Background—Myocyte hypertrophy accompanies many forms of heart disease, but its contribution to electrical remodeling is unknown.

Methods and Results—We studied mouse hearts subjected to pressure overload by surgical thoracic aortic banding. In unbanded control hearts, action potential duration (APD) was significantly longer in subendocardial myocytes compared with subepicardial myocytes. Hypertrophy-associated APD prolongation was significantly greater in subendocardial myocytes compared with subepicardial myocytes, indicating stress-induced amplification of repolarization dispersion. To investigate the underlying basis, we performed voltage-clamp recordings on dissociated myocytes. Under control unoperated conditions, subendocardial myocytes exhibited significantly less transient outward current (I_{to}) than did subepicardial cells. Hypertrophy was not associated with significant changes in I_{to}, sustained current, or inward rectifier current densities, but peak L-type Ca^{2+} current density (I_{Ca,L}) increased 26% (P<0.05). Recovery from I_{Ca,L} inactivation was accelerated in hypertrophied myocytes. Inhibition of calcineurin with cyclosporin A prevented increases in heart mass and myocyte size but was associated with an intermediate APD. The hypertrophy-associated increase in I_{Ca,L} and the accelerated recovery from inactivation were blocked by cyclosporin A.

Conclusions—These data reveal regional variation in the electrophysiological response within the left ventricle by way of a mechanism involving upregulated Ca^{2+} current and calcineurin. Furthermore, these results reveal partial uncoupling of electrophysiological and structural remodeling in hypertrophy. (Circulation. 2001;104:1657-1663.)

Key Words: calcium \( \bullet \) electrophysiology \( \bullet \) action potentials \( \bullet \) arrhythmia \( \bullet \) hypertrophy
dysfunction. We addressed 3 questions: (1) Is hypertrophy associated with electrical remodeling, and if so, what is the underlying electrophysiological basis? (2) Does hypertrophic transformation amplify the regional variation of recovery of electrical excitability in the LV? (3) What is the role of a recently described calcineurin transcriptional pathway?

Methods

Pressure-Overload Hypertrophy Model

Male mice (C57BL/6, 6 to 8 weeks old) were subjected to pressure overload by thoracic aortic banding (TAB). Some mice were subjected to a sham operation in which the aortic arch was visualized but not banded. Perioperative (24-hour) mortality was <10%. On the morning of postoperative day 1, TAB or sham-operated mice were randomized to receive either cyclosporin A (CsA) 25 mg/kg or vehicle (Veh) subcutaneously twice daily.

Myocyte Isolation

LV cardiomyocytes were isolated by using a protocol slightly modified from that of Xu et al. In brief, after retrograde perfusion with Ca²⁺-free buffer followed by collagenase solution, the apex was removed and the LV midwall was divided into subendocardial- and subepicardial-enriched fractions. After whole-cell currents were recorded, cells were plated and studied within 6 to 8 hours.

Electrophysiological Recordings

Myocytes were superfused at 1 to 2 mL/min (21°C to 23°C) with Tyrode’s solution containing (in mmol/L) 137 NaCl, 5.4 KCl, 1.0 MgCl₂, 1.0 CaCl₂, 10.0 dextrose, and 10.0 HEPES (pH 7.4). Calcium-tolerant quiescent myocytes with a typical rod-shaped appearance and clear cross striations were chosen for experimentation. Borosilicate glass capillaries (7052 glass, 1.65-mm OD, A-M Systems) were prepared with tip resistances of 1.5 to 3.0 MΩ. Voltage-clamp action potentials were recorded with an Axopatch 200B amplifier (Axon Instruments) under computer control with Clampex 7.0 (Axon Instruments) and analyzed with Clampfit 9.0 (Axon Instruments). The holding potential of −40 mV was used to inactivate rapid Na⁺ currents. To record Ito and Isus, a holding potential of −50 mV was applied. Ito was defined as the current remaining at the end of the pulse. Ito was defined as the difference between peak current (Ipeak) and Irest. To measure ICa,L, cells were continuously superfused with a solution containing (in mmol/L) CaCl₂, MgCl₂, tetraethylammonium chloride, glucose, and HEPES (pH 7.3). The pipette-filling solution contained (in mmol/L) 100.0 cesium aspartate, 20.0 CsCl, 1.0 MgCl₂, Mg₂ATP, 0.5 GTP, 5.0 EGTA, and 5.0 HEPES (pH 7.3).

Statistical Methods

Averaged data are reported as mean±SEM. Sample sizes are listed as n=xy/n to denote x cells from y mice. Statistical significance was analyzed with a Student’s unpaired t test or 1-way ANOVA followed by Bonferroni’s method for post hoc pairwise multiple comparisons. Quality of exponential fits was analyzed with a χ² test statistic.

Results

Recent studies have revealed heterogeneity in the electrophysiological architecture of the mammalian heart in physiological and pathological conditions. In large mammals, the transient outward current (Ito) is more prominent in subepicardial myocytes than in other cells. To investigate this in mice, short-axis slices of LV were microdissected into subepicardial- and subendocardial-enriched fractions. Current-clamp action potentials recorded in subepicardial (Figure 1A) and subendocardial (Figure 1C) myocytes from unoperated control mice were similar in morphology, displaying relatively little phase 2 plateau. APD in subendocardial myocytes was statistically significantly greater than that recorded in subepicardial cells (Table 1).

Figure 1. Representative action potentials recorded from ventricular myocytes isolated from subepicardial (A) or subendocardial (C) fraction of control unoperated LV. Outward K⁺ currents recorded by stepping from −50 mV to voltages between −40 to +60 mV in myocytes isolated from subepicardial (B) or subendocardial (D) fraction. Action potentials recorded from myocytes isolated from sham-operated mice were identical.

TABLE 1. APDs and Whole-Cell Current Densities Measured at +40 mV

<table>
<thead>
<tr>
<th></th>
<th>Sham + Veh</th>
<th>Subepicardial</th>
<th>Subendocardial</th>
<th>TAB + Veh</th>
<th>Subepicardial</th>
<th>Subendocardial</th>
<th>TAB + CsA</th>
<th>Subepicardial</th>
<th>Subendocardial</th>
</tr>
</thead>
<tbody>
<tr>
<td>APD₀, ms</td>
<td>16.9±1.5</td>
<td>27±2.5</td>
<td>14.6±0.7</td>
<td>19.2±2</td>
<td>12.3±0.5</td>
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<tr>
<td>APD₃₀, ms</td>
<td>7.1±0.6</td>
<td>12±2.1</td>
<td>4.7±0.4</td>
<td>7.3±0.5</td>
<td>4.5±0.4</td>
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<tr>
<td>I_to, pA/pF</td>
<td>5.4±0.9</td>
<td>16±3.3</td>
<td>6.9±0.8</td>
<td>8±2</td>
<td>13±2</td>
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<tr>
<td>I_to, pA/pF</td>
<td>7.0±0.6</td>
<td>10.1±0.5</td>
<td>6.6±0.5</td>
<td>8±1</td>
<td>10±1</td>
<td></td>
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*P<0.05, †P<0.01 compared with Sham + Veh.
‡P<0.01 compared with Sham + Veh subendocardial (Subendo) cells.
To investigate the basis for the differences in APD, whole-cell depolarization-activated K+ currents were recorded under conditions in which inward Na+ and Ca2+ currents were inhibited (Table 1). In the majority of myocytes (16/22 cells from 10 mice, 73%) isolated from the subepicardial fraction of unoperated control LV, voltage-clamp recordings revealed a large I\textsubscript{K1} (Figure 1B). In contrast, the majority of cells (15/23 cells from 11 mice, 65%) isolated from the subendocardial fraction did not reveal substantial I\textsubscript{to} (Figure 1D). In contrast, I\textsubscript{sus} densities were similar in these 2 cell fractions.

**Role of Calcineurin in Spatial Dispersion of Hypertrophic Electrical Remodeling**

To determine whether hypertrophic electrical remodeling is heterogeneous within the mouse LV, mice were subjected to surgical aortic banding for 3 weeks. Heart weight normalized to body weight increased 59% (P<0.05) in TAB+Veh-treated hearts compared with controls (Sham+Veh; Table 2). Isolated ventricular myocytes were enlarged significantly in TAB+Veh hearts compared with Sham+Veh hearts assayed in terms of whole-cell membrane capacitance (increase of 33%, P<0.01) and 2-dimensional cell surface area (increase of 52%, P<0.01; Table 2). Within each treatment group, cell capacitances and 2-dimensional cell surface areas were similar in subendocardial and subepicardial myocytes (Table 2).

We have shown previously that calcineurin inhibition with CsA blocks the increases in heart mass and myocyte size associated with pressure overload in this model.13 Because it is not known whether the electrophysiological and structural phenotypes of hypertrophy are causally linked, we examined whether they could be dissociated by calcineurin inhibition. To control for the influence of hypertrophic growth on electrophysiological remodeling, mice were randomized on the morning after TAB or sham operation to receive either CsA (25 mg/kg SC BID) or vehicle. In hearts exposed to CsA (TAB+CsA; negative data not shown), heart mass, myocyte surface area, and myocyte capacitance were not significantly different from baseline (Table 2), thereby confirming the efficacy of hypertrophy abrogation. Calcineurin phosphatase activity in the sham-operated LV was 349±18 pmol · mg\textsuperscript{-1} · min\textsuperscript{-1} (n=8), which was not statistically significantly different from TAB (260±40 pmol · mg\textsuperscript{-1} · min\textsuperscript{-1}, n=7, P=NS). In the presence of CsA, however, calcineurin activity (49±7 pmol · mg\textsuperscript{-1} · min\textsuperscript{-1}, n=7) was significantly diminished compared with sham-operated (P<0.01) or TAB (P<0.01) conditions.

**TABLE 1 Continued**

<table>
<thead>
<tr>
<th></th>
<th>Sham+CsA</th>
<th>Subepicardial</th>
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<tr>
<td>Subendocardial</td>
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<tr>
<td>14.2±0.6† (n=8/4)</td>
<td>11.2±0.7 (n=7/3)</td>
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<tr>
<td>6.1±0.5* (n=8/4)</td>
<td>4.0±0.5 (n=7/3)</td>
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<tr>
<td>6±2 (n=5/3)</td>
<td>17±3 (n=5/3)</td>
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<tr>
<td>8±2 (n=5/3)</td>
<td>9.2±0.4 (n=5/3)</td>
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Current-clamp action potentials revealed significant differences in the electrophysiological response to pressure overload in cells derived from 2 regions of the LV wall (Table 1). Action potentials recorded in hypertrophied (TAB+Veh) subepicardial myocytes (Figure 2A) were prolonged 23% (P<0.05). Significantly greater effects were observed in subendocardial myocytes, in which a phase 2 shoulder began to appear (Figure 2B), and the APD increased 60% (P<0.01) at 3 weeks (Figure 2C). Comparison of subepicardial and subendocardial responses to hemodynamic stress (Figure 2D) revealed a strikingly greater degree of action potential prolongation (measured as APD\textsubscript{90} or APD\textsubscript{50}) in subendocardial cells (P<0.01). Current- and voltage-clamp recordings in sham-operated mice were similar to those of unoperated controls.

Calcineurin inhibition completely blocked the increases in cell size associated with TAB (Table 2). Despite elimination of the structural changes of hypertrophy, APD prolongation was blunted though not normalized by calcineurin inhibition (Figure 2, Table 1), suggesting that the structural and electrophysiological components of remodeling in hypertrophy are partially dissociable.

**Repolarizing K+ Currents**

I\textsubscript{K1} is a major determinant of repolarization in murine ventricular myocytes and is frequently implicated in the electrical remodeling of heart failure.17 We studied the effects of TAB+Veh and TAB+CsA on I\textsubscript{K1} and on the current that persists at 300 ms (I\textsubscript{sus}). At baseline, I\textsubscript{K1} was relatively large in myocytes isolated from the subepicardial aspect of the LV (Figure 3A). With hypertrophy, I\textsubscript{K1} and I\textsubscript{sus} did not change significantly (Figure 3B, Table 1), and the proportion of subendocardial cells exhibiting substantial I\textsubscript{K1} (32/43 cells from 16 mice, 74%) was unchanged. Inhibition of hypertrophy with calcineurin blockade (TAB+CsA) was associated with similar I\textsubscript{K1} and I\textsubscript{sus} waveforms (Figure 3C) and densities (Figures 3E and 3F), as was exposure to CsA alone (Figures 3D through 3F, Table 1). Calcineurin inhibition (TAB+CsA) did not alter the proportion of subepicardial myocytes exhibiting I\textsubscript{K1} (17/27 cells from 10 mice, 63%). I\textsubscript{K1} and I\textsubscript{sus} densities were similar under conditions of TAB+CsA and Sham+CsA (Figures 3E through 3F, Table 1).

In contrast with subepicardial cells, LV myocytes isolated from the subendocardial fraction exhibited relatively little I\textsubscript{K1} at baseline (see online Figure IA at www.circulationaha.org). Similar to subepicardial cells, hypertrophy (TAB+Veh) had no effect on I\textsubscript{K1} or I\textsubscript{sus} waveforms (online Figure IB) and densities (online Figures IE through IF, Table 1). The proportion of cells exhibiting substantial I\textsubscript{K1} was not altered by hypertrophy (TAB+Veh, 12/36 cells from 13 mice, 33%) or by exposure to CsA (TAB+CsA, 10/22 cells from 9 mice, 45%). I\textsubscript{K1} and I\textsubscript{sus} densities were similar under conditions of TAB+CsA and Sham+CsA (online Figures IE and IF, Table 1).

Inward rectifier current (I\textsubscript{K1}) densities and kinetics did not differ between subepicardial and subendocardial cells. I\textsubscript{K1} recorded from hypertrophied myocytes (TAB+Veh) was similar to baseline (control and sham operated) and after exposure to CsA (TAB+CsA, Sham+CsA; negative data not shown).
Hypertrophic Upregulation of Inward Ca\textsuperscript{2+} Current

Because intracellular Ca\textsuperscript{2+} is increasingly viewed as a central point of regulation in the pathogenesis of hypertrophy\textsuperscript{18} and disease-related electrical remodeling,\textsuperscript{19,20} we measured the L-type Ca\textsuperscript{2+} current (I\textsubscript{Ca,L}) in the presence (TAB + Veh) and absence (Sham + Veh, TAB + CsA, Sham + CsA) of hypertrophy. No differences in I\textsubscript{Ca,L} amplitude or kinetics were observed between subendocardial (Figure 4A) and subepicardial (online Figure II) cell fractions. The amplitude of I\textsubscript{Ca,L} in hypertrophied myocytes (TAB + Veh), however, was larger than in sham-operated controls (Figure 4B). Normalization to cell size (capacitance) revealed that peak I\textsubscript{Ca,L} density increased 26\% (P<0.05) in TAB + Veh myocytes (14±1 pA/pF) compared with Sham + Veh cells (11.1±0.4 pA/pF). I\textsubscript{Ca,L} density was increased in TAB + Veh cells at all potentials ranging from −20 to +50 mV (Figure 4F).

Peak I\textsubscript{Ca,L} and current-voltage relations in cells exposed to TAB + CsA (Figure 4C) were not significantly different from sham-operated controls. The peak I\textsubscript{Ca,L} density measured in Sham + CsA cells was slightly less than that measured in Sham + Veh myocytes (9.3±0.9 pA/pF, P<0.05).

To determine whether changes in channel availability underlie the differences in peak I\textsubscript{Ca,L}, we examined the voltage dependence of activation and inactivation (Figures 5A through 5C). This analysis revealed no significant differences in steady-state voltage-dependent activation or inactivation of I\textsubscript{Ca,L} among any of the 4 treatment groups (Figure 5C).

Because intracellular Ca\textsuperscript{2+}, acting through calmodulin,\textsuperscript{21} modulates the kinetics of L-type Ca\textsuperscript{2+} channel inactivation, we examined the time dependence of recovery from steady-state I\textsubscript{Ca,L} inactivation by using a paired-pulse protocol (Figure 5D). The time constants of recovery from inactivation for TAB + Veh and Sham + Veh cells were 65±7 ms (χ\textsuperscript{2}=0.0055) and 168±11 ms (χ\textsuperscript{2}=0.0067), respectively (P<0.05), revealing a significant acceleration of recovery from inactivation in hypertrophied myocytes. The faster recovery from I\textsubscript{Ca,L} inactivation was absent in nonhypertrophied banded hearts (TAB + CsA, Figure 5F). The time constants of recovery from steady-state inactivation in TAB + CsA (149±14 ms, χ\textsuperscript{2}=0.0033) and Sham + CsA (130±13 ms, χ\textsuperscript{2}=0.0031) cells were not significantly different from sham-operated controls.

Discussion

In recent years, electrophysiological remodeling has emerged as an important pathophysiological mechanism in many types of cardiac pathology. Because clinical heart disease often involves both hypertrophic and failure cellular phenotypes, we sought to elucidate the contribution of hypertrophy in isolation. Working with a model of pressure overload that induces cardiac hypertrophy without LV dysfunction, we evaluated action potential characteristics across the LV and

**Figure 2.** Representative action potentials (stimulation cycle length, 200 ms) recorded from isolated ventricular myocytes (A, subepicardial fraction; B, subendocardial fraction) from hearts isolated under sham-operated (A), TAB + CsA (B), or TAB + Veh (C) conditions. C, APD\textsubscript{90} (±SEM) measured in subepicardial (subepi) and subendocardial (subendo) myocytes from hearts treated as listed. D, Percent change in APD\textsubscript{90} and APD\textsubscript{50} between hypertrophied myocytes (TAB + Veh) and control (Sham + Veh). *P<0.05, **P<0.01.
studied the major currents involved in repolarization. We identified differences within the LV in cellular APD that are amplified by hemodynamic stress, and we addressed the role of a recently described calcineurin signaling pathway.

Accumulating evidence points to disorders of ventricular refractoriness underlying ventricular tachyarrhythmias. Data from clinical electrophysiological studies, surface electrocardiography, and animal models all point to dispersion of ventricular refractoriness as a major predisposing factor in the development of reentrant ventricular arrhythmias. At a basic level, it is becoming increasingly apparent that the electrophysiological characteristics of ventricular tissue are heterogeneous across the ventricular wall, including murine ventricle, and that these regional differences are altered in hypertrophy. It will be important in the future to determine why myocytes from different regions of the LV wall display markedly different electrophysiological phenotypes.

The cellular and molecular phenotype of hypertrophy is modulated by the degree of hypertrophy and the primary lesion that induces hypertrophy. In the TAB hypertrophy model, outward K currents (I, ) do not change, whereas the inward current (I, ) increases, thus inducing greater APD prolongation in subendocardial myocytes, given their relative lack of repolarizing I. Other models of moderate hypertrophy also manifest increased I,. These findings contrast with numerous models of heart failure in which outward K currents, especially I, are decreased. Differences in mechanisms underlying action potential prolongation in hypertrophy and heart failure may have significant implications.
in devising therapeutic approaches to treating arrhythmias in these diseases.

TAB in mice induces ≈50% cardiac hypertrophy at 3 to 5 weeks, and this process is blocked by calcineurin inhibition (see Table 2 and Hill et al13). We have exploited calcineurin inhibition with CsA to control for the structural changes of hypertrophic growth. Calcineurin blockade eliminated the increases in heart mass and myocyte size associated with pressure overload and attenuated the APD prolongation of hypertrophy in both subepicardial and subendocardial myocytes. Similarly, calcineurin blockade prevented the upregulation of ICa,L, and prevented the accelerated recovery from ICa,L inactivation seen with hypertrophy. At present, we do not know whether the CsA-mediated changes in ICa,L are secondary to the abrogated structural changes of hypertrophy, vice versa, or are separate phenomena. Because the action potential prolongation did not normalize completely with CsA, electrogenic transport processes in addition to ICa,L must be involved. Nevertheless, it is possible that the modulatory effects of CsA on hypertrophy are mediated via the L-type channel; a model could be proposed wherein CsA decreases ICa,L, which diminishes intracellular Ca2+, a central point in several hypertrophic signaling pathways.18,33

Calcineurin was inhibited with CsA at a dose that produced CsA trough levels of 2.6±0.2 μg/mL,13 which inhibited ≈90% of total calcineurin phosphatase activity in the heart. Our assay of calcineurin activity relies on myocyte disruption with provision of exogenous Ca2+ and calmodulin, thereby activating total myocyte stores of calcineurin. Although documenting the efficacy of calcineurin inhibition by CsA, this assay does not distinguish between activated and inactive calcineurin within the cell.

The central role of intracellular calcium signaling in hypertrophy is established. In the heart, a small amount of Ca2+ entering through L-type channels triggers the release of much larger amounts of Ca2+ from intracellular stores. Thus, modest changes in Ca2+ influx are amplified within the cell. We predict that hypertrophy-associated ICa,L upregulation may generate positive feedback in the hypertrophic cascade. CsA-sensitive changes in ICa,L inactivation kinetics are suggestive of calcineurin-mediated regulation of the Ca2+ channel complex, as has been reported for a number of proteins involved in Ca2+ homeostasis, including the 1,4,5-triphosphate and ryanodine receptors, and the neuronal and cardiac Na+-Ca2+ exchangers (reviewed in Carafoli et al35).

Cardiac hypertrophy has been viewed as a pathological milestone in disease progression leading to heart failure, a syndrome in which compensatory responses such as vasoconstriction, neurohumoral recruitment, and cytokine activation are maladaptive in the long term. To this list, we must add ICa,L upregulation and action potential prolongation.19,32 Although an increased influx of Ca2+ may prove beneficial by maximizing inotropic potential in the short run, the resulting action potential prolongation heightens dispersion of refractoriness and predisposes to afterdepolarizations and triggered automaticity, reentry, and arrhythmogenesis.

Acknowledgments

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Electrical Remodeling in Pressure-Overload Cardiac Hypertrophy: Role of Calcineurin
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