Inhibition of Calcineurin and Sarcolemmal Ca$^{2+}$ Influx Protects Cardiac Morphology and Ventricular Function in $K_v4.2N$ Transgenic Mice

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**Background**—Cardiac-targeted expression of truncated $K_v4.2$ subunit ($K_v4.2N$) reduces transient outward current ($I_{to}$) density, prolongs action potentials (APs), and enhances contractility in 3- to 4-week-old transgenic mice. By 13 to 15 weeks of age, these mice develop severely impaired cardiac function and signs of heart failure. In this study, we examined whether augmented contractility in $K_v4.2N$ mice results from elevations in intracellular calcium ([Ca$^{2+}$]) secondary to AP prolongation and investigated the putative roles of calcineurin activation in heart disease development of $K_v4.2N$ mice.

**Methods and Results**—At 3 to 4 weeks of age, L-type Ca$^{2+}$ influx and peak [Ca$^{2+}$], were significantly elevated in $K_v4.2N$ myocytes compared with control because of AP prolongation. Cardiac calcineurin activity was also significantly elevated in $K_v4.2N$ mice by 5 weeks of age relative to controls and increased progressively as heart disease developed. This was associated with activation of protein kinase C (PKC)-α and PKC-θ but not PKC-ε, as well as increases in β-myosin heavy chain (β-MHC) and reductions in sarcoplasmic/endoplasmic reticulum Ca$^{2+}$-ATPase (SERCA)-2α expression. Treatment with either cyclosporin A or verapamil prevented increases in heart weight to body weight ratios, interstitial fibrosis, impaired contractility, PKC activation, and changes in the expression patterns of β-MHC and SERCA2α.

**Conclusions**—Our results demonstrate that AP prolongation caused by $I_{to}$ reduction results in enhanced Ca$^{2+}$ cycling and hypercontractility in mice and suggests that elevations in [Ca$^{2+}$], via $I_{Ca,L}$ and activation of calcineurin play a central role in disease development after $I_{to}$ reduction using the $K_v4.2N$ construct. (*Circulation*. 2002;105:1850-1856.)

**Key Words:** action potentials ■ contractility ■ cardiomyopathy ■ ion channels

The cardiac Ca$^{2+}$-independent transient outward current ($I_{to}$) is encoded by $K_v4.2$, $K_v4.3$, and $K_v1.4$ genes. Reduction in $I_{to}$, as typically occurs in heart disease, results in prolongation of the early phase of the cardiac action potential (AP), Electrophysiological consequences of increased AP duration in heart disease include QT prolongation and increased arrhythmias. Indeed, reduced $I_{Ca,L}$ currents in transgenic mice can cause AP prolongation, lengthened QT intervals, and ventricular tachyarrhythmias. AP prolongation as a result of $I_{to}$ reduction in rodent hearts also elevates intracellular Ca$^{2+}$ transients via increased L-type Ca$^{2+}$ influx ($I_{Ca,L}$), thereby leading to enhanced myocardial contractility. The finding that $I_{to}$ downregulation and associated AP prolongation occur early in the disease process suggests that this may represent a compensatory mechanism to enhance contractility of the compromised myocardium. In addition to enhancing contractility, increased Ca$^{2+}$ entry via $I_{Ca,L}$ after AP prolongation might also contribute to the myocyte hypertrophy commonly seen in heart disease by acting as a stimulus for cellular growth through the activation of cell-signaling pathways, such as the calcineurin-dependent pathway or the interconnected protein kinase C (PKC)-α-dependent pathway. Recently, this Ca$^{2+}$ hypothesis was directly supported in mice overexpressing the L-type Ca$^{2+}$ channel in the heart, in which a sustained increase in $I_{Ca,L}$ resulted in severe cardiomyopathy by 8 months of age via PKC-α activation.

Previously, we showed that overexpressing an N-terminal fragment of $K_v4.2$ ($K_v4.2N$) reduced $I_{to}$ and prolonged APs in transgenic mice in a dominant-negative manner. The hearts of $K_v4.2N$ mice were hypercontractile at 2 to 4 weeks of age and progressively developed cardiac hypertrophy with cham-
ber dilatation by 13 to 15 weeks of age. In this study, we demonstrate that reductions in $I_{\text{Na}}$ and resultant AP prolongation enhance both transsarcolemmal $\text{Ca}^{2+}$ influx and intracellular $\text{Ca}^{2+}$ in K,4.2N myocytes. Furthermore, the transition from hypercontractility to cardiomyopathy in K,4.2N hearts can be prevented by either $\text{Ca}^{2+}$ channel blockade or calcineurin inhibition, supporting the notion that aberrant $\text{Ca}^{2+}$ cycling leading to activation of calcineurin may contribute to the initiation of heart disease.

**Methods**

**K,4.2N Transgenic Mice**

Transgenic mice overexpressing a truncated K,4,2 subunit (K,4.2N) were generated as previously reported.

**Cardiomyocyte Isolation**

Mouse ventricular myocytes were isolated from 2- to 4-week-old control and transgenic mice as previously described.

**Electrophysiology and Intracellular $\text{Ca}^{2+}$ Measurements**

Electrophysiological and intracellular $\text{Ca}^{2+}$ recordings were performed using the whole-cell patch-clamp technique as previously described. For AP and intracellular $\text{Ca}^{2+}$ transient measurements, the extracellular solution was composed of (mmol/L): 140 NaCl, 4 KCl, 10 HEPES, 1 MgCl$_2$, 2 CaCl$_2$, and 10 d-glucose, adjusted to pH 7.4 with NaOH. For $I_{\text{Ca,L}}$ measurements, the extracellular solution contained (mmol/L): 135 choline-Cl, 10 HEPES, 1 MgCl$_2$, 2 CaCl$_2$, and 10 d-glucose, adjusted to pH 7.4 with CsOH. The pipette solution for AP measurements contained (mmol/L): 140 KCl, 10 HEPES, 1 MgCl$_2$, 10 NaCl, 7 Mg-ATP, and 0.1 BAPTA, adjusted to pH 7.2 with KOH. The same internal solution was used for $\text{Ca}^{2+}$ measurements, but 100 $\mu$mol/L BAPTA was replaced with 60 $\mu$mol/L fur 2 pentapotassium salt. Pipette solutions for $I_{\text{Ca,L}}$ contained (mmol/L): 120 aspartic acid, 120 CsOH, 10 HEPES, 1 MgCl$_2$, 7 Mg-ATP, 10 mmol/L TEA-Cl, and 10 EGTA, adjusted to pH 7.2 with CsOH. APs were evoked in current-clamp mode by a brief 5-ms current injection. Intracellular $\text{Ca}^{2+}$ and $I_{\text{Ca,L}}$ were measured with current-clamp and AP-clamp recordings after steady-state stimulation at a frequency of 0.25 Hz.

**Calcineurin Activity**

Calcineurin phosphatase enzymatic activity was measured induplicate from cardiac protein extracts obtained from 3 to 5 hearts as described previously.

**Treatment Protocol**

Male transgenic K,4.2N mice and littermate controls were divided into 4 groups at 2 to 3 weeks of age for either cyclosporin A (CsA) or verapamil treatment: control+vehicle, control+CsA (or verapamil), K,4.2N+vehicle, and K,4.2N+CsA (or verapamil). The vehicle used for CsA was the castor oil–based solvent cremophor (Sigma), and for verapamil, 3% dextrose was used. CsA-treated mice were injected once daily subcutaneously with a dose of 30 mg/kg of CsA (Calbiochem), whereas vehicle-treated mice received an equivalent volume of cremophor. Verapamil (Sigma) was administered through drinking water (0.025 mg/mL). CsA was withdrawn for 48 hours, and verapamil was withdrawn for 24 hours before assessment of ventricular function. Direct effects of CsA on contractile function were assessed in control mice after 4 weeks of treatment without drug withdrawal.

**Histology**

After each mouse was euthanized, hearts were removed and either snap-frozen in liquid nitrogen for Western analysis or calcineurin assays or fixed in 10% buffered formalin for histopathology. At least 3 hearts from each group were bisected midway between base and apex and embedded in paraffin. Thin sections (5 $\mu$mol/L) were cut on a rotary microtome and stained with hematoxylin/eosin or elastic trichrome.

**Hemodynamic Indexes**

Mice were anesthetized with ketamine (50 to 100 mg/kg IP) and xylazine (3 to 6 mg/kg IP). The right common carotid was cannulated with a 1.4F Millar catheter (Millar Inc), which was advanced into the proximal aorta and into the left ventricular cavity. Pressure recordings were filtered at 300 Hz and sampled at 2 kHz (2801A, Data Translation). The time constant for isovolumic relaxation, $\tau$ (s), was determined by fitting the bottom half of the left ventricular pressure traces with a monoeponential function, $P(t)=P_e^{-\tau} t^{-\rho}$.

**Echocardiography**

After anesthesia (see above), transthoracic 2D, M-mode, and Doppler echocardiographic examination was performed with an Acuson Sequoia C256 system equipped with a 15-MHz linear transducer (15L8) (version 4.0, Acuson Corp).

**Western Analysis and PKC Translocation**

Protein (10 $\mu$g) from the total homogenates of individual mouse hearts were analyzed by Western blot as previously described with monoclonal antibodies to sarcoplasmic/endoplasmic reticulum $\text{Ca}^{2+}$-ATPase (SERCA)-2a (Santa Cruz Biotechnology), $\beta$-myosin heavy chain ($\beta$-MHC) (kindly provided by Dr Jeffery Robbins, Cincinnati, Ohio), and calsequestrin (Santa Cruz Biotechnology), which was used as an internal standard. Membrane and cytosolic fractions of heart homogenates were isolated as previously described. Proteins were separated on an 8% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were stained with Ponceau S to check for equal protein loading and then cut into 2 halves: the upper half was probed with PKC-α, PKC-θ, or PKC-ε antibodies, and the bottom half was probed with calsequestrin antibody (Santa Cruz Biotechnology), which was used as an internal standard.

**Statistical Analysis**

A 1-way ANOVA was used to test for overall significance, followed by the Student-Newman-Keuls test for multiple comparison testing between the various groups. The unpaired $t$ test was used to compare the means of 2 groups. All statistical analyses were performed with the SPSS software (version 10.1). A value of $P<0.05$ was considered statistically significant. Data are presented as mean±SEM; $n$ refers to the sample size.

**Results**

**Effects of K,4,2N Overexpression on AP Duration, $\text{Ca}^{2+}$ Influx, and [Ca$^{2+}$], Transients in 3- to 5-Week-Old Mice**

As reported previously,6 overexpressing a dominant-negative truncated mutant K,4,2 subunit prolongs AP duration (APD; Figure 1A) in K,4,2N myocytes (APD$_{50,KV4.2N}$=9.9±0.7 ms, APD$_{50,KV4.2N}$=35.2±5.1 ms; $n=4$) compared with controls (APD$_{50,CON}$=3.1±0.4 ms, APD$_{50,CON}$=20.2±3.6 ms; $n=8$). The increased APD$_{50}$ in K,4,2N myocytes is consistent with the dominant contribution of $I_{\text{Ca,L}}$ to repolarization in mouse myocardium.17 As expected from previous reports,8,18 total $\text{Ca}^{2+}$ entry through L-type $\text{Ca}^{2+}$ channels ($Q_{\text{Ca,L}}$) was elevated ($P<0.01$) nearly 3-fold in transgenic myocytes ($Q_{\text{Ca,L}}$=14.8±1.7 picocoulombs, $n=6$) stimulated with typical K,4.2N APs compared with control myocytes ($Q_{\text{Ca,L}}$=5.1±0.9 picocoulombs, $n=7$) driven by control APs (Figure 1B). Despite these differences in integrated $\text{Ca}^{2+}$ entry per heat, $I_{\text{Ca,L}}$ densities evoked by voltage steps to $+10$ mV did not differ significantly between control ($15.9±1.9$ pA/pF, $n=6$) and K,4.2N ($15.2±1.5$ pA/pF,
The above results suggest that cardiac hypercontractility in early-stage Kv4.2N hearts results from AP prolongation and increased Ca\(^{2+}\) influx through \(I_{\text{Ca,SL}}\). These sustained elevations in Ca\(^{2+}\) and \(I_{\text{Ca,SL}}\) might also contribute to the transition from hyperfunction to heart failure in the Kv4.2N mice via activation of Ca\(^{2+}\)-dependent signaling pathways.\(^{11}\) Recently, the Ca\(^{2+}\)-activated phosphatase calcineurin has been identified as an important signaling protein for the induction of hypertrophy in numerous animal models\(^{19,20}\) as well as in humans.\(^{21}\) Therefore, calcineurin activity was measured in both control and Kv4.2N mice before (5 to 8 weeks of age) and after (13 to 15 weeks of age) the development of overt heart disease in the Kv4.2N mice. Figure 2, A through C, shows that the calcineurin activity is elevated in Kv4.2N hearts, precedes disease development, and increases progressively (\(P<0.05\)) with age. To establish a causal relationship between calcineurin activation and disease initiation, Kv4.2N mice were treated with the calcineurin inhibitor CsA for 12 weeks beginning at 3 weeks of age. Figure 3A shows that calcineurin inhibition effectively reduced (\(P<0.05\)) the ratio of heart weight (HW) to body weight (BW) of Kv4.2N mice (HW/BW\(_{\text{Kv4.2N,CsA}}\) = 0.57 ± 0.06, \(n = 10\); HW/BW\(_{\text{Kv4.2N,Veh}}\) = 0.44 ± 0.02, \(n = 8\)) to control levels. Furthermore, treatment with CsA did not affect body weight in either control or Kv4.2N mice (data not shown).

Trichrome staining shows that extensive cardiomyocyte vacuolization and interstitial fibrosis accompany myocyte hypertrophy as assessed by cell-membrane capacitance (232 ± 16 pF; \(n = 16\)) in vehicle-treated 15-week-old Kv4.2N hearts (Figure 4B) compared with vehicle-treated controls (156 ± 6 pF; \(n = 23\); Figure 4A). The causes of the excessive collagen deposition in late-stage Kv4.2N hearts are unclear but are consistent with myocyte dropout,\(^{6}\) potentially arising from calcineurin-dependent apoptosis.\(^{22}\) By contrast, CsA treatment of Kv4.2N mice markedly reduced these pathological changes (Figure 4C).

**Figure 1.** AP records, L-type Ca\(^{2+}\) currents, and intracellular [Ca\(^{2+}\)] transients in control and Kv4.2N mice. A, APs recorded from Kv4.2N myocytes are lengthened relative to control. B, Calcium currents measured in control (left) and Kv4.2N (right) myocytes under AP-clamp conditions using the APs shown in A. C, \([\text{Ca}^{2+}]_i\) transients measured in current-clamp mode from control and Kv4.2N myocytes driven under their own short and long APs, respectively. D, Peak \([\text{Ca}^{2+}]_i\) measured in AP-clamp mode from a control myocyte stimulated with a long Kv4.2N AP (left) and a Kv4.2N myocyte stimulated with a short control AP (right).

**Figure 2.** Calcineurin activity in Kv4.2N mice throughout development. Calcineurin activity measured in Kv4.2N mice at 5, 8, and 13 weeks of age was elevated 1.53-fold, 1.85-fold, and 2.92-fold, respectively, relative to littermate controls. *\(P<0.05\) between control and Kv4.2N, †\(P<0.001\) between 13-week-old and 5- and 8-week-old Kv4.2N mice.

**Figure 3.** HW/BW ratios after CsA (A) and verapamil (B) treatment. Neither CsA nor verapamil changed HW/BW ratios of control mice, whereas both were effective at normalizing HW/BW ratios of Kv4.2N mice. *\(P<0.05\) between untreated Kv4.2N mice and all other groups.
Functional characterization of hearts from 13- to 15-week-old vehicle-treated and CsA-treated Kv4.2N mice showed that systolic and diastolic ventricular function was significantly improved with CsA treatment. In fact, fractional shortening, heart-rate–corrected velocity of fractional shortening (Table 1), and \( \frac{\text{d}P}{\text{d}t} \) (Table 2) were all enhanced (\( P<0.05 \)) relative to control, as observed in untreated Kv4.2N mice before the onset of pathology. These findings did not appear to result from direct effects of CsA, because CsA treatment reduced contractility in 7-week-old control mice, consistent with previous findings,23 whereas Kv4.2N treated with CsA mice at this age remained hypercontractile (data not shown). These results demonstrate that enhanced ventricular function in 13- to 15-week-old, CsA-treated Kv4.2N mice reflects a genuine hypercontractility, probably arising from AP prolongation. Although diastolic function was not enhanced in CsA-treated Kv4.2N mice, normalization of the passive versus active components of mitral flow (E/A ratios),24 as well as restoration of \(-\frac{\text{d}P}{\text{d}t}\) and rate of ventricular pressure decay (\( \tau \)) (Tables 1 and 2), collectively suggest that normal diastolic function was preserved in CsA-treated Kv4.2N mice.

Effects of Verapamil Treatment on Cardiac Morphology and Ventricular Function

Having established that calcineurin activation is critical for disease progression in Kv4.2N mice, we next tested whether the heart disease process depended on I\(_{\text{Ca,L}}\). Because the total integrated I\(_{\text{Ca,L}}\) (Q\(_{\text{Ca,L}}\)) is increased \( \approx \)3-fold as a result of AP prolongation in Kv4.2N myocytes and because the activity of numerous hypertrophic signaling pathways, including the calcineurin pathway, are Ca\(^{2+}\)-dependent,25 we examined whether treatment of Kv4.2N mice with L-type Ca\(^{2+}\) channel blockers could modify the course of the disease. As seen with CsA, verapamil treatment also normalized HW/BW ratios (HW/BW\(_{\text{Kv4.2N-Veh}} = 0.51 \pm 0.03\), \( n=6 \); HW/BW\(_{\text{Kv4.2N-Verap}} = 0.35 \pm 0.02\), \( n=8 \); HW/BW\(_{\text{CON-Veh}} = 0.38 \pm 0.01\) (Figure 3B) and improved the cardiac morphology of Kv4.2N mice by reducing interstitial fibrosis and cardiomyocyte vacuolization (Figure 4D) compared with vehicle-treated Kv4.2N hearts (Figure 4B).

Both systolic and diastolic ventricular function as assessed by echocardiographic and hemodynamic measures (Tables 1 and 2) were also restored to control levels in verapamil-

**TABLE 1. Echocardiographic Characterization of 13- to 15-Week-Old Verapamil- and CsA-Treated Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Diastolic Dimension, cm</th>
<th>PW, mm</th>
<th>HR, bpm</th>
<th>FS, %</th>
<th>PAV, m/s</th>
<th>VFS(_c), %/ms</th>
<th>E/A Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control+Vehicle (n=6)</td>
<td>0.350±0.011</td>
<td>0.635±0.001*</td>
<td>268±13</td>
<td>33.8±1.3</td>
<td>0.781±0.017</td>
<td>0.50±0.02</td>
<td>3.1±0.4</td>
</tr>
<tr>
<td>K(_{\text{v4.2N}})+Vehicle (n=16)</td>
<td>0.434±0.016*</td>
<td>0.544±0.001*</td>
<td>272±14</td>
<td>19.9±1.4*</td>
<td>0.647±0.028</td>
<td>0.28±0.03*</td>
<td>4.6±0.4*</td>
</tr>
<tr>
<td>K(_{\text{v4.2N}})+Verapamil (n=8)</td>
<td>0.379±0.008</td>
<td>0.592±0.001</td>
<td>265±24</td>
<td>28.5±2.3</td>
<td>0.777±0.066</td>
<td>0.44±0.03</td>
<td>3.3±0.3</td>
</tr>
<tr>
<td>K(_{\text{v4.2N}})+CsA (n=8)</td>
<td>0.337±0.010</td>
<td>0.584±0.001</td>
<td>270±19</td>
<td>40.0±1.9*</td>
<td>0.709±0.038</td>
<td>0.60±0.03*</td>
<td>3.0±0.4</td>
</tr>
</tbody>
</table>

PW indicates posterior wall thickness; HR, heart rate; FS, fractional shortening; PAV, peak aortic velocity; VFS\(_c\), velocity of fractional shortening corrected for heart rate; and E/A ratio, ratio of E-wave (passive, early filling) to A-wave (active, atrial filling) derived from mitral valve Doppler flows.

*Statistically different, \( P<0.05 \), from all other groups.
treated Kv4.2N mice but were not hypercontractile as observed with CsA treatment. This suggests that L-type Ca\(^{2+}\) channel inhibition at the doses in our studies could not protect Kv4.2N mice sufficiently against disease to preserve the hyperfunctional state.

### Expression of SERCA2a and β-MHC and PKC Activation

Reduced expression of SERCA2a and enhanced expression of β-MHC are universal molecular markers of cardiac dysfunction and disease (Figure 5).\(^{26,27}\) In untreated Kv4.2N mice at 13 to 15 weeks of age, cardiac SERCA2a protein levels were significantly \((P<0.05)\) reduced \((17.5\pm1.8\text{ arbitrary units [AU], } n=8)\) compared with control SERCA2a levels \((38.7\pm2.1\text{ AU, } n=4)\), and this reduction was prevented after 10 to 13 weeks of treatment with either verapamil \((39.9\pm1.4\text{ AU, } n=4)\) or CsA \((36.9\pm2.0\text{ AU, } n=4)\) (Figure 5A and 5B). Similarly, β-MHC protein expression was elevated \(8.2\)-fold \((P<0.05)\) in untreated 13- to 15-week-old Kv4.2N mice \((4.38\pm0.70\text{ AU, } n=8)\) relative to controls \((0.54\pm0.35\text{ AU, } n=4)\). This increase was significantly reduced \((P<0.05)\) in both verapamil-treated \((1.39\pm1.00\text{ AU, } n=4)\) and CsA-treated \((0.98\pm0.63\text{ AU, } n=4)\) Kv4.2N mice to levels indistinguishable from control \((P>0.05)\;\text{Figure 5A and 5C)}\).

Because calcineurin activation is associated with a characteristic pattern of PKC isoform activation,\(^{14}\) the membrane translocation patterns of the PKC-α, -ε, and -θ isoforms were examined. The membrane-to-cytosol ratios of the PKC-α and PKC-θ isoforms were increased \(6.1\)- and \(2.4\)-fold, respectively, in Kv4.2N mice relative to controls (Figure 6, A through C). Consistent with a connection between PKC and calcineurin pathways, treatment of Kv4.2N mice with either verapamil or CsA normalized both PKC-α and -θ expression patterns (Figure 6, A through C). In contrast, PKC-ε activation did not differ among any of the groups studied (Figure 6, A and D).

### Discussion

Diminished \(I_{\text{Ca,L}}\) density and altered AP profile have been associated with heart disease in humans and in numerous animal models.\(^{1,2,18,28}\) This \(I_{\text{Ca,L}}\) reduction occurs early in the disease process,\(^{10}\) suggesting that \(I_{\text{Ca,L}}\) may play a role in the initiation of disease.\(^{11}\) Previously, we found that primary \(I_{\text{Ca,L}}\) reduction achieved by cardiac-restricted overexpression of a truncated Kv4.2N peptide in mice causes enhanced ventricular function that progresses to failure by 15 weeks of age.\(^{6}\) In this study, we investigated the potential role of Ca\(^{2+}\) in the enhanced contractility and progression of disease in Kv4.2N overexpressing mice.

Consistent with previous studies in rat myocytes,\(^{8,9,18}\) AP prolongation in Kv4.2N transgenic myocytes increased peak [Ca\(^{2+}\)], supporting the notion that \(I_{\text{Ca,L}}\) plays an important role in influencing Ca\(^{2+}\) handling and contractility in situations, such as heart disease, in which \(I_{\text{Ca,L}}\) densities are reduced.\(^{8,9,18}\) Our results are the first to demonstrate enhanced Ca\(^{2+}\) transients and inotropy via altered AP profile in transgenic mice with selective \(I_{\text{Ca,L}}\) reductions.

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### Table 2. Hemodynamic Characterization of 13- to 15-Week-Old Verapamil- and CsA-Treated Mice

<table>
<thead>
<tr>
<th>Group</th>
<th>HR, bpm</th>
<th>SBP, mm Hg</th>
<th>DBP, mm Hg</th>
<th>LVEDP, mm Hg</th>
<th>(r), ms</th>
<th>(+dP/dt, mm Hg/s)</th>
<th>((-dP/dt)/HR), mm Hg/s</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + Vehicle ((n=6))</td>
<td>242±8</td>
<td>99±3</td>
<td>58±3</td>
<td>6.3±0.5</td>
<td>8.0±0.2</td>
<td>5541±281</td>
<td>23.0±1.1</td>
</tr>
<tr>
<td>K(_{\text{v4.2N}})+ Vehicle ((n=13))</td>
<td>266±9</td>
<td>66±4*</td>
<td>30±4*</td>
<td>12.3±2.4*</td>
<td>14.7±1.7 †</td>
<td>3131±309*</td>
<td>11.8±1.2*</td>
</tr>
<tr>
<td>K(_{\text{v4.2N}})+ Verapamil ((n=6))</td>
<td>244±5</td>
<td>83±5</td>
<td>48±4</td>
<td>7.5±0.5</td>
<td>9.7±0.7</td>
<td>4319±426</td>
<td>17.9±2.0</td>
</tr>
<tr>
<td>K(_{\text{v4.2N}})+ CsA ((n=6))</td>
<td>229±9*</td>
<td>86±2</td>
<td>51±3</td>
<td>7.6±1.7</td>
<td>11.2±1.5</td>
<td>6163±551*</td>
<td>26.7±1.9*</td>
</tr>
</tbody>
</table>

HR indicates heart rate; SBP, systolic blood pressure; DBP, diastolic blood pressure; LVEDP, left ventricular end-diastolic pressure; \(r\), time constant of left ventricular pressure decay; \(+dP/dt\), peak positive rate of change of LV pressure over time; \((-dP/dt)/HR\), HR normalized \(+dP/dt\); and \((-dP/dt\), peak negative rate of change of LV pressure over time.

*Statistically different, \(P<0.05\), from all other groups; †\(P<0.05\), from Control + Vehicle and K\(_{\text{v4.2N}}\)+ Verapamil.

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Figure 5. Western blots of SERCA2a and β-MHC protein. SERCA2a protein (A and B) is reduced by \(~55\%) and β-MHC increased \(8\)-fold (A and C) in 15-week-old vehicle-treated K\(_{\text{v4.2N}}\) hearts relative to vehicle-treated controls. Treatment of K\(_{\text{v4.2N}}\) mice with either CsA or verapamil completely restored the level of SERCA2a expression to normal and decreased β-MHC expression to near control levels. Blots were probed with calcequestrin antibody to demonstrate equal protein loading. \(*P<0.05\) between K\(_{\text{v4.2N}}\) and all other groups.
Associated with elevated intracellular Ca\(^{2+}\), calcineurin activity increased with disease progression, consistent with previous studies establishing a central role for calcineurin activation in animal models of cardiac hypertrophy\(^{12,19,20}\) and during the hypertrophic phase of human heart disease.\(^{21}\) The finding that calcineurin activity was elevated in K\(_v\)4.2N mice before the onset of disease prompted us to attempt to prevent disease development by inhibiting I\(_{Ca,L}\) or calcineurin. The high degree of protection conferred to K\(_v\)4.2N transgenic mice by verapamil or CsA suggests that the transition from hyperfunction to failure in K\(_v\)4.2N mice is Ca\(^{2+}\)-dependent.

Recently, Muth et al\(^{13,29}\) showed that cardiac-specific overexpression of voltage-dependent L-type Ca\(^{2+}\) channels (L-VDCCs) resulted in increased Ca\(^{2+}\) influx and enhanced basal contractility in mice at 8 weeks of age\(^{29}\) and progression to cardiomyopathy by 8 months of age.\(^{13}\) The striking similarity in activation of signaling pathways and overall phenotype between the L-VDCCs and the K\(_v\)4.2N mice suggests common mechanisms. Indeed, both transgenic models generate chronically elevated I\(_{Ca,L}\) (by 50% in L-VDCC mice\(^{13}\) and by 200% in K\(_v\)4.2N mice\(^{13}\)) and show activation of Ca\(^{2+}\)-dependent PKC-\(\alpha\) but not PKC-\(\epsilon\). Although calcineurin signaling was not examined in L-VDCC mice,\(^{13}\) calcineurin activation has recently been associated with a characteristic activation profile of PKC isoforms (PKC-\(\alpha\) and -\(\theta\) and not -\(\epsilon\), -\(\beta\), or -\(\lambda\)) and mitogen-activated protein kinase signaling pathways (JNK and not p38 or ERK1/2).\(^{14}\) The patterns of PKC isoform activation observed in our studies in association with calcineurin activation are consistent with these findings and suggest that the calcineurin pathway is the dominant hypertrophic signaling pathway in K\(_v\)4.2N mice.

Regardless of the specific hypertrophic signaling pathways involved, the phenotypes observed in both K\(_v\)4.2N and L-VDCC transgenic mice implicate Ca\(^{2+}\)-dependent mechanisms.\(^{11}\) These observations may be clinically relevant, because the initial stage of heart disease is typically characterized by I\(_{Ca,L}\) downregulation\(^{2,10}\) and neurohumoral activation,\(^{30}\) both of which may elevate Ca\(^{2+}\) influx and intracellular Ca\(^{2+}\), thereby helping to maintain adequate cardiac output from the diseased heart. Our results suggest that normalization of Ca\(^{2+}\) cycling via I\(_{Ca,L}\) blockade or decoupling of the beneficial inotropic effects of Ca\(^{2+}\) from its detrimental hypertrophic effects via inhibition of Ca\(^{2+}\)-dependent signaling pathways may present a useful therapeutic strategy for heart disease treatment.

Interestingly, the absence of cardiac pathology in the phospholamban knockout mouse, which also exhibits elevated intracellular [Ca\(^{2+}\)], via enhancements in SR Ca\(^{2+}\) uptake,\(^{31}\) suggests that the source of the Ca\(^{2+}\) imbalance (I\(_{Ca,L}\) versus SERCA2a:phospholamban ratio) might be an important factor in disease initiation. The subsarcolemmal colocalization of calcineurin\(^{32}\) and PKC with the L-type Ca\(^{2+}\) channel via the A-kinase anchoring protein AKAP79\(^{33}\) supports the notion that these molecules may respond preferentially to local elevations in submembrane Ca\(^{2+}\), as expected after enhancements in I\(_{Ca,L}\) versus bulk cytosolic Ca\(^{2+}\).

The discrepancy in the phenotypes observed in the K\(_v\)4.2N and K\(_v\)4.2W362F mice,\(^{7}\) despite similar electrophysiological findings, suggests that secondary effects of K\(_v\)4.2N peptide expression,\(^{6}\) in addition to enhanced Ca\(^{2+}\) cycling, may contribute in part to the cardiomyopathy in K\(_v\)4.2N mice. Recent studies in our laboratory, however, have demonstrated that overexpression of either K\(_v\)4.2N or K\(_v\)4.2W362F transgenes in neonatal rat ventricular myocytes both result in similar levels of Ca\(^{2+}\)-calcineurin–dependent hypertrophy.\(^{34}\) Furthermore, consistent with our findings, additional studies have now revealed that pressure-overload–induced cardiac hypertrophy is enhanced in K\(_v\)4.2W362FxKv1.4\(^{1+}\) mice relative to controls,\(^{35}\) suggesting that I\(_{Ca,L}\) reduction and AP prolongation do, in fact, contribute to myocyte hypertrophy.

In summary, we have shown that AP prolongation as a result of I\(_{Ca,L}\) reduction by overexpression of the K\(_v\)4.2N peptide...
increases contractility in early-stage transgenic myocytes by increasing $I_{Ca,L}$ and intracellular [Ca$^{2+}$]. Progression to failure then occurs via a Ca$^{2+}$- and calcineurin-dependent process that is associated with activation of PKC-$\alpha$ and -b but not -e and can be inhibited by verapamil and CsA. Although a secondary, indirect effect of K$_{4.2}$N expression may also contribute to disease in these hearts, our data clearly show that $I_{Ca,L}$, [Ca$^{2+}$], and calcineurin are dominant players in the pathology observed after AP prolongation in K$_{4.2}$N hearts. Furthermore, these findings are consistent with a “Ca$^{2+}$” hypothesis” of hypertrophic gene expression induced by sustained elevations in Ca$^{2+}$ influx and/or intracellular [Ca$^{2+}$].

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References


Inhibition of Calcineurin and Sarcolemmal Ca\(^{2+}\) Influx Protects Cardiac Morphology and Ventricular Function in K\(_{\text{ATP}}\)4.2N Transgenic Mice

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