Identification of the T-Type Calcium Channel (Ca_v3.1d) in Developing Mouse Heart

Leanne L. Cribbs, Beverly L. Martin, Elizabeth A. Schroder, Bradley B. Keller, Brian P. Delisle, Jonathan Satin

Abstract—During cardiac development, there is a reciprocal relationship between cardiac morphogenesis and force production (contractility). In the early embryonic myocardium, the sarcoplasmic reticulum is poorly developed, and plasma membrane calcium (Ca^{2+}) channels are critical for maintaining both contractility and excitability. In the present study, we identified the Ca_v3.1d mRNA expressed in embryonic day 14 (E14) mouse heart. Ca_v3.1d is a splice variant of the α1G, T-type Ca^{2+} channel. Immunohistochemical localization showed expression of α1G Ca^{2+} channels in E14 myocardium, and staining of isolated ventricular myocytes revealed membrane localization of the α1G channels. Dihydropyridine-resistant inward Ba^{2+} or Ca^{2+} currents were present in all fetal ventricular myocytes tested. Regardless of charge carrier, inward current inactivated with sustained depolarization and mirrored steady-state inactivation voltage dependence of the α1G channel expressed in human embryonic kidney-293 cells. Ni^{2+} blockade discriminates among T-type Ca^{2+} channel isoforms and is a relatively selective blocker of T-type channels over other cardiac plasma membrane Ca^{2+} handling proteins. We demonstrate that 100 μmol/L Ni^{2+} partially blocked α1G currents under physiological external Ca^{2+}. We conclude that α1G T-type Ca^{2+} channels are functional in midgestational fetal myocardium. (Circ Res. 2001;88:403-407.)

Key Words: calcium channel ■ cardiac development ■ low-voltage-activated Ca^{2+} channel

L-type and T-type calcium (Ca^{2+}) channels comprise two broadly classified families of voltage-gated Ca^{2+} channels. L-type Ca^{2+} channel expression and function are well characterized in cardiac myocytes, where L-type channels mediate plasma membrane influx of extracellular Ca^{2+}. Plasma membrane influx of Ca^{2+} leads to Ca^{2+}-induced Ca^{2+} release, which in turn regulates cardiac contractility. The L-type Ca^{2+} channels also are an important therapeutic target for management of a variety of cardiovascular disorders. In contrast to L-type channels, T-type Ca^{2+} channel expression and function are poorly understood, and the therapeutic potential of T-type Ca^{2+} channels in mature myocardium is unknown. Nonetheless, earlier studies showed that de novo expression of T-type Ca^{2+} channels may contribute to the cardiac hypertrophic phenotype. More recently, Nattel and colleagues suggested that T-type Ca^{2+} current might contribute to arrhythmogenesis in patients exhibiting atrial fibrillation.

L-type and T-type Ca^{2+} channels are perhaps most clearly distinguished by their respective voltage range of activation. L-type Ca^{2+} channels activate only with strong depolarizations (≥−50 mV), corresponding to the plateau of the action potential. In contrast, T-type Ca^{2+} channels activate with weak depolarizations (≥−80 mV). In fact, we recently demonstrated that T-type Ca^{2+} channels activate at slightly more negative potentials than even cardiac Na^{+} channel currents. This low-voltage-activation range of T-type Ca^{2+} channels allows them to provide a substantial inward, depolarizing current in the late phase of diastole. Thus it is likely that T-type Ca^{2+} current contributes to the initiation of the action potential upstroke in pacemaker cells.

Embryonic ventricular myocytes are capable of automatic activity, in contrast to normal adult myocardium. Studies on embryonic chick ventricular myocytes clearly show the expression of T-type Ca^{2+} current; however, the literature from embryonic mouse heart is controversial. Nuss and Marbán showed substantial T-type Ca^{2+} channel expression, but Davies et al were unable to detect T-type current. The recent molecular identification of T-type Ca^{2+} channels facilitates unequivocal detection of channel gene expression. The α1G channel is encoded by the Ca_v3.1 gene, and there are 4 distinct splice variants in the domain III-IV connecting loop in rodents and humans. Our recent studies showed that mouse atrial tumor (AT-1) cells functionally express α1G-d (Ca_v3.1d). AT-1 cells have an excitability pattern that parallels embryonic ventricular myocytes, suggesting that developing myocardium may also express α1G channels.

Original received October 17, 2000; revision received December 28, 2000; accepted December 28, 2000.
From the Cardiovascular Institute (Leanne L. Cribbs, PhD, B.L.M.), Loyola University Medical Center, Maywood, Ill, and the Departments of Pediatrics (Elizabeth A. Schroder, PhD, B.P.D.) and Physiology (B.F.D., J.S.), University of Kentucky College of Medicine, Lexington, Ky.
Correspondence to Jonathan Satin, PhD, Department of Physiology, MS-508, University of Kentucky College of Medicine, Lexington, KY 40536-0298; E-mail jsatin1@pop.uky.edu
© 2001 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org
We undertook the present study to evaluate the expression of T-type Ca\(^{2+}\) channels in embryonic myocytes. We show that the T-type Ca\(^{2+}\) channel splice variant \(\alpha 1G-d\) is expressed in embryonic day 13 (E13) to E14.5 mouse ventricle, and we present data consistent with the hypothesis that Ca\(^{2+}\) entry via the T-type channel may contribute to excitability in the developing myocardium.

Materials and Methods

Tissue Harvest

E12.5 to E13 mouse hearts were dissected free of connective tissues, and ventricles were separated from atria. For isolation of total RNA, whole ventricles were immersed in Trizol reagent (GIBCO-BRL) and homogenized. For immunohistochemistry, E13 hearts were removed, embedded in OCT compound, and rapidly frozen in a dry-ice bath. For cell isolation for patch-clamp recordings and immunocytochemistry, ventricles from 3 to 8 embryos were minced and quickly placed into nominally Ca\(^{2+}\)-free digestion buffer containing 0.5 mg/mL collagenase (type II, Worthington) and 1 mg/mL pancreatin (GIBCO-BRL) for two 15-minute cycles.

Molecular Characterization

Total E13 ventricular RNA was characterized by reverse transcriptase–polymerase chain reaction (RT-PCR), as described elsewhere.\(^1\) We undertook the present study to evaluate the expression of T-type Ca\(^{2+}\) channels in embryonic myocytes. We show that the T-type Ca\(^{2+}\) channel splice variant \(\alpha 1G-d\) is expressed in embryonic day 13 (E13) to E14.5 mouse ventricle, and we present data consistent with the hypothesis that Ca\(^{2+}\) entry via the T-type channel may contribute to excitability in the developing myocardium.

Immunostaining

Type-specific antibodies 2×G1 (anti-\(\alpha 1G\)) and 2×H1 (anti-\(\alpha 1H\)), prepared against peptides derived from the \(\alpha 1G\) and \(\alpha 1H\) T-type channel sequences\(^2,1,3\) (for 2×G1: FVCGEDTRTNKSD-CAEAS and 2×H1: YYCEGPDTRNSTKACQRAAH), were immunooaffinity-purified commercially (Bethyl Laboratories). Monoclonal antibody MF 20 (Developmental Studies Hybridoma Bank, University of Iowa) was used to stain myofibrils using a rhodamine-conjugated secondary antibody (Molecular Probes). For additional details, see the online data supplement available at http://www.circresaha.org.

Results

Assay of total E12 to E14 ventricular myocyte RNA by RT-PCR and DNA sequencing allows an unequivocal assessment of T-type Ca\(^{2+}\) channel mRNA expression. We used a strategy identical to that used by Satin and Cribbs\(^1\) to identify T-type Ca\(^{2+}\) channels in fetal mouse ventricle. Mouse E12.5 to E14 ventricular myocytes express the \(\alpha 1G-d\) splice variant, as shown previously for AT-1 cells (Figure 1). Even though the PCR primers used can amplify all 3 known T-type channel variants were discovered.\(^19\) No other low-voltage-activated channels or splice variants were discovered. The expression of mRNA does not always correlate with protein expression. To confirm protein expression, we used antibodies (2×G1 and 2×H1) to evaluate expression and localization of T-type channels in embryonic heart. Figure 2 shows a sagittal section of ventricle with vigorous staining for \(\alpha 1G\). We also noted staining of vasculature and atrial walls...
Figure 3. Immunostain of cultured E13 mouse ventricular myocyte. Isolated cells were stained with either MF 20 (myosin heavy chain monoclonal antibody, rhodamine-conjugated secondary antibody) (A) or 2×G1 (FITC-conjugated secondary antibody) (B). Magnification is ×1120.

(Figure 2B). Preimmune controls (Figures 2A and 2C) and anti-αH antibodies did not stain (not shown). The staining of α1G was blocked completely by 2×G1 peptide–absorbed antibody (Figure 2E), whereas 2×H1 peptide had no effect on 2×G1 staining (Figure 2F). To additionally control for antibody specificity, 2×G1 clearly stained human embryonic kidney (HEK) cells stable-transfected with α1G; in contrast, HEK cells expressing α1H or untransfected HEK cells did not show 2×G1 staining (see the online data supplement). We also stained isolated ventricular myocytes using the 2×G1 antibody (Figure 3). Cells were cotained with MF 20, a monoclonal antibody to myosin heavy chain, to confirm their identity as myocytes (Figure 3A). Figure 3B shows the expected plasma membrane localization of α1G, a staining pattern distinct from the myofibrillar staining with MF 20. Taken together, the RT-PCR and immunostaining results show that embryonic ventricular myocardium expresses the α1G-d splice variant.

We performed whole-cell mode patch-clamp recordings of embryonic ventricular myocytes to evaluate α1G-d channel function. To isolate T-type channel current from voltage-gated Na⁺, L-type Ca²⁺, and K⁺ currents, we bathed cells in Na⁺-free bath containing 2.5 mmol/L Ba²⁺, 30 μmol/L TTX, and 100 μmol/L nifedipine. Figure 4 shows representative recordings of T-type Ca²⁺ current. Channel kinetics (Figure 4A) and the steady-state inactivation voltage dependence are consistent with scoring this current as a T-type Ca²⁺ channel current. The voltage dependence of activation is depolarization-shifted (midpoint = −22 mV; Figure 4B), but this is a consistent trend observed for voltage-gated Na⁺ and Ca²⁺ channels in developing myocardium. The presence of inactivating Ba²⁺ current in 100 μmol/L nifedipine (Figures 4A and 4C) is unequivocal evidence that the current is not through L-type Ca²⁺ channels.

We used Ni²⁺ blockade as an indication of T-type isoform expression in fetal cardiomyocytes. Ni²⁺, 200 μmol/L, blocks the α1G Ca²⁺ current channel by ~50%; in contrast, ~10 μmol/L Ni²⁺ is sufficient for half-block of the α1H T-type Ca²⁺ current. In E12.5 to E14 ventricular myocytes, 100 μmol/L Ni²⁺ blocks T-type Ca²⁺ current by 38.3 ± 5.8% (Figure 4D; n = 3). Ni²⁺-blocked and -unblocked currents have a comparable voltage dependence of inactivation consistent with a uniform dihydropyridine (DHP)-resistant channel population. On the basis of Ni²⁺ blockade and current kinetics coupled with the molecular identification and immunohistochemical data (Figures 1 through 3), we assign the T-type Ca²⁺ current in embryonic ventricular myocardium as an α1G-d current.

Discussion

Ca₃.3.1d is a splice variant of the α1G, T-type Ca²⁺ channel. In this study we demonstrate for the first time, to our
knowledge, the following observations: (1) α1G mRNA and protein are expressed in developing fetal cardiac myocytes; (2) α1G is functionally expressed, as evidenced by T-type Ca\(^{2+}\) currents in E12.5 to E14 ventricular myocytes; and (3) α1G is DHP resistant 30, 32. We increased the nifedipine dose to 100 μmol/L to completely block L-type Ca\(^{2+}\) current 24, 25 and exposed the cells to drug for >5 minutes to prevent confusion of use-dependent blockade of L-type channels with I\(_{\text{Ca}}\). 26 Therefore, the inward Ca\(^{2+}\) or Ba\(^{2+}\) current that we observe is DHP resistant, thereby excluding L-type channels. Furthermore, the current we measure inactivates both by sustained depolarization and as a function of steady-state holding potential. Similarly, we observed a 100 μmol/L nifedipine-resistant inactivating Ba\(^{2+}\) current. Furthermore, the steady-state inactivation voltage dependence was similar to our steady-state inactivation measurements of α1G expressed heterologously in HEK293 cells. 4, 17 We increased the nifedipine dose to 100 μmol/L to completely block L-type Ca\(^{2+}\) current 24, 25 and exposed the cells to drug for >5 minutes to prevent confusion of use-dependent blockade of L-type channels with I\(_{\text{Ca}}\). 26 Therefore, the inward Ca\(^{2+}\) or Ba\(^{2+}\) current that we observe is DHP resistant, thereby excluding L-type channels. Furthermore, the current we measure inactivates both by sustained depolarization and as a function of steady-state holding potential; these properties exclude the unidentified current in fetal myocytes in the α1C knockout mouse. 27 Finally, the activity of this current in the presence of 30 μmol/L TTX excludes a contribution from \(I_{\text{Ca,TTX}}\). 28 We also suggest that the novel fetal \(I_{\text{Ca,TTX}}\) is in fact α1G T-type current. Given that heterologously expressed current through α1G is DHP resistant 30 and that kinetic and steady-state inactivation properties from fetal ventricular myocytes mirror our earlier studies of α1G current, we conclude that fetal ventricular myocytes express functional T-type Ca\(^{2+}\) current. This assignment is based on a combination of the exclusion of known inward current carriers and parallels the reported properties of heterologously expressed T-type channel current.

One procedure common between the present study and the study by Nuss and Marbán 10 is that spontaneously active cells were selected for study. Automatic activity is a well-described property of neonatal ventricular myocytes. 31, 32 We find it interesting to note the ongoing correlation of T-type Ca\(^{2+}\) channel expression with pacemaker cells from heart 6, 8 and noncardiac tissues. 33 Furthermore, the α1G-d splice variant that we now identify in fetal ventricular myocytes is the same splice variant that we previously identified in the murine AT-1 cell line. 17 As with fetal myocytes, AT-1 cells in culture fire spontaneous action potentials. 34 Finally, our present studies and the study by Nuss and Marbán 10 corroborate earlier findings from chick that \(I_{\text{f}}\) in early embryonic development has a depolarizing shifted voltage dependence of the macroscopic \(I-V\) curve. 8 This creates an overlap of T- and L-type Ca\(^{2+}\) currents in the absence of DHP. In any case, our results unequivocally show that a particular splice variant of T-type Ca\(^{2+}\) channels is expressed in E13 ventricle and is a likely pathway for inward, DHP-resistant Ca\(^{2+}\) current.

There are three major plasma membrane transport mechanisms for inward Ca\(^{2+}\) flux in fetal cardiomyocytes: \(I_{\text{Ca,L}}\), \(I_{\text{f}}\), and reverse-mode Na\(^{+}\)-Ca\(^{2+}\) exchange. Unequivocal assessment of the relative contribution of these mechanisms is hindered by the lack of selective pharmacological agents. Ni\(^{2+}\) is relatively selective for \(I_{\text{f}}\) compared with \(I_{\text{Ca,L}}\) and Na\(^{+}\)-Ca\(^{2+}\) exchange, but it may also have subtle effects, particularly on \(I_{\text{Ca,L}}\) in the range of 100 μmol/L used in this study. 35 Therefore, in the absence of selective T-type channel blockers, establishment of a role for T-type Ca\(^{2+}\) channels in developing myocardium may ultimately require a combination of molecular genetic approaches, including their complete ablation using knockout strategies and overexpression of α1G-d in mouse heart.

**Acknowledgments**

This work was supported by the American Heart Association (grant 0051434Z to L.L.C) and the National Institutes of Health (National Research Service Award F32-HL-10200-02 to E.S. and grant HL63416 to J.S.). The authors thank Lindsay Burns and Joe Tinney for excellent technical assistance.

**References**

terization of $\alpha_1H$ from human heart, a member of the T-type calcium channel gene family. *Circ Res*. 1998;83:103–109.


21. Cribbs et al. T-Type Calcium Channels in Fetal Ventricle


Identification of the T-Type Calcium Channel (Ca\textsubscript{v}3.1d) in Developing Mouse Heart
Leanne L. Cribbs, Beverly L. Martin, Elizabeth A. Schroder, Bradley B. Keller, Brian P. Delisle
and Jonathan Satin

doi: 10.1161/01.RES.88.4.403

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/88/4/403

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2001/02/21/88.4.403.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Circulation Research* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Circulation Research* is online at:
http://circres.ahajournals.org/subscriptions/
Materials & Methods.

Immunostaining. Both antibodies were immunoadsorbed to the common “DTRNI” peptide to eliminate potential cross-reactivity due to that epitope. E14 mouse heart cryosections (14 μm) were fixed in 4% paraformaldehyde/PBS, then blocked with 1% normal goat serum/PBS. Sections were incubated with preimmune serum (negative control) or primary antibody (diluted in PBS/0.1% Triton X-100) for 1 hr. In some cases, primary antiserum was pre-absorbed with 50 μM antigenic peptide as a control for specificity. Immunodetection was with ABC Elite reagents with VIP as the chromogenic substrate (Vector Laboratories). For immunocytochemistry, isolated cells were cultured up to 48 hrs on glass chamber slides then fixed with 2% paraformaldehyde, permeabilized with 0.5% Triton X-100/PBS, blocked with 1% normal goat serum and incubated with primary antibodies. “2xG1” staining was detected with FITC-conjugated secondary antibody (Amersham-Pharmacia).

Supplementary Figure Legends

Supplementary Figure #1. Control for specificity of α1G antibody. Upper panel) Stable-transfected HEK cells with α1G stain positive. Middle panel) Stable-transfected HEK cells with α1H, or untransfected HEK cells (lower panel) show no immunolabelling with α1G antibody used in this study.

Supplementary Figure #2. Control for specificity of α1H antibody. Upper panel) Stable-transfected HEK cells with α1G stain positive. Middle panel) Stable-transfected
HEK cells with α1H, or untransfected HEK cells (lower panel) show no immunolabelling with α1G antibody used in this study.

**Supplementary Figure #3.** Fast inward current is observed only in the absence of TTX under ionic conditions used in this study. Family of inward currents with cardiac Na channel kinetics in the absence of TTX with 2.5 mM Ba2+ and 140 mM TEA external ionic conditions. The current decay is well fitted by a single exponential with τ ~2 ms for V_{test} = 0 mV (not shown). This is about 10 fold slower than that for α1G current or for the DHP-resistant and TTX-resistant inward current we report in fetal ventricular myocytes.
supplementary figure #1. 2xG1 antibody specificity for α1G in HEK cells

α1G

α1H

HEK - untransfected
supplementary figure #2. 2xH1 antibody specificity for α1H in HEK cells

α1G

α1H

HEK - untransfected
supplementary figure #3. Na channel kinetics are distinct from TTX,DHP-R currents

Inward current with Na channel kinetics in the absence of TTX with 2.5 Ba, 140 TEA external ionic conditions.

Current decay $\tau$ is $\sim$2 ms for $V_{test}$ 0 mV.