Ca\textsuperscript{2+} Influx Through T- and L-Type Ca\textsuperscript{2+} Channels Have Different Effects on Myocyte Contractility and Induce Unique Cardiac Phenotypes

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Abstract—T-type Ca\textsuperscript{2+} channels (TTCCs) are expressed in the developing heart, are not present in the adult ventricle, and are reexpressed in cardiac diseases involving cardiac dysfunction and premature, arrhythmogenic death. The goal of this study was to determine the functional role of increased Ca\textsuperscript{2+} influx through reexpressed TTCCs in the adult heart. A mouse line with cardiac-specific, conditional expression of the α1G-TTCC was used to increase Ca\textsuperscript{2+} influx through TTCCs. α1G hearts had mild increases in contractility but no cardiac histopathology or premature death. This contrasts with the pathological phenotype of a previously studied mouse with increased Ca\textsuperscript{2+} influx through the L-type Ca\textsuperscript{2+} channel (LTCC) secondary to overexpression of its β2a subunit. Although α1G and β2a myocytes had similar increases in Ca\textsuperscript{2+} influx, α1G myocytes had smaller increases in contraction magnitude, and, unlike β2a myocytes, there were no increases in sarcoplasmic reticulum Ca\textsuperscript{2+} loading. Ca\textsuperscript{2+} influx through TTCCs also did not induce normal sarcoplasmic reticulum Ca\textsuperscript{2+} release. α1G myocytes had changes in LTCC, SERCA2a, and phospholamban abundance, which appear to be adaptations that help maintain Ca\textsuperscript{2+} homeostasis. Immunostaining suggested that the majority of α1G-TTCCs were on the surface membrane. Osmotic shock, which selectively eliminates T-tubules, induced a greater reduction in L-versus TTCC currents. These studies suggest that T- and LTCCs are in different portions of the sarcolemma (surface membrane versus T-tubules) and that Ca\textsuperscript{2+} influx through these channels induce different effects on myocyte contractility and lead to distinct cardiac phenotypes. (Circ Res. 2008;103:1109-1119.)

Key Words: T-type Ca\textsuperscript{2+} channels ■ myocyte death ■ EC coupling

Ca\textsuperscript{2+} influx through voltage-regulated ion channels is essential for initiating and regulating cardiac function.\textsuperscript{1,2} Changes in cytosolic [Ca\textsuperscript{2+}] also influence a variety of signaling pathways,\textsuperscript{3,4} regulate normal cardiac metabolism,\textsuperscript{5} and cause both physiological and pathological hypertrophy.\textsuperscript{6–9} The primary pathway for Ca\textsuperscript{2+} influx in the adult heart is via the L-type Ca\textsuperscript{2+} channel (LTCC). This Ca\textsuperscript{2+} influx pathway is essential for triggering sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release and is the major source of Ca\textsuperscript{2+} to load the SR.\textsuperscript{10} Excess Ca\textsuperscript{2+} influx through LTCCs has been linked to necrotic myocyte death, cardiac dysfunction, and premature death.\textsuperscript{11}

Ca\textsuperscript{2+} can also enter cardiac myocytes through the voltage-operated T-type Ca\textsuperscript{2+} channel (TTCC). These channels are expressed throughout cardiac development until the end of the fetal/neonatal period.\textsuperscript{12–14} TTCC expression decreases soon after birth, with little or no expression in normal adult ventricular myocytes (VMs). TTCCs are reexpressed when the heart is subjected to pathological stressors that induce cardiac hypertrophy and failure\textsuperscript{15–19} and are associated with depressed cardiac function and sudden death.\textsuperscript{20} However, the functional significance of TTCC reexpression in adult VMs has not been established and is the focus of this study. Our major goal was to determine whether Ca\textsuperscript{2+} influx through T- and L-type Ca\textsuperscript{2+} channels induce different cardiac phenotypes.

Three TTCC genes have been identified, but only 2, Ca\textsubscript{3.1} (α1G) and Ca\textsubscript{3.2} (α1H), have been found in the heart.\textsuperscript{12,21,22} All TTCCs have similar biophysical properties, including activation at more negative voltages and faster inactivation than LTCCs. Based on studies in Purkinje cells and guinea pig VMs, TTCC current (I\textsubscript{Ca,T}) appears to be less efficient than LTCC current (I\textsubscript{Ca,L}) in excitation–contraction (EC) coupling.\textsuperscript{23,24} Electrophysiological assessment of I\textsubscript{Ca,T} is technically difficult because of its small amplitude. Additionally, it is difficult to extrapolate findings in Purkinje cells to VMs...
because of the limited T-tubule and ryanodine receptor (RyR) organization of Purkinje cells.\textsuperscript{25}

\(\alpha1G\) and \(\alpha1H\) are reexpressed in the adult ventricle under pathological stress,\textsuperscript{15–19} but the involvement of these channels in cardiac dysfunction, pathological remodeling and arrhythmias is not known. To understand the function of these channels in the myocardium, we generated mice with inducible, cardiac-specific expression of the \(\alpha1G\)-TTCC. Increased Ca\(^{2+}\) influx through TTCCs should augment myocyte contractility and activate Ca\(^{2+}\)-regulated signaling pathways.\textsuperscript{11,26} Recently, we showed that increasing Ca\(^{2+}\) influx through LTCCs, by overexpressing its \(\beta2a\) subunit, increased myocyte contractility but eventually induced SR Ca\(^{2+}\) overload and Ca\(^{2+}\)-mediated myocyte necrosis, leading to heart failure and premature death.\textsuperscript{11} In the present study, we asked whether excess Ca\(^{2+}\) influx via TTCCs also causes alterations in contractility, cardiac dysfunction, and premature death.

Our experiments show that mice with cardiac-specific \(\alpha1G\)-TTCC expression have increased Ca\(^{2+}\) influx through functional TTCCs. Hearts from \(\alpha1G\) animals were mildly hypercontractile, but there was no cardiac pathology or premature death. Myocytes isolated from \(\alpha1G\) hearts had increased Ca\(^{2+}\) influx, contractions, and systolic [Ca\(^{2+}\)]\(_i\) transients, but the SR Ca\(^{2+}\) load was not increased. The duration of the [Ca\(^{2+}\)]\(_i\) transient and contractions were shortened, secondary to what appears to be adaptive changes in Ca\(^{2+}\) regulatory proteins. Experiments with electrophysiological and immunostaining approaches suggest that \(\alpha1G\)-TTCCs are more highly concentrated in the surface membrane than in T-tubules and their associated junctional SR. These findings explain why \(I_{\text{Ca,T}}\) is an inefficient trigger for SR Ca\(^{2+}\) release and an inefficient source of Ca\(^{2+}\) to load the SR. These studies also show that unlike LTCCs, Ca\(^{2+}\) influx through TTCCs does not induce Ca\(^{2+}\)-mediated cardiac pathologies or arrhythmogenic sudden death. Taken together, our new findings suggest that the source of the Ca\(^{2+}\) influx pathway can be a critical determinant of the induced cardiac phenotype.

\section*{Materials and Methods}

All experiments involving animals were approved by the Institutional Animal Care and Use Committee of Temple University.

\subsection*{Mice}

Transgenic mice with conditional and cardiac-specific expression of mouse \(\alpha1G\) subunit or rat \(\beta2a\) subunit were generated using a modified murine \(\alpha\)-myosin heavy chain (\(\alpha\)-MHC) promoter expression vector.\textsuperscript{11,26} Animals between 10 to 16 weeks of age were used for experiments.

\subsection*{Echocardiography}

Whole heart morphology and function were assessed via echocardiography. Hearts were viewed in short-axis and analyzed in M-mode.

\subsection*{Histology}

Hearts from animals (9 months old) were fixed in formalin, and longitudinal sections were evaluated for gross morphology and fibrosis.
Western Blot Analysis
Abundance/phosphorylation status of Ca\(^{2+}\) regulatory proteins in ventricular tissue was assessed as described previously.\(^{27}\)

Electrophysiology
Ca\(^{2+}\) transients (Fluo-3 epifluorescence), fractional shortening, and SR Ca\(^{2+}\) content of isolated VMs were assessed at 0.5 Hz, 35°C.\(^{28}\) Thapsigargin (1 mmol/L) was used to inhibit SR function and assess net Ca\(^{2+}\) influx. Action potentials (APs), Ca\(^{2+}\) currents, and Na\(^{+}/\)Ca\(^{2+}\) exchange (NCX) currents were measured via a Axopatch 2B voltage-clamp amplifier as described previously.\(^{11,29}\)

Immunostaining
Isolated VMs were fixed, permeabilized, and stained to assess membrane localization of \(\alpha\)1G, \(\alpha\)1C, or RyR2.

Detubulation
Isolated VMs were incubated in a formamide solution (1.5 mol/L) for 15 minutes. Cells were quickly transferred to a formamide-free Tyrode’s solution to induce detubulation.

Figure 2. Ca\(^{2+}\) influx in \(\alpha\)1G and \(\beta\)2\(\alpha\) myocytes. A, Representative \(I_{\text{Ca,T}}\) from \(-50\) mV holding potential was subtracted from total Ca\(^{2+}\) current recorded from \(-90\) mV holding potential in control and \(\alpha\)1G VMs. B, Voltage–current relationship of \(I_{\text{Ca,L}}\) (subtraction as in A) in control (n=10) and \(\alpha\)1G (n=15) myocytes. C, Time course of \(I_{\text{Ca,T}}\) recovery from inactivation (n=7) and representative example of \(I_{\text{Ca,T}}\) recovery from inactivation (inset). D, Representative example of total Ca\(^{2+}\) currents under AP voltage clamp in control, \(\beta\)2\(\alpha\), and \(\alpha\)1G VMs at 0.5 Hz. E, Integral of total Ca\(^{2+}\) current (without drugs) at different pacing frequencies in control (n=6), \(\beta\)2\(\alpha\) (n=8), and \(\alpha\)1G VMs (n=6). F, Representative example of [Ca\(^{2+}\)]\(_i\) transients at baseline and after thapsigargin application (1 mmol/L) at 1 mmol/L and 3 mmol/L bath Ca\(^{2+}\). G, Peak [Ca\(^{2+}\)]\(_i\) transient and rise rate of the [Ca\(^{2+}\)]\(_i\) transient after thapsigargin treatment (in 3 mmol/L Ca) in control (n=20), \(\beta\)2\(\alpha\) (n=20), and \(\alpha\)1G VMs (n=25).

Results
Phenotype of Adult Mice With \(\alpha\)1G-TTCC Overexpression
Previously\(^{11}\) we showed that inducible, cardiac-specific overexpression of the \(\beta\)2\(\alpha\) subunit of the LTCC increases Ca\(^{2+}\) influx and myocyte contractility but eventually leads to depressed pump function, fibrosis, myocyte necrosis, cardiac hypertrophy, and premature death.\(^{11}\) In the present study, we asked whether increases in Ca\(^{2+}\) influx of a similar magnitude through TTCCs, a class of Ca\(^{2+}\) channels reexpressed during cardiac disease, produced a similar excess Ca\(^{2+}\) phenotype. Mice with cardiac-specific, inducible \(\alpha\)1G expression were generated to define the functional role of these channels in the myocardium. A bitransgenic system was used in which a standard \(\alpha\)-MHC promoter-driven expression of tTA (regu-
lated by doxycycline) is coupled to a modified α-MHC promoter containing the tet-operon for regulated expression of the α1G cDNA (Figure 1A). α1G mice, unlike the β2a mice,11 had no premature death during the first 12 months of life. Similarly, there were no signs of histopathology (Figure 1B), cardiac dysfunction (Figure 1C), ventricular remodeling, or increases in myocyte death. For comparison, β2a hearts showed robust histopathology by 5 months of age (Figure 1B), as well as premature lethality.11 Provocatively, not only were α1G mice without disease, they also showed enhanced ventricular performance (fractional shortening) compared with control animals (Figure 1D). These data show that expression of α1G-TTCCs induces a fundamentally different cardiac phenotype from overexpression of LTCC-β2a subunit.11

**Ca**

**2**

**Influx via T- and LTCCs in α1G Mice**

One possible explanation for the pathological phenotype of β2a versus α1G mice is that Ca**2** influx is increased to a greater extent in β2a than α1G myocytes, resulting in Ca**2**-mediated myocyte death. To address this possibility, we measured total Ca**2** influx in myocytes from α1G and β2a myocytes. I$_{Ca,T}$ and I$_{Ca,L}$ were measured (Figures 2 and 3) using standard biophysical conditions. Control VMs had no I$_{Ca,T}$, as expected, but I$_{Ca,T}$ was present in every α1G VM. Peak I$_{Ca,T}$ density (Figure 2B) was similar to peak I$_{Ca,L}$ density in the β2a mice (Figure 3B). The voltage dependence of I$_{Ca,T}$ was similar to published values observed in wild-type channels, with a peak near −30 mV (Figure 2B). I$_{Ca,T}$ also had normal recovery from inactivation, with 80% of TTCCs fully recovered within 100 ms (Figure 2C). Therefore, at the normal heart rates of α1G mice, TTCCs will be fully available for activation. I$_{Ca,T}$ was less sensitive to Ca**2** than I$_{Ca,L}$ and was blocked by Ni**2** (Figure 2D), consistent with the known properties of wild-type α1G-TTCCs. These results show that the expressed α1G has functional and pharmacological properties like native I$_{Ca,T}$.

AP voltage clamp was used to measure and compare the total Ca**2** influx through both T- and LTCCs during a mouse AP (Figure 2D). Net Ca**2** influx during an AP was similar in α1G and β2a VMs and 3-fold greater than in control VMs (Figure 2E). These studies show that the amount of excess Ca**2** entry via TTCCs in α1G mice is approximately equal to the excess Ca**2** entry via LTCCs in the β2a mice.

Differences in Ca**2** influx in control, β2a, and α1G VMs were also compared by measuring the field-stimulated [Ca**2**], transient after inhibition of SR function with thapsigargin (1 μmol/L) (Figure 2F and 2G). Peak [Ca**2**], transients after thapsigargin treatment were similar in α1G and β2a VMs and significantly greater than control VMs. Peak rising rate of the [Ca**2**], transient, which is an index of Ca**2** flux, was also similar in α1G and β2a VMs and greater than control VMs. Collectively, these results show that Ca**2** entry is increased to a similar extent in α1G and β2a mice. Therefore, differences in Ca**2** influx magnitude do not explain the different phenotypes of α1G and β2a mice.

I$_{Ca,L}$ density was significantly smaller in α1G versus control myocytes (Figure 3A through 3C), and its voltage dependence of activation was shifted in the depolarized direction (Figure 3D and 3E). These changes may be a compensation
(reduced Ca\textsuperscript{2+} influx via the normal influx pathway) for the increased Ca\textsuperscript{2+} influx through \(\alpha 1G\)-TTCCs.

**AP, Contraction, and [Ca\textsuperscript{2+}] Transients in \(\alpha 1G\) Myocytes**

We have previously shown that excess Ca\textsuperscript{2+} influx through the LTCC increases myocyte contractions and [Ca\textsuperscript{2+}] transients.\textsuperscript{11} \(\alpha 1G\) VMs had longer AP durations at 50\%, 70\%, and 90\% repolarization and a more prominent plateau phase than control VMs (Figure 4A and 4B). Peak systolic [Ca\textsuperscript{2+}], transients were greater in \(\alpha 1G\) versus control myocytes, and the rate of [Ca\textsuperscript{2+}], transient decay was accelerated (Figure 4C and 4D). Contraction magnitude of \(\alpha 1G\) myocytes was significantly greater than control myocytes. The duration of contraction was significantly shorter, and relengthening kinetics were accelerated in \(\alpha 1G\) versus control VMs (Figure 4E and 4F). These results show that increases in Ca\textsuperscript{2+} influx through \(\alpha 1G\) induces an increase in [Ca\textsuperscript{2+}], transient and myocyte contraction but also suggest substantial changes in fundamental aspects of Ca\textsuperscript{2+} handling in \(\alpha 1G\) myocytes. The increased [Ca\textsuperscript{2+}], transient and contractility in \(\alpha 1G\) myocytes was significantly smaller than in \(\beta 2a\) myocytes (Figure 4).

One possible explanation for these results is that increased Ca\textsuperscript{2+} entry through TTCCs induces adaptations in myocyte Ca\textsuperscript{2+} handling such as reducing Ca\textsuperscript{2+} influx through the LTCCs (see above).

**SR Calcium Load and \(I_{NCX}\)**

Our data suggest that excess Ca\textsuperscript{2+} entry through TTCCs causes a much more modest increase in contractility than a similar increase in Ca\textsuperscript{2+} entry through LTCCs. One possible explanation is that the 2 sources of Ca\textsuperscript{2+} entry cause different degrees of SR Ca\textsuperscript{2+} loading. To test this idea, we measured SR Ca\textsuperscript{2+} content, defined as the caffeine-induced [Ca\textsuperscript{2+}], transient. SR Ca\textsuperscript{2+} content was not different in \(\alpha 1G\) versus control VMs (Figure 5A and 5B), whereas SR Ca\textsuperscript{2+} content was increased significantly in \(\beta 2a\) myocytes (Figure 5A and 5B). The decay phase of the caffeine-induced [Ca\textsuperscript{2+}], transient primarily results from NCX-mediated Ca\textsuperscript{2+} efflux and was not different between control and \(\alpha 1G\) VMs. \(I_{NCX}\) measured at +60 mV and −80 mV (Figure 5C and 5D) was also similar in \(\alpha 1G\) and control myocytes. These results suggest that there are no significant changes in the density or [Ca\textsuperscript{2+}]-dependent activity of the NCX in \(\alpha 1G\) myocytes, unlike what we have observed in \(\beta 2a\) myocytes.\textsuperscript{11} These results suggest that increased Ca\textsuperscript{2+} influx through T- and LTCCs are not equivalent in their respective abilities to load the SR.

**Ca\textsuperscript{2+} Regulatory Proteins in \(\alpha 1G\) Hearts**

The rate of decay of the systolic [Ca\textsuperscript{2+}], transient and its duration were significantly shorter in \(\alpha 1G\) versus control VMs, suggesting significant alterations in myocyte Ca\textsuperscript{2+} handling. To address this possibility, we measured Ca\textsuperscript{2+} regulatory protein abundance and phosphorylation state in control and \(\alpha 1G\) hearts. Western blot analysis of ventricular tissue confirmed expression of \(\alpha 1G\)-TTCCs in \(\alpha 1G\) hearts and found no detectable \(\alpha 1G\) protein in control tissue (Figure 6A). \(\alpha 1G\) expression was associated with reduced \(\alpha 1C\)
protein abundance, consistent with reduced \( I_{\text{Ca,L}} \) amplitude (see Figure 3).

SERCA2a abundance was significantly greater, and phospholamban (PLB) abundance was reduced with increased phosphorylation of PLB-Ser16 and PLB-Thr17 in \( \beta^{1G} \) versus control ventricles (Figure 6A and 6B). These changes in SR protein abundance and phosphorylation can account for the enhanced rate of relaxation and more rapid decay of the \([Ca^{2+}]_i\) transient in \( \beta^{1G} \) myocytes. No differences in RyR abundance or RyR-Ser2808 phosphorylation were observed in \( \beta^{1G} \) hearts. NCX abundance was slightly but significantly greater in \( \beta^{1G} \) versus control ventricles.

**I\(_{\text{Ca,T}}\) and EC Coupling**

We next studied whether \( Ca^{2+} \) influx through both T- and LTCCs were equally effective triggers of SR \( Ca^{2+} \) release. \([Ca^{2+}]_i\) transients and contractions induced by \( I_{\text{Ca,L}} \) and \( I_{\text{Ca,T}} \) in \( \beta^{1G} \) VMs were measured in Na\(^+\)- and K\(^+\)-free conditions to eliminate Na\(^+\) and NCX currents and to minimize loss of voltage control. Voltage steps from \(-90\) to \(-40\) mV resulted in an \( I_{\text{Ca,T}} \) with a 3-fold larger amplitude than the \( I_{\text{Ca,L}} \) induced by voltage steps from \(-50\) to \(+10\) mV (Figure 7). However, \( I_{\text{Ca,T}} \) induced significantly smaller \([Ca^{2+}]_i\) transients than \( I_{\text{Ca,L}} \) (Figure 7C), and these transients had a slower rate of rise than those induced by \( I_{\text{Ca,L}} \) (Figure 7F). Contractions induced by \( I_{\text{Ca,T}} \) were smaller than those induced by \( I_{\text{Ca,L}} \), and the kinetics of contractions were slower (Figure 7D and 7G). EC coupling gain (the ratio of peak \([Ca^{2+}]_i\) transient to peak \( Ca^{2+} \) current) was nearly 3-fold smaller for \( I_{\text{Ca,T}} \) versus \( I_{\text{Ca,L}} \) (Figure 7E).

Similar experiments in control VMs showed no \( I_{\text{Ca,T}} \), \([Ca^{2+}]_i\) transients, or contractions from \(-90\) to \(-40\) mV, whereas \( I_{\text{Ca,L}} \) induced \([Ca^{2+}]_i\) transients and contractions from \(-50\) to \(+10\) mV (data not shown). These findings show that \( Ca^{2+} \) influx through \( \alpha^{1G}\)-TTCCs is not an effective trigger of SR \( Ca^{2+} \) release, at least under our conditions.

**TTCC Membrane Localization**

One reason why \( \alpha^{1G}\)-TTCCs may be ineffective triggers of SR \( Ca^{2+} \) release is that these channels are not localized to regions of the sarcolemma near the junctional SR. Localization of \( \alpha^{1G} \) on the VM membrane was determined using an \( \alpha^{1G} \)-specific antibody validated in previous studies. This antibody labeled \( \alpha^{1G} \)-TTCCs that were primarily on the surface sarcolemma of VMs, with less staining within the T-tubules (Figure 8A). By comparison, the staining pattern of \( \alpha^{1C} \), the pore-forming subunit of the LTCC, was primarily within the T-tubules, producing a similar pattern of coincident localization as RyR2 (Figure 8C and 8D). Membrane localization of T- and LTCCs was performed using confocal imaging at the level of the nucleus to ensure that similar intracellular and membrane surface regions were examined in every myocyte. This staining pattern supports the idea that \( I_{\text{Ca,T}} \) is an ineffective trigger of SR \( Ca^{2+} \) release because...
TTCCs are concentrated in the surface membrane away from the Ca\(^{2+}\) release channels (RyR2).

Using antibodies to define membrane localization of ion channels can produce variable results.\(^3\) Therefore, we used another independent technique to confirm that TTCCs are more concentrated in the surface membrane than in the T-tubules of \(\alpha1G\) VMs. Formamide-induced osmotic shock produces rapid changes in cellular volume that causes T-tubules to detach from the surface membrane.\(^3\) This detubulation technique has been used to confirm the principal localization of LTCCs within the T-tubule system.\(^3\) We used this approach to examine the idea that TTCCs are not in high density within the T-tubules. Membrane staining with di-8-ANEPPS confirmed the presence and loss of T-tubules in normal and detubulated \(\alpha1G\) VMs, respectively (Figure 8E and 8F). Voltage-clamp techniques were then used to measure \(I_{Ca,L}\) and \(I_{Ca,T}\) in \(\alpha1G\) VMs with and without detubulation (Figure 8G). Detubulation in \(\alpha1G\) myocytes was associated with a 72% reduction in \(I_{Ca,L}\) and a significantly smaller, 32% decline in \(I_{Ca,T}\), consistent with the antibody studies (Figure 8H). Collectively, these data support the idea that \(\alpha1G\)-TTCCs are primarily on the surface membrane of VMs.

**Discussion**

The objective of this study was to define the functional role of TTCCs in adult VMs. TTCCs are expressed early in the developing heart,\(^12\)-\(^14\) where they are thought to contribute to pacemaking and the induction of contraction in immature myocytes. TTCC expression decreases after birth and few, if
any of these channels exist in adult VMs. Cardiovascular diseases that increase systolic wall stress in the ventricle, such as hypertension and myocardial infarction, induce reexpression of TTCCs, which was hypothesized to contribute to cardiac dysfunction, arrhythmias, and sudden death. However, a direct link between Ca\textsuperscript{2+}/H\textsuperscript{1+} influx through TTCCs and these processes has not been causally established. To explore the role of TTCCs in the adult heart, we generated a mouse model with inducible expression of Ca\textsuperscript{2+}/H\textsuperscript{1+} TTCCs. Mice had functional TTCCs that caused an increased Ca\textsuperscript{2+}/H\textsuperscript{1+} influx (Figure 2). Previously, we showed that increasing Ca\textsuperscript{2+}/H\textsuperscript{1+} influx through LTCCs by expressing its Ca\textsuperscript{2+}/H\textsuperscript{1+} subunit increases myocyte contractility but over time causes cardiac dysfunction by inducing myocyte death from SR Ca\textsuperscript{2+} overload–induced necrosis. These findings strongly support the idea that persistent increases in Ca\textsuperscript{2+}/H\textsuperscript{1+} influx eventually cause cardiomyopathy by reducing the number of functional cardiac myocytes, consistent with the known benefit of LTCC blockers in select forms of heart disease.

The Ca\textsuperscript{2+}/H\textsuperscript{1+} mice used in the present experiments had increases in Ca\textsuperscript{2+}/H\textsuperscript{1+} influx that were similar to or greater than in Ca\textsuperscript{2+}/H\textsuperscript{1+} mice, but, surprisingly, they did not develop a pathological cardiac phenotype. These hearts and their resident myocytes were hypercontractile, but we could not detect evidence for cardiac histopathology or sudden death, at least during the first 12 months of life. These new studies show that increasing Ca\textsuperscript{2+}/H\textsuperscript{1+} influx through T- and LTCCs induces such fundamentally different phenotypes in the adult heart is not clear and will be a major topic for future studies. Our current studies suggest that the location of the Ca\textsuperscript{2+}/H\textsuperscript{1+} influx pathway appears to be the critical determinant of the induced cardiac phenotype.

Expression of Ca\textsuperscript{2+}/H\textsuperscript{1+}-TTCC Induces Alterations in Myocyte Ca\textsuperscript{2+} Handling

Ca\textsuperscript{2+} influx was ∼3-fold greater than normal in both Ca\textsuperscript{2+}/H\textsuperscript{1+} and Ca\textsuperscript{2+}/H\textsuperscript{1+} VMs. In the absence of compensatory changes in other Ca\textsuperscript{2+} regulatory processes, this increase in Ca\textsuperscript{2+} entry should cause similar increases in myocyte contractility and SR Ca\textsuperscript{2+} loading in myocytes from both transgenic mice. However, myocyte contractions and [Ca\textsuperscript{2+}]/H\textsuperscript{1+} transients were smaller in Ca\textsuperscript{2+}/H\textsuperscript{1+} versus Ca\textsuperscript{2+}/H\textsuperscript{1+} myocytes (Figure 4 and

\begin{itemize}
\item Figure 7. \textit{I}_{Ca,L} is less effective than \textit{I}_{Ca,T} in inducing SR Ca\textsuperscript{2+} release (\textit{n}=10). A, Representative example of peak \textit{I}_{Ca,L} and \textit{I}_{Ca,T} and their corresponding [Ca\textsuperscript{2+}], transients and contractions. B, Average \textit{I}_{Ca,L} (at +10 mV) and \textit{I}_{Ca,T} (at −40 mV), as measured in 1 mmol/L bath Ca\textsuperscript{2+}. C and D, Peak [Ca\textsuperscript{2+}], transients (C) and peak contractions (D) triggered by \textit{I}_{Ca,L} and \textit{I}_{Ca,T}. E, EC coupling gain, determined as the ratio of peak [Ca\textsuperscript{2+}] transients/peak current for \textit{I}_{Ca,L} and \textit{I}_{Ca,T}. F, Maximum rate of rise of the [Ca\textsuperscript{2+}] transient induced by \textit{I}_{Ca,L} and \textit{I}_{Ca,T}. G, Maximum rate of contraction attributable to \textit{I}_{Ca,L} and \textit{I}_{Ca,T}. **\textit{P}<0.01.
\end{itemize}
This appears to result from the fact that the SR Ca\(^{2+}\) load was not increased in 1G VMs (Figure 5), suggesting that T- and L-type Ca\(^{2+}\) fluxes are handled differently by cardiac myocytes. In this regard, we observed a significant decrease in the duration of contraction and the \([\text{Ca}^{2+}]_i\) transient in 1G myocytes (Figure 4C and 4E), suggesting that 1G-TTCC expression and the associated increase in Ca\(^{2+}\) influx induced remodeling of other aspects of myocyte Ca\(^{2+}\) handling. We explored these compensatory changes using cellular biophysical techniques and Western blot analysis.

Western blot analysis of ventricular tissue showed that the abundance and phosphorylation state of certain Ca\(^{2+}\)-handling proteins were altered in 1G hearts. The protein abundance of the LTCC-\(\alpha1C\) pore-forming subunit was significantly smaller in 1G versus control hearts. \(I_{\text{Ca,L}}\) density was also smaller in 1G versus control VMs, consistent with the Western blot analysis. These results suggest that the increased Ca\(^{2+}\) influx through the \(\alpha1G\)-TTCCs induces a reduction in LTCC density. This compensatory change could reduce the likelihood of SR Ca\(^{2+}\) overload and associated pathologies in \(\alpha1G\) hearts. Western blot analysis also showed an increased SERCA2a expression level and a reduced PLN abundance with increased phosphorylation at PLB-Ser16 and PLN-Thr17 in 1G versus control hearts. These changes will promote Ca\(^{2+}\) uptake by the SR and can explain the shortened \([\text{Ca}^{2+}]_i\) transient that we observed in 1G mice. Western blot analysis showed an increase in NCX abundance, but we did not find an increase in Ca\(^{2+}\)-mediated NCX activity in \(\alpha1G\) VMs in response to caffeine-induced SR Ca\(^{2+}\) release.

Peak systolic Ca\(^{2+}\) was increased in \(\alpha1G\) versus control myocytes, but their SR Ca\(^{2+}\) loads were not greater than in controls. We speculate that the increased systolic Ca\(^{2+}\) in \(\alpha1G\) myocytes results from an increase in Ca\(^{2+}\) influx through TTCCs, together with a similar (to that in control VMs) Ca\(^{2+}\) release from the SR (consistent with our finding that the Ca\(^{2+}\) load is normal). Why an increase in \(\alpha1G\)-mediated sarcolemmal Ca\(^{2+}\) influx does not result in an increase in SR Ca\(^{2+}\) loading is not clear, because increasing Ca\(^{2+}\) influx via
the LTCCs caused a significant increase in SR Ca$^{2+}$ loading (Figure 5).

**TTCCs Are on the Surface Sarcolemma, and LTCCs Are in T-Tubules**

Our experiments demonstrate that Ca$^{2+}$ influx through T- and LTCCs have a different capacity to load the SR (Figure 5) and to induce SR Ca$^{2+}$ release (Figure 7). $I_{\text{Ca,L}}$ induced [Ca$^{2+}$], transients with smaller amplitudes and slower rates of rise than those caused by $I_{\text{Ca,T}}$, suggesting that TTCCs are not proximal to the RyRs to induce efficient SR Ca$^{2+}$ release. Our results also suggest that Ca$^{2+}$ entry via TTCCs is not taken up by the SR to the same extent as the Ca$^{2+}$ influx via LTCCs. We therefore went on to test the idea that T- and LTCCs are localized to different regions of the surface membrane.

Immunostaining showed that α1G-TTCCs are localized to the surface membrane and are not significantly localized within the T-tubules, the principal site of LTCC localization (Figure 8). The T-tubule system is composed of extensive invaginations of the cell membrane that allow rapid transmission of electric signals into the interior of the VM for spatial uniformity of SR Ca$^{2+}$ release for myocyte contraction. The fraction of cell membrane comprised by the T-tubular system is very species-dependent and has been reported to be between 50% and 64% for murine VMs.33–37 TTCCs are localized to regions of the T-tubules (Figure 8) that are in close apposition to the junctional SR.30 To confirm that Ca$^{2+}$ entry via TTCCs is not taken up by the SR to the same extent as the Ca$^{2+}$ influx via LTCCs. We therefore went on to test the idea that T- and LTCCs are localized to different regions of the surface membrane.

We conclude that Ca$^{2+}$ transport out of the cell, so that only a small fraction of this Ca$^{2+}$ enters the SR. This, coupled with a compensatory reduction in Ca$^{2+}$ entry through LTCCs, could explain the modest increase in contractility, with no significant change in SR Ca$^{2+}$ loading. Similar increases in total Ca$^{2+}$ entry through the LTCCs in β2a mice caused increased SR Ca$^{2+}$ loading, [Ca$^{2+}$], transients, and contractility. Our most important finding is that increased Ca$^{2+}$ entry through TTCCs did not induce cardiac dysfunction, histopathology, or premature death. These results show that the Ca$^{2+}$ entry pathway, the location of the Ca$^{2+}$ channels within the membrane, as well as the Ca$^{2+}$ influx magnitude, determines the resultant cardiac phenotype. Additional studies are needed to determine the specific role of increased Ca$^{2+}$ influx via TTCCs under pathological conditions.

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**Disclosures**

None.

**References**


Ca$^{2+}$ Influx Through T- and L-Type Ca$^{2+}$ Channels Have Different Effects on Myocyte Contractility and Induce Unique Cardiac Phenotypes

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EXPANDED MATERIALS AND METHODS

Generation of α1G and β2a transgenic mice: Transgenic mice with conditional (Tet-off) and cardiac-specific expression of mouse TTCC α1G subunit were generated using a modified murine α-MHC promoter expression vector containing the tet operon. These mice were crossed with transgenic mice expressing the α-MHC promoter-driven tetracycline-transactivator protein (tTA) to generate double transgenic (DTG) mice expressing both the α1G and tTA transgenes (termed α1G mice in this report). Mice between 10-16 weeks of age were used. β2a mice were generated using a similar strategy as described previously.

Echocardiography: Whole heart morphology and function were assessed via echocardiography (VisualSonics Vevo 770). Mice were anesthetized with isoflurane at 2% initially and at 1.5% during the procedure. Hearts were viewed at the level of the papillary muscles in short-axis and analyzed in M-mode to assess cardiac function, chamber dimensions and wall thicknesses.

Myocyte Isolation: Mouse VMs were isolated using a constant-pressure Langendorff apparatus. Animals were anesthetized with sodium pentobarbital (0.1 mL/100 g), and hearts were digested via retrograde perfusion of a Tyrodes solution containing collagenase (290 U/mL) and (mM): CaCl₂ 0.02, glucose 10, HEPES 5, KCl 5.4, MgCl₂ 1.2, NaCl 150, sodium pyruvate 2, pH 7.4. After 8-10 min, the ventricles were minced and isolated VMs were equilibrated in a Tyrodes solution containing 0.2 mM CaCl₂ and 0.5% bovine serum albumin.

Histological Analysis: Hearts from animals (9 months old) were excised, fixed in 10% formalin, and processed and embedded in paraffin. Serial 7μm longitudinal sections were
stained with hematoxylin/eosin or Masson’s trichrome to evaluate gross morphology, fiber integrity and fibrosis.

**Electrophysiology:** Myocytes were placed in a heated chamber (35°C) on the stage of an inverted microscope and perfused with Tyrode’s solution containing 1 mM CaCl₂. Myocytes were loaded with Fluo-3AM to measure [Ca²⁺], and myocyte fractional shortening was measured with edge detection. Myocytes were paced at 0.5 Hz. Rapid application of caffeine (10 mM) via a Picospritzer was used to induce SR Ca²⁺ release and to assess SR calcium content. Total transmembrane Ca²⁺ influx was assessed under field stimulation (0.5 Hz) after inactivating the SR via thapsigargin (1 μM). Peak [Ca]ᵢ and the rate of rise of the [Ca]ᵢ were evaluated in the presence of thapsigargin with 1 mM or 3 mM bath Ca²⁺. Ca²⁺ influx was also determined under AP clamp conditions, with pipettes containing (mM): CsOH 130, aspartic acid 130, EGTA 10, MgCl₂ 1, NMDG 10, HEPES 10, TEA-Cl 20, Tris-ATP 5, pH 7.2. The voltage profile of a typical AP recorded from a control VM was applied at various frequencies (0.5, 2.0, 4.0, and 8.0 Hz). Capacitative currents were subtracted manually or via P/4 subtraction, and the resulting Ca²⁺ currents were integrated (20-ms) to assess Ca²⁺ influx.

Whole-cell patch clamp techniques were used to measure action potentials (APs), in current clamp mode with pipettes (>4 MΩ) containing (mM): KOH 120, aspartic acid 120, KCl 20, Na₂ATP 5, MgCl₂ 1, HEPES 10, pH 7.2. APs were evoked with current pulses (<5 nA amplitude, <5ms duration) at 2.0 Hz. Ca²⁺ currents were measured with whole cell voltage clamp techniques with pipettes (3-4 MΩ) containing (mM): CsOH 130, aspartic acid 130, EGTA 10, MgCl₂ 1, NMDG 10, HEPES 10, TEA-Cl 20, Tris-ATP 5, pH 7.2. After 10 minutes of dialysis, cells were perfused with a Na-free, K-free solution containing (mM): CaCl₂ 2, 4-AP 2, CsCl₂ 5.4, Glucose 10, HEPES 5, MgCl₂ 1.2, NMDG 150, pH 7.4. Total calcium current was measured from a -90 mV holding potential using square wave pulses from -70 to +60 mV. I_Ca,L was measured from a -50
mV holding potential using similar test pulses. $I_{\text{Ca},T}$ was determined by subtracting $I_{\text{Ca},L}$ from total current. $I_{\text{NCX}}$ was measured as described previously. All experiments used the Axopatch 2B voltage-clamp amplifier and pClamp8 software.

$I_{\text{Ca},T}$ and $I_{\text{Ca},L}$ induced SR $\text{Ca}^{2+}$ release was measured using Na-, K-, and EGTA-free conditions. The pipette solution consisted of (mM): Fluo-3K5 0.1, CsOH 130, aspartic acid 130, MgCl$_2$ 1, NMDG 10, HEPES 10, TEA-Cl 20, Tris-ATP 5, pH 7.2. The bath solution contained (mM): CaCl$_2$ 1, 4-AP 2, CsCl$_2$ 5.4, Glucose 10, HEPES 5, MgCl$_2$ 1.2, NMDG 150, pH 7.4. Myocytes was conditioned with three 120-ms square pulses to +10 mV followed by a test pulse from -90 to -40 mV to measure $I_{\text{Ca},T}$ or -50 to +10 mV for $I_{\text{Ca},L}$. $\text{Ca}^{2+}$ currents and their accompanying $\text{Ca}^{2+}$ transients and contractions were recorded for offline analysis with pClamp8 software.

**Immunostaining:** Isolated VMs were fixed in either 4% paraformaldehyde or 1.5% gluteraldehyde solution and permeabilized in 0.05% Triton X-100. Immunostaining of Cav3.1 was performed using an $\alpha$1G-specific antibody (from our collaborator, Dr. Leanne Cribbs). LTCC staining was done with a Cav1.2 ($\alpha$1C) specific antibody (BD Biosciences). RyR immunostaining was performed using a RyR2-specific antibody (Affinity BioReagents).

**Detubulation:** Isolated VMs were incubated in a Tyrodes solution containing formamide (1.5 mol/L) for 15 minutes. Thereafter, cells were quickly placed in a formamide-free Tyrode’s solution. The re-exposure to isosmotic conditions results in cellular swelling and detubulation as reported previously. To confirm detubulation, cells were stained with 5.0 µM Di-8-ANNEPS to label the cell membrane and then imaged with confocal microscopy.
**Western blot analysis:** Cytoplasmic and membrane protein were isolated from ventricular tissue using PBS lysis buffer containing: 0.5% Triton X-100, 5 mM EDTA (pH 7.4), phosphatase inhibitors (10 mM NaF and 0.1 mM NaVO4), proteinase inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin A, 8 μg/ml calpain inhibitor I & II, and 200 μg/ml benzamidine). Cardiac actin was isolated from resulting pellet using PBS lysis buffer containing 2% sodium dodecyl sulfate, SDS (FisherBiotech), 1% IGEPAL CA-630 (Sigma), 0.5% deoxycholate (Sigma), 5 mM EDTA (pH 7.4), and proteinase inhibitors. Protein abundance and phosphorylation levels in isolated protein were analyzed with Western blot analysis as described previously. Target antigens were probed with the following antibodies: SERCA (Sigma), phospholamban (PLB) (Upstate Biotechnology), RyR (Research Diagnostics), α-sarcomeric actin (Sigma), LTCC-α1C subunit (Chemicon), sodium calcium exchanger (NCX) (Swant), GAPDH (Serotec), PS2809-RyR, PS16-PLB, and PT17-PLB (Badrilla).

**Statistics:** Data are presented as mean±SEM. Between-group comparisons were performed by using the t-test. For all tests, statistical significance was set at P<0.05.
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