Reduction of $I_{to}$ Causes Hypertrophy in Neonatal Rat Ventricular Myocytes

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Abstract—Prolonged action potential duration (APD) and decreased transient outward K$^+$ current ($I_{to}$) as a result of decreased expression of $K_v4.2$ and $K_v4.3$ genes are commonly observed in heart disease. We found that treatment of cultured neonatal rat ventricular myocytes with Heteropoda Toxin, a blocker of cardiac $I_{to}$, induced hypertrophy as measured using cell membrane capacitance and $^3$H-leucine uptake. To dissect the role of specific $I_{to}$-encoding genes in hypertrophy, $I_{to}$ was selectively reduced by coexpressing mutant dominant-negative (DN) transgenes. $I_{to}$ amplitude was reduced equally (by about 50%) by overexpression of DN $K_v1.4$ ($K_v1.4$N) or DN $K_v4.2$ (either $K_v4.2$N or $K_v4.3$W362F), but only DN $K_v4.2$ prolonged APD duration (at 1 Hz) and induced myocyte hypertrophy. This hypertrophy was prevented by coexpressing wild-type $K_v4.2$ channels ($K_v4.2$F) with the DN $K_v4.2$ genes, suggesting the hypertrophy is due to $I_{to}$ reduction and not nonspecific effects of transgene overexpression. The hypertrophy caused by reductions of $K_v4.3$-based $I_{to}$ was associated with increased activity of the calcium-dependent phosphatase, calcineurin, and could be prevented by coinfection with Ad-CAIN, a specific calcineurin inhibitor. The hypertrophy and calcineurin activation induced by $K_v4.2$N infection were prevented by blocking Ca$^{2+}$ entry and excitability with verapamil or high [K$^+$]o. Our studies suggest that reductions of $K_v4.2$-based $I_{to}$ play a role in hypertrophy signaling by activation of calcineurin. (Circ Res. 2002;90:578-585.)

Key Words: cardiac myocyte $\bullet$ [Ca$^{2+}$]i $\bullet$ transient outward K$^+$ current $\bullet$ hypertrophy

One prominent feature of diseased cardiac muscle is prolongation of action potential duration (APD). Although changes in many ionic currents have been reported in heart disease, reductions in transient outward K$^+$ current ($I_{to}$) have consistently been observed in heart disease regardless of species. In mammalian hearts, $I_{to}$ has been shown to be encoded by $K_v1.4$, $K_v4.2$, and $K_v4.3$ potassium channel genes, although the relative contribution of these genes varies between species. While a number of changes in cardiac function and gene expression occur in diseased hearts, decreased expression of $K_v4.2$ and $K_v4.3$ genes and associated changes in both $I_{to}$ density and AP profile are commonly observed in myocytes from many animal models of heart disease and human heart failure. Moreover, the magnitude of $I_{to}$ and the level of expression of $K_v4.2$ and $K_v4.3$ channels are also reduced by both acute and long-term activation of various receptor-mediated pathways in response to neurohumoral factors known to be involved in initiating cardiac hypertrophy, such as angiotensin II and phenylephrine. Despite the correlation between reduced $K_v4.2$ expression and heart disease, the link between reductions in $I_{to}$, $K_v4.2$, expression, and cardiac hypertrophy is still unclear, although reductions in $I_{to}$ density and $K_v4.2$ expression occur very early after myocardial infarction in rats. APD prolongation following $I_{to}$ reduction can increase Ca$^{2+}$ influx through voltage-dependent L-type Ca$^{2+}$ channels ($I_{Ca,L}$), thereby elevating [Ca$^{2+}$]i. Because Ca$^{2+}$ is an essential cofactor for several hypertrophy signaling pathways, including calcineurin, mitogen-activated protein kinases (MAPK), and protein kinase C, it is conceivable that increased Ca$^{2+}$ influx by $I_{to}$ reduction might modulate hypertrophy signaling in myocardium. One particularly attractive candidate pathway, linking reductions in $I_{to}$ to hypertrophy, is the Ca$^{2+}$/calmodulin-activated cytoplasmic serine/threonine phosphatase calcineurin that dephosphorylates NFAT3 leading to nuclear translocation and transcriptional activation of numerous hypertrophy genes. However, calcineurin has been shown to play an important role in triggering hypertrophy signaling. In this study, the connection between $I_{to}$ reduction and hypertrophy was investigated in cultured neonatal rat ventricular myocytes. Using DN overexpression methods, reductions in $K_v4.2$-based $I_{to}$, but not $K_v1.4$-based $I_{to}$, causes myocyte hypertrophy that appears to require calcineurin activation.

Materials and Methods

Neonatal Rat Ventricular Myocyte Isolation
Neonatal Sprague-Dawley (1 to 2 days old) rat ventricular myocytes (Charles River, Montreal, Canada) were isolated and cultured as...
described previously. For adenoviral infection studies, myocytes were infected (5 to 10 PFU/cell) for 4 to 6 hours immediately following replacement of serum-containing culture medium with serum-free medium 24 hours after isolation. This titer of viral infection did not cause any detectable cell death, which is consistent with previous studies. Electrophysiological recordings were performed at least 30 hours after the myocytes were serum-starved and infected with adenoviruses.

**Voltage Constructs**

Recombinant adenoviruses were generated (using pAd-Easy) by inserting the green fluorescent protein (GFP) gene with or without the K+ channel transgenes into the E1 transcription region of adenovirus backbone under a CMV promoter. Using this technique, we constructed adenoviruses expressing full-length Kv1.4 and Kv1.4 N (amino acids 1 to 311), Kv4.2 M (Kv4.2 W362F) (kindly provided by Dr J. Nerbonne, Washington University, St. Louis, Mo), and Kv4.2 N (amino acids 1 to 385) transgenes. Viruses were purified by CsCl-gradient banding after plaque-purification. Expression of these genes or transgenes in cultured myocytes was confirmed by Western blot analysis (data not shown). The production of adenoviruses expressing CAIN (Ad-CAIN) has been described previously.

**Rhodamine-Conjugated Phalloidin Staining**

Actin filaments were visualized in cultured myocytes using Rhodamine-conjugated Phalloidin and imaged using a laser scanning confocal microscope (Bio-Rad MRC 600).

**Electrophysiological Recordings**

Whole cell patch-clamp recordings were done as described previously at room temperature, 30 to 70 hours after the myocytes were serum-starved and infected with adenoviruses. Infected myocytes were identified by their GFP fluorescence.

**3H-Leucine Uptake Experiments**

To evaluate the rate of protein synthesis, serum-starved and infected myocytes were incubated in 1 μCi 3H-Leucine (per 2 × 10^6 cells) overnight. Next, the cells were washed with PBS, treated with Trichloroacetic acid (TCA), harvested by 0.5 mol/L NaOH, and their radioactivity was recorded using a scintillation counter.

**Calcineurin Activity**

Calcineurin phosphatase enzymatic activity was determined as described previously.

**Luciferase Assay to Measure NFAT Activity**

To measure NFAT activity, as a representative of calcineurin activity, we measured luciferase activity of myocytes 48 hours after infection did not cause any detectable cell death, which is consistent with previous studies. Electrophysiological recordings were performed at least 30 hours after the myocytes were serum-starved and infected with adenoviruses.

**Statistical Analysis**

All averaged data are presented as mean ± SEM. Statistical significance was determined using t test to compare 2 groups, and ANOVA to compare multiple groups. Statistical significance was realized at P < 0.05.

An expanded Materials and Methods section can be found in the online data supplement available at http://www.circresaha.org.

**Results**

**Blockade of I_ca Using Pharmacological Agents**

To examine the consequences of long-term I_ca reduction on cellular hypertrophy, we used Heteropoda toxin-3 (HpTx3), a peptide spider toxin that blocks I_ca in rat cardiac myocytes. Application of HpTx3 (400 nmol/L) to cultured neonatal rat ventricular myocytes (NRVM) for 30 to 70 hours increased (P < 0.05) cell membrane capacitance (21.5 ± 0.8 pF, n = 10, versus 16.4 ± 0.5 pF, n = 34, in control myocytes), as well as 3H-Leucine incorporation by 23.4 ± 8.4% (Figure 1). Long-term treatment with HpTx3 also prolonged (P < 0.01) APD (APD20 = 206.6 ± 27.5 versus 76.7 ± 16.5 ms, n = 6) and reduced (P < 0.05) I_ca amplitude (73.1 ± 13.1 pA, n = 10, versus 141.0 ± 18.4 pA, n = 34) recorded in the absence of HpTx3. Long-term treatment with HpTx3 also reduced (P < 0.01) Kv4.2 protein levels (by 34.5 ± 3.6%), although not when myocyte excitability was blocked by elevated [K+]o (data not shown). These results suggest that prolonged I_ca reduction by HpTx3 induces hypertrophy as well as decreasing expression of K_v4.2 protein.

**Electrophysiological Effects of DN K+ Channel Transgene Overexpression**

Because cardiac I_ca is encoded by K_v1.4, K_v4.2, and K_v4.3 K+ channel genes, dominant-negative (DN) transgenes were overexpressed in NRVM to dissect the relative contributions of these different K+ channel families to I_ca and myocyte hypertrophy. K_v1.4/N overexpression was used to selectively reduce the portion of I_ca encoded by members of the K_v1.4 family gene, which have been directly linked to the slow component of I_ca (I_ca,s), as underlies the fast component of I_ca (I_ca,f), 23–25 and K_v4.2-based I_ca, which underlies the fast component of I_ca (I_ca,f), 23–25 was reduced using two DN K_v4.2 transgenes, a truncated K_v4.2 gene (K_v4.2N), and a pore mutant (K_v4.2 W362F or K_v4.2 M), as used previously in transgenic mice models. 27,28 Figure 2A shows typical traces recorded from GFP, K_v1.4 N-, K_v4.2 N-, or K_v4.2 M-infected myocytes in response to voltage steps to +60 mV from a holding potential of −90 mV. The corresponding average I_ca amplitudes (per cell) are summarized in Figure 2B. This figure shows that overexpression of K_v1.4/N decreased...
(P<0.001) $I_{to}$ amplitude by almost 50% from 180.4±10.0 pA (10.2±0.5 pA/pF, n=28) in GFP myocytes to 94.1±6.8 pA (5.5±0.4 pA/pF, n=32) by eliminating the slow-recovering component, $I_{to,s}$. Similar reductions (P<0.001) were observed with overexpression of $K_v4.2$ N (85.5±10.4 pA or 3.3±0.4 pA/pF, n=15) or $K_v4.2$ M (80.9±7.4 pA or 3.2±0.3 pA/pF, n=22), but these constructs abolished the fast-recovering component, $I_{to,f}$. The relative reductions of $I_{to}$ by $K_v1.4$ N versus $K_v4.2$ N and $K_v4.2$ M matches closely the relative proportions of $I_{to,s}$ versus $I_{to,f}$ that exist in NRVM. 

Because $I_{to}$ is an important current for the early repolarization of rat cardiac action potentials (APs), we anticipated that reductions in $I_{to}$ would cause prolongation of APD. Typical APs recorded at 1 Hz stimulation rate from GFP-, $K_v1.4$ N-, $K_v4.2$ N-, and $K_v4.2$ M-infected myocytes are presented in Figure 2C with Figure 2D summarizing averaged APD$_{50}$ and APD$_{90}$. Despite causing significant reductions in $I_{to}$, overexpression of $K_v1.4$ N transgene did not alter the APD$_{50}$ or APD$_{90}$ (P>0.4) (97.6±13.3 and 190.4±13.6 ms, n=11) compared with GFP (84.5±13.3 and 168.2±19.9 ms, n=11). On the other hand, overexpression of $K_v4.2$ N or $K_v4.2$ M transgenes prolonged (P<0.001) APD$_{50}$ (266.7±28.8 or 270.9±31.9 ms, n=11) as well as APD$_{90}$ (362.6±30.1 or 353.7±40.8 ms, respectively). Prolongation of APD in $K_v4.2$ N- and $K_v4.2$ M-, but not in $K_v1.4$ N-infected myocytes establishes that $K_v1.4$-based $I_{to,s}$ makes minor contributions to APD as expected from the slow kinetics of recovery from inactivation of $I_{to,s}$ ($\tau_{rec,s}$=3 to 4 s) compared with $K_v4.2$-based $I_{to}$ ($\tau_{rec,f}$=80 to 100 ms). 

**Selective Effects of DN Channel Expression on Myocyte Hypertrophy**

In addition to alterations in $I_{to}$ current magnitude and APD, myocytes infected with $K_v4.2$ N or $K_v4.2$ M were noticeably larger than myocytes infected with either GFP or $K_v1.4$ N when...
assessed using confocal microscopy (Figure 3A). Figure 3B shows that the averaged cell membrane capacitance in Kv4.2 N- and Kv4.2 M-infected myocytes (25.9 ± 2.8 pF, n = 19, and 24.8 ± 0.9 pF, n = 21, respectively) were larger (P < 0.001) than in GFP- (16.7 ± 0.6 pF, n = 28) or Kv1.4 N- (17.1 ± 2.7 pF, n = 31) infected myocytes. Moreover, Figure 3C shows that 3 H-Leucine incorporation was also increased (P < 0.001) following Kv4.2 N (by 39.3 ± 5.6%, n = 15) or Kv4.2 M (by 37.9 ± 4.9%, n = 22) infection compared with GFP- or Kv1.4 N-infected myocytes. Staining NRVM with Rhodamine-conjugated phalloidin, an F-actin label, revealed that this hypertrophy did not affect the normal packing and alignment of the myofilaments (Figure 3D). On the other hand, overexpression of full-length Kv4.2 or Kv4.2 (Kv4.2 F, Kv4.2 F) genes did not affect cell membrane capacitance (15.3 ± 2.5 pF, n = 10, or 18.4 ± 1.3 pF, n = 11, respectively) or 3 H-leucine uptake (91.9 ± 4.1%, n = 9, or 95.1 ± 3.7%, n = 12, respectively) compared with GFP-infected myocytes (P > 0.3), suggesting that hypertrophy observed in Kv4.2 N- and Kv4.2 M-infected myocytes is related to changes in Ito levels and APD.

Because contractile activity can have major effects on cell growth and hypertrophy signaling pathways,29,30 it is conceivable that Ito reductions could induce hypertrophy secondary to changing the spontaneous firing rates of AP in the NRVM. However, no difference (P > 0.2) in spontaneous contraction rates were observed between the different groups (0.67 ± 0.06 Hz in GFP-infected compared with 0.57 ± 0.05 and 0.63 ± 0.07 Hz in Kv4.2 N- and Kv4.2 M-infected myocytes). Moreover, overexpression of DN Kv4.2 in myocytes electrically paced at 2 Hz (for 48 hours) also enhanced 3 H-leucine uptake (by 40.3 ± 5.3% for Kv4.2 N and by 31.5 ± 6.2% for Kv4.2 M) relative to chronically paced GFP-infected myocytes (P < 0.001), despite the fact that electrical pacing alone increased (P < 0.001) 3 H-leucine uptake in GFP myocytes by 57.1 ± 2.6% compared with nonpaced GFP myocytes (data not shown).

Reversal of Hypertrophy Induced By DN Kv4.2 Using K4.2 F

To explore further whether hypertrophy induced by Kv4.2 N and Kv4.2 M infection was related to reductions in Ito, DN

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**Figure 4.** NFAT activity (a measure of calcineurin activity) was determined using a Dual Luciferase Reporter (DLR) assay in cultured myocytes infected with adenoviruses expressing GFP (n = 19), GFP + CAIN (a specific inhibitor of calcineurin) (n = 12), Kv4.2 N (n = 19), Kv4.2 N + CAIN (n = 12), Kv4.2 M (n = 19), or Kv4.2 M + CAIN (n = 12). Columns are mean ± SEM; *P < 0.05 compared with GFP.

**Figure 5.** Average effects of GFP, CAIN, Kv4.2 N, and Kv4.2 N + CAIN (n = 14) on Ito amplitude (A) and cell membrane capacitance (B). C, The effects of DN Kv4.2 overexpression and coexpression with CAIN on cell 3 H-leucine uptake. D, Typical images of myocytes infected with GFP, CAIN, Kv4.2 N, and Kv4.2 N + CAIN. Columns are mean ± SEM; *P < 0.05 compared with GFP.
Kv4.2-infected NRVMs were coinfectected with K<sub>a2</sub>F. As expected, K<sub>a2</sub>F coexpression increased (P<0.001) I<sub>0</sub> amplitude (1453.4±302.4 pA, n=15 for K<sub>a2</sub>N+K<sub>a2</sub>F and 1129.5±271.5 pA, n=13 for K<sub>a2</sub>M+K<sub>a2</sub>F) and abbreviated APD (APD<sub>50</sub>=2.2±0.4 ms, APD<sub>90</sub>=10.8±3.2 ms, n=18 for K<sub>a2</sub>N+K<sub>a2</sub>F and APD<sub>50</sub>=2.8±0.8 and 12.4±2.1 ms, n=15 for K<sub>a2</sub>M+K<sub>a2</sub>F) compared with GFP, K<sub>a2</sub>N or K<sub>a2</sub>M infection alone. More importantly, K<sub>a2</sub>F coinfection prevented increases in cell capacitance (18.9±1.0 pF, n=18 for K<sub>a2</sub>N and 18.1±0.9 pF, n=15 for K<sub>a2</sub>M) and 'H-leucine uptake (91.8±7.0% for K<sub>a2</sub>N and 97.1±6.1% for K<sub>a2</sub>M) compared with GFP-infected myocytes (P>0.4). As expected, overexpression of K<sub>a2</sub>F alone elevated (P<0.001) I<sub>0</sub> amplitude (1,808.3±473.6 pA, n=10) and shortened (P<0.001) both APD<sub>50</sub> (2.7±0.5 ms) and APD<sub>90</sub> (12.7±2.9 ms) at 1 Hz recording rate without affecting cell membrane capacitance or 'H-leucine uptake (as stated earlier). Taken together, these findings suggest that hypertrophy induced by K<sub>a2</sub>N or K<sub>a2</sub>M infection requires APD prolongation following reductions in I<sub>0</sub>.

**Hypertrophy Pathway: Calcineurin**

We investigated the possible role of calcineurin in DN Kv4.2-induced hypertrophy by measuring the level of activation of NFAT (DLR assay), a calcineurin-activated transcription factor downstream from calcineurin, and by measuring the specific phosphatase activity of calcineurin (see Materials and Methods). Compared with GFP-infected myocytes, activity of NFAT was increased (P<0.001) by 68.7±8.2% in K<sub>a2</sub>N- and by 66.4±10.4% in K<sub>a2</sub>M-infected myocytes (n=19) (Figure 4). These increases are comparable to the 63.5±5.9% increase in calcineurin phosphatase enzymatic activity following K<sub>a2</sub>N infection (data not shown). Coinfection of NRVM with Ad-CAIN (calcineurin inhibitor), a specific noncompetitive inhibitor of calcineurin, predicted significant increases (P<0.2) in cell capacitance (18.5±2.1 pF, n=11) (Figure 5B) and 'H-leucine uptake (97.7±3.7%) (Figure 5C), as well as changes in cell morphology (Figure 5D) in K<sub>a2</sub>N-infected NRVM compared with GFP, without altering the degree of I<sub>0</sub> reduction (81.7±17.2 pA, n=14) (Figure 5A). Overexpression of CAIN plus GFP did not measurably alter I<sub>0</sub> density, cell capacitance, protein synthesis, or cell morphology. Similar results were found using 1000 ng/mL cyclosporine A (CsA), a nonspecific inhibitor of calcineurin (data not shown). These findings demonstrate that the hypertrophy response induced by I<sub>0</sub> reduction is dependent on Ca<sup>2+</sup>-activated calcineurin.

**Blockade of Myocyte Excitability and Inhibition of Ca<sup>2+</sup> Influx**

If calcineurin activation and induction of hypertrophy by DN K<sub>a2</sub> overexpression results from increased Ca<sup>2+</sup> entry in response to APD prolongation, blockade of Ca<sup>2+</sup> entry and excitability should prevent these effects. Application of verapamil (10 µmol/L) or high [K<sup>+</sup>]o (50 mmol/L) eliminated spontaneous beating of NRVM, and they both prevented cell hypertrophy induced by DN K<sub>a2</sub>N (Figure 6). Treatment with verapamil reduced 'H-leucine uptake in K<sub>a2</sub>N-infected myocytes by 41.6±6.9% (n=6) relative to untreated K<sub>a2</sub>N-infected myocytes to levels indistinguishable (P>0.1) from GFP-infected myocytes in the presence of verapamil. Similarly, application of 50 mmol/L external K<sup>+</sup> reduced 'H-leucine incorporation in K<sub>a2</sub>N-infected myocytes by 36.1±2.3% (n=4) relative to untreated K<sub>a2</sub>N-infected myocytes to levels that were slightly elevated (14.01±2.13%, P=0.055) over that measured in GFP-infected myocytes in the presence of 50 mmol/L external K<sup>+</sup>. Despite these effects on cell growth, verapamil and high [K<sup>+</sup>]o did not interfere with the reduction in I<sub>0</sub> and APD prolongation after DN K<sub>a2</sub> overexpression. These observations demonstrate that cellular hypertrophy induced by DN K<sub>a2</sub>N depends critically on spontaneous beating of myocytes as expected if these changes resulted from APD prolongation leading to elevated Ca<sup>2+</sup> entry. Indeed, calcineurin activity and NFAT activation were both prevented in K<sub>a2</sub>N-infected NRVM by verapamil and elevated [K<sup>+</sup>]o (data not shown).

**Discussion**

Different models of cardiac disease in mammals and humans are associated with prolonged action potential duration and reduced I<sub>0</sub>.

2.7 Previous studies have established that K<sub>a4</sub>, K<sub>a2</sub>, and K<sub>a1</sub> channels encode for I<sub>0</sub> currents,<sup>3,4,34</sup> and are expressed in mammalian myocardium, although the relative contribution of these genes to I<sub>0</sub> varies among different...
species in a developmental and region-specific fashion.\textsuperscript{24,35–37} In addition, K\textsubscript{v4.3} channels also encode for \(I_{\text{to}}\) currents and are expressed in mammalian heart,\textsuperscript{25} although their contribution to cardiac \(I_{\text{to}}\) is unclear. In heart disease and hypertrophy, the reductions in \(I_{\text{to}}\) are associated with decreased K\textsubscript{v4.2} and K\textsubscript{v4.3} expression.\textsuperscript{5,6,9} The primary purpose of this study was to explore the possible contribution of reductions in \(I_{\text{to}}\) and K\textsubscript{v4.2} expression in triggering hypertrophy in cardiac myocytes.

Adenoviral infection of cultured NRVM with DN K\textsubscript{v4.2} and DN K\textsubscript{v4.3} transgenes were used to selectively eliminate currents generated by \(I_{\text{to}}\)-encoding cardiac genes.\textsuperscript{7} Taking advantage of the subfamily-specific nature of voltage-gated K\textsuperscript{+} channel assembly,\textsuperscript{38} \(I_{\text{to}}\) currents generated by K\textsubscript{v1.4} (and possibly K\textsubscript{v1.7}) were reduced using a truncated K\textsubscript{v1.4} transgene (K\textsubscript{v1.4}N) and those generated by K\textsubscript{v4.2/3} channel genes were reduced using truncated (K\textsubscript{v2.3}N or K\textsubscript{v2.3}M) K\textsubscript{v2.3} transgenes.\textsuperscript{18,20} K\textsubscript{v1.4}N overexpression in cultured NRVM reduced \(I_{\text{to}}\) by about 50%, abolishing selectively slow \(I_{\text{to}}\) (\(I_{\text{t,slow}}\)).\textsuperscript{27} Overexpression of K\textsubscript{v4.2}N or K\textsubscript{v4.2}M also reduced \(I_{\text{to}}\) by about 50% but selectively eliminated the fast component of \(I_{\text{to}}\) (\(I_{\text{t,fast}}\)) in NRVM.\textsuperscript{27} Despite the similar reductions in \(I_{\text{to}}\) only K\textsubscript{v4.2}N/M induced measurable APD prolongation in myocytes at 1 Hz stimulation rates, a rate similar to the intrinsic beating rate of our cultured NRVM (0.55 to 0.65 Hz). These observations are anticipated because the slow rate of recovery from inactivation of \(I_{\text{to}}\) (i.e., \(\tau_{\text{t,slow}}=2\) to 3 s) limits its contribution to membrane repolarization at 1 Hz as reported previously.\textsuperscript{15,27,39} By contrast, K\textsubscript{v2.3} channels recover rapidly from inactivation (\(\tau_{\text{t,fast}}=80\) to 100 ms)\textsuperscript{19} and are therefore expected to contribute disproportionately greater to membrane repolarization and APD in beating NRVM (discussed later).

**Myocyte Hypertrophy in Response to Reductions in K\textsubscript{v4.2/3}-Based \(I_{\text{to}}\)**

Our studies show that chronic treatment of cultured NRVM with HpTx3, a blocker of K\textsubscript{v4.2} and K\textsubscript{v4.3} channels,\textsuperscript{21} caused myocyte hypertrophy. An interesting feature of this hypertrophy is that it is APD prolongation that induces hypertrophy in beating NRVMs with reduced \(I_{\text{to}}\). Indeed, elimination of \(I_{\text{to}}\) by K\textsubscript{v4.2}N is expected to only reduce total \(I_{\text{to}}\) amplitude by 10% to 20% when cells are beating spontaneously versus more than 80% reduction in \(I_{\text{to}}\) amplitude by DN K\textsubscript{v4.2} infection. These observations are consistent with the results from several rodent models of cardiac disease showing that K\textsubscript{v4.2} expression is reduced while K\textsubscript{v1.4} expression is increased\textsuperscript{2–18} and results in NRVM treated with phenylephrine (an \(\beta\)-adrenoreceptor agonist).\textsuperscript{10}

**Mechanism of Hypertrophy**

We have demonstrated previously that reductions in K\textsubscript{v4.2/3}-based \(I_{\text{to}}\) channel expression can prolong APD, increase Ca\textsuperscript{2+} influx through L-type Ca\textsuperscript{2+} channels, and elevate [Ca\textsuperscript{2+}]o transients in each cardiac cycle.\textsuperscript{2,41} These increases in [Ca\textsuperscript{2+}]o could potentially modulate the activation of several Ca\textsuperscript{2+}-activated hypertrophy pathways,\textsuperscript{13} such as PKC (\(\alpha\) and \(\beta\)), MAPK pathways (SAPK, ERK, and P38), and/or calcineurin (see Molkentin and Dorn\textsuperscript{42} for review). Our studies strongly support a connection between APD prolongation, Ca\textsuperscript{2+} entry, and cell growth in our DN K\textsubscript{v4.2}-infected myocytes for several reasons. First, blocking excitability and Ca\textsuperscript{2+} entry with verapamil or elevated [K\textsuperscript{+}]o prevented the hypertrophy induced by DN K\textsubscript{v4.2} overexpression without averting the reductions of \(I_{\text{to}}\) in cultured NRVM. Moreover, \(\text{H}^{-}\)-leucine incorporation following blockade of excitability was normalized between GFP- and DN K\textsubscript{v4.2}–infected myocytes and reduced to below that observed in GFP-infected NRVM without blockade. Second, a strong connection between APD prolongation and hypertrophy in NRVM following \(I_{\text{to}}\) reduction was further supported by the ability of K\textsubscript{v4.2}F coinfusion to elevate \(I_{\text{to}}\), shorten APD, and prevent myocyte hypertrophy induced by DN K\textsubscript{v4.2}, as well as the lack of hypertrophy in NRVM overexpressing K\textsubscript{v4.1}N (which does not prolong APD). Third, overexpression of DN K\textsubscript{v4.2} transgenes in electrically stimulated cultured myocytes (at 2 Hz) increased \(\text{H}^{-}\)-leucine incorporation over and above that caused by electrical stimulation alone, which has been reported previously.\textsuperscript{30} The putative link between elevated Ca\textsuperscript{2+} influx and the induction of hypertrophy is consistent with the hypertrophy that occurs in TG mice overexpressing cardiac L-type Ca\textsuperscript{2+} channels\textsuperscript{43} and the K\textsubscript{v4.2}N construct.\textsuperscript{33}

Although numerous cell signaling pathways might be involved in transduction of \(I_{\text{to}}\) reductions into a hypertrophy signal, we initially focused on the possible role of calcineurin, a cytoplasmic phosphatase that is activated by calcium and Ca/CaM complex.\textsuperscript{44} Cardiac expression of a constitutively active form of calcineurin in TG mice caused severe cardiac interventions on beating rate because spontaneous beating rates were not different between K\textsubscript{v4.1}N-, K\textsubscript{v4.2}N-, K\textsubscript{v4.2}M-, and GFP-infected myocytes (see Mechanism of Hypertrophy section). Furthermore, DN K\textsubscript{v4.2} infection also induced hypertrophy in NRVM electrically stimulated at 2 Hz. By contrast, despite reducing \(I_{\text{to}}\) densities by 50%, DN K\textsubscript{v4.1} infection did not induce cardiac hypertrophy in spontaneously beating cultured NRVMs (at about 0.6 Hz). The presence of cardiac hypertrophy with DN K\textsubscript{v4.2} infection, combined with the absence of hypertrophy when K\textsubscript{v1.4}-based currents were reduced, suggest that it is APD prolongation that induces hypertrophy in beating NRVMs with reduced \(I_{\text{to}}\)
hypertrophy that was blocked by pharmacological inhibition of calcineurin in vivo and in vitro. Calcineurin has also been shown to be involved in development of hypertrophy induced by angiotensin II, phenylephrine, and pressure overload. In addition, TG mice expressing inhibitory domain of either CAIN or AKA79 (calcineurin inhibiting peptides) partially reduced catecholamine- and pressure overload–induced cardiac hypertrophy. These and several other studies establish that calcineurin is a strong candidate contributing to cardiac myocyte hypertrophy possibly by integrating with other signaling pathways. Consistent with a prominent role for calcineurin in hypertrophy, NFAT activity and calcineurin’s phosphatase activity was increased after overexpression of Kvs4.2 N or Kvs4.2 M. Treatment with the nonspecific calcineurin inhibitor, cyclosporin A (CsA) (data not shown), or coinfection with the specific peptide inhibitor of calcineurin, CAIN, completely blocked the increases in NFAT activity as well as the hypertrophy in NRVM infected with DN Kvs4.2 genes, suggesting that these increases in NFAT3 activity were the consequence of increased calcineurin activity with no, or minimal, interference from other hypertrophy pathways. These results are also consistent with our findings in TG mice expressing Kvs4.2 N, where cardiac hypertrophy and fibrosis were prevented and cardiac function maintained when treated with CsA or verapamil.

It is conceivable that infection of myocytes with recombinant adenoviruses might be linked to nonspecific or toxic actions in NRVM. Indeed, increased cell death can occur when cultured myocytes are infected with high titers of adenoviruses. For this reason, myocytes in our studies were infected with 5 to 10 PFU/cell of purified virus for 4 to 6 hours and experiments were done 30 to 70 hours after infection. This titer consistently resulted in greater than 90% transfection efficiency with no detectable cell death as reported previously. In studies involving coinfection with Kvs4.2 N or Kvs4.2 M along with full length Kvs4.2 F, the number of PFUs per cell was increased (15 to 20 PFU/cell) and produced no evidence of cell death. More importantly, coinfection with Kvs4.2 F elevated I, shortened APD, and prevented the myocyte hypertrophy induced by DN Kvs4.2. Although I was increased well above the control levels following Kvs4.2 F coinfection, attempt to reduce the I levels by reducing viral titers below 5 to 10 PFU/cell (of each construct) resulted in reduction in infection efficiency, making interpretation of our studies on cell populations problematic. Regardless, the ability of Kvs4.2 F to prevent hypertrophy suggests that the hypertrophy was not due to nonspecific or toxic effects of these viruses, or that hypertrophy induced by nonspecific effects of overexpression depends critically on APD prolongation.

In conclusion, our results show that reductions in I produced by Kvs4.2/3 channel genes can trigger hypertrophy in NRVM by prolonging APD, which appears to depend critically on calcineurin activation. The extent to which these observations are relevant to other species, with AP profiles different from NRVMs, is uncertain and clearly warrants further investigation.

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