Modulation of Ca2+ Signaling by Microtubule Disruption in Rat Ventricular Myocytes and Its Dependence on the Ruptured Patch-Clamp Configuration

S.C. Calaghan, J.-Y. Le Guennec, E. White

Abstract—In the absence of hypertrophic proliferation of microtubules, microtubule disruption by colchicine does not modulate contraction of adult cardiac myocytes. However, Gomez et al (Circ Res. 2000;86:30–36) recently reported that disruption of microtubules by colchicine in ruptured patch-clamped myocytes increased $I_{Ca,L}$ density and [Ca2+], transient amplitude and depressed the response of these parameters to the β-adrenoceptor agonist isoproterenol. These effects were ascribed to stimulation of adenyl cyclase by increased intracellular free tubulin. In the present study, we show that in intact rat ventricular myocytes, 2 to 4 hours of exposure to 10 μmol/L colchicine had no effect on shortening or [Ca2+] transient amplitude or on the amplitude of $I_{Ca,L}$ in perforated patch-clamped cells, under basal conditions and after stimulation with 1 μmol/L isoproterenol. However, in ruptured patch-clamped myocytes, basal $I_{Ca,L}$ was 2-fold higher after treatment with colchicine compared with vehicle and, in contrast to vehicle-treated cells, $I_{Ca,L}$ did not increase in response to isoproterenol. Cell width decreased during ruptured patch-clamp experiments in colchicine-treated but not vehicle-treated myocytes. We conclude that in cells with intact sarcolemma, colchicine does not modulate Ca2+ signaling or the response to β stimulation. However, the combination of microtubule disruption by colchicine and the ruptured patch configuration activates $I_{Ca,L}$ and attenuates the response to β stimulation. We propose that these effects may be due to loss of free tubulin by intracellular dialysis or to increased sensitivity to mechanical stimulation as a result of microtubule disruption. These findings have important implications for cardiomyopathies associated with decreased free tubulin or a diminished microtubular network. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2001;88:e32-e37.)

Key Words: cardiac myocyte | contraction | calcium signaling | microtubules | colchicine

Microtubules are hollow protein cylinders of α- and β-tubulin heterodimers. Although they form a major component of the cardiac cell cytoskeleton, their functional role within the myocyte remains unclear. Microtubules have been implicated in pathological conditions including ischemia,1 cardiac hypertrophy, and failure.2 In some (but not all) cardiac hypertrophies, microtubules have been shown to be proliferated.3–6 Such microtubule proliferation is associated with a depression of cardiac contractile function,3–7 which has been shown to be normalized by the microtubule-disrupter colchicine,3–6 thereby directly implicating microtubules in the modulation of contraction. It has been suggested that microtubule proliferation may modify contraction by increasing sarcomeric load,3 although contractile depression associated with chemical proliferation of the microtubules by taxol is accompanied by a decrease in the amplitude of the intracellular [Ca2+], transient.7

In the absence of prior hypertrophic proliferation, a number of studies have shown that microtubule disruption by colchicine does not increase contractility in either multicellular preparations or single myocytes in the rat,5,8–10 cat,3,4,6,11,12 guinea pig,13 and dog.14 However, the recent study by Gomez et al15 published in Circulation Research showed that in ruptured patch-clamped rat ventricular myocytes, disruption of microtubules by colchicine increased $I_{Ca,L}$ density and the amplitude of the [Ca2+] transient and depressed the response of these parameters to the β-adrenoceptor agonist isoproterenol. These effects were ascribed to stimulation of adenyl cyclase by free tubulin within the cell. Given the involvement of microtubules in some models of hypertrophic contractile dysfunction and the central role of β-adrenergic stimulation in cardiac function, the work by Gomez et al15 raises interesting questions about the possible role of microtubules in the heart. However, the increase in [Ca2+] and $I_{Ca,L}$ seen by Gomez et al15 appears inconsistent with the lack of contractile effect of colchicine reported by others.

To date, no single study has measured the response of contraction, [Ca2+], and $I_{Ca,L}$ to colchicine or the effect of colchicine on the response of these parameters to β-adrenergic stimulation. We have undertaken such an inves-
supplied by Central Biomedical Services at the University of Leeds were used in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986.

**Immunofluorescence Confocal Microscopy**

The microtubules were labeled with monoclonal antibody to β-tubulin (1:200; Sigma Chemical Co) and FITC-conjugated donkey anti-mouse IgG (1:50; Jackson), as described by Howarth et al. Sections of ~2 μm thickness taken longitudinally through the cell at the level of the nucleus were imaged using confocal laser microscopy (Leica True Confocal Scanner SP). Settings were identical for imaging of vehicle- and colchicine-treated cells.

**Measurement of Contraction and [Ca\(^{2+}\)]\(_i\)**

Contraction and [Ca\(^{2+}\)]\(_i\) were measured in cells loaded with fura-2-AM (Molecular Probes). Cells were electrically stimulated at 0.5 Hz, and cell shortening was monitored using a video edge-detection system (Crescent Electronics). Myocytes were alternately illuminated by 340 and 380 nm light, and the fluorescent emission at 510 nm was recorded. The ratio of the emitted fluorescence at the two excitation wavelengths (340/380 ratio) was calculated to provide an index of [Ca\(^{2+}\)]\(_i\). Experiments were performed at 22°C to 25°C.

**Perforated and Ruptured Patch-Clamp Studies**

For perforated patch-clamp studies, pipettes were filled with PP solution (see Solutions) containing 400 μg/mL amphotericin B. Access resistance using this configuration was 13.8±0.7 MΩ, and time to peak of \(I_{\text{Ca,L}}\), an indicator of good access, was 7.5±0.5 ms. For ruptured patch experiments, pipettes were filled with RP solution (see Solutions). Cell capacitance and series resistance were electronically compensated by ~60%. Pipette tip resistance was 2 to 3 MΩ. \(I_{\text{Ca,L}}\) was elicited from a holding potential of ~80 mV by a 300-ms step depolarization to ~40 mV (to inactivate \(I_{\text{Ca}}\), followed by a 300-ms depolarization to 0 mV (at a stimulation frequency of 0.5 Hz). Current-voltage relationships for \(I_{\text{Ca,L}}\) were measured using a conventional protocol that was preceded at each potential by 4 priming depolarizations to 0 mV. In ruptured patch experiments, cell size was also recorded from a video image of the cell at selected time points after establishment of the whole-cell configuration.

**Materials and Methods**

Single ventricular myocytes were isolated enzymatically from hearts of male Wistar rats (200 to 250 g) as described previously. Rats were used in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986.

**Effect of Colchicine (1 and 10 μmol/L) on Contraction and [Ca\(^{2+}\)]\(_i\), Transient in Rat Ventricular Myocytes**

<table>
<thead>
<tr>
<th></th>
<th>Contraction</th>
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<tr>
<td></td>
<td><strong>Resting Length, μm</strong></td>
<td><strong>Shortening, % of Resting Length</strong></td>
<td><strong>Time to Peak, ms</strong></td>
</tr>
<tr>
<td>0.01% Veh (39)</td>
<td>116±2</td>
<td>3.74±0.36</td>
<td>223±8</td>
</tr>
<tr>
<td>1 μmol/L Col (43)</td>
<td>117±2</td>
<td>4.02±0.30</td>
<td>224±7</td>
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<tr>
<td>0.1% Veh (28)</td>
<td>117±2</td>
<td>4.79±0.50</td>
<td>239±11</td>
</tr>
<tr>
<td>10 μmol/L Col (42)</td>
<td>115±2</td>
<td>4.56±0.36</td>
<td>215±8</td>
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<tr>
<th></th>
<th><strong>Diastolic [Ca(^{2+})](_i), RU</strong></th>
<th><strong>[Ca(^{2+})](_i) Transient Amplitude, RU</strong></th>
<th><strong>Time to Peak, ms</strong></th>
<th><strong>Time to Half-Decay, ms</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01% Veh (33)</td>
<td>0.055±0.002</td>
<td>0.045±0.004</td>
<td>76±5</td>
<td>221±10</td>
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<tr>
<td>1 μmol/L Col (42)</td>
<td>0.052±0.001</td>
<td>0.043±0.003</td>
<td>72±4</td>
<td>233±5</td>
</tr>
<tr>
<td>0.1% Veh (20)</td>
<td>0.050±0.002</td>
<td>0.044±0.004</td>
<td>77±6</td>
<td>244±9</td>
</tr>
<tr>
<td>10 μmol/L Col (30)</td>
<td>0.060±0.004*</td>
<td>0.035±0.004</td>
<td>82±4</td>
<td>199±61</td>
</tr>
</tbody>
</table>

*Cells were exposed to colchicine (Col) or vehicle (Veh) for 2 to 4 hours. Values are mean±SEM with the number of cells given in parentheses. RU indicates fura-2 ratio units.

*\(P<0.01\), †\(P<0.001\) compared with respective values in vehicle-treated cells (unpaired Student's t test).
cells was reduced to 57% of that recorded in vehicle-treated sections taken at the level of the nucleus in colchicine-treated myocytes from each heart were always used to study the effects of ducted in the presence of colchicine or vehicle as appropriate. All experiments were conducted in a further 2-hour period. All experiments were performed using the Student’s t test for multiple comparisons. The only significant effect of colchicine was a reduction in the time to peak of the transient in the presence of isoproterenol (+P<0.05 compared with respective vehicle-treated group).

Figure 2. Effect of colchicine on contraction of rat ventricular myocytes. Myocytes were exposed to either vehicle (Veh) or 10 μmol/L colchicine (Col) for 2 hours and contraction was studied at room temperature under basal conditions or at steady state after perfusion with 1 μmol/L isoproterenol. A, Representative records of shortening in cells exposed to either vehicle or colchicine under basal conditions (+) and in the presence of isoproterenol (•). Shortening under basal conditions with colchicine is normalized to that under basal conditions in the vehicle-treated cell for ease of comparison. B, Shortening expressed as a percentage of resting cell length. C, Time to peak of the contraction. D, Time to half-relaxation. Open bars are under basal conditions, and hatched bars are in the presence of isoproterenol. All bars are mean±SEM of 11 to 12 observations. *P<0.05, **P<0.01, ***P<0.001 compared with respective basal conditions (paired Student’s t test). There were no significant differences between colchicine- and vehicle-treated cells.

Statistics

Results are expressed as mean±SEM of n observations. Statistical analysis was performed using the Student’s t test (paired and unpaired) or 2-way repeated-measures ANOVA followed by Bonferroni i test for multiple comparisons.

Results

Effect of Colchicine on Microtubules

Immunofluorescence techniques were used to establish the effect of colchicine on the microtubules in rat ventricular myocytes. Figure 1 shows the effect of 2-hour exposure of myocytes to 10 μmol/L colchicine. Fluorescence intensity in sections taken at the level of the nucleus in colchicine-treated cells was reduced to 57% of that recorded in vehicle-treated cells (P<0.01) confirming that, in our study, colchicine caused disruption of the microtubules.

Effect of Colchicine on Contraction and [Ca2+]

Initially, a study of the effect of colchicine (at the two concentrations used most widely in the literature) on contraction and [Ca2+]; in myocytes with intact sarcolemma was carried out. Exposure of rat ventricular myocytes to 1 and 10 μmol/L colchicine for 2 hours had no effect on either the magnitude or kinetics of cell shortening (Table, top). Diastolic [Ca2+], was significantly higher (P<0.01) in cells exposed to 10 μmol/L colchicine; however, at neither concentration of colchicine did we observe any significant effect on [Ca2+]; transient amplitude (Table, bottom). The only effect of colchicine on transient kinetics was a reduction (P<0.01) in the t1/2 of the transient at 10 μmol/L colchicine (Table, bottom).

Additional studies examining the effect of colchicine on the response to β-adrenergic stimulation were then performed. Figure 2 shows the effect of 10 μmol/L colchicine on the contractile response to the β-adrenergic agonist isoproterenol. Colchicine did not modify the effect of isoproterenol on either the magnitude or kinetics of contraction. Neither did colchicine affect the increase in [Ca2+]; transient amplitude with isoproterenol (Figure 3). In this series of experiments, the only effect of colchicine on the [Ca2+]; transient was an attenuation of the increase in time to peak of the transient observed in the presence of isoproterenol (Figure 3).

Solutions

HEPES-based bathing solution contained (mmol/L) NaCl 113, HEPES 5, Na2HPO4 1, MgSO4 ·7H2O 1, KCl 5, CaCl2 1, glucose 10, sodium acetate 20, insulin 5 U/L; pH 7.4. PP pipette solution contained (mmol/L) KCl 10, NaCl 10, MgCl2 · 6H2O 1, CaCl2 1, potassium glutamate 110, HEPES 5; pH 7.2 with KOH. RF pipette solution contained (mmol/L) KCl 130, MgCl2 · 6H2O 1, NaH2PO4 1, Na2 phosphocreatine 3.6, MgATP 5, HEPES 10, EGTA 0.1; pH 7.2 with KOH. Stock solutions of colchicine were dissolved in methanol. The final concentration of methanol in colchicine-containing solutions was 0.01% or 0.1%. Cells were exposed to colchicine (1 or 10 μmol/L) or vehicle (0.01% or 0.1% vol/vol) for 2 hours and then studied within a further 2-hour period. All experiments were conducted in the presence of colchicine or vehicle as appropriate. Myocytes from each heart were always used to study the effects of both vehicle and colchicine in parallel. Contraction, [Ca2+];, and fCa,L were measured under basal conditions at steady state after perfusion with 1 μmol/L isoproterenol.

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Effect of Colchicine on \( I_{Ca,L} \)

The effect of colchicine on \( I_{Ca,L} \) was measured using both amphotericin perforated patch-clamp and ruptured patch-clamp techniques. Basal \( I_{Ca,L} \) was similar in perforated patch-clamped cells in the presence of vehicle and colchicine (8.7 ± 1.5 versus 10.0 ± 1.9 pA/pF; n = 8), and, in both groups of cells, \( I_{Ca,L} \) increased 2-fold (\( P < 0.05 \)) in response to isoproterenol (Figure 4C). However, in ruptured patch-clamped myocytes, basal \( I_{Ca,L} \) was significantly higher (\( P < 0.05 \)) in colchicine-treated cells (11.8 ± 2.0; n = 6) than in vehicle-treated cells (6.4 ± 0.7; n = 6). Furthermore, in the ruptured patch-clamp configuration, although we observed a significant increase in \( I_{Ca,L} \) in vehicle-treated cells in response to isoproterenol, in cells treated with colchicine, we saw no significant increase (\( P > 0.05 \)) in \( I_{Ca,L} \) after exposure to isoproterenol (Figure 4D). By contrast to the study of Gomez et al\(^{15} \) performed in the ruptured patch configuration, using perforated patch there was no difference (\( P > 0.05 \)) in current-voltage relationships under basal conditions between vehicle-treated cells and colchicine-treated cells (Figure 5). There was a tendency for the \( I_{Ca,L} \) response to isoproterenol to be enhanced in cells treated with colchicine; however, this difference was not significant (2-way repeated-measures ANOVA).

Figure 4. Comparison of the effect of colchicine on \( I_{Ca,L} \) recorded using the perforated and ruptured configuration of the patch-clamp technique in rat ventricular myocytes. A and B, Individual traces of \( I_{Ca,L} \) recorded in amphotericin-perforated (A) and ruptured (B) patch-clamped myocytes. Membrane potential was stepped to 0 mV from a holding potential of −80 mV after a 300-ms prepulse to −40 mV to inactivate \( I_{Ca,L} \). \( I_{Ca,L} \) was recorded in cells after exposure to vehicle (Veh) or 10 \( \mu \)mol/L colchicine (Col) for 2 to 4 hours under basal conditions (●) and at steady state after perfusion with 1 \( \mu \)mol/L isoproterenol (●). C and D, Mean data in perforated (C) and ruptured (D) patch-clamped myocytes. Open bars are under basal conditions, and hatched bars are in the presence of 1 \( \mu \)mol/L isoproterenol. All data are mean ± SEM of 6 to 8 observations. \( *P < 0.05, \#P < 0.01 \) compared with respective group under basal conditions; + \( P < 0.05 \) compared with respective vehicle-treated group (Student’s t test). A significant increase in \( I_{Ca,L} \) in response to isoproterenol was present in all cells except colchicine-treated cells studied using the ruptured patch-clamp technique.

Figure 5 summarizes the effects of colchicine on the response of contraction, \([Ca^{2+}]_c\), transient, \( I_{Ca,L} \) measured in perforated patch, and \( I_{Ca,L} \) measured in ruptured patch configuration to isoproterenol. This figure shows clearly that in cells with an intact sarcolemma (measurements of contraction, \([Ca^{2+}]_c\), transient, and \( I_{Ca,L} \) under perforated patch-clamp conditions), colchicine had no effect on the ratio of isoproterenol-stimulated to basal values of the parameters. In fact there is a (nonsignificant) tendency for this ratio to be increased in colchicine-treated cells (\( P > 0.05 \)). However, in ruptured patch-clamped cells, the ratio of isoproterenol-stimulated to basal value is significantly reduced (\( P < 0.05 \)) in colchicine-treated cells compared with vehicle-treated cells.

Effect of Colchicine on Cell Size in Ruptured Patch Configuration

The ruptured patch configuration may cause dialysis of the cell with the contents of the microelectrode; this can lead to dilution of intracellular contents and alterations in cell shape. To test whether an interaction between the effects of colchicine and a
change in cell shape might account for the results obtained in ruptured patch, cell size was recorded immediately and at 8 to 15 minutes after rupture of the membrane patch (at 10.4±1.2 and 13.3±1.1 minutes in vehicle- and colchicine-treated cells, respectively; \( P>0.05 \)). In vehicle-treated cells, neither resting cell length (105±8 versus 104±7 \( \mu m \); \( n=5 \)) nor cell width (18.1±2.9 versus 17.5±2.6 \( \mu m \)) changed during the course of the experiment (\( P>0.05 \)). In colchicine-treated cells, although there was no significant change (\( P>0.05 \)) in resting cell length (99.0±3.1 versus 97.8±3.0 \( \mu m \); \( n=6 \)), cell width was significantly smaller (\( P<0.001 \)) at the later time point (25.2±1.5 versus 22.8±1.4 \( \mu m \)).

**Discussion**

When the dynamic network of microtubules within the cardiac cell is proliferated either pathologically (in some models of pressure-overload hypertrophy) or chemically (by taxol), contractility is reduced.\(^3\)–\(^7\) However, in the absence of prior hypertrophic proliferation, evidence strongly suggests that microtubule disruption by colchicine does not modulate contraction.\(^3\)–\(^6\),\(^8\)–\(^14\) However, Gomez et al\(^15\) recently presented data in *Circulation Research* showing that in ruptured patch-clamped myocytes, microtubule disruption by colchicine can increase \( I_{Ca,L} \) and the \([Ca^{2+}]_i\), transient, effects that would be expected to increase contraction, unless myofilament sensitivity was concurrently depressed (for review, see Calaghan and White\(^16\)). We have addressed the apparent inconsistency highlighted by the findings of Gomez et al\(^15\) by undertaking a comprehensive investigation of the effect of colchicine on contraction, \([Ca^{2+}]_i\), and \( I_{Ca,L} \).

The ability of colchicine to disrupt microtubules under the conditions of our experiments was confirmed by the use of immunofluorescence confocal microscopy. We saw a 43% reduction in immunofluorescence intensity in cells treated with 10 \( \mu mol/L \) colchicine for 2 hours; this degree of reduction in immunofluorescence intensity is comparable with the 38% reduction observed in the study of Gomez et al\(^15\) after a 2-hour incubation of rat ventricular myocytes with 1 \( \mu mol/L \) colchicine.

Our findings that microtubule disruption had no effect on the amplitude of the \([Ca^{2+}]_i\), transient or contraction in intact cells nor on the amplitude of \( I_{Ca,L} \) under perforated patch-clamp conditions are consistent with those of many contractile studies reported in the literature.\(^3\)–\(^6\),\(^8\)–\(^14\) In addition, we saw no effect of colchicine on the change in amplitude of contraction, \([Ca^{2+}]_i\), transient (see Palmer et al\(^19\)), or \( I_{Ca,L} \), in response to the \( \beta\)-adrenoceptor agonist isoproterenol. We conclude that in cells with an intact sarcolemma and cytosol, microtubule disruption by colchicine does not activate adenylyl cyclase. These results contrast with measurements of \([Ca^{2+}]_i\), transients and \( I_{Ca,L} \) obtained by Gomez et al\(^15\) in ruptured patch-clamp cells.

However, our own observations on \( I_{Ca,L} \) under ruptured patch conditions agree with those of Gomez et al.\(^15\) In cells treated with colchicine, basal \( I_{Ca,L} \) is enhanced and the response to isoproterenol is blunted. Therefore, it appears that these data and those of Gomez et al\(^15\) are related to some aspect of the ruptured patch-clamp configuration. Our use of \( K^+\)-based pipette solution for the ruptured patch experiments allows us to exclude the possibility that \( Cs^+\)-based solutions\(^15\) contributed to these effects.\(^20\)

Gomez et al\(^15\) proposed that, on microtubule disruption, adenylyl cyclase is activated via nucleotide exchange to \( G_i \) protein by accumulation of free tubulin within the cell. Our lack of response to colchicine in intact cells and under perforated patch conditions argues against this explanation. In ruptured patch conditions, inhibitors of adenylyl cyclase blunt the response to colchicine\(^15\) suggesting that the combination of microtubule disruption and the ruptured patch configuration work together to activate adenylyl cyclase. This configuration can cause more dialysis of the intracellular milieu with the contents of the recording microelectrode than is seen in the perforated patch configuration.\(^21\),\(^22\) Indeed run-down of \( I_{Ca,L} \) in ruptured patch-clamped cells is a common observation that has been related to intracellular dialysis.\(^23\) One possible mechanism that could account for the effect of colchicine in ruptured patch-clamped cells is the loss, through dialysis, of cellular tubulin (or a tubulin-\( G_i \) complex) resulting in decreased cellular activity of \( G_i \). In support of this hypothesis, there is evidence that tubulin can activate \( G^{24,25} \) and, in the cardiac cell, \( G_i \) is in excess of \( G_s \).\(^26\) In our experiments, colchicine appeared to oppose the effect of the ruptured configuration on \( I_{Ca,L} \) run-down.

The ruptured patch-clamp configuration may also be associated with changes in cell shape, and an alternative explanation to account for the effect of colchicine on ruptured patch-clamped myocytes is based on our observation that disruption of the microtubular network by colchicine was associated with a reduction in cell width under ruptured patch conditions. Shrinkage of rat cardiac myocytes has been shown to activate adenylyl cyclase.\(^27\) Alterations in cell size might reflect changes in stress on the sarcolemma and t-tubular system, and there is evidence that adenylyl cyclase is mechanosensitive.\(^28,29\) Early time-dependent potentiation of \( I_{Ca,L} \) after establishment of the whole-cell configuration in ruptured patch has been ascribed to mechanical effects of this configuration.\(^30\)

There are several reports in the literature of pathological conditions (including ischemia, hypothyroidism, and chronic pressure-overload hypertrophy)\(^19,31,32\) in which the cardiac microtubule network is diminished and in which cellular levels of tubulin may be decreased. Based on our observations and re-interpretation of the findings of Gomez et al,\(^15\) the possibility that the loss of cellular tubulin activates adenylyl cyclase or that microtubule disruption renders adenylyl cyclase more susceptible to mechanical stimulation has important implications for such conditions.

**Acknowledgments**

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**References**


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