Inotropic and Calcium Kinetic Effects of Calcium Channel Agonist and Antagonist in Isolated Cardiac Myocytes From Cardiomyopathic Hamsters

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The mechanism by which heart cells of cardiomyopathic (CM) hamsters become calcium overloaded is not known. We examined the number of slow calcium channels, calcium uptake via slow calcium channels, calcium pool sizes, and the contractile response to Bay K 8644, verapamil, and nifedipine using isolated cardiac myocytes from 8–9-month-old CM hamsters (BIO 14.6) and age-matched normal controls. The number of dihydropyridine binding sites as assessed by specific binding of [3H]PN200-110 was similar in the two groups (control hearts: B\textsubscript{max} = 333±89 [mean±SD] fmol/mg; CM hearts: B\textsubscript{max} = 357±75 fmol/mg; n=5 experiments, p=0.6). Current density through L-type calcium channels was determined using the whole-cell clamp technique (at -50 mV holding potential and -10 mV test potential) and was the same in CM myocytes (17.8±1.5 [mean±SD] pA/pF) and control myocytes (18.6±2.1 pA/pF) (n=5 experiments, p=0.5). The current-voltage relation (test potentials varied from -40 to +50 mV) was also the same in CM and control cells, as was apparent threshold, peak current, and reversal potential. However, the initial rate of \(^{45}\)Ca influx as well as the size of the rapidly exchangeable calcium pool was significantly greater in myocytes obtained from CM than from normal hamsters. In both myocyte preparations, Bay K 8644 increased the rate of \(^{45}\)Ca uptake by 25% at 60 seconds; verapamil decreased \(^{45}\)Ca uptake at 60 seconds by 16% and 17% in normal and CM hamsters, respectively. A similar inhibitory effect was observed with nifedipine. The amplitude of cell motion in cells driven at 1.5 Hz as assessed by an optical-video system increased progressively with increasing concentrations of extracellular calcium or Bay K 8644 in cardiac myocytes from normal or CM hamsters. However, the concentration-effect curves for the two effectors were shifted to the left in CM cells compared with cells from normal hamsters. Both preparations demonstrated similar contractile responses to verapamil and nifedipine. These findings demonstrate that single enzymatically dissociated cardiac myocytes from CM hamsters have impaired contractile properties analogous to those seen in the intact heart and thus provide a useful experimental system in which to study underlying cellular mechanisms operative in this model of heart failure. Our results further indicate that calcium overload in CM hamster cardiac myocytes may not be due to increased calcium influx via dihydropyridine-sensitive calcium channels, as suggested previously, but rather to abnormalities of intracellular calcium homeostasis. (Circulation Research 1990;67:599–608)

The Syrian cardiomyopathic hamster is known to develop a genetically determined cardiomyopathy, with progressive development of congestive heart failure.\textsuperscript{1,2} This model has been much used for studies of fundamental cellular abnormalities in heart failure. Several sites of cellular dysfunction have been reported in the cardiomyopathic hamster myocardium.\textsuperscript{3} These include defects in mitochondria,\textsuperscript{4} sarcoplasmic reticulum,\textsuperscript{5} myofibrils,\textsuperscript{6} and sarcolemma.\textsuperscript{7} However, the primary defect initiating the chain of events leading to overt heart failure and early death has not been identified.\textsuperscript{8,9} The myocardium of the cardiomyopathic hamster, BIO 14.6 strain, shows evidence of calcium overload, and it is believed that an excess of free intracellular calcium ([Ca\textsubscript{i}]) plays an important role in the pathogenesis of

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this disease.\textsuperscript{10} Compatible with this theory, studies of Rouleau et al\textsuperscript{11} demonstrated that verapamil reduced the degree of myocardial damage and preserved the contractility of the cardiomyopathic Syrian hamster heart. There was a weak correlation between the maximum velocity of shortening and the actin-activated myosin ATPase activity. Verapamil is thought to prevent damage in the cardiomyopathic hamster by decreasing intracellular calcium and thus preventing the deleterious effects of calcium overload.\textsuperscript{12} However, verapamil has many coronary and peripheral vascular effects in addition to direct effects on myocardial calcium homeostasis. Thus, the mechanism or mechanisms of injury in the cardiomyopathic hamster heart remain to be clarified.\textsuperscript{13}

A recent study\textsuperscript{14} reported that cardiomyopathic Syrian hamsters have 50\% more slow calcium channels (dihydropyridine [DHP] binding sites) than age-and sex-matched controls. Wagner et al\textsuperscript{15} reported that the number of receptor binding sites for calcium antagonists in heart, brain, skeletal muscle, and smooth muscle were markedly increased in cardiomyopathic hamsters. However, other investigators\textsuperscript{16,17} have found a decrease in DHP binding sites in ventricles from cardiomyopathic hamsters with heart failure.

In this study, we investigated DHP binding and current density of L-type calcium channels, as well as the effects of verapamil, nifedipine, and Bay K 8644 on [Ca\textsubscript{i}], contractile state, and calcium pool sizes, using isolated cardiac myocytes from 8–9-month-old cardiomyopathic hamsters (BIO 14.6) and age- and sex-matched normal control hamsters.

**Materials and Methods**

**Cell Isolation**

Eight- to 9-month-old cardiomyopathic hamsters (BIO 14.6, Bio Research, Cambridge, Mass.) and age-matched normal hamsters (FIB) were anesthetized with ether. The hearts were rapidly removed and, after cannulation of the aorta, were perfused with oxygenated (37\°C) Krebs-Henseleit (K-H) bicarbonate buffered solution (pH 7.30) containing (mM) NaCl 118, KCl 4.7, CaCl\textsubscript{2} 0.6, MgSO\textsubscript{4} 1.20, KH\textsubscript{2}PO\textsubscript{4} 1.20, NaHCO\textsubscript{3} 25, and glucose 15. Hearts were perfused at 5 ml/min for approximately 8 minutes until completely cleared of blood. Hearts were then perfused with the same solution but without CaCl\textsubscript{2} for 5 minutes. Collagenase (0.03\%) and hyaluronidase (0.015\%) were added to this Ca\textsuperscript{2+}-free-buffered medium and recirculated for an additional 25 minutes. The ventricular muscle was removed from the perfusion apparatus, cut into 3-mm\textsuperscript{3} pieces, and placed in a 10-ml flask with 0.03\% collagenase, 0.015\% hyaluronidase, 0.0015\% trypsin, 0.0015\% deoxyribonuclease, and 1 mM CaCl\textsubscript{2} in the K-H buffer. The flask was shaken at 37\°C for 15 minutes. Tissue pieces were transferred to the Ca\textsuperscript{2+}-free enzyme buffer with 2.0\% bovine serum albumin added. Tissue pieces were mechanically dissociated by gently triturating 10–15 times with a 5-ml pipette (tip diameter, 3 mm). The isolated myocytes were filtered through Nitex mesh (Tetco Co., Elmsford, N.Y.) and collected in a centrifuge tube. Cells were centrifuged two times at 500 rpm for 1 minute in Ca\textsuperscript{2+}-free K-H buffer to remove dissociation enzymes. The isolated myocytes were resuspended in 0.6 mM Ca\textsuperscript{2+} solution. Approximately 80\% of cells exhibited rod-shaped morphology with clear cross-striations and excluded trypan blue. Cells used for [Ca\textsubscript{i}] and contractility measurements were permitted to attach to 12-mm circular glass coverslips coated with collagen.

**Contractile Response Measurements**

To determine the contractile response of cardiomyopathic and normal hamster myocytes to calcium channel agonist and antagonists, measurements of contractility were performed by using an optical-video system as described by Barry and Smith.\textsuperscript{18} A 12-mm circular glass coverslip with attached myocytes was placed on the stage of an inverted phase-contrast microscope (Lietz-Diavert, The Lietz Co., Overland Park, Kan.) and perfused at a rate of 1 ml/min with physiological solution containing (mM) HEPES 5, CaCl\textsubscript{2} 0.6, KCl 4.0, NaCl 140, MgCl\textsubscript{2} 0.5, and glucose 11 (pH 7.35). After a 10-minute equilibration period, cells were superfused with graded concentrations of (±)-Bay K 8644, verapamil, or nifedipine. The microscope and perfusion chamber were enclosed in a thermostated Lucite box (37±0.5\°C). The cells were electrically stimulated (3-msec duration) at a frequency of 1.5 Hz by use of platinum electrodes. Movement of a border on the axis of cell shortening was tracked with a video motion detector that provided new position data every 16 msec. Cells chosen for experimentation always contracted toward an attachment point at the center of the cell, such that both freely moving ends of the cell shortened with stimulation. The amplitude of cell motion remained stable for the duration of control experiments, indicating the stability of the preparation.

[Ca\textsubscript{i}] Measurements

Mean [Ca\textsubscript{i}] of isolated cardiac myocytes was measured using the calcium-sensitive fluorescent dye fura 2, as previously described by Kim et al.\textsuperscript{19} Cells were exposed to a 3-\muM concentration of the acetoxymethyl ester of fura 2 (fura 2-AM) for 10 minutes at room temperature and then washed for 5 minutes with HEPES-buffered K-H medium. A glass coverslip with attached isolated heart cells was placed in a specially designed perfusion chamber on an inverted microscope (Nikon Inc., Garden City, N.Y.). The cells were perfused with oxygenated HEPES-buffered solution (37\°C). Excitation wavelengths were 340 and 380 nm, and emission wavelength was 505 nm. Excitation wavelengths alternated at 30 Hz. Fluorescence signals from cells were acquired at 0.1- or 1-second intervals and stored separately by the SPEX microcomputer (SPEX Industries, Inc., Edison, N.J.). The cell was electrically stimulated by
using platinum electrodes at desired frequencies, and the contractions that followed the stimulation were confirmed before, during, and after each experiment. After equilibration of the fluorescence signal from each cell studied, that cell was perfused with a test drug, and the change in fluorescence signal was continuously monitored. At the end of an experiment, background autofluorescence from cells not loaded with fura 2 was subtracted from the initial signals. In most cases, the background signal was less than 1% of the fura 2 signal from the cell. To calibrate the fluorescence signal to represent actual [Ca], values, cells were perfused with 3 μM ionomycin solution until a peak 340-nm signal was present; the cells were then perfused with 3 mM EGTA solution to obtain the minimum value. The equation of Grynkiewicz et al.\(^{21}\) determined the fluorescence ratio (340/380), which was used to minimize problems with variations in dye concentration, dye leakage, and cell thickness. Under the conditions of our studies, 3 μM fura 2 exposure for 10 minutes reduced the amplitude of cell motion by approximately 15%. Washing of cells produced no further significant decline in cell motion.

**Calcium Flux Measurements**

For determination of calcium influx, cells were resuspended in HEPES-buffered K-H medium containing 0.6 mM calcium after being washed with calcium-free buffer. Then \(^{45}\)Ca (5 μCi/ml) medium with or without added Bay K 8644, verapamil, or nifedipine was added to the cell suspension as indicated in individual experiments. Cells were incubated for 15, 30, 45, or 60 seconds at 37° C. They were then washed by vacuum filtration using three rapid (10-second) washes with ice-cold HEPES buffer containing no radiolabeled ions. Filters were dried and counted in an LKB liquid scintillation counter (Gaithersburg, Md.).

**Radioligand Binding Experiments**

For determination of equilibrium binding properties of (+)-\(^{3}H\)PN200-110, the isolated myocytes were resuspended in HEPES-buffered solution at 37° C. Graded concentrations of radioligand and unlabeled (+)-PN200-110 were added, and the isolated cells were then incubated at 37° C for 40 minutes in the dark. Control experiments confirmed that binding equilibrium was achieved. The reaction was terminated by rapid vacuum filtration over GF/B glass fiber filters (Whatman Inc., Clifton, N.J.), followed by four rapid washes with 4 ml wash buffer (50 mM KH₂PO₄, pH 7.5, 4° C) to remove unbound radioligand. Radioactivity was measured by liquid scintillation spectrometry (LKB). Equilibrium binding data and displacement curves were analyzed by using a modification of the method of Munson and Rodbard as previously described.\(^{21}\)

**Voltage-Clamp Recording**

Calcium current was obtained with patch-clamp techniques in the whole-cell mode.\(^{22}\) The currents were recorded with a patch-clamp amplifier with a cutoff frequency of 1 kHz (model EP-7, Adams and List, New York) and analyzed on a PDP-11.73 computer (Digital Equipment Corp., Maynard, Mass.). Whole-cell recordings were obtained with the following solutions: the external solution contained (mM) barium acetate 20, tetraethylammonium chloride aspartate 135, and HEPES 10, pH 7.5, and the internal solution contained cesium aspartate 135, Cs₂EGTA 10, HEPES 10, ATP 4, and MgCl₂ 5, pH 7.5. In experiments kept for analysis, the series resistance remaining after compensation did not exceed 2.5 MΩ. The stimulation frequency was 0.2 Hz. A specific membrane capacitance of 1 μF/cm² was assumed for the calculation of the current density. All recordings were obtained at room temperature (20–22° C). Linear components of capacitance and leak currents have been subtracted digitally from all records shown by using scaled current from signal-averaged small pulses that elicited no ionic current.

**Materials**

The (+) enantiomer of \(^{3}H\)PN200-110 was obtained from Amersham, Arlington Heights, Ill. Unlabeled (+)-PN200-110 was a gift from Sandoz, Ltd., Basel, Switzerland. \(^{45}\)Ca was obtained from New England Nuclear, Boston. (+)-Bay K 8644 was the generous gift of Dr. A. Scriabine, Miles Laboratories, West Haven, Conn. Verapamil was a gift from G.D. Searle and Co., Skokie, Ill. Phorbol myristate acetate, phorbol dihexanoate, hyaluronidase, and trypsin were from Sigma Chemical Co., St. Louis. Collagenase and deoxyribonuclease were from Worthington Biochemical, Malvern, Pa.

**Statistical Analysis**

Statistical analysis was performed by analysis of variance (ANOVA). Where appropriate, simple comparisons were made by paired t test. When multiple comparisons were made, the Bonferroni correction was applied. Concentration-effect curve slopes were analyzed by log-logit transform and linear regression analysis. Results are expressed as mean±1 SD, as an index of dispersion of values around the mean.

**Results**

**Number of DHP Binding Sites**

To determine whether the binding of a calcium channel antagonist as a measure of calcium channel number was related to calcium flux and contractile state in myocytes from cardiomyopathic hamster hearts, we examined the number and affinity of DHP binding sites on intact isolated cells using (+)-\(^{3}H\)PN200-110.\(^{23}\) Isolated myocytes were equilibrated in HEPES-buffered medium (pH 7.4) containing 0.6 mM Ca²⁺ for 5 minutes and were counted at...
binding was 333±89 fmol/mg protein in normal cells, and a statistically similar value of 357±75 fmol/mg protein was observed in intact cells from cardiomyopathic animals. The binding affinity of this DHP calcium channel antagonist was not significantly different in the two cell types, with $K_d$ values in the 2.3–2.4 nM range for both normal and cardiomyopathic cells. To avoid the possibility of variation in the fraction of depolarized cells affecting the binding results, we also measured the number of (+)-[H]$^3$IPN200-110 binding sites on isolated myocytes under depolarized conditions, using buffer containing 100 mM KCl with an equimolar reduction in [NaCl]. The $B_{\text{max}}$ for both cardiomyopathic and normal cells was increased to 543±53 and 582±41 fmol/mg protein, respectively; the $K_d$ values were unchanged. There was no significant difference in $B_{\text{max}}$ ($p=0.6$) or in $K_d$ ($p=0.7$; $n=3$ experiments) values between myopathic and normal cells. Thus, our findings do not confirm earlier reports of significant differences in the numbers of DHP binding sites in cells from cardiomyopathic hamster hearts.14,15

### Whole-Cell L-Type Calcium Channel Current Density

To characterize further the DHP-sensitive calcium channel properties in normal and cardiomyopathic myocytes, we used the patch-clamp technique to obtain direct evidence for the current density of L-type calcium channels in cardiac myocytes from both cardiomyopathic and age-matched control hamsters. The L-type calcium channel current was determined in ventricular cells by the whole-cell clamp technique, using Ba$^{2+}$ as the charge carrier. Outward currents were minimized by replacing intracellular

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**Figure 1.** Plot showing displacement of (+)-[H]$^3$IPN200-110 by unlabeled (+)-PN200-110 in isolated cardiac myocytes from cardiomyopathic hamsters (CM) and control hamsters (NH). (+)-[H]$^3$IPN200-110 and graded concentrations of (+)-PN200-110 were incubated with intact cells for 40 minutes. Each point is the mean of five replicates. The vertical axis indicates ratio of bound (+)-[H]$^3$IPN200-110 to total (+)-[H]$^3$IPN200-110. This curve is representative of five similar experiments.

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**Figure 2.** Tracings and plot showing current density of L-type calcium channels, which was determined in cardiac myocytes from cardiomyopathic hamsters (CM) and age-matched normal controls (NH) using the whole-cell patch-clamp technique. Panel A: Whole-cell currents carried by Ba$^{2+}$ at −50 mV holding potential with −40, −30, −20, and −10 mV test potentials from CM cells and NH cells. The cell capacitance is 215 pF in the NH cell and 219 pF in the CM cell. Panel B: Current-voltage relations in CM cells and NH cells. Each point is the mean of five replicates. The test potentials varied from −40 to +50 mV. Calcium currents were measured as peak inward currents in reference to zero current. As an index of current density, we used the cell current divided by cell capacitance (pA/pF).
K+ with Cs+. Membrane holding potential was −50 mV. For the current-voltage relations in Figure 2, test potentials varied from −40 to +50 mV. On step depolarizations from −10 to +50 mV, the slow channel current had a density of 18.6±2.1 pA/pF and 17.8±1.5 pA/pF (mean±SD, n=5 experiments) in normal and cardiomyopathic myocytes, respectively. The current-voltage relation showed an apparent threshold at −40 mV, a maximum at −10 mV, and an apparent reversal potential at +40 mV in both cardiomyopathic myocytes and age-matched normal control cells (Figure 2). There was no statistically significant difference in each test potential–induced current change between myopathic and normal cells. These results further confirmed that there was no measurable difference in the properties of L-type, DHP-sensitive channels in ventricular myocytes from cardiomyopathic hamsters compared with normal hamsters. However, the average cell capacitance was significantly higher in myopathic cells (236±52 pF, n=32 experiments) than in normal cells (181±47 pF, n=32 experiments; p<0.01).

Contractile Properties

Contractile properties were investigated in isolated single ventricular cells driven at 1.5 Hz. The normal and cardiomyopathic cells were superfused with oxygenated HEPES-buffered medium. Bay K 8644 produced a similar concentration-dependent increase in amplitude of cell motion for control and cardiomyopathic cells. We have previously reported that baseline amplitude of contraction of cardiomyopathic myocytes (10.8±2.4 μm, n=10 experiments) was significantly lower than that for control cells (16.7±3.2 μm, n=10 experiments; p<0.01, with Bonferroni correction) in the same extracellular calcium concentration ([Ca]o) buffer. The contractile responses of myocytes to graded concentrations of Ca2+ with Bay K 8644, nifedipine, and verapamil are shown in Figure 3. The amplitude of contraction of cardiomyopathic myocytes was significantly lower than control while being perfused with comparable concentrations of calcium channel agonist and antagonists. Both preparations demonstrated a concentration-dependent positive inotropic response to Bay K 8644. The Bay K 8644 concentration-effect curve tended to be leftward shifted with an early plateau for cardiomyopathic hamster cells compared with controls (Figure 4).

**Figure 3.** Concentration-effect curves showing the contractile response of myocytes to various concentrations of extracellular calcium ([Ca]o), Bay K 8644, and nifedipine. The amplitude of contraction of the myocytes from cardiomyopathic hearts was significantly lower than that of myocytes from control hearts at every [Ca]o tested. Contractile performance in 0.6 mM [Ca]o was reduced at all concentrations of calcium agonist and antagonists. This representative experiment was replicated eight times.

**Figure 4.** Plot showing contractile response to Bay K 8644. Concentration-effect curve for contractile response of isolated cardiomyopathic (CM) myocytes and normal controls (NH) to graded concentrations of Bay K 8644. Values are expressed as percent of initial value of normal cells before Bay K 8644 exposure. It is evident that the initial contractile amplitude of CM cells at zero Bay K 8644 was 68% of that for control cells (see Figure 3). At 10−4 M Bay K 8644, CM cell contractile amplitude is somewhat less than that for normal cells. Each point and vertical bar indicate the mean±SD of 10 determinations. When no standard deviation bar is indicated, the bar is smaller than the size of the symbol.
4). The increment in amplitude of cell motion over control was significantly greater in myopathic cells than that in normal cells for Bay K 8644 concentrations from $10^{-9}$ to $10^{-6}$ M (ANOVA, $p<0.05$). The maximal absolute amplitude of cell motion for cardiomyopathic myocytes in response to $10^{-4}$ M Bay K 8644 was significantly lower than that for control cells ($p<0.01$, with Bonferroni correction) (Figure 3).

The negative inotropic effects of verapamil and nifedipine were demonstrable in both groups of myocytes. Nifedipine produced a concentration-dependent decrease in the amplitude of contraction of myopathic cells (Figure 5) that was similar to that observed in control cells. There was a biphasic change in contractile amplitude produced by verapamil in cardiomyopathic cells. For both calcium channel blocking drugs the negative inotropic effect of $10^{-3}$ M drug was the same as that in normal cells. Thus, calcium channel antagonists decrease the contractility of both myopathic cardiac myocytes and control cells to a similar degree.

Systolic and diastolic velocities were measured in normal and cardiomyopathic cells. In the cardiomyopathic myocytes, the maximum systolic velocity ($V_{\text{max}}$) was lower ($V_{\text{max}}=87\pm11$ $\mu$m/sec) than in controls ($V_{\text{max}}=114\pm24$ $\mu$m/sec; $p<0.05$, $n=8$ experiments). The systolic velocity increased with increasing $[\text{Ca}_i]_0$, as well as with increasing Bay K 8644 concentrations (at $[\text{Ca}_i]_0=0.6$ mM) both in normal cells and in cardiomyopathic cells. However, in contrast to cells from normal control animals, Bay K 8644 failed to produce a further increase in systolic velocity of cardiomyopathic cells at concentrations in the $10^{-6}$–$10^{-5}$ M range. The blunted systolic contractile response to $[\text{Ca}_i]_0$ was also found at $[\text{Ca}_i]_0$ greater than 1.2 mM in myopathic cells. By using $10^{-7}$–$10^{-6}$ M Bay K 8644, the change in systolic velocity in myopathic cells was larger than that of normal control cells ($p<0.05$, $n=8$ experiments). However, the diastolic velocity was similar in cardiomyopathic cells ($V_{\text{max}}=82\pm22$ $\mu$m/sec) and normal cells ($V_{\text{max}}=90\pm28$ $\mu$m/sec; $n=8$ experiments, $p>0.05$) at $[\text{Ca}_i]_0$ of 0.6 mM. The diastolic velocity increased with graded increases in Bay K 8644 concentration, and responses were similar in normal control and cardiomyopathic cells. The effect of bathing calcium concentration on diastolic velocity was also not significantly different in myopathic and control myocytes. These results demonstrate an abnormality in systolic function without any apparent abnormality in diastolic function in cardiomyopathic hamster heart cells over the ranges of $[\text{Ca}_i]_0$ and Bay K 8644 examined.

**Modulation of $^{45}$Ca Influx by Calcium Channel Agonists and Antagonists**

The uptake of $^{45}$Ca in response to Bay K 8644, verapamil, and nifedipine was studied in cardiomyopathic and normal myocytes. If the putative augmented number of DHP binding sites reflected an increase in the number of functional voltage-dependent calcium channels, one might anticipate an increase in calcium influx rate in cells with more channels. Accordingly, we measured the uptake of $^{45}$Ca in $10^{-6}$ M Bay K 8644–treated cells isolated from normal and cardiomyopathic hamster hearts (Figure 6). Under normal conditions, $^{45}$Ca content was higher at all time points for cardiomyopathic than for normal cells. Bay K 8644 increased the $^{45}$Ca content by 25% at 60 seconds in normal myocytes, as well as myopathic cells ($p=0.5$). At 5 minutes, the $^{45}$Ca content in myopathic cells (5.9±0.1 nmol/mg protein) was significantly greater than in control cells (4.6±0.1 nmol/mg protein; $p<0.01$, with Bonferroni correction). In cardiomyopathic cells, the increment in the rapidly exchangeable $^{45}$Ca pool compared with control (at 5 minutes) induced by Bay K 8644 was significantly greater than that in normal cells ($p<0.05$, with Bonferroni correction). Thus, at the...
concentration of Bay K 8644 tested, the two populations of cells showed a difference in response to this calcium channel agonist. These results are consistent with the findings from our contractility studies. In contrast, verapamil (10⁻⁶ M) decreased the ⁴⁵Ca uptake similarly at 60 seconds, by 20% and 21% in normal and cardiomyopathic hamster cells, respectively. A similar inhibitory effect was also observed with nifedipine. At time points later than 1 minute, the decrease in ⁴⁵Ca uptake produced by graded doses of verapamil or nifedipine was similar for both groups of myocytes. Thus, cardiomyopathic cells display a greater response to a saturating concentration of a calcium channel activator than do control cells, but no difference in response to calcium channel blockers was evident.

[Ca]ᵢ Studies

We examined the effects of Bay K 8644, verapamil, and nifedipine on time-averaged [Ca]ᵢ in cardiomyopathic and normal cells. To determine whether the differing positive inotropic actions of Bay K 8644 in normal and cardiomyopathic cells are due to different effects on [Ca]ᵢ, we measured the changes in fluorescence intensity ratio (340 nm/380 nm) from cells loaded with fura 2. Fura 2–loaded cells were electrically stimulated at 1.5 Hz, and their contractions were visually confirmed. Time-averaged [Ca]ᵢ was 472±30 nM in cardiomyopathic myocytes, which was substantially higher than the mean level in control cells (270±19 nM; p<0.01), as shown in Figure 7A. Without electrical stimulation, [Ca]ᵢ was 260±34 nM in myopathic cells, significantly higher than in normal cells (121±28 nM; p<0.01).

As shown in Figure 7A, when cells were exposed to Bay K 8644 there was an early plateau of [Ca]ᵢ in the cardiomyopathic myocytes as Bay K 8644 concentration was increased, compared with normal cells. In 0.6 mM [Ca]ᵢ medium, Bay K 8644 (10⁻⁵ M), on average, produced a significant increase in time-averaged [Ca]ᵢ in myopathic cells from 415±42 to 478±28 nM (p<0.05 after Bonferroni correction; ANOVA demonstrated overall difference for curve). At the same concentration, Bay K 8644 did not produce a measurable change in [Ca]ᵢ in normal myocytes. At concentrations greater than 10⁻⁷ M, a strong dependence of [Ca]ᵢ on Bay K 8644 concentration was seen in normal myocytes, with [Ca]ᵢ rising from 240±35 to 380±47 nM as Bay K 8644 concentration was increased from 10⁻⁶ to 10⁻³ M (p<0.01). However, in the cardiomyopathic myocytes, Bay K 8644 (10⁻⁶–10⁻³ M) did not further increase [Ca]ᵢ.

Graded concentrations of Bay K 8644 caused a gradual rise in [Ca]ᵢ over a time course of 4.2±0.1 minutes (n=10 experiments) in normal myocytes (Figure 7B). New steady-state levels were reached at 1.7±0.04 minutes (n=10 experiments) in cardiomyopathic cells, which was significantly more rapid than in controls (p<0.01). Washout of the drug produced a slow decrease in [Ca]ᵢ, over an average of 6 minutes in normal cells. In the cardiomyopathic cells, the [Ca]ᵢ recovery to control levels appeared to be slightly but not significantly more rapid, averaging 5 minutes.

Nifedipine (10⁻⁶ M) reduced [Ca]ᵢ by the same amount in cardiomyopathic cells as in controls (Figure 8). At concentrations of nifedipine of 10⁻⁵ M, time-averaged [Ca]ᵢ was decreased in 18% in both groups of myocytes. Verapamil (10⁻⁶–10⁻⁵ M) also produced an equivalent decrease in [Ca]ᵢ for control and myopathic cells (Figure 8).

Discussion

The experiments described here yield eight interrelated observations that bear on the loci of abnormalities in excitation-contraction coupling in the Syrian hamster (BIO 14.6 strain) model of cardiomyopathy.

First, when DHP binding was studied in intact myocytes from normal and cardiomyopathic hearts,
there was no difference in either the affinity for antagonist ligand or the number of DHP binding sites that could be identified. This finding in intact cells (the first such study of which we are aware) differs from that of some other laboratories\textsuperscript{14,15} that examined DHP binding in membrane fractions from homogenized preparations. The reason for this difference in findings is not certain. The present experiments were highly reproducible, and the binding curves were of good quality (Figure 1). No difference in $K_d$ or $B_{\text{max}}$ between normal and cardiomyopathic cells was found, whether cells were polarized or depolarized. Of note, other laboratories\textsuperscript{16,17} have also reported findings that differ from the initial report by Wagner et al.\textsuperscript{15} of a marked increase in DHP binding sites in membranes from myopathic hearts.

Second, by using standard techniques\textsuperscript{26,27} our whole-cell clamp studies comparing the L-type calcium current density in cardiac myocytes from cardiomyopathic and age-matched normal hamsters yielded indistinguishable results at each test potential. Current-voltage relations were also similar in myopathic and normal myocytes, as were the threshold potential, peak current potential, and the reversal potential. These findings support the view that DHP-sensitive voltage-dependent calcium channels are neither qualitatively nor quantitatively abnormal at this mature developmental stage of the BIO 14.6 strain of cardiomyopathic hamsters.

The third observation is that at a given $[\text{Ca}]_o$, contractile amplitude was lower for cardiomyopathic cells. Other laboratories\textsuperscript{28,29} have previously reported analogous findings. Possible causes are hypothesized as follows: 1) less triggering calcium enters the cell through the slow calcium channel during each depolarization, although the results cited in the last paragraph suggest that this is unlikely, 2) for an equivalent amount of triggering transsarcolemmal calcium flux, sarcoplasmic reticulum calcium release is decreased in myopathic cells, as suggested by Lossnitzer et al.\textsuperscript{28} or 3) there is decreased contractile protein sensitivity to equivalent increases in $[\text{Ca}]_i$ in cardiomyopathic compared with normal cells, as suggested for another cardiomyopathic hamster strain by Malhotra et al.\textsuperscript{29}

The fourth observation is that the contractile response to elevated $[\text{Ca}]_i$, and to graded concentrations of Bay K 8644 reached a plateau at lower concentrations in myopathic cells than in normal cells. Thus, regardless of how transsarcolemmal calcium flux is altered, the contractile response reached a maximum at a lower extracellular concentration of effector. This observation makes hypothesis 1 above even less likely but leaves open the possibility of abnormal sarcoplasmic reticulum calcium release or abnormal contractile protein sensitivity to $[\text{Ca}]_i$ in myopathic cells.

The fifth experimental observation is that there was similar contractile sensitivity and efficacy for normal and myopathic cells to two calcium channel blockers, nifedipine and verapamil. The former is a DHP; the latter is a phenylalkylamine. The similar contractile response to decreasing transsarcolemmal calcium flux that occurs as progressively more calcium channels are blocked at higher drug concentrations is consistent with the view that there is no important difference in the number or conductance of functional calcium channels in the sarcolemma of myopathic cells compared with controls. This functional observation is in accord with the radioligand binding and whole-cell clamp data. One would expect that, if there were a large excess of calcium channels in cardiomyopathic cells,\textsuperscript{15} contractile state might be somewhat less sensitive to graded concentrations of a calcium channel blocker. This was not observed.

The sixth experimental observation is that maximal systolic velocity of shortening ($+V_{\text{max}}$) was less in cardiomyopathic cells than in normal cells. Conversely, there was no measurable difference in rate of relaxation ($-V_{\text{max}}$) under the experimental conditions used. The difference in $+V_{\text{max}}$ favors hypothesis 2, abnormalities in sarcoplasmic reticulum calcium release, or hypothesis 3, an abnormality in contractile protein sensitivity to cytosolic calcium. The similarity in relaxation velocities tends to argue against a major abnormality in calcium uptake by sarcoplasmic reticulum. A limitation of this interpretation, however, is the implicit but untested assumption that the loading properties for both contraction and relaxation of the isolated myocytes attached to a glass coverslip are the same for control and myopathic cells.

The seventh observation is that $^{45}$Ca content under control conditions was higher in cardiomyopathic than in normal cells at all points from 15 seconds to 5 minutes after $^{45}$Ca exposure (see Figure 6). Moreover, Bay K 8644 at a saturating concentration pro-
duced a greater change in $^{45}$Ca content and also a greater absolute $^{45}$Ca content at 5 minutes in cardiomyopathic than in normal cells. However, calcium channel blockers produced equivalent effects in cardiomyopathic and normal cells (Figure 6). The fact that $^{45}$Ca content increased more in cardiomyopathic cells in response to Bay K 8644 raises at least three possibilities: 1) there are different calcium channel gating properties in cardiomyopathic cells (unlikely in view of the patch-clamp results), 2) there is greater calcium sequestration in the sarcoplasmic reticulum, or 3) the ability of the cell to extrude calcium in response to calcium loading conditions is abnormal. This could be due to either an abnormal sarcolemmal calcium transport ATPase or abnormal sodium-calcium exchange. Importantly, the higher $^{45}$Ca content at 5 minutes in cardiomyopathic cells under control conditions helps explain the earlier plateau of contractile response to increases in calcium influx produced either by elevated [Ca]$^\text{+}$, or by Bay K 8644. One need not postulate different contractile protein sensitivity to [Ca]$^\text{+}$; one may simply be starting from a higher plateau in calcium content before calcium-loading interventions.

The eighth major observation is that [Ca]$^\text{+}$, not only starts at a substantially higher value in stimulated or unstimulated myopathic cells than in normal cells but is augmented and reaches a plateau at lower concentrations of Bay K 8644 in myopathic cells than in normal cells. This observation is consistent with the observed augmentation of $^{45}$Ca content and with observations regarding contractile sensitivity to effectors that elevate transsarcolemmal calcium flux ([Ca]$^\text{+}$, and Bay K 8644). Normal cells clearly defend their low cytosolic calcium concentrations more effectively than do cardiomyopathic cells. Five mechanisms by which this might occur include the following: 1) normal cells may inhibit calcium channel-dependent calcium influx at lower [Ca]$^\text{+}$, values, 2) normal cells may have more active sarcoplasmic reticulum calcium sequestration to defend cytosolic calcium, 3) calcium efflux via the Na-Ca exchanger may have higher sensitivity and/or capacity in normal cells, or [Na]$^-$, may differ in myopathic cells, 4) the calcium transport ATPase of the sarcolemma may have a lower $K_m$ or a higher capacity for calcium extrusion in normal cells, or 5) increased calcium entry in myopathic cells (Figure 6) may be extruded relatively normally by the low affinity Na-Ca exchange mechanism but overwhelms a limited transport capacity of the high-affinity sarcolemmal Ca-ATPase extrusion mechanism. Mechanism 2 is less likely because $^{45}$Ca content as well as cytosolic calcium content is elevated in myopathic cells. The absolute values of time-averaged [Ca]$^\text{+}$, in normal and myopathic cells must be interpreted with caution, as Yue et al$^{31}$ point out, since time-averaged fluorescence ratios as reported here may underestimate the time integral of individual [Ca]$^\text{+}$, transients. Nevertheless, time-averaged [Ca]$^\text{+}$, provides an objective measure of relative changes in [Ca]$^\text{+}$, and permits comparisons between two populations of cells.

It is of interest that, despite the increase in diastolic [Ca$^{2+}$], in cardiomyopathic cells, resting cell length is greater in myopathic cells (178±26 μm) than for normal cells (160±15 μm, n=20 experiments, p<0.01) (L. Sen, D. Kim, J.D. Marsh, T.W. Smith, submitted manuscript). The greater cell length reflects, in part, hypertrophy of myopathic cells. To the extent that elevated diastolic [Ca$^{2+}$], may produce incomplete relaxation, myopathic cell length may be even larger.

These experimental observations should be considered in the context of other studies in the cardiomyopathic hamster heart-failure model.$^{15}$ A number of laboratories have reported that calcium channel antagonists, when administered in vivo, help preserve contractile function in myopathic hearts.$^{11}$ There are a number of coronary and peripheral vascular sites of action of calcium channel antagonists that could contribute to the salutary effect, but the current observations are consistent with a direct myocyte effect of verapamil.$^{32}$ Given the substantial body of experimental evidence supporting the existence of abnormal calcium loading in cardiomyopathic myocytes, any agent (including calcium channel blockers) that decreases calcium loading may well be of benefit, even if the calcium channel to which the effector binds is not the primary site of the abnormality.$^{13}$ It is important to note that the cardiomyopathic Syrian hamster constitutes a dynamic model of cardiac hypertrophy and failure. Thus, the abnormalities of calcium homeostasis identified in this study can strictly be attributed to this model only at the developmental stage examined. It is likely that abnormalities that appear only at later stages reflect secondary changes and are not the genetically determined primary biological defect. Nevertheless, secondary changes may also inform our understanding of the pathophysiology of myocyte dysfunction leading to or accompanying heart failure.

The current experimental data permit several conclusions regarding calcium homeostasis in the BIO 14.6 cardiomyopathic hamster heart at the stage studied. First, L-type calcium channels show no demonstrable abnormality as judged by the absence of any major change in number of DHP binding sites. The affinity for a DHP antagonist ligand is normal, consistent with the absence of any major change in the structure of the channel, at least in the DHP binding domain. Whole-cell clamp studies corroborate this provisional conclusion, supporting the view that there is no noteworthy abnormality of the DHP-sensitive voltage-dependent calcium channel in this model. Second, there are major abnormalities in calcium homeostasis. These are manifested both as abnormal time-averaged cytosolic free calcium concentrations and abnormal magnitude of the calcium pool labeled from 15 seconds to 5 minutes by $^{45}$Ca. Our current data do not permit us to exclude definitively the possibility of abnormal calcium channel
gating properties, including reduced inhibition of calcium influx by elevated \([Ca^+]_o\). Finally, one need not invoke abnormal contractile protein sensitivity to \([Ca]_o\), to explain our experimental findings. In this model of cardiomyopathy, abnormalities in mechanism(s) that maintain \([Ca]_o\), in the physiological range appear to be of central importance.

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