βAR-Gs-AC signaling pathway (1–9). However, the chronic augmentation of βAR signaling in the model with overexpression of cardiac Gsα 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 Gsα can alter L-type Ca^{2+} 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 Gsα 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 Gsα (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 Ca^{2+} channel activity in adult cells, and (*b*) it is well known that βAR-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 βAR-Gs-AC pathway and the extent to which myocyte contractile function is altered by overexpression

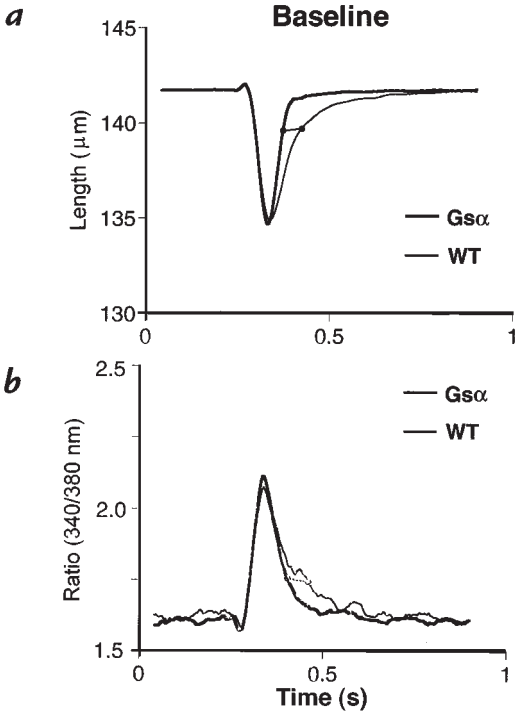

Figure 1
Superimposed representative length (***a***) and Ca^{2+} transient (***b***) recordings from a WT myocyte (*thin line*) and Gsα-overexpressed myocyte (*thick line*). Neither myocyte contractile function nor systolic fura-2 signaling was affected in overexpressed Gsα myocytes. However, the recovery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

of cardiac Gsα, 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 βAR stimulation involved an action on the Ca^{2+} channel, potentially independent of cAMP. This was accomplished using a dual approach. First, Ca^{2+} channel function was assessed directly using patch-clamp techniques. Second, the effects of βAR 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 βAR stimulation of AC via Gs.

Methods

Preparation of LV myocytes. Cardiac myocytes were prepared from WT and Gsα-overexpressed (Gsα) mice 15 ± 2 weeks old, as described previously (18). In brief, the heart was rapidly excised and submerged in Ca^{2+}-free Tyrode's solution containing (in mmol/l): 140 NaCl, 5.4 KCl, 1 $MgCl_2$, 0.33 NaH_2PO_4, 10 glucose, 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% O_2 and 5% CO_2 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 $MgCl_2$, 1.2 KH_2PO_4, 11 glucose, 5 HEPES, 25 $NaHCO_3$, 2 taurine, 1 pyruvate, and 1 $CaCl_2$. 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 ×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

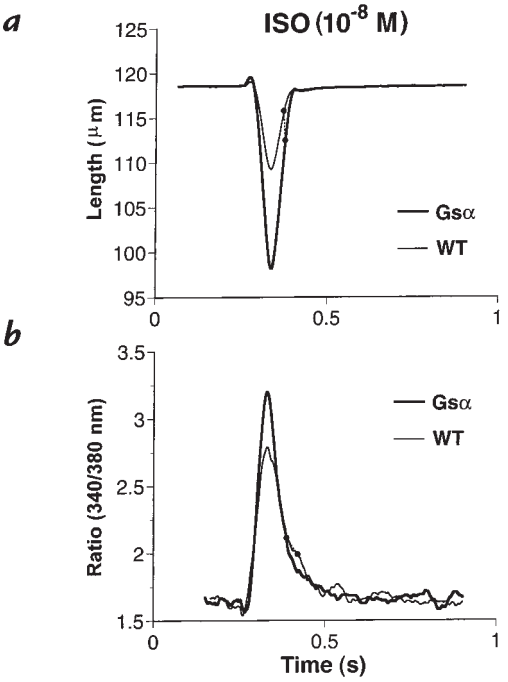

Figure 2
Superimposed representative contraction (***a***) and Ca^{2+} transient (***b***) recordings in response to ISO (10^{-8} M) in WT (*thin line*) and Gsα (*thick line*). In response to ISO, the enhanced contractile function was associated with an increased Ca^{2+} transient in Gsα. Note that differences 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 Gsα. *ISO,* isoproterenol.

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

	Baseline	ISO	Baseline	ISO
Myocyte contractile and relaxation				
Diastolic length (μm)	139 ± 6	136 ± 5	138 ± 4	141 ± 6
Systolic length (μm)	130 ± 6	125 ± 5	130 ± 4	121 ± 5
Contraction (%)	6.2 ± 0.8	8.4 ± 1.1[B]	6.1 ± 0.3	14.2 ± 0.9[A,B]
$-dL/dt_{max}$ (μm/s)	−369 ± 56	−532 ± 84[B]	−376 ± 28	−885 ± 114[A,B]
$+dL/dt_{max}$ (μm/s)	317 ± 48	465 ± 55[B]	341 ± 38	808 ± 90[A,B]
TR 70% (ms)	57 ± 7	43 ± 2[B]	46 ± 7[A]	47 ± 6
n	6	6	6	6
Myocyte Ca^{2+} transient				
Baseline (340/380 nm)	1.56 ± 0.04	1.61 ± 0.05	1.66 ± 0.09	1.62 ± 0.04
Amplitude (340/380 nm)	0.51 ± 0.06	0.67 ± 0.04[B]	0.55 ± 0.09	0.87 ± 0.08[A,B]
TRC 70% (ms)	139 ± 17	112 ± 11[B]	94 ± 9[A]	82 ± 5[A]
n	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 Ca^{2+} transient. *ISO*, isoproterenol. *WT*, wild-type. Values are means ± 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 ± 0.08 μm, $n = 3$; Gsα: 1.90 ± 0.09 μm, $n = 3$).

Myocyte contractile and relaxation function in WT and Gsα mice was assessed (*a*) with isoproterenol (ISO; 10^{-9} to 10^{-7} M) to determine the extent to which βAR function is altered (10 myocytes from 6 WT, and 9 myocytes from 6 Gsα); (*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 Gsα); (*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 Gsα) and forskolin (10 myocytes from 4 WT, and 11 myocytes from 4 Gsα) in Gsα mice is solely due to an enhanced βAR-Gs-AC signaling pathway or whether a cAMP-independent mechanism is also operative (7 myocytes from 5 WT, and 10 myocytes from 5 Gsα); and (*d*) with $CaCl_2$ (2 and 3 mM) to determine whether the inotropic and lusitropic responses of Gsα myocytes to non–βAR-mediated stimulation were altered (9 myocytes from 6 WT, and 11 myocytes from 6 Gsα). 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.*, Ca^{2+} (20, 21).

Measurement of myocyte Ca^{2+} 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 Ca^{2+}. 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 Photoscan dual-beam spectrofluorophotometer (Photon Technology International, Monmouth Junction, New Jersey, USA). Intracellular-free Ca^{2+} 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 Gsα mice.

Measurement of L-type Ca^{2+} channel current. Whole-cell currents were recorded using patch-clamp techniques in WT ($n = 5$) and

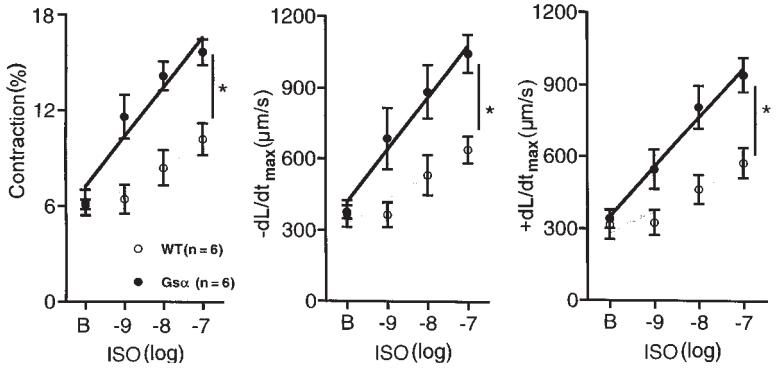

Figure 3
Enhanced contractile (percent contraction, –dL/dt) and relaxation (+dL/dt) function in response to ISO in Gsα compared with WT, and the upward shift of the dose–response curves. *$P < 0.05$ vs. respective WT.

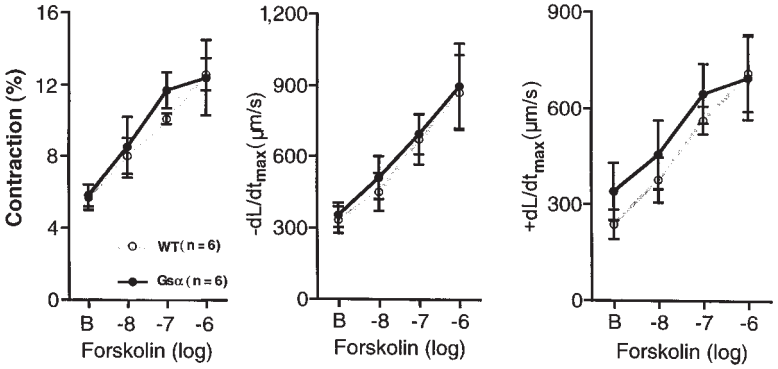

Figure 4
Contraction and relaxation in response to forskolin (10^{-8} to 10^{-6} M). Forskolin elicited similar increases in contractile and relaxation function in Gsα and WT.

Gsα ($n = 5$) mice as described previously (23). The patch pipettes had a resistance of 2 MΩ 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 $CaCl_2$, 1 $MgCl_2$, 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 $MgCl_2$, 2 MgATP, 0.5 GTP, 5 EGTA or 10 BAPTA, and 5 HEPES (pH 7.3). These external and internal solutions provided isolation of Ca^{2+} channel currents (I_{Ca}) from other membrane currents, such as Na^+ and K^+ channel currents, and also from Ca^{2+} flux through the Na^+/Ca^{2+} exchanger (25). In the initial characterization of basic I_{Ca} kinetics, cells were dialyzed with 5 mM EGTA, as we have shown previously that Ca^{2+}-dependent inactivation properties can be reliably measured under these experimental conditions (23, 26). To determine responses to βAR stimulation, myocytes were dialyzed with the faster Ca^{2+} chelator BAPTA to minimize Ca^{2+}-dependent inactivation and subsequent negative regulation of Ca^{2+} 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 ± 3.1 pF, $n = 40$; Gsα: 115.0 ± 3.0 pF, $n = 53$). Myocyte Ca^{2+} channel function in WT and Gsα mice was assessed with ISO (10^{-9} to 10^{-6} M), forskolin (5×10^{-6} M), and Rp-cAMP (100 μM). All experiments were done at room temperature.

Determination of cAMP levels. Whole hearts from both Gsα ($n = 7$) 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[^{125}I] RIA kit; Du Pont NEN Research Products, Boston, Massachusetts, USA) (27).

Western blot analysis for phospholamban phosphorylation. Cardiac myocytes were isolated from both Gsα 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 Ser[16] (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: $LVEF = [(LVIDd)^3 - (LVIDs)^3]/LVIDs^3$.

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

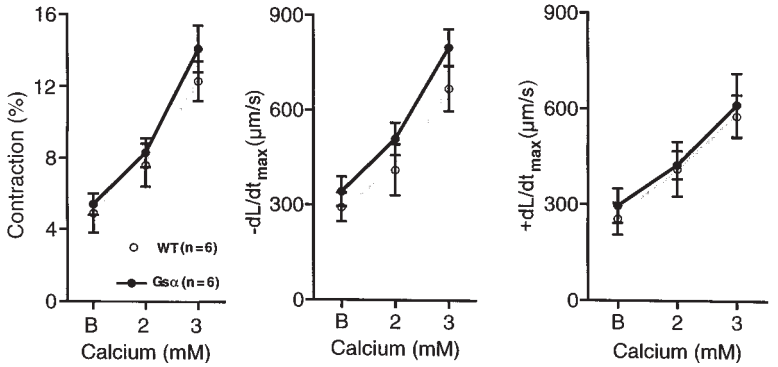

Figure 5
Contraction and relaxation in response to $CaCl_2$ (2 and 3 mM). $CaCl_2$ elicited similar increases in contractile and relaxation function in Gsα and WT.

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

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

–dL/dtmax, the maximum rate of contraction. *+dL/dtmax*, 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 ± SE. Comparison of the data between WT and Gsα 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 Gsα using regression analysis.

Results

Baseline contractile and relaxation function. Figure 1 shows representative contraction/relaxation and Ca^{2+} transient recordings at baseline in WT and Gsα. Although peak amplitude of contraction and Ca^{2+} transients were not different from WT myocytes, the late relaxation phase in Gsα myocytes was significantly shorter; this was associated with accelerated Ca^{2+} uptake. As summarized in Table 1, indices of systolic function (percent contraction and –dL/dt) were similar in Gsα 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 Gsα: the time for 70% recovery of relaxation was less ($P < 0.05$) in Gsα (46 ± 7 ms) compared with WT (57 ± 7 ms) (Table 1). Similar data were observed for the Ca^{2+} transients. The amplitude of systolic Ca^{2+} uptake function was similar at baseline for WT myocytes (0.51 ± 0.06) and Gsα myocytes (0.55 ± 0.09), whereas the late recovery was significantly faster in Gsα-overexpressed myocytes. For example, the time to 70% recovery of the Ca^{2+} transient was less ($P < 0.05$) in Gsα myocytes (94 ± 9 ms) compared with myocytes from WT mice (139 ± 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 (Gsα: 1.29 ± 0.04; WT: 1.19 ± 0.02). However, ejection fraction as an index of LV systolic function *in vivo* was not different at baseline in the Gsα and WT mice (Gsα: 71 ± 2%, $n = 3$; WT: 73 ± 2%, $n = 3$).

Contraction and relaxation responses to isoproterenol, forskolin, and $CaCl_2$. Figure 2 shows representative contraction/relaxation and Ca^{2+} transient recordings in response to isoproterenol (ISO) in Gsα and WT mice. Peak systolic contraction and fura-2 signaling were enhanced in Gsα after ISO. The differences in late diastolic recovery between Gsα and WT mice were no longer apparent, because this part of relaxation was accelerated by ISO in WT but not in Gsα mice. Figure 3 compares dose–response data for contractile function (percent contraction, $-dL/dt_{max}$) and relaxation function ($+dL/dt_{max}$) in Gsα 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 Gsα 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 Gsα myocytes ($P < 0.05$) compared with WT myocytes. For example, at ISO 10^{-8} M, percent contraction in Gsα myocytes was increased ($P < 0.05$) (14.2 ± 0.9 vs. 8.4 ± 1.1%) and the maximum rate of contraction ($-dL/dt_{max}$) was increased ($P < 0.05$) (–885 ± 114 vs. –532 ± 84 μm/s), compared with myocytes from WT. The maximum rate of relaxation ($+dL/dt_{max}$) in Gsα myocytes was also increased ($P < 0.05$) compared with WT myocytes (808 ± 90 vs. 465 ± 55 μm/s) (Table 1). Of note, the enhanced contractile responses to ISO were associated with increased Ca^{2+} transients measured by fura-2 (Figure 2). The amplitude of the Ca^{2+} signal in Gsα myocytes was significantly increased (from 0.55 ± 0.09 to 0.87 ± 0.08; $P < 0.05$) compared with WT controls (from 0.51 ± 0.06 to 0.67 ± 0.04; $P < 0.05$). ISO reduced the time to 70% recovery of relaxation in WT (from 57 ± 7 to 43 ± 2 ms) but did not further reduce the time to 70% recovery of relaxation in Gsα (from 46 ± 7 to 47 ± 6 ms). Similarly, the time for 70% decay of the Ca^{2+} transient was

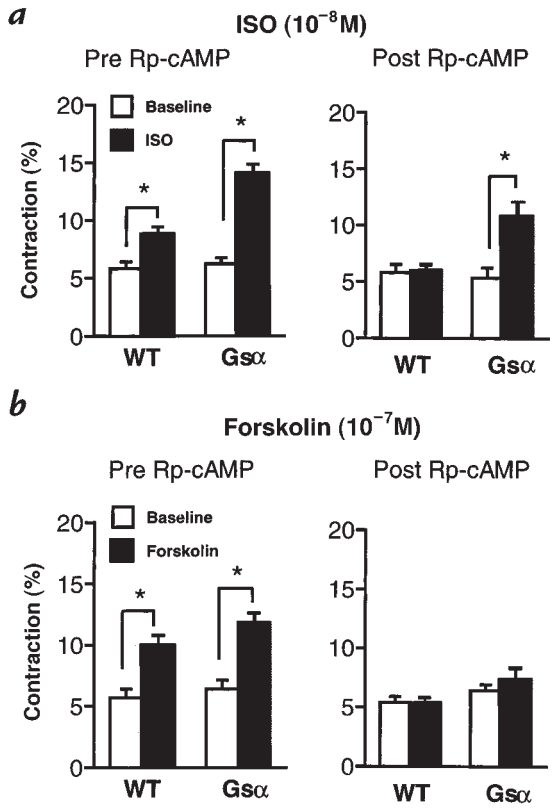

Figure 6
Contractile function in response to ISO (***a***) and forskolin (***b***) in the presence of Rp-cAMP. Rp-cAMP blocked the increase in percent contraction in response to ISO in WT and to forskolin in both WT and Gsα, but not the response to ISO in Gsα myocytes. *$P < 0.05$ vs. respective baseline.

reduced ($P < 0.05$) with ISO in WT myocytes (from 139 ± 17 to 112 ± 11 ms) but not in Gsα myocytes (from 94 ± 9 to 82 ± 5 ms.). To determine whether the enhanced ISO responses observed in Gsα 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 Gsα and WT mice (Figure 4). Furthermore, non–βAR-mediated inotropic stimulation by $CaCl_2$ was not altered in Gsα 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 Gsα myocytes. Interestingly, after Rp-cAMP, the differences in the time to 70% recovery of the relaxation between WT and Gsα myocytes at baseline were abolished (WT: 62 ± 14 ms ; Gsα: 57 ± 4 ms) because Rp-cAMP prolonged the time for 70% recovery in Gsα myocytes (from 47 ± 3 to 57 ± 4 ms) ($P < 0.005$). However, increases in contractile function in both Gsα 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 Gsα myocytes in response to ISO is not simply a result of enhanced AC activity but, rather, may involve a cAMP-independent mechanism. We examined the L-type Ca^{2+} channel to determine whether its regulation was similarly altered in Gsα myocytes.

Ca^{2+} 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 Gsα myocytes, Ca^{2+} channel activity was measured in myocytes from Gsα ($n = 5$) and WT ($n = 5$) mice. Although the current–voltage (I–V) relationships in Gsα myocytes are similar to those in WT myocytes, the Ca^{2+} 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 μM Bay K 8644 (a dihydropyridine agonist) increased I_{Ca} and also shifted the I–V relationship to negative potentials (Gsα: 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 μM), reduced I_{Ca} amplitude to 9.59 ± 0.03% of baseline in Gsα ($n = 5$), similar to that observed in WT (11.20 ± 0.02% of baseline, $n = 5$). Interestingly, the maximal I_{Ca} response to ISO in Gsα was significantly higher compared with WT myocytes (Gsα: 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 μM) (Gsα: 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 Gsα. To support this hypothesis, the effects of ISO were reexamined in the presence of Rp-cAMP (100 μM). 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 Gsα ($P < 0.001$) 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 Gsα mice (1.21 ± 0.08

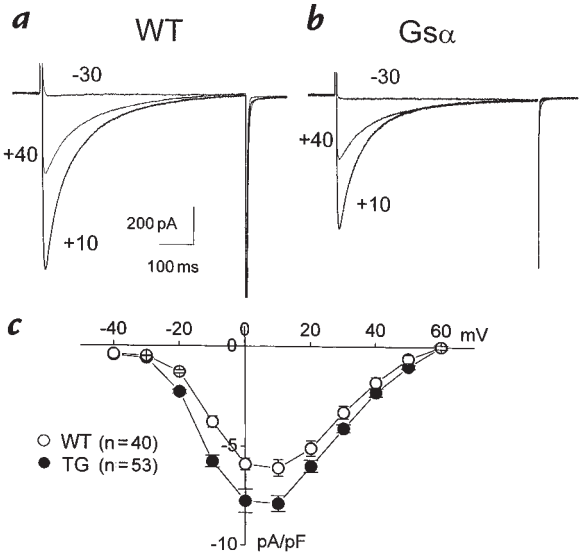

Figure 7
Representative I_{Ca} recordings in WT (***a***) and Gsα (***b***) mice. Currents were elicited from a holding potential of –50mV to the indicated test potentials. (***c***) A comparison of the current–voltage relationships in WT and Gsa mice. I_{Ca} was normalized to the cell capacitance to give current densities (pA/pF). Average peak I_{Ca} densities for WT and Gsα mice were 8.4 ± 0.5 (pA/pF) and 6.6 ± 0.3 (pA/pF), respectively. Numbers correspond to number of cells. I_{Ca}, Ca^{2+} channel currents.

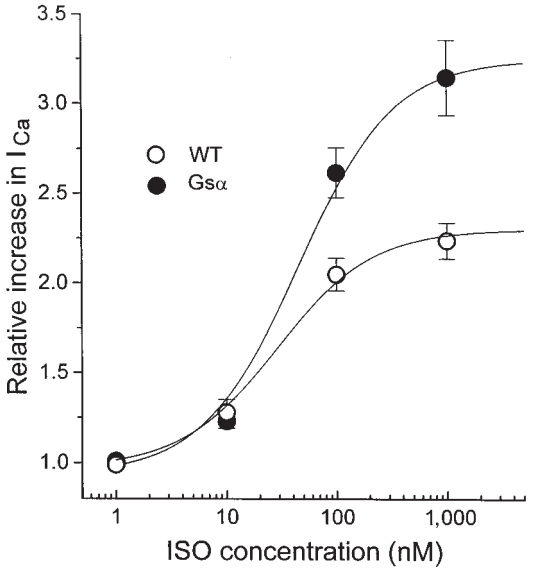

Figure 8
Concentration-dependent effects of ISO on I_{Ca} in WT and Gsα mice. The increase of current amplitude relative to baseline was plotted against ISO concentration. The increase in I_{Ca} amplitude in Gsα myocytes in response to ISO was significantly higher than in WT myocytes ($P < 0.005$). Data are mean ± 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 (Ser^{16}) was higher at baseline in myocytes from Gsα 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 βAR 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 Gsα, a component of the βAR signaling pathway (1–7). Recent studies in our laboratory on this model demonstrated that cardiac Gsα 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 βAR-Gs-AC pathway and the extent to which myocyte contraction and relaxation is altered by overexpression of Gsα, 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 Gsα 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 Gsα. Although baseline contractile function was not altered in Gsα 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 βAR, elicited similar increases in contractile and relaxation function and Ca^{2+}channel activity in both Gsα 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 Gsα myocytes in their contractile responses, also support the position that augmented inotropic responses to βAR stimulation with ISO in Gsα myocytes is a consequence of enhanced signaling via the βAR 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 βAR stimulation in Gsα myocytes was due solely to increased cAMP production.

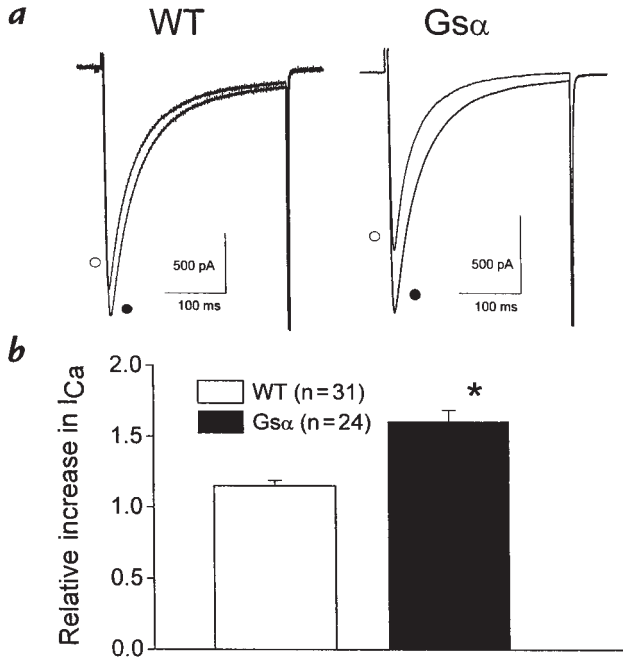

Figure 9
Change to I_{Ca} response in the presence of Rp-cAMP. (***a***) Current traces were recorded in WT and Gsα myocytes from a holding potential of –50 mV to 0 mV and were superimposed before (*open circles*) and after ISO (*filled circles*). (***b***) Mean increase of I_{Ca} elicited by ISO assessed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

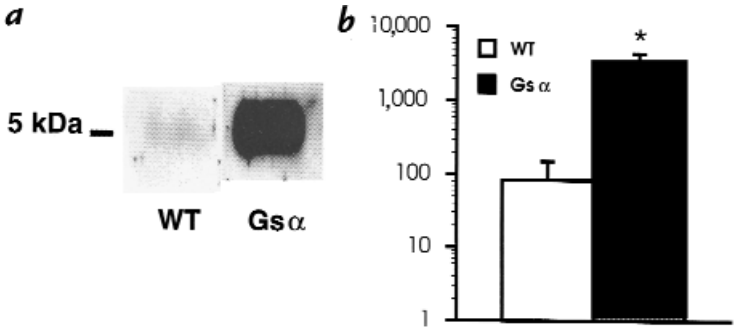

Figure 10
(***a***) Western blots of baseline phospholamban phosphorylation at Ser^{16} in isolated cardiac myocytes from WT and Gsα mice . (***b***) Baseline phospholamban phosphorylation at Ser^{16} in Gsα myocytes (n = 3) is increased compared with WT (n = 3). *Y* axis is in arbitrary units.

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 Gsα myocytes did not respond to forskolin with increased contraction after Rp-cAMP. In contrast, the Gsα myocytes still responded to ISO with enhanced contraction in the presence of Rp-cAMP. These experiments suggested that overexpressed Gsα permitted ISO to exert a positive inotropic effect independent of cAMP, *e.g.*, potentially by an action directly or indirectly on the Ca^{2+} channel. In support of this hypothesis, measurement of I_{Ca} demonstrated a significantly increased response to ISO in the presence of Rp-cAMP in Gsα myocytes but not in WT myocytes. Interestingly, a recent study by Muntz *et al*. (6) demonstrated that the Gsα protein was localized in the T-tubules and intercalated disks in the Gsα myocytes. These and other data indicate that colocalization of the various components of both the βAR signaling unit (βAR-Gs-AC) and its targets, particularly those regulating Ca^{2+} handling, allows efficient and rapid activation of all components necessary to enhance contractility in response to βAR stimulation. Although Hartzell *et al*. (14) demonstrated that inotropic response to βAR stimulation was exclusively due to a cAMP-dependent pathway, other studies have identified another pathway by which βAR agonists can increase Ca^{2+} 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 Ca^{2+} currents in this investigation differ in certain ways from a recent study by Lader *et al*. (7), which also used our Gsα 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 Gsα myocytes. The study by Lader *et al*. found enhanced I_{Ca} at baseline in Gsα 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 Gsα 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 Ca^{2+} currents were also higher (31), but Ca^{2+} channel activity in adult β_2-AR–overexpressed myocytes is reduced compared with WT myocytes (32). Thus, the discrepancy between Ca^{2+} channel activity in neonatal and adult myocytes appears consistent for both β_2-AR overexpression and Gsα overexpression and points to an important limitation in extrapolating from neonatal or fetal to adult physiological regulation. This is particularly relevant to βAR-Gs-AC regulation, which is well recognized to be different in neonatal animals (15–17).

As already noted, prior studies in the Gsα 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 Gsα. The recovery of the late phase of diastole for both the myocyte length and fura signal was accelerated in the overexpressed Gsα myocytes at baseline. This suggests an interesting possibility that Ca^{2+} reuptake during late diastole is regulated by Gsα even at baseline. In further support of this possibility, after Rp-cAMP, the differences in recovery during late diastole were no longer different in Gsα and WT myocytes. The cellular basis for the accelerated relaxation in Gsα myocytes is not clear. It is possible that there is an enhanced SR Ca^{2+} uptake due to an increase in phosphorylation of phospholamban (33) in Gsα myocytes. Indeed, both increased cAMP levels at baseline and enhanced phospholamban phosphorylation were observed in the present study. It is also possible that other Ca^{2+} regulatory proteins are altered in this transgenic model, independent from, but potentially in response to, the actual genetic perturbation.

In conclusion, overexpression of Gsα 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 βAR stimulation are enhanced in Gsα myocytes. The enhanced inotropic response to βAR stimulation partly reflects increased Ca^{2+} 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.

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