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Expression of a G_i-coupled receptor in the heart causes impaired Ca²⁺ handling, myofilament injury, and dilated cardiomyopathy

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Abstract

Increased signaling by G_i-coupled receptors has been implicated in dilated cardiomyopathy. To investigate the mechanisms, we used transgenic mice that develop dilated cardiomyopathy after conditional expression of a cardiac-targeted G_i-coupled receptor (Ro1). Activation of G_i signaling by the Ro1 agonist spiradoline caused decreased cellular cAMP levels and bradycardia in Langendorff-perfused hearts. However, acute termination of Ro1 signaling with the antagonist *nor*-binaltorphimine did not reverse the Ro1-induced contractile dysfunction, indicating that Ro1 cardiomyopathy was not due to acute effects of receptor signaling. Early after initiation of Ro1 expression, there was a 40% reduction in the abundance of the sarcoplasmic reticulum Ca²⁺-ATPase ($P < 0.05$); thereafter, there was progressive impairment of both Ca²⁺ handling and force development assessed with ventricular trabeculae. Six weeks after initiation of Ro1 expression, systolic Ca²⁺ concentration was reduced to 0.61 ± 0.08 vs. 0.91 ± 0.07 μM for control ($n = 6-8$; $P < 0.05$), diastolic Ca²⁺ concentration was elevated to 0.41 ± 0.07 vs. 0.23 ± 0.06 μM for control ($n = 6-8$; $P < 0.01$), and the decline phase of the Ca²⁺ transient (time from peak to 50% decline) was slowed to 0.25 ± 0.02 s vs. 0.13 ± 0.02 s for control ($n = 6-8$; $P < 0.01$). Early after initiation of Ro1 expression, there was a ninefold elevation of matrix metalloproteinase-2 ($P < 0.01$), which is known to cause myofilament injury. Consistent with this, 6 wk after initiation of Ro1 expression, Ca²⁺-saturated myofilament force in skinned trabeculae was reduced to 21 ± 2 vs. 38 ± 0.1 mN/mm² for controls ($n = 3$; $P < 0.01$). Furthermore, electron micrographs revealed extensive myofilament damage. These findings may have implications for some forms of human heart failure in which increased activity of G_i-coupled receptors leads to impaired Ca²⁺ handling and myofilament injury, contributing to impaired ventricular pump function and heart failure.

Keywords

matrix metalloproteinase-2; sarco(endo)plasmic reticulum Ca²⁺-ATPase; contraction; κ -opioid receptor; conditional expression

Increased signaling by G_i-coupled receptors has been implicated in human heart failure. For example, increased levels of muscarinic receptors were found in patients with idiopathic dilated cardiomyopathy (DCM) (19). Moreover, a subgroup of patients with idiopathic DCM had autoantibodies against muscarinic receptors, which exerted an agonist-like effect (12, 21). Levels of G α_i were increased in ventricles of patients with idiopathic DCM (4, 8, 27).

Furthermore, decreased adenylyl cyclase activity (an effect of G_i signaling) was found in hearts of patients with DCM (8, 9, 11). Despite the association between increased G_i signaling and DCM, the mechanisms involved remain unclear. One difficulty has been a lack of pharmacological agents that are specific for G_i -coupled cardiac receptors but do not cause undesirable side effects. Therefore, to investigate the mechanisms involved in DCM caused by G_i -coupled receptors, we used a transgenic mouse that conditionally expresses a G_i -coupled receptor (Ro1) and that subsequently develops a lethal DCM (32).

We found that, early after initiation of Ro1 expression and before functional impairment had occurred, Ro1 expression caused reduced sarcoplasmic reticulum Ca^{2+} -ATPase (SERCA) and elevated matrix metalloproteinase-2 (MMP-2) levels. Consistent with these effects, continued Ro1 expression caused progressive impairment of Ca^{2+} handling, and significant myofilament injury developed. Thus increased levels of a G_i -coupled receptor in the heart leads to abnormalities of two primary determinants of contractile function: Ca^{2+} transients and myofilament function.

METHODS

Transgenic mouse model

The Animal Studies Subcommittee of the San Francisco Veterans Affairs Medical Center approved all procedures.

Two lines of mice were used to obtain cardiac-targeted doxycycline-regulated expression of the G_i -coupled receptor Ro1 (32). In one line, the cardiac α -myosin heavy-chain promoter (α -MHC) drives expression of a transcription activator (tTA) (47). Transactivation by tTA is inhibited by doxycycline, which prevents tTA binding to DNA (13). α -MHC-tTA mice are crossed with a second line of mice containing the tTA-responsive promoter tetO linked to the Ro1 transgene. This cross yields double-transgenic mice having doxycycline-repressed expression of Ro1 in the heart (31). Doxycycline added to the animal chow (200 mg/kg, no. S3888; Bio-Serve, French-town, NJ) prevents Ro1 expression, and withdrawal of doxycycline induces Ro1 expression. Chronic Ro1 expression results in lethal cardiomyopathy with 90% mortality at 16 wk (32).

For ex vivo assays, to study early effects of Ro1 expression, we used 2 wk of doxycycline withdrawal, where Ro1 expression begins around *day 11* (31); for longer term effects of Ro1 expression, we used 4–6 wk of doxycycline withdrawal. Control animals were the single transgenic α -MHC-tTA littermates exposed to the same doxycycline dosing. Mice were maintained in an FVB/N background for over 10 generations.

In vivo and ex vivo hemodynamics

For recordings of in vivo hemodynamics, we used echocardiography in conscious mice as previously described (17). Two-dimensional long-axis images of the left ventricle (LV) were obtained in parasternal long- and short-axis views, with guided M-mode recordings at the midventricular level in both views.

For recordings of ex vivo hemodynamics, we used the Langendorff-perfused mouse heart model. Hearts were excised, mounted, and perfused, and LV pressure was recorded as previously described (42).

In vitro force and Ca^{2+} transients

Contraction force and Ca^{2+} transients were measured with ventricular trabeculae as previously described (22–24). Briefly, ultrathin right ventricular trabeculae were mounted to

a force transducer and superfused with a modified Krebs-Henseleit solution. The Ca^{2+} indicator fura 2 was loaded into the cytosol by iontophoretic injection (2). To minimize rundown of the preparation, experiments were performed at low temperature (22°C) and low pacing rate (0.5 Hz). The width and thickness of trabeculae from Ro1 mice (189 ± 12 and $107 \pm 8 \mu\text{m}$, respectively; $n = 30$) were not statistically different ($P > 0.05$) compared with trabeculae from control mice (160 ± 13 and $111 \pm 10 \mu\text{m}$, respectively; $n = 15$).

To assess myofilament function, as previously described (44), trabeculae were demembranated with 1% Triton X-100, and steady-state contractions were measured using activating solutions with various bath Ca^{2+} concentrations (44).

Biochemical analyses

As previously described, we used Western blots to monitor levels of SERCA, MMP-2, and the control protein GAPDH (3, 24).

Levels of cAMP were monitored in isolated myocytes. Myocytes from Ro1-expressing or control hearts were isolated by collagenase digestion as previously described (41). Cells were cultured overnight with or without 500 ng/ml pertussis toxin, then treated with or without $1 \mu\text{M}$ spiradoline (Ro1 agonist) for 15 min, and finally treated with or without $1 \mu\text{M}$ isoproterenol or with or without $100 \mu\text{M}$ forskolin for 10 min. Cells were washed twice with ice-cold PBS, and cAMP was extracted with 1 ml of 100% ethanol and measured by RIA (28).

Electron microscopy

Hearts were perfused with ice-cold PBS, and blocks of the LV free wall were fixed in modified Karnovsky solution. Ultrathin sections were stained with lead citrate-uranyl acetate by standard methodology.

Statistical analysis

Data are presented as means $\pm$ SE. Statistical comparisons between groups were made using *t*-test and ANOVA. The level of significance was set at $P < 0.05$.

RESULTS

Reduced cAMP production with Ro1 expression

Ro1 is a G_i -coupled receptor based on the human κ -opioid receptor that was engineered to be minimally responsive to endogenous ligands but activatable by the small molecule drug spiradoline (6). Prolonged Ro1 expression caused a lethal DCM over 16 wk, even in the absence of spiradoline, suggesting some basal signaling (32). To assay G_i signaling before cardiomyopathy developed, we measured cAMP production in myocytes isolated from Ro1 hearts 2 wk after doxycycline withdrawal, which is shortly after Ro1 expression begins (around *day 11*) (31). Figure 1A shows that the Ro1 agonist spiradoline had no effect on cAMP production in myocytes from control hearts. However, for myocytes expressing Ro1, as expected, spiradoline markedly reduced isoproterenol-stimulated cAMP generation. Moreover, the effect of spiradoline was abolished by inhibition of G_i signaling with pertussis toxin. This confirms G_i signaling mediated by Ro1.

In the absence of spiradoline, Fig. 1B shows that forskolin-stimulated cAMP production was lower in Ro1-expressing vs. control myocytes, suggesting that Ro1 expression increased basal G_i signaling. Consistent with this, in the presence of pertussis toxin, forskolin-stimulated cAMP production was not lower in Ro1-expressing vs. control myocytes. Figure 1 also shows that isoproterenol-stimulated cAMP production was lower in Ro1-expressing

vs. control cells, both with and without pertussis toxin, suggesting that β -adrenergic signaling was downregulated independent of G_i signaling.

Ro1 expression caused progressive impairment of cardiac function

In vivo echocardiography (Fig. 2A) showed that initiation of Ro1 expression by removal of doxycycline from the diet caused a progressive decrease in fractional shortening. Functional deterioration was accompanied by progressive ventricular dilation ($P < 0.001$), assessed echocardiographically from a rise of LV end-diastolic volume (Fig. 2A, *inset*). Moreover, Ro1 expression caused a progressive reduction in peak contraction force of in vitro ventricular trabeculae (Fig. 2B). Relatively little functional change had occurred in vivo or in vitro at 2 wk after doxycycline withdrawal, but there was appreciable impairment beyond 4 wk.

To investigate the role of acute regulatory effects of Ro1 signaling, we used the specific κ -opioid receptor antagonist *nor*-binaltorphimine dihydrochloride (*nor*-BNI) (Tocris Cookson, Ballwin, MO). Figure 3A shows that, for trabeculae from hearts with 0–6 wk of Ro1 expression, incubation with *nor*-BNI (20 μ M for 4 h) did not affect force development, suggesting that acute regulatory effects of Ro1 signaling were not involved in the progressive impairment of myocardial force caused by Ro1 expression.

As a positive control for inhibition of Ro1 signaling by *nor*-BNI, we studied bradycardia (a known effect of G_i signaling) induced by stimulating Ro1 with spiradoline (0.5 μ M for 10 min) using unpaced Langendorff-perfused hearts 2 wk after doxycycline withdrawal (before functional impairment occurred). Figure 3B shows that *nor*-BNI prevented the profound bradycardia induced by stimulation of Ro1 with spiradoline.

Ro1 expression caused impaired Ca^{2+} handling and Ca^{2+} responsiveness

To investigate the basis for impaired contractile function induced by Ro1 expression, we assayed Ca^{2+} handling and myofilament function. Levels of SERCA were reduced in myocytes early after initiation of Ro1 expression (2 wk after doxycycline withdrawal; Fig. 4, A and B) and before the ejection fraction and myocardial force development were impaired (Fig. 2). By 6 wk after doxycycline withdrawal, Ro1 expression had caused Ca^{2+} transients to become grossly abnormal (Fig. 4C), with decreased peak cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$), slowing of the decline phase of the Ca^{2+} transient, and increased diastolic $[Ca^{2+}]_c$. Impairment of Ca^{2+} handling worsened with the duration of Ro1 expression (Fig. 4, D–F, Table 1).

We investigated the relationship between impaired Ca^{2+} handling and impaired contraction induced by Ro1 expression, and Figure 5 shows peak $[Ca^{2+}]_c$ and peak force during contractions of ventricular trabeculae over a range of activation levels achieved by varying extracellular Ca^{2+} concentration ($[Ca^{2+}]_e$). Compared with controls, 6 wk after doxycycline withdrawal, Ro1 expression caused reduced peak $[Ca^{2+}]_c$ and peak force when $[Ca^{2+}]_e$ was between 1 and 2 mM. Maximum force of Ro1 trabeculae (22 ± 3 mN/mm²) was lower than that for controls (39 ± 2 mN/mm²) ($P < 0.001$, $n = 11$ and 7, respectively); in addition, maximal systolic $[Ca^{2+}]_c$ for Ro1 trabeculae (612 ± 76 nM) was lower than that for controls (912 ± 65 nM) ($P < 0.05$, $n = 8$ and 6, respectively).

The relationship between peak force and peak $[Ca^{2+}]_c$ for both control and Ro1 trabeculae over the full range of activation (Fig. 5C) suggests that reduced Ca^{2+} transients with Ro1 expression contributed to the reduced force vs. that shown for controls. Moreover, there was a trend for the data points for Ro1 trabeculae to lie to the right (i.e., at higher Ca^{2+} levels) of the controls, suggesting that decreased myofilament Ca^{2+} responsiveness was also involved. To investigate this possibility directly, we measured myofilament function.

Ro1 expression caused impaired myofilament force

To assess myofilament function, we measured the force of skinned trabeculae using activating solutions of different Ca^{2+} concentrations (Fig. 6A). Fitting to the Hill equation showed that the maximum myofilament force after 6 wk of Ro1 expression (21 ± 2 mN/mm²) was ~50% lower than that for controls (38 ± 0.1 mN/mm²; $P < 0.01$, $n = 3/\text{group}$). Thus, with Ro1 expression, the lower myofilament force parallels the lower force measured in intact Ro1 trabeculae. The other fit parameters of the Hill equation (EC_{50} and n), were not significantly different between Ro1 expressers and controls (not shown).

Our recent studies (3, 44) suggest that upregulation of MMP-2 leads to myofilament injury and impaired force development, suggesting a critical role for MMP-2 in the progression to heart failure. Consistent with this, we found that MMP-2 was appreciably upregulated by Ro1 expression. Figure 6B shows that MMP-2 levels were significantly increased early after Ro1 induction (2 wk off doxycycline; $n = 8-10$) and before in vivo or in vitro contractions were impaired (see Fig. 2). With longer term Ro1 expression (6 wk off doxycycline), MMP-2 levels increased further (Fig. 6B).

Myofilament injury due to Ro1 expression was evident at the ultrastructural level. Figure 6C shows that, compared with control (*left*), Ro1 expression (*right*) caused a significant decrease in myofilament volume with areas of myofilament lysis. Mitochondria appeared heterogeneous in size and contained areas of cristae disruption. This phenotype closely resembled our recent findings in a cardiac transgenic mouse expressing active MMP-2 (3).

DISCUSSION

The major findings in this study were that initiating expression of a G_i -coupled receptor (Ro1) in the heart caused an early decrease in SERCA protein levels and subsequent progressively impaired Ca^{2+} handling. Moreover, there was an early increase in MMP-2 and subsequent myofilament injury. Thus increased levels of a G_i -coupled receptor in the heart leads to abnormalities of two primary determinants of contractile function: Ca^{2+} transients and myofilament function.

Impaired Ca^{2+} transients and myofilament dysfunction

Heart failure is associated with multiple abnormalities of Ca^{2+} handling (14, 16). For example, patients with idiopathic DCM and patients with postinfarction cardiomyopathy had reduced SERCA activity, reduced sarcoplasmic reticulum Ca^{2+} uptake rate, and slowed relaxation (10, 37). The mechanisms causing impaired Ca^{2+} handling in failing myocardium remain unclear. Some studies of human heart failure found that the abundance of SERCA was decreased (15, 25, 34, 37, 40); in contrast, others found that SERCA abundance was unchanged (10, 20, 26, 35, 36). We found that initiation of Ro1 expression caused an early reduction in SERCA abundance along with slowing of the decline phase of the Ca^{2+} transient. However, at least initially, the systolic and diastolic Ca^{2+} levels were not changed, which may imply some mechanism of compensation. For example, decreased SERCA abundance in early-stage phenylephrine-induced hypertrophy caused more modest effects on Ca^{2+} signaling, suggesting compensation by other Ca^{2+} transport mechanisms (30). Ultimately, Ro1 expression did lead to elevated diastolic Ca^{2+} levels and reduced systolic Ca^{2+} levels. Moreover, the relationship between the amplitudes of the Ca^{2+} transient and the resultant force response suggested that smaller Ca^{2+} transients caused by sustained Ro1 expression contributed to the reduced myocardial contractile force.

Previous studies suggest that Ca^{2+} homeostasis is an important regulator of MMP-2 expression and activity (18, 45). Thus, with Ro1 expression, impaired Ca^{2+} clearance from the cytosol may contribute to our finding of elevated MMP-2 levels. Increased MMP-2

activity and expression are known to occur in human and animal models of heart failure (1, 29, 33, 39). Moreover, our group (3, 44) recently found that cardiac-specific expression of constitutively active MMP-2 caused myofilament dysfunction and heart failure. These findings identify MMP-2 as a critical mediator of myofilament injury and heart failure. In the present study, we found that initiation of Ro1 expression caused an early elevation of MMP-2, before functional impairment had occurred. Subsequently, Ro1 expression led to markedly impaired myofilament force and ultrastructural evidence for myofilament injury. These findings suggest that Ro1 expression leads to upregulation of MMP-2, which contributes to myofilament injury and heart failure. Our findings are consistent with a recent study demonstrating that heart failure involves impaired myofilament function manifest as a reduction in Ca^{2+} -saturated steady-state force development (7). Together, our findings suggest that impaired Ca^{2+} handling and myofilament dysfunction contribute to impaired ventricular pump function during Ro1-induced heart failure.

G_i-coupled receptor signaling and heart failure

Previous studies suggest that an antiadrenergic effect of elevated G_i signaling could counteract the hyperadrenergic state typically found in failing hearts. In contrast, other studies suggest that upregulation of G_i signaling per se may contribute to disease. Therefore, we expressed Ro1 in the heart to study the role of G_i-coupled receptor signaling in heart failure. A limitation of this transgenic approach is that it is unclear whether the artificial receptor Ro1 truly relates to endogenous G_i function in health and disease.

Ro1 expression caused a lethal DCM (32), which was prevented by inhibiting Ro1 signaling with chronic administration of the antagonist *nor*-BNI (5), suggesting that chronic Ro1 signaling was responsible for the cardiomyopathy. In the present study, we found that acutely terminating Ro1 signaling with *nor*-BNI did not reverse the contractile dysfunction of failing myocardium, suggesting that contractile dysfunction in failing myocardium did not involve acute regulatory effects of Ro1 signaling. Consistent with this, we found that Ro1 expression caused structural damage to myofilaments, which likely contributed to contractile dysfunction.

Although Ro1-induced cardiomyopathy depends on signaling by Ro1, the role of G_i signaling remains unclear. Activation of G_i signaling by Ro1 was evidenced by several findings. Stimulation of Ro1 with spiradoline caused bradycardia (a physiological effect of increased G_i signaling) in Langendorff hearts that could be inhibited by the Ro1 antagonist *nor*-BNI. Moreover, stimulation of Ro1 with spiradoline lowered isoproterenol-stimulated cAMP levels in myocytes, and this effect was prevented by inhibition of G_i signaling with pertussis toxin. Finally, with forskolin stimulation, cAMP levels were lower in myocytes expressing Ro1 than in control myocytes, and this difference was abolished by pertussis toxin.

However, Ro1 may mediate other effects that play a role in cardiomyopathy. For example, with isoproterenol stimulation, myocyte cAMP levels were lower with Ro1 expression vs. those in controls, and this difference was not abolished by inhibition of G_i signaling with pertussis toxin, suggesting that β -adrenergic signaling was downregulated independent of G_i signaling.

Interestingly, κ -opioid receptors (on which Ro1 is based) have been shown to inhibit cardiac myocyte growth and excitation-contraction coupling (38, 43). Moreover, κ -opioid receptors have also been shown to inhibit the responses of β -adrenoceptors (46). These effects of κ -opioid receptors parallel those of the modified κ -opioid receptor Ro1, which caused a reduction in the volume fraction of myofilaments, impaired ec-coupling, and decreased β -adrenergic signaling.

In conclusion, the findings of this study may have implications for the progression of some forms of DCM, where increased activity of G_i-coupled receptors in the heart may lead to impairment of both Ca²⁺ handling and myofilament function, which contribute to impaired ventricular pump function.

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Fig. 1.

Reduced cAMP production in myocytes early after initiation of Ro1 expression (2 wk doxycycline withdrawal). **A:** isoproterenol-stimulated cAMP production in myocytes in the absence (vehicle) or presence of the Ro1 agonist spiradoline and in the absence or presence of pertussis toxin ($n = 6-8/\text{group}$). $**P < 0.01$. **B:** cAMP production stimulated by isoproterenol or forskolin, in the absence or presence of pertussis toxin ($n = 9-13/\text{group}$). $*P < 0.05$, $**P < 0.01$.

Fig. 2.

Ro1 expression initiated by doxycycline withdrawal, caused left ventricular (LV) dilation (A, *inset*) and progressive impairment of cardiac function. In vivo fractional shortening (A; $n = 13-14$) and in vitro contraction force of ventricular trabeculae with 2 mM bath Ca^{2+} (B; $n = 7-11$) were significantly reduced after 4 wk of doxycycline withdrawal ($P < 0.01$).

Fig. 3.

Acute inhibition of Ro1 signaling with *nor*-binaltorphimine dihydrochloride (*nor*-BNI). **A:** for ventricular trabeculae, *nor*-BNI did not prevent the lowered contraction force caused by several weeks of Ro1 expression ($n = 2-5$). **B:** as a positive control for inhibition of Ro1 signaling by *nor*-BNI, *nor*-BNI prevented spiradoline-induced bradycardia in un-paced Langendorff-perfused Ro1-expressing hearts. Un-paced heart rate without drug was 247 ± 23 beats/min ($n = 11$). * $P < 0.05$ and ** $P < 0.01$, comparing with (+) *nor*-BNI vs. without (-) *nor*-BNI.

Fig. 4. Reduced sarcoplasmic reticulum Ca²⁺-ATPase (SERCA) and impaired Ca²⁺ handling with Ro1 expression. *A*: representative Western blots assessing SERCA and the control protein GAPDH in samples from myocytes obtained from control hearts and early after Ro1 induction (2 wk doxycycline withdrawal). *B*: summary of SERCA levels normalized to GAPDH. arb, Arbitrary unit. **P* < 0.05, *n* = 11/group. *C*: Ca²⁺ transients measured in trabeculae from control and Ro1-expressing hearts 6 wk after doxycycline withdrawal. With increasing duration of Ro1 expression, there was worsening of the reduction of systolic cytosolic Ca²⁺ concentration ([Ca²⁺]_i) (*D*), slowing of the Ca²⁺ transient decline (RT₅₀, time to 50% decline) (*E*), and rise of diastolic [Ca²⁺]_i (*F*) (*n* = 6–8/group).

Fig. 5.

Ro1 expression reduced maximum systolic $[Ca^{2+}]_c$ and force. Systolic $[Ca^{2+}]_c$ (A) and peak force (B) measured at various extracellular Ca^{2+} concentrations ($[Ca^{2+}]_e$) from trabeculae (controls and 6 wk Ro1 expression) are shown. Ro1 expression caused reduced systolic Ca^{2+} concentration ($P < 0.05$) and peak force ($P < 0.001$, $n = 6-11$ /group). C: relationship of peak force vs. peak $[Ca^{2+}]_c$. $[Ca^{2+}]_e$, extracellular Ca^{2+} concentration.

Fig. 6.

Ro1 expression caused myofilament injury. *A*: myofilament force of skinned trabeculae from control and Ro1-expressing hearts using activating solutions of various Ca²⁺ concentrations ($n = 3/\text{group}$). *B*: matrix metalloproteinase-2 (MMP-2) abundance in control and Ro1 hearts at different times after doxycycline withdrawal. $**P < 0.01$. *C*: electron micrographs of myocardium from control (*left*) and Ro1-expressing hearts (*right*) 4 wk off doxycycline. White arrow, area of myofilament lysis; black arrows, areas of cristae disruption. Original magnification = $\times 11,500$.

Table 1Effect of Ro1 expression on Ca^{2+} transient kinetics

	Time, wk			
	0	2	4	6
Time to peak, ms	61±7	68±4	67±5	66±6
RT ₅₀ , ms	129±22	226±17 [*]	337±24 [‡]	249±20 [‡]

Values are means ± SE for Ca^{2+} transient time to peak and RT₅₀ (time from peak to 50% decline) for various durations of Ro1 expression initiated by doxycycline withdrawal ($n = 6-8$) group. Ro1 expression caused slowing of the decline phase of the Ca^{2+} transient but did not affect the time to peak. Statistical differences are shown for multiple comparisons vs. *time 0* group:

^{*} $P < 0.05$,

[‡] $P < 0.01$,

[†] $P < 0.001$, using Bonferroni *t*-test.