Calcineurin Increases Cardiac Transient Outward K⁺ Currents via Transcriptional Up-regulation of Kv4.2 Channel Subunits*

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Fast transient outward potassium currents (I_{to,f}) are critical determinants of regional heterogeneity of cardiomyocyte repolarization as well as cardiomyocyte contractility. Additionally, I_{to,f} densities are markedly down-regulated in cardiac hypertrophy and heart disease, conditions associated with activation of the serine/threonine phosphatase calcineurin (Cn). In this study, we investigated the regulation of I_{to,f} expression by Cn in cultured neonatal rat ventricular myocytes (NRVMs) with and without α₁-adrenoceptor stimulation with phenylephrine (PE). Overexpression of constitutively active Cn in NRVMs induced hypertrophy and caused profound increases in I_{to,f} density as well as Kv4.2 mRNA and protein expression and promoter activity, without affecting Kv4.3 or KChIP2 levels. The effects of Cn on hypertrophy, I_{to,f}, and Kv4.2 transcription were associated with NFAT activation and were abrogated by NFAT inhibition. Despite activating Cn and inducing hypertrophy in NRVMs, PE resulted in profound down-regulation of I_{to,f} densities as well as Kv4.2, Kv4.3, and KChIP2 expression. Although hypertrophy and NFAT activation were inhibited by the Cn inhibitory peptide CAIN, I_{to,f} and Kv4.2 expression were further reduced by CAIN, whereas Cn overexpression eliminated PE-induced reductions in I_{to,f} and Kv4.2 expression without affecting Kv4.3 or KChIP2 levels. We conclude that Cn increases cardiac I_{to,f} densities by positively regulating Kv4.2 gene transcription. Consistent with this conclusion, we found that I_{to,f} was increased in myocytes isolated from young mice overexpressing Cn prior to the development of heart disease. This positive regulation of Kv4.2 transcription by Cn activation is expected to minimize the reductions in I_{to,f} and Kv4.2 expression observed in hypertrophic cardiomyocytes.

Normal cardiac repolarization is orchestrated by coordinated activity of many K⁺ currents, which vary between regions of the heart as well as between different species. One K⁺ current of particular interest in many mammalian species, including humans, is the cardiac transient outward K⁺ current, which is composed of fast (I_{to,f}) and slow types. I_{to,f} is encoded by pore-forming α-subunits (i.e. Kv4.2 and Kv4.3 in rodents and Kv4.3 in humans) and auxiliary KChIP2 subunits. Variations in I_{to,f} as a result of regional differences in Kv4.2 (rodents) (1, 2) or Kv4.3 or KChIP2 (humans and dogs) (3, 4) are major contributors to electrical heterogeneity of repolarization in the heart, which is strongly linked to arrhythmias, particularly the Brugada syndrome (5). I_{to,f} densities also have profound effects on the strength and timing of myocyte contractions (6, 7).

Reductions in I_{to,f} as well as Kv4.2, Kv4.3, and KChIP2 expression are commonly observed in conjunction with action potential duration prolongation following treatment of cardiomyocytes with hypertrophic agents such as α₁-adrenoceptor (α₁, AR) agonists (8) and angiotensin II receptor agonists (9) as well as in cardiac hypertrophy (10–12) and heart disease (13–15). The mechanisms responsible for altered I_{to,f} levels induced by α₁,AR stimulation in myocytes (8) as well as in heart disease (13–15) are unclear. Recent studies concluded that calcineurin (Cn), a Ca²⁺/calmodulin-activated serine/threonine phosphatase that dephosphorylates NFATc1–4 (16), causes reductions in I_{to,f} (17, 18). This is a particularly attractive mechanism because Cn is necessary and sufficient to induce hypertrophy in rodents with pressure overload (19, 20) as well as in myocytes treated with α₁,AR agonists (21, 22). However, K⁺ currents are reported to be unchanged in young mice with cardiac overexpression of Cn, despite significant hypertrophy prior to the development of cardiomyopathy (23). Moreover, I_{to,f} is increased in NFATc4 transgenic mice (24). Thus, despite the importance of I_{to,f} and Cn in heart disease, the precise role of Cn in the regulation of I_{to,f} remains poorly understood.

Our experiments were designed to elucidate the regulation of I_{to,f} by Cn in neonatal rat ventricular myocytes (NRVMs). Our

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*This work was supported in part by the Canadian Institutes of Health Research (to P. H. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by postdoctoral fellowships from the Heart and Stroke Foundation of Canada, the Canadian Institutes of Health Research Tailored Advanced Collaborative Training in Cardiovascular Science Program, and the Faculty of Medicine at the University of Toronto.

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4 The abbreviations used are: I_{to,f}, fast transient outward K⁺ current; α₁,AR, α₁-adrenoceptor; Cn, calcineurin; NFAT, nuclear factor of activated T-cells; NRVMs, neonatal rat ventricular myocytes; PE, phenylephrine; CAIN, calcineurin inhibitory peptide; GFP, green fluorescent protein; G_max, maximal chord conductance; V_{1/2}, voltage for half-maximal activation of I_{to,f}; G_slope, slope conductance; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; I_{load}, sustained current; pF, picofarad.
results establish that Cn overexpression strongly enhanced $I_{\text{to,f}}$ and Kv4.2 transcription in an NFAT-dependent manner. In addition, Cn overexpression reversed $I_{\text{to,f}}$ reductions induced by $\alpha_1$AR stimulation with phenylephrine (PE) via increases in Kv4.2 mRNA and protein, whereas Cn inhibition with the Cn inhibitory peptide CAIN (21) caused further reductions in $I_{\text{to,f}}$ and Kv4.2 transcription observed with PE treatment. These results demonstrate that Cn/NFAT is a positive regulator of $I_{\text{to,f}}$ via transcriptional up-regulation of Kv4.2 in myocytes and thus minimizes the degree of $I_{\text{to,f}}$ reductions induced in cardiac hypertrophy.

**EXPERIMENTAL PROCEDURES**

**Isolation, Culture, and Intervention of NRVMs—**Day 1 or 2 Sprague-Dawley NRVMs (Charles River Laboratories, Inc., Montreal, Canada) were isolated and cultured in medium containing 5% fetal bovine serum in the presence of 0.1 mmol/liter bromodeoxyuridine (Sigma) following removal of non-myocytes by pre-plating (37 °C, 1 h) as described previously (25). After 24 h, the myocytes were cultured in the absence of serum (and supplemented with insulin-transferrin-selenium-X (Invitrogen), 25 µg/ml ascorbic acid, and 1 mmol/liter LiCl) and infected with adenoviruses (green fluorescent protein (GFP), CAIN, constitutively active Cn, ΔNFATc3, or VIVIT (a peptide consisting of Met-Ala-Gly-Pro-His-Pro-Val-Ile-Val-Ile-Thr-Gly-Pro-His-Glu-Glu designed to inhibit Cn-mediated NFAT activation (32); VIVIT adenoviruses were kindly provided by Dr. S. D. Kraner) at a multiplicity of infection of 5–10 for 16 h. Myocyte cultures were then washed and incubated in the presence or absence of the $\alpha_1$AR agonist PE (100 µmol/liter) for 48 h. Infection efficiency was typically >98%, with minimal cell death at these levels of multiplicity of infection. Myocytes were plated on laminin-coated coverslips at a density of 1.5 × 10^6 cells/ml for experiments involving immunofluorescence staining and electrophysiological recordings and at 5 × 10^6 cells/ml for all other experiments.

**Immunofluorescence Staining of NRVMs—**Immunofluorescent fluorescence was used to assess myocyte size and numbers. To perform these experiments, cultured cells were fixed and permeabilized with methanol (−20 °C, 15 min). After washing with phosphate-buffered saline, cells were incubated with 1:200 anti-α-actinin monoclonal antibody (sarcomeric; Sigma) in phosphate-buffered saline with 1% bovine serum albumin at room temperature for 1 h. Phosphate-buffered saline-washed cells were subsequently incubated at room temperature for 1 h. Phosphate-buffered saline-washed cells were subsequently incubated at room temperature for 30 min with 1:400 Alexa Fluor® 488-labeled goat anti-mouse IgG antibody (H + L; Molecular Probes) and viewed under a confocal microscope. Typically, >98% of the cells were α-actinin-positive 96 h after NRVM isolation, confirming the dominance of myocytes in the culture.

**[3H]Leucine Uptake Experiments—**The rate of protein synthesis was measured by [3H]leucine uptake experiments. Cells were cultured in leucine-free Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (Sigma) in the presence of 1 µCi/ml [3H]leucine with or without 100 µmol/liter PE for 48 h. Cells were then incubated with 10% trichloroacetic acid at 4 °C for 2 h, followed by cell lysis with 1 N NaOH. Each of the lysed samples was added to 7 ml of ScintiSafe™ 30% liquid scintillation mixture (Fisher) in a 20-ml polyethylene scintillation vial (Fisher) and subjected to liquid scintillation counting.

**Electrophysiological Recordings in NRVMs—**Whole-cell voltage-clamp recordings were done as described previously (8, 25) at room temperature. Whole-cell currents were filtered at 2 kHz (Axon 200A amplifier). Myocytes were perfused with drug-free extracellular solution for at least 15 min before measurements were performed. The extracellular solution contained 140 mmol/liter NaCl, 4 mmol/liter KCl, 2 mmol/liter CaCl2, 1 mmol/liter MgCl2, 0.5 mmol/liter CdCl2, 10 mmol/liter HEPES, and 10 mmol/liter glucose (pH 7.4). The intracellular solution contained 140 mmol/liter KCl, 1 mmol/liter MgCl2, 10 mmol/liter EGTA, 10 mmol/liter HEPES, and 5 mmol/liter MgATP (pH 7.25).

Chord conductance ($G$) was calculated according to the following equation: $G = I/(V_m - E_K)$, where $I$ is $I_{\text{to,f}}$ recorded in response to step depolarization to $V_m$ and $E_K$ represents the Nernst potential for $K^+$ ions. Fits of the relationship between $G$ and $V_m$ to the Boltzmann equation allow estimation of the maximal chord conductance ($G_{\text{max}}$) and the voltage for half-maximal activation of $I_{\text{to,f}}$ (i.e. $V_{1/2}$). Slope conductance ($G_{\text{slope}}$) was estimated by linear regression of the relationship between peak $I_{\text{to,f}}$ and $V_m$ at step voltages ranging from +10 to +60 mV.

**Electrophysiological Recordings in Cardiomyocytes from Young Mouse Hearts—**Cardiomyocytes were dissociated from the ventricular apices of 3–4-week-old non-transgenic and Cn-overexpressing transgenic mice according to the technique described previously (23). Whole-cell voltage-clamp recordings were done as described previously (26) at room temperature. Cardiomyocytes were perfused with normal Tyrode’s solution containing 138 mmol/liter NaCl, 4 mmol/liter KCl, 2 mmol/liter CaCl2, 1 mmol/liter MgCl2, 10 mmol/liter glucose, 10 mmol/liter HEPES, and 0.33 mmol/liter NaH2PO4 adjusted to pH 7.4 with NaOH. L-type Ca2+ current was eliminated by 0.3 mmol/liter CdCl2 included in the recording solution. The pipette solution contained 120 mmol/liter potassium glutamate, 10 mmol/liter KCl, 2 mmol/liter MgCl2, 10 mmol/liter HEPES, 5 mmol/liter EGTA, and 2 mmol/liter MgATP (adjusted to pH 7.2 with KOH).

**RNA Extraction and Quantitative Real-time PCR Analysis—**Total RNA was extracted from NRVMs using TRIzol reagent (Invitrogen) as recommended by the manufacturer and treated with RNase-free DNase I. CDNA was synthesized from 2 µg of total RNA using SuperScript™ (Invitrogen) and amplified by real-time PCR (Applied Biosystems). For KClIP2, TaqMan probe and primers (assay ID Rn01411445_g1) were used for mRNA quantification. Kv4.2, Kv4.3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified using SYBR Green PCR Master Mix (Applied Biosystems). The primers for Kv4.2 were 5’-GTGTCAGGAACTAGCATAGCCG-3’ (forward) and 5’-TTCAAAACACAGACCCTGTA-3’ (reverse). The primers for Kv4.3 were 5’-CACCACCTGACTACGTCTAAAGC-3’ (forward) and 5’-TCTGTCTGATCAATATACCTGTGGTT-3’ (reverse). The primers for GAPDH were 5’-TGCACCATCAGACACTGTTACG-3’ (forward) and 5’-GATGCAGGGATGATGTTCTG-3’ (reverse). mRNA determinations were done in duplicate using standard curves, and specificity was confirmed using 2% agarose gel and/or melting curves.
Role of Calcineurin in Regulation of \( \text{I}_{\text{to,f}} \)

Western Immunoblot Analysis—Cells were collected and lysed in Laemmli buffer. Equal amounts of protein (determined using the Bio-Rad RC DC assay) were loaded, separated on 12% SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes, and probed using rabbit polyclonal antibodies against Kv4.2 or Kv4.3 (Chemicon) or KChIP2 (Affinity BioReagents). GAPDH was detected by a mouse monoclonal antibody (Research Diagnostics, Inc.). Anti-rabbit or antimouse antibodies conjugated with horseradish peroxidase (Amersham Biosciences) were used as secondary antibodies to allow protein visualization with enhanced chemiluminescence (Amersham Biosciences).

Isolation of the KChIP2 Promoter and Cloning into the pGL3-Basic Vector—Rat genomic DNA was isolated from Sprague-Dawley rat livers using previously described methods (27). The KChIP2 promoter region (−1524 to +312) was isolated from rat genomic DNA by Advantage™-GC genomic PCR (BD Biosciences) using primers designed according to the nucleotide sequence of NW_043419. The primers used were 5′-TAAGCTGTGGGTTGACTTATCTATTTACA-3′ (forward) with the addition of an NheI site and 5′-AGCTGTGGGGTGCATCATCTCTATTCA-3′ (reverse) with the addition of an SmaI site at the 5′-end. Following purification, the amplified DNA was digested with NheI and SmaI and sub-cloned into the pGL3-Basic vector (Promega Corp.). Positive clones containing the KChIP2 promoter were confirmed by nucleotide sequencing and Dual-Luciferase assay.

Transfection and Promoter Activity Measurements—For promoter experiments, myocytes were transfected 16 h after viral infection with pGL3 with a firefly luciferase reporter gene driven by the Kv4.2 (kindly provided by Dr. K. Takimoto) (28), Kv4.3 (kindly provided by Dr. E. S. Levitan) (29), or KChIP2 promoter using Lipofectamine 2000 (Invitrogen). pRL-TK encoding Renilla luciferase was cotransfected as a control to correct for transfection efficiency. Cell lysates were obtained 48 h after transfection to measure Dual-Luciferase activity (Promega Corp.). In the experiments to measure the activation of Cn/NFAT, NRVMs were transfected with pGL3 with a firefly luciferase reporter gene driven by a promoter containing three NFAT cis-elements (kindly provided by Dr. A. Rao).

Statistical Analysis—All data are expressed as the means ± S.E. Statistical significance was calculated using Student’s \( t \) test to compare two groups and analysis of variance to compare multiple groups. \( p \) values <0.05 were considered statistically significant.

RESULTS

Cn Increases \( \text{I}_{\text{to,f}} \) via NFAT-dependent Increases in Kv4.2 Transcription—To examine the regulation of \( \text{I}_{\text{to,f}} \) by Cn, we overexpressed constitutively active Cn in NRVMs. As reported previously (30, 31), Cn overexpression induced marked myocyte hypertrophy as determined by cell microscopy, cell capacitance data, and \([\text{H}]\)leucine uptake experiments (Fig. 1A). Rather than decreasing \( \text{I}_{\text{to,f}} \), as might be expected from previous studies (17, 18), Cn caused a 2-fold increase in \( \text{I}_{\text{to,f}} \) density, which was quantified as the difference in the peak current minus the sustained current (\( \text{I}_{\text{sus}} \)) at the end of the pulse divided by the cell capacitance. \( \text{I}_{\text{to,f}} \) density recorded at +60 mV (Fig. 1, B and C; and Table 1) was increased in myocytes overexpressing Cn compared with control myocytes. As expected, \( G_{\text{slope}} \), which gives a more direct measure of the extent of \( \text{I}_{\text{to,f}} \) enhancement, was also increased in myocytes overexpressing Cn compared with control myocytes, along with increased \( G_{\text{max}} \) of \( \text{I}_{\text{to,f}} \) (Table 1). The increases in \( \text{I}_{\text{to,f}} \) density and conductance were not accompanied by shifts (\( p = 0.57 \)) in the voltage dependence of \( \text{I}_{\text{to,f}} \) activation as assessed by estimates of the voltages required for \( \text{I}_{\text{to,f}} \) to reach 50% of the maximal conductance (\( V_{1/2} \)) (data not shown). In addition, there were no changes in the time course of \( \text{I}_{\text{to,f}} \) inactivation (data not shown). The effect of Cn overexpression on \( \text{I}_{\text{to,f}} \) appeared to be specific because the current density remaining at the end of the voltage step (i.e. \( \text{I}_{\text{sus}} \))
was not changed ($p > 0.50$) in myocytes by overexpression of Cn (Table 1).

To test whether the increases in $I_{to,f}$ induced by Cn were associated with changes in the expression of genes encoding $I_{to,f}$, we measured the expression of Kv4.2, Kv4.3, and KChIP2. As shown in Fig. 2, Cn overexpression increased Kv4.2 mRNA and protein, without affecting Kv4.3 or KChIP2 expression compared with control cells. To investigate whether the increases in Kv4.2 mRNA were related to increased transcription activity (versus changes in mRNA stability), NRVMs were transfected with Kv4.2 (28), Kv4.3 (29), and KChIP2 promoter constructs. Fig. 3A shows that overexpression of Cn increased the activity of the Kv4.2EB promoter (−3162 to +592). This increase in Kv4.2EB promoter activity, as well as the changes in $I_{to,f}$ appeared to be mediated by NFAT dephosphorylation because $I_{to,f}$ recorded at +60 mV in NRVMs upon overexpression of Cn was reduced to 8.71 ± 2.25 pA/pF (pF) ($n = 6$) when co-infected with adenoviral VIVIT (32), a selective inhibitory peptide of Cn-mediated NFAT dephosphorylation. VIVIT overexpression alone did not alter $I_{to,f}$ (11.52 ± 1.86 pA/pF, $n = 5$). Cn-mediated Kv4.2 promoter activity was abolished by co-infection with VIVIT, which alone had no effect on basal Kv4.2 promoter activity (Fig. 3A), and Cn overexpression induced a 6-fold increase in NFAT activity (Fig. 3B), whereas overexpression of ΔNFATc3 (i.e. constitutively active NFATc3) (33) stimulated Kv4.2EB promoter activity by similar amounts (Fig. 3A). By contrast, neither Cn nor ΔNFATc3 overexpression altered the activity of the Kv4.3 (−2337 to +54), Kv4.3 (−663 to +54), or KChIP2 (−1524 to +312) promoter (data not shown), consistent with mRNA and protein results (Fig. 2). These results establish that Cn increases $I_{to,f}$ via NFAT-dependent increases in Kv4.2 transcription.

Cn Minimizes $I_{to,f}$ Reductions following Chronic $\alpha_1$AR Stimulation—Because several previous studies have suggested that Cn activation in cardiac hypertrophy is responsible for reductions in $I_{to,f}$ (8, 21), we investigated the regulation of $I_{to,f}$ by Cn in an in vitro hypertrophic model induced by $\alpha_1$AR stimulation. To achieve this, we overexpressed either CAIN or constitutively active Cn in NRVMs in the presence of 100 μmol/liter PE for 48 h. As expected from a previous study (8), PE treatment induced marked myocyte hypertrophy based on cell microscopy, cell capacitance data, and $[3H]$leucine uptake experiments (Fig. 4A) and increased NFAT activity by 4-fold compared with myocytes not treated with PE (Fig. 4B). In addition, overexpression of CAIN (21), a specific inhibitory peptide of Cn, inhibited NFAT activation and prevented hypertrophy following treatment with PE, confirming the essential role of Cn signaling in $\alpha_1$AR-induced hypertrophy. Consistent with previous studies (8, 34), Fig. 4 (C and D) and Table 1 show that $I_{to,f}$ densities along with $G_{to,f}$ and $G_{max}$ were decreased in PE-treated myocytes compared with control myocytes without PE. However, contrary to expectations based on studies concluding that Cn is responsible for $I_{to,f}$ reductions (17, 18), CAIN overexpression in PE-treated myocytes further reduced $I_{to,f}$ densi-

**TABLE 1**

|                     | GFp  | Cn  | GFp + PE | CAIN + PE | Cn + PE |
|---------------------|------|-----|----------|-----------|---------|
| $I_{to,f}$ (pA/pF) at +60 mV | 16.3 ± 3.7 | 34.5 ± 5.4$a$ | 1.7 ± 0.3$a$ | 0.5 ± 0.3$ab$ | 20.6 ± 5.4$ab$
| $C_{to,f}$ of $I_{to,f}$ (pA/pF-mV) | 0.226 ± 0.062 | 0.497 ± 0.084$ab$ | 0.033 ± 0.006$ab$ | 0.015 ± 0.004$ab$ | 0.293 ± 0.082$ab$
| $C_{to,f}$ of $I_{to,f}$ (pA/pF-mV) | 0.112 ± 0.024 | 0.238 ± 0.034$ab$ | 0.012 ± 0.002$ab$ | 0.003 ± 0.002$ab$ | 0.142 ± 0.034$ab$
| $G_{max}$ (mV)     | 6.8 ± 1.3 | 6.8 ± 0.9 | 5.8 ± 0.8 | 7.5 ± 2.0 | 5.5 ± 1.0

$a_p < 0.05$ versus GFp. $b_p < 0.05$ versus GFp + PE.
Role of Calcineurin in Regulation of $I_{to,f}$

**FIGURE 3.** Activity of the $Kv4.2$ promoter in NRVMs overexpressing GFP, Cn, ΔNFATc3, or VIVIT (A) and activity of a promoter containing three NFAT cis-elements in NRVMs overexpressing GFP or Cn (B). Sixteen hours after viral infection, cells were transfected with pGL3 with the firefly luciferase gene driven by the $Kv4.2$ promoter or a promoter containing three NFAT cis-elements. pRL-TK encoding Renilla luciferase was cotransfected as a control for transfection efficiency. Cell lysates were obtained 48 h after transfection and subjected to Dual-Luciferase assay. Firefly luciferase activity normalized to Renilla luciferase activity is plotted for each category of NRVMs. The basal activity of the promoter in NRVMs infected with adenoviral GFP was artificially set as 1. Error bars represent the means ± S.E. ($n$ ≥ 3). *, $p < 0.05$ versus GFP.

In agreement with the changes in $I_{to,f}$ density, $G_{slope}$ and $G_{max}$ (Fig. 4, C and D; and Table 1). On the other hand, CAIN overexpression did not alter ($p > 0.40$) the basal levels of $I_{to,f}$ densities, $G_{slope}$ or $G_{max}$ (data not shown). Remarkably, as shown in Fig. 4 (C and D) and Table 1, overexpression of constitutively active Cn (31) completely reversed the PE-induced reductions in $I_{to,f}$ densities as well as $G_{slope}$ and $G_{max}$ without affecting the activation-gating properties of $I_{to,f}$ as assessed by estimates of $V_{1/2}$. Neither PE treatment nor Cn overexpression altered the kinetics of $I_{to,f}$ inactivation. The effects of Cn and CAIN overexpression on $I_{to,f}$ appeared to be specific because $I_{to,f}$ density was not changed ($p > 0.50$) in PE-treated myocytes by overexpression of Cn or CAIN (Table 1).

In agreement with the changes in $I_{to,f}$ density, PE reduced $Kv4.2$, $Kv4.3$, and KChIP2 mRNA levels as summarized in Fig. 5, with similar reductions in $Kv4.2$, $Kv4.3$, and KChIP2 protein levels compared with the control (Fig. 6). Overexpression of CAIN had no effect on the basal mRNA and protein levels of $Kv4.2$, $Kv4.3$, and KChIP2 (data not shown), but further reduced $Kv4.2$ mRNA and protein expression induced by PE treatment, without affecting $Kv4.3$ and KChIP2 expression (Figs. 5 and 6). On the other hand, overexpression of Cn following PE treatment increased $Kv4.2$ mRNA and protein levels compared with the control while having no effect ($p > 0.12$) on $Kv4.3$ and KChIP2 mRNA and protein levels (Figs. 5 and 6). It is important to note that the differences in $Kv4.3$ mRNA between PE and CAIN/PE or between PE and Cn/PE as shown in Fig. 5B are not significant. At first glance, it may seem surprising that the level of $I_{to,f}$ in the PE-treated myocytes expressing Cn was not increased ($p = 0.53$) above that in the control (i.e. myocytes not treated with PE) given that Cn induced a nearly 2-fold increase in $Kv4.2$ protein expression above the control when overexpressed in PE-treated myocytes. However, this lack of concordance between $I_{to,f}$ levels and $Kv4.2$ protein levels is consistent with the large reductions in $Kv4.3$ and KChIP2 expression because rodent $I_{to,f}$ channels are formed primarily as heterotetramers of $Kv4.2$ and $Kv4.3$ channels (1) and because $I_{to,f}$ channels require KChIP2 for membrane insertion of functional channels (35). Therefore, taken together, these results establish that Cn is a positive regulator of $Kv4.2$ expression and $I_{to,f}$ density when $I_{to,f}$ is reduced by PE.

The above results establish that $I_{to,f}$ is positively regulated by Cn in cultured neonatal myocytes. To explore whether Cn also regulates $I_{to,f}$ in intact myocardium, we measured $I_{to,f}$ in myo-

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**FIGURE 4.** Shown are typical immunofluorescence images and cell capacitance and [3H]leucine uptake data (A) and $K^+$ currents (B) obtained from NRVMs overexpressing GFP, CAIN, or Cn following PE treatment. The activity of a promoter containing three NFAT cis-elements was enhanced in NRVMs treated with PE. In C, representative families of outward potassium current density are recorded. The voltage protocols were as described in the legend to Fig. 1. In D, $I_{to,f}$ density-voltage relationships are displayed as described in the legend to Fig. 1. Cell capacitance ($n = 16$), [3H]leucine uptake data ($n = 4$), and $G_{slope}$ (between +10 and +60 mV; $n = 6$ cells/group) were compared by analysis of variance analysis between groups. *, $p < 0.05$ versus GFP; γ, $p < 0.05$ versus GFP + PE. CTL control.

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cytes isolated from mice overexpressing Cn. We performed these experiments in mice that were 3–4 weeks of age to reduce the complexities associated with cardiac hypertrophy and heart disease that become apparent in older mice (30). As summarized in Fig. 7 and Table 2, myocytes isolated from mice overexpressing Cn in the heart had higher \( I_{to,f} \) densities and \( G_{slope} \) than age-matched non-transgenic littermate control mice. These results demonstrate that \( I_{to,f} \) is positively regulated by Cn in intact adult myocardium.

DISCUSSION

Our study has confirmed that overexpression of constitutively active Cn induces hypertrophy. Surprisingly, the Cn-induced hypertrophy in cultured NRVMs was associated with marked increases in \( I_{to,f} \) that correlated strongly with increases in the mRNA, protein, and promoter activities of Kv4.2, but not the other molecular constituents of \( I_{to,f} \) (i.e. Kv4.3 and KChIP2). The increases in Kv4.2 mRNA were associated with enhanced promoter activity, suggesting that Cn induces increases in Kv4.2 transcription activity versus altered mRNA stability. Positive regulation of \( I_{to,f} \) by Cn was also observed in very young (3–4 weeks old) mice overexpressing Cn, suggesting that this positive regulation of \( I_{to,f} \) is not specific to cultured neonatal myocytes.
Role of Calcineurin in Regulation of $I_{to,f}$

**FIGURE 7.** Typical K$^+$ currents obtained from cardiomyocytes dissociated from the ventricular apices of 3–4-week-old non-transgenic (NTG) and Cn-overexpressing transgenic (Cn TG) mice. A, representative families of outward potassium currents recorded in dissociated cardiomyocytes in response to step depolarization from −40 to +60 mV (increments of 10 mV) for 4000 ms from a holding potential of −80 mV. $I_{to,f}$ was estimated by fitting the decay phase of outward K$^+$ currents with bi- or triexponential function using Clampfit Version 9.0, and $G_{\text{K}_{\text{ch}}}$ (pA/pF) was determined by dividing $I_{to,f}$ by the cell capacitance (Cm). B, $I_{to,f}$ density plotted as a function of the step potential. $G_{\text{K}_{\text{ch}}}$ values (between +10 and +60 mV) are compared by Student’s t test between groups. *p < 0.05 versus NTG.

**TABLE 2**
Electrophysiological properties of $I_{to,f}$ recorded in cardiomyocytes dissociated from the ventricular apices of 3–4-week-old non-transgenic and Cn-overexpressing transgenic mice

|                     | Non-transgenic mice | Cn-overexpressing transgenic mice |
|---------------------|---------------------|----------------------------------|
| $I_{to,f}$ (pA/pF) at +60 mV | 10.52 ± 1.79 (n = 6) | 16.77 ± 1.37 (n = 14)* |
| $G_{\text{K}_{\text{ch}}}$ (pA/pF/MV) | 0.15 ± 0.02 (n = 6) | 0.29 ± 0.04 (n = 8)* |

*p < 0.05 versus non-transgenic mice.

Consistent with previous studies concluding that the hypertrophy induced by Cn requires dephosphorylation of NFAT (probably NFATc3 and NFATc4) (30, 36), which leads to nuclear translocation and transcriptional alterations in gene expression (30), NFAT inhibition (with VIVIT) blocked both the Cn-induced hypertrophy and the enhancement of $I_{to,f}$ and Kv4.2 promoter activity. On the other hand, changes in NTG did not affect Kv4.3 or KChIP2 promoter activity. These results suggest that Cn/NFAT has no effect on Kv4.2 transcription or $I_{to,f}$ under basal conditions because VIVIT alone had no effect on $I_{to,f}$ or Kv4.2 promoter activity, whereas CAIN alone did not alter $I_{to,f}$ or Kv4.2 expression.

It is important to note that only the 3.5-kb Kv4.2EB promoter (−3162 to +592) activity was increased by Cn and δNFATc3, whereas the shorter promoters Kv4.2HB (−1094 to +592) and Kv4.2SB (−432 to +592) (28) were not activated by Cn or δNFATc3. These observations are consistent with the existence of 13 consensus NFAT-binding sites (i.e. GGAAA cis-elements) between −3162 and −1095 in the Kv4.2 promoter versus only three NFAT-binding sites between −1094 and 0. Similarly, the inability of Cn or δNFATc3 to increase Kv4.3 or KChIP2 transcription correlates with the presence of only six putative NFAT sites in the Kv4.3 promoter (−2337 to +54) and five NFAT sites in the KChIP2 promoter (−1524 to +312). Although the ability of Cn and NFAT to increase Kv4.2 promoter activity correlates with the number of NFAT-binding sites, NFAT activation can be modulated by other transcription factors (37), which could also conceivably contribute to or be responsible for the enhanced Kv4.2 transcription by Cn.

We further investigated the role of Cn in the regulation of $I_{to,f}$ in an *in vitro* hypertrophic model of cardiomyocytes induced by α1AR stimulation. Our experiments confirmed that chronic α1AR stimulation in cultured NRVMs decreased $I_{to,f}$ in association with reductions in Kv4.2, Kv4.3, and KChIP2 mRNA and protein levels (8, 34). We have also demonstrated that Cn inhibition (with CAIN overexpression) blocked α1AR-mediated hypertrophy in cultured NRVMs, as reported previously (21). Previous studies established that hypertrophy induced by α1AR stimulation in cultured NRVMs is associated with Cn activation (8, 21) and $I_{to,f}$ reductions (8). Similarly, Cn activation (19, 38, 39) and $I_{to,f}$ reductions (10, 11, 14, 18) are commonly observed in various animal models of cardiac hypertrophy and heart disease as well as in heart disease patients (40, 41). Collectively, these observations have led to the suggestion that Cn activation is responsible for the reductions in $I_{to,f}$ (17, 18) as well as hypertrophy. However, rather unexpectedly (17, 18), our results showed that, in cultured NRVMs chronically treated with α1AR agonists, inhibition of Cn (using CAIN) caused further reductions in $I_{to,f}$ despite blocking hypertrophy, whereas overexpression of constitutively active Cn induced large increases in $I_{to,f}$ density without inhibiting myocyte hypertrophy. These changes in $I_{to,f}$ were associated with changes in mRNA and protein expression of Kv4.2, but not Kv4.3 or KChIP2, a pattern identical to that observed in the absence of α1AR stimulation. Thus, in the presence of cardiac hypertrophy, we conclude that Cn activation will minimize the degree of reductions in $I_{to,f}$ by driving Kv4.2 transcription (see the proposed model in Fig. 8).

In contrast to our findings, a previous study showed that inhibition of Cn with cyclosporin or knock out of NFATc3 prevents $I_{to,f}$ reductions induced by myocardial infarction in adult mice (18). The differences between our experiments using NRVMs and those using infarcted (18) or normal (42) adult mouse hearts may reflect age-dependent differences in signaling or differences between cultured isolated myocytes and intact myocardium. Alternatively, because the reductions in $I_{to,f}$ following myocardial infarction in adult mice also require βAR stimulation (18), it is plausible that βAR and α1AR stimulation activates distinct signaling pathways capable of modulating the net effects of Cn. The potential differential Cn-mediated regulation of $I_{to,f}$ activated by α1AR and βAR stimulation is in keeping with the complex regulation of Cn signaling in the myocardium. For example, Cn/NFAT-mediated tumor necro-
The enhanced \( I_{\text{to,f}} \) by Cn observed in our study may represent an important feedback pathway operating under conditions of cardiac hypertrophy and heart disease. Specifically, as already mentioned, many hypertrophic stimuli, including \( \alpha_1 \text{AR} \) stimulation and heart disease, reduce \( I_{\text{to,f}} \) as well as Kv4.2, Kv4.3, and KChIP2 expression (8, 45, 46). These conditions have also been linked to Cn activation (8, 45, 47), which is expected from our results to limit the extent of \( I_{\text{to,f}} \) down-regulation. Preventing excessive \( I_{\text{to,f}} \) reductions may be of critical importance because changes in \( I_{\text{to,f}} \) can lead to altered electrical and contractile properties of the myocardium, which may contribute to impaired pump function and arrhythmias (6, 48–51). For example, we (48, 52) and others (53) have shown previously that not only is \( I_{\text{to,f}} \) an important determinant of action potential profile changes occurring in heart disease and regional differences in action potential profile, but \( I_{\text{to,f}} \) is also a major regulator of the amplitude and kinetics of cardiac contraction in rodents and larger mammals by modulating Ca\(^{2+}\) entry via L-type Ca\(^{2+}\) channels. Thus, Cn activation not only induces hypertrophy, but also modulates the extent of the electrical and contractile changes induced as a result of \( I_{\text{to,f}} \) reduction caused by hypertrophic stimuli. In addition, a recent study found that increased \( I_{\text{to,f}} \) by adenoviral overexpression of Kv4.3 can prevent hypertrophy (45), whereas increased \( I_{\text{to,f}} \) in NRVMs treated with PE prevents both myocyte hypertrophy and Cn activation (8), suggesting that, at least in these models, increased \( I_{\text{to,f}} \) by Cn could limit the degree of hypertrophy.

In conclusion, we have found that Cn/NFAT potently increases Kv4.2 transcription and \( I_{\text{to,f}} \) levels in rodent myocardium. This regulation provides a potentially useful mechanism for modulating the cellular, electrical, and contractile responses of the myocardium to cardiac hypertrophy and heart disease. The relevance of Cn-mediated regulation of Kv4.2 to larger mammalian species such as humans and dogs is uncertain because, in these species, \( I_{\text{to,f}} \) is encoded by Kv4.3 and KChIP2 subunits. Additional studies will be required to completely define the role of Cn in the regulation of \( I_{\text{to,f}} \) in non-rodents.

Acknowledgments—We thank D. Zhao and Z. Kassiri for amplification of the recombinant adenoviruses. We acknowledge the kind gifts of Kv4.2 promoters from Dr. K. Takimoto, Kv4.3 promoters from Dr. E. S. Levinan, the \( 3\times\text{NFAT-luc} \) vector from Dr. A. Rao, and VIVIT adenoviruses from Dr. S. D. Kramer.
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