Response to Research Commentary

Microtubule Disruption by Colchicine Reversibly Enhances Calcium Signaling in Intact Rat Cardiac Myocytes

B.G. Kerfant, G. Vassort, A.M. Gómez

Abstract—Using the whole-cell patch-clamp configuration in rat ventricular myocytes, we recently reported that microtubule disruption increases calcium current ($I_Ca$) and [Ca$^{2+}]_i$, transient and accelerates their kinetics by adenylyl cyclase activation. In the present report, we further analyzed the effects of microtubule disruption by 1 μmol/L colchicine on Ca$^{2+}$ signaling in cardiac myocytes with intact sarcolemma. In quiescent intact cells, it is possible to investigate ryanodine receptor (RyR) activity by analyzing the characteristics of spontaneous Ca$^{2+}$ sparks. Colchicine treatment decreased Ca$^{2+}$ spark amplitude ($F/F_0$: 1.78±0.01, n=983, versus 1.64±0.01, n=1660, recorded in control versus colchicine-treated cells; $P<0.0001$) without modifying the sarcoplasmic reticulum Ca$^{2+}$ load and enhanced their time to peak (in ms: 6.85±0.09, n=1185, versus 7.33±0.13, n=1647; $P<0.0001$). Microtubule disruption also induced the appearance of Ca$^{2+}$ sparks in doublets. These alterations may reflect RyR phosphorylation. To further investigate Ca$^{2+}$ signaling in cardiac myocytes with intact sarcolemma, we analyzed [Ca$^{2+}]_i$, transient evoked by field stimulation. Cells were loaded with the fluorescence Ca$^{2+}$ indicator, Fluo-3 cell permeant, and stimulated at 1 Hz. [Ca$^{2+}]_i$, transient amplitude was greater and its decay was accelerated in colchicine-treated, field-stimulated myocytes. This effect is reversible. When colchicine-treated myocytes were placed in a colchicine-free solution for 30 minutes, tubulin was repolymerized into microtubules, as shown by immunofluorescence, and the increase in [Ca$^{2+}]_i$ transient was reversed. In summary, we demonstrate that microtubule disruption by colchicine reversibly modulates Ca$^{2+}$ signaling in cardiac cells with intact sarcolemma. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2001;88: e59-e65.)

Key Words: microtubules ■ calcium transient ■ calcium sparks ■ adenylyl cyclase

Microtubules, components of the cytoskeleton, are involved in cell locomotion and mitosis. In adult cardiac cells, which do not undergo cell division, the role of microtubules has long been ignored, although interest began to focus on them after Tsutsui et al\(^1\) showed that microtubule tubules has long been ignored, although interest began to focus on them after Tsutsui et al\(^1\) showed that microtubule tubules has long been ignored, although interest began to focus on them after Tsutsui et al\(^1\) showed that microtubule disruption increases Ca$^{2+}$ signaling in cardiac myocytes with intact sarcolemma. In quiescent intact cells, it is possible to investigate ryanodine receptor (RyR) activity by analyzing the characteristics of spontaneous Ca$^{2+}$ sparks. Colchicine treatment decreased Ca$^{2+}$ spark amplitude ($F/F_0$: 1.78±0.01, n=983, versus 1.64±0.01, n=1660, recorded in control versus colchicine-treated cells; $P<0.0001$) without modifying the sarcoplasmic reticulum Ca$^{2+}$ load and enhanced their time to peak (in ms: 6.85±0.09, n=1185, versus 7.33±0.13, n=1647; $P<0.0001$). Microtubule disruption also induced the appearance of Ca$^{2+}$ sparks in doublets. These alterations may reflect RyR phosphorylation. To further investigate Ca$^{2+}$ signaling in cardiac myocytes with intact sarcolemma, we analyzed [Ca$^{2+}]_i$, transient evoked by field stimulation. Cells were loaded with the fluorescence Ca$^{2+}$ indicator, Fluo-3 cell permeant, and stimulated at 1 Hz. [Ca$^{2+}]_i$, transient amplitude was greater and its decay was accelerated in colchicine-treated, field-stimulated myocytes. This effect is reversible. When colchicine-treated myocytes were placed in a colchicine-free solution for 30 minutes, tubulin was repolymerized into microtubules, as shown by immunofluorescence, and the increase in [Ca$^{2+}]_i$ transient was reversed. In summary, we demonstrate that microtubule disruption by colchicine reversibly modulates Ca$^{2+}$ signaling in cardiac cells with intact sarcolemma. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2001;88: e59-e65.)

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The Research Commentary from Calaghan et al\(^20\) analyzes the effects of colchicine treatment on contraction, [Ca$^{2+}]_i$, transient, and $I_Ca$ in adult rat cardiac myocytes in various experimental settings. Their results confirm our data obtained on myocytes under whole-cell or ruptured patch-clamp. However, under perforated patch-clamp, they found no effect of colchicine. Therefore, they suggested that membrane rupture under the patch pipette tip had influenced the colchicine effects.

In the present study, we demonstrate that colchicine treatment modulated Ca$^{2+}$ signaling in freshly isolated intact myocytes. Although the former studies on adult cells\(^1-7\) did not analyze [Ca$^{2+}]_i$, transient, contraction measurements should follow [Ca$^{2+}]_i$, transient unless microtubule disruption modifies myofilament Ca$^{2+}$ sensitivity. To our knowledge, the only publications presenting an analysis of [Ca$^{2+}]_i$, transient during microtubule alteration in adult cardiac myocytes are our recent report, in which an increase in [Ca$^{2+}]_i$, transient after longer colchicine treatment was demonstrated,\(^18\) and one by Howarth et al,\(^19\) who showed a decrease in [Ca$^{2+}]_i$, transient after microtubule stabilization with taxol, suggesting that microtubules may be important regulators of Ca$^{2+}$ handling in the heart.

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cardiac myocytes: the characteristics of spontaneous Ca\(^{2+}\) sparks were altered in a way that is consistent with ryanodine receptor (RyR) phosphorylation. Moreover, the increase in the [Ca\(^{2+}\)] transient induced by microtubule disruption was reversible on colchicine washout.

**Materials and Methods**

**Cell Preparation**

Adult male Wistar rats (250 to 350 g) were anesthetized with sodium pentobarbital (100 mg/kg IP). Hearts were removed, and single ventricular myocytes were enzymatically (collagenase Worthington II, 1 mg/mL) isolated as previously described. Myocytes were stored in a Tyrode solution containing 1 mmol/L CaCl\(_2\) and maintained at room temperature. Some cells were supplemented with 1 μmol/L colchicine (dissolved in DMSO at 0.01% final DMSO concentration) at least 2 hours until used for experiments (2 to 6 hours). Experiments were performed on rod-shaped quiescent Ca\(^{2+}\)-tolerant myocytes at room temperature (24°C to 25°C).

For experiments, myocytes were perfused with a Tyrode solution containing, in mmol/L: NaCl 140, MgCl\(_2\) 0.5, KCl 5, glucose 5.5, HEPES 5, and CaCl\(_2\) 1.8; pH was adjusted to 7.4 with NaOH.

**Imaging of Ca\(^{2+}\) Sparks**

Imaging was performed on Fluo-3–loaded myocytes using a laser scanning confocal microscope (Zeiss LSM 510), coupled to an inverted microscope (Axiovert 100M, Zeiss) and equipped with a 488-nm line of an argon ion laser. Emitted fluorescence was measured at wavelengths over 515 nm. Image acquisition was made in the line-scan mode. A single myocyte was scanned repetitively along a line parallel to the longitudinal cell axis every 1.5 ms. Image processing and analysis of Ca\(^{2+}\) sparks were performed using IDL software (Research Systems), as previously described. Briefly, each image was background-subtracted. The fluorescence transient was obtained by averaging the fluorescence values in a 1.4-μm frame over time. Amplitude was measured as the maximum value of F/F\(_0\), where F is the fluorescence signal and F\(_0\) is the basal fluorescence (measured as the average of the 50 lowest values on the fluorescence transient). Decay time was calculated by fitting the descending phase of the fluorescence trace to a single exponential. Time to peak was measured in two ways: (1) absolute time to peak was measured as the time between the maximum of the second derivative and the peak F/F\(_0\), and (2) time to first peak was measured as the time between the maximum and minimum values of the second derivative. Half-width was measured from a transversal fluorescence transient obtained by averaging the fluorescence values in a 15 ms over space and fitted to a Gaussian function. These IDL routines allowed us to analyze a large number of Ca\(^{2+}\) sparks. When failure to converge occurred, the parameter was disregarded.

**Field-Stimulation Experiments**

Myocytes were loaded with the fluorescence membrane-permeant Ca\(^{2+}\) dye Fluo-3 AM, as previously described. Cells were placed on a Nikon microscope fitted with an epifluorescence attachment. Excitation was provided with a xenon lamp as previously described. The fluorescence signal was digitized (TL-1, Axon Instruments) and acquired at a rate of 200 μs using pClamp 6. Cells were stimulated at a 1.5 excitation threshold through two platinum electrodes at 1 Hz. Data were acquired after 2 to 3 minutes of stabilization.

**Immunolabeling**

Single ventricular myocytes were permeabilized in a microtubule protective buffer, followed by 3% formaldehyde fixation. Antibodies were used as previously described. Confocal images were acquired with the Lasersharp program. Analysis was performed with IDL (Research Systems). 3D reconstruction of the Z-stack was achieved with Imaris (Bitplane).

**Statistics**

Data are presented as mean±SEM. An unpaired Student’s t test was performed to compare control and colchicine-treated cells, and a paired Student’s t test was used to compare drugs effects within each group. P<0.05 was considered significant.

**Results**

**Effects of Microtubule Disruption on Ca\(^{2+}\) Sparks**

Cardiac excitation-contraction (EC) coupling is activated by the calcium-induced calcium release mechanism: membrane depolarization during each action potential activates sarcolemmal L-type calcium currents (I\(_{\text{Ca,L}}\)), leading to a local increase in [Ca\(^{2+}\)], around the neighboring sarcoplasmic reticulum (SR) Ca\(^{2+}\) release channels (RyRs). The activation of RyRs by Ca\(^{2+}\) triggers a large release of Ca\(^{2+}\) that activates cell contraction. The global [Ca\(^{2+}\)] transient is constituted by the spatial and temporal summation of its elementary units, Ca\(^{2+}\) sparks. Ca\(^{2+}\) sparks are localized Ca\(^{2+}\) transients that can be visualized in cardiac myocytes by using confocal imaging and appropriate Ca\(^{2+}\) dyes such as Fluo-3. They correspond to the synchronous activation of a cluster of RyRs. Consequently, in intact cells, RyR activity can be studied by analyzing Ca\(^{2+}\) spark properties.

Spontaneous Ca\(^{2+}\) sparks were analyzed in colchicine-treated myocytes and compared with those recorded in control myocytes. Figure 1 shows averaged line-scan images corresponding to Ca\(^{2+}\) sparks recorded in a cell stored in the absence (Figure 1A) or presence (Figure 1B) of 1 μmol/L colchicine. To provide a better impression of average Ca\(^{2+}\) spark fluorescence intensity before and after colchicine treat-
ment, each image is also represented by its 3D projection. It is clear that the fluorescence intensity decreased after microtubule disruption. Figure 1C shows the bar graph of the averaged Ca\(^{2+}\) spark amplitude. After colchicine treatment, Ca\(^{2+}\) spark amplitude was significantly smaller. The frequency histogram (Figure 1D) shows a shift toward Ca\(^{2+}\) sparks of lesser amplitudes. The decrease in Ca\(^{2+}\) spark amplitude could be due to a smaller SR Ca\(^{2+}\) load. We analyzed the SR Ca\(^{2+}\) load by rapid caffeine application. Figure 2A shows line-scan images of caffeine-induced Ca\(^{2+}\) release in a control (top) and a colchicine-treated cell (bottom). Averaged data (Figure 2B) show that microtubule disruption did not significantly modify SR Ca\(^{2+}\) content in quiescent cells, although there is a tendency to increase. Fluo-3 is a highly sensitive Ca\(^{2+}\) dye, and the fluorescence changes begin to be nonlinear at very high [Ca\(^{2+}\)]. Therefore, it is possible that even if the fluorescence ratio increase did not reach statistical significance after colchicine treatment, there may be an increase in SR Ca\(^{2+}\) load, but in no case would there be a decrease. Thus, the decrease in Ca\(^{2+}\) spark amplitude was not due to a reduction in the SR Ca\(^{2+}\) content.

The characteristics of Ca\(^{2+}\) sparks are given in the Table. In addition to Ca\(^{2+}\) spark amplitude, the only other parameter significantly altered by microtubule disruption was the time to peak, which was prolonged (Figure 3C). Further analysis of the lengthening in time to peak after colchicine treatment led us to observe [Ca\(^{2+}\)] peaks in doublets (Figures 3A and 3B). Then, when we analyzed the time to the first peak instead of the time to the absolute peak, no significant difference was found (Table). More than one peak (generally two) was observed in 9.6% of the Ca\(^{2+}\) sparks recorded after microtubule disruption. The frequency histograms corresponding to the distance (left) and the time (right) between 2 consecutive peaks, when present, in colchicine-treated myocytes.

**Field-Stimulated [Ca\(^{2+}\)] Transients**

We previously reported that microtubule disruption by colchicine increases global [Ca\(^{2+}\)], transient under whole-cell

| Effects of Cell Incubation With 1 \(\mu\)mol/L Colchicine on Ca\(^{2+}\) Spark Characteristics |
|---------------------------------|-----------------|-----------------|
| Amplitude, F/F\(_0\)             | Control n (62)  | Colchicine n (65) |
| Amplitude, F/F\(_0\)             | 1.785±0.014     | 1.641±0.010\(^*\) |
| Decay time, ms                   | 31.868±1.627    | 30.864±1.140    |
| Half-width, \(\mu\)m             | 0.871±0.014     | 0.883±0.014     |
| Time to peak, ms                 | 6.857±0.089     | 7.332±0.128\(^*\) |
| Time to first peak, ms           | 6.314±0.086     | 6.276±0.073     |

\(^*P<0.0001\).

n indicates the number of individual Ca\(^{2+}\) sparks; the number of cells is in parentheses.

Figure 2. SR Ca\(^{2+}\) load is not significantly modified after microtubule disruption in quiescent myocytes. A, Line-scan images obtained during rapid application of 10 mmol/L caffeine in a control (top) and a colchicine-treated (bottom) myocyte. The color-coded scale bar indicates values of F/F\(_0\) ranging from 0 (black) to 5 (white). B, Bar graph corresponding to the caffeine-induced fluorescence [Ca\(^{2+}\)]\(_i\) transients in 29 control cells (white bar) and 20 colchicine-treated myocytes (blue bar).

Figure 3. Colchicine treatment induces Ca\(^{2+}\) sparks in doublets. Examples of a Ca\(^{2+}\) spark recorded in a control (A) and a colchicine-treated (B) myocyte. Below each image, the fluorescence trace (F/F\(_0\), measured as in Figure 1) obtained in the portion of the image indicated by the red line is shown. The color-coded scale is the same as in Figure 1. The image from the colchicine-treated myocyte presents 2 peaks. C, Bar graph showing the time to peak of the fluorescence signal in control (white bar, n=1185) and colchicine-treated cells (blue bar, n=1647); *P<0.0001. D, Frequency histograms indicating the distance (left) and the time (right) between 2 consecutive peaks, when present, in colchicine-treated myocytes.
patch-clamp. This result has been challenged by Calaghan et al. They suggested that the increase in [Ca\(^{2+}\)], transient is related to the whole-cell patch-clamp technique, which implies the rupture of the membrane under the pipette tip. To further examine whether microtubule disruption could modulate Ca\(^{2+}\) signaling in intact myocytes, we analyzed [Ca\(^{2+}\)], transients in field-stimulated myocytes. Figure 4 compares sample traces of fluorescence [Ca\(^{2+}\)], transients obtained in a control and a colchicine-treated myocyte. Isoproterenol application increased the [Ca\(^{2+}\)], transient in the control but was without effect on the colchicine-treated myocyte that originally demonstrated a larger [Ca\(^{2+}\)], transient. Pooled data are shown in Figure 4C. On average, [Ca\(^{2+}\)], transient amplitude increased 1.4-fold after microtubule disruption, whereas isoproterenol (1 \(\mu\)mol/L) significantly increased [Ca\(^{2+}\)], transient in the control myocyte, but it had no effect on the colchicine-treated cell. The total duration of the experiment is shown on the x-axis. The time base of individual [Ca\(^{2+}\)], transients is indicated by the calibration bar. C, Bar graph showing averaged [Ca\(^{2+}\)] transient peaks in 9 control myocytes (white bar) before (open bar) and after (hatched bar) addition of 1 \(\mu\)mol/L ISO. Black bars represent values obtained in 7 colchicine-treated myocytes before (solid bar) and after (hatched bar) ISO application. The fluorescence signal (F) was normalized to the signal before stimulation (F0). D, Time-constant decay of the [Ca\(^{2+}\)] transient (\(\tau\)) obtained by fitting the fluorescence trace to a single exponential. Bars are the same as in panel C for 7 control and 9 colchicine-treated myocytes bathed in a solution containing 1 mmol/L CaCl\(_2\). Colchicine treatment accelerates [Ca\(^{2+}\)], transient decay. ISO accelerates the decay only in control myocytes; *P<0.05, **P<0.01.

**Figure 4.** Colchicine treatment increases [Ca\(^{2+}\)], transient in intact myocytes and prevents \(\beta\)-adrenergic stimulation. Sample traces of [Ca\(^{2+}\)], transient (F, in arbitrary units, a.u.) obtained under field stimulation at 1 Hz in a control (A) and a colchicine-treated (B) ventricular cardiac myocyte in a solution containing 1.8 mmol/L CaCl\(_2\). Isoproterenol (ISO, 1 \(\mu\)mol/L) significantly increased [Ca\(^{2+}\)], transient in the control myocyte, but it had no effect on the colchicine-treated cell. The total duration of the experiment is shown on the x-axis. The time base of individual [Ca\(^{2+}\)], transients is indicated by the calibration bar. C, Bar graph showing averaged [Ca\(^{2+}\)] transient peaks in 9 control myocytes (white bar) before (open bar) and after (hatched bar) addition of 1 \(\mu\)mol/L ISO. Black bars represent values obtained in 7 colchicine-treated myocytes before (solid bar) and after (hatched bar) ISO application. The fluorescence signal (F) was normalized to the signal before stimulation (F0). D, Time-constant decay of the [Ca\(^{2+}\)] transient (\(\tau\)) obtained by fitting the fluorescence trace to a single exponential. Bars are the same as in panel C for 7 control and 9 colchicine-treated myocytes bathed in a solution containing 1 mmol/L CaCl\(_2\). Colchicine treatment accelerates [Ca\(^{2+}\)], transient decay. ISO accelerates the decay only in control myocytes; *P<0.05, **P<0.01.

The decay time (\(\tau\)) of the [Ca\(^{2+}\)], transient was estimated by fitting to a first-order exponential function. Similarly to the decay of the \(I_{Ca}\)-evoked [Ca\(^{2+}\)], transient, the field-stimulated [Ca\(^{2+}\)], transient decay was accelerated in colchicine-treated cells (reduced to \(~75\%\) (Figure 4D). This is consistent with the reduction to 80% reported by Calaghan et al\(^{20}\) at the same [Ca\(^{2+}\)], (see their Table). Sarcoplasmic reticulum (SR) Ca\(^{2+}\)-ATPase (SERCA) is the main factor contributing to [Ca\(^{2+}\)], transient decay in rat cardiac myocytes.\(^{26}\) Its activity is enhanced after phospholamban phosphorylation by protein kinase A (PKA).\(^{26}\) The faster [Ca\(^{2+}\)], transient decay after colchicine treatment is in line with such a PKA activation. Furthermore, as is shown in Figure 4D, isoproterenol reduced the [Ca\(^{2+}\)], transient decay time in control cells, but it had no significant effect after microtubule disruption.

These results are consistent with our previous report on patch-clamped myocytes showing both an increase in [Ca\(^{2+}\)], transient and a loss of \(\beta\)-adrenergic effect after colchicine treatment, as well as with the proposal that PKA is activated after G\(_i\) protein stimulation by free tubulin.\(^{18}\)

**Rescue of the Microtubule Network**

It is often assumed that microtubule disruption by colchicine is poorly reversible; thus, most experiments are performed in control conditions on colchicine-treated cells. Figure 5A shows anti-\(\beta\)-tubulin–labeled images of a control (top) and a colchicine-treated (middle) myocyte. Colchicine treatment markedly disrupted the microtubules, which was reflected by a decrease in fluorescence (Figure 5B). However, the immunolabeling of cells that were similarly treated by colchicine and then allowed a 30-minute washout demonstrated polymerized microtubules (Figure 5A, bottom), which was reflected by an increase in fluorescence (Figure 5B).

Microtubule repolymerization after colchicine removal may have functional consequences. This is illustrated by the comparison of [Ca\(^{2+}\)], transients evoked by field stimulation on a colchicine-treated cell at the beginning of the experiment (Figure 5C, a\(_1\)) and after a 30-minute colchicine washout (Figure 5C, a\(_2\)). After this delay, [Ca\(^{2+}\)], transients had diminished toward control values. However, when colchicine was present throughout the experiment, the fluorescence [Ca\(^{2+}\)], transient remained about constant over the 30-minute period (Figure 5C, b). Average variations are presented in Figure 5C, bottom.

**Discussion**

Microtubules, which are components of the cytoskeleton, have a structural role and are generally acknowledged to be important to cell locomotion and mitosis. In adult cardiac myocytes, their role has been neglected because of their relatively low abundance. Tubulin concentration in these cells represents \(~0.01\%\) of total myocyte protein.\(^{27,28}\) However, a role as a second messenger could be important, because free tubulin has been shown to bind and activate G proteins.\(^{29–33}\) In general, the biological function of microtubules is based on the ability of tubulin to polymerize and depolymerize. Proper control of microtubule dynamics is essential for many microtubule-dependent processes. The present work extends our previous data.\(^{18}\) In the present study, we demonstrate that microtubule disruption modulates Ca\(^{2+}\) signaling in intact rat...
ventricular myocytes. Microtubule depolymerization by colchicine modulates spontaneous Ca\textsuperscript{2+} spark characteristics in quiescent cells and increases [Ca\textsuperscript{2+}], transient in field-stimulated cells. Furthermore, we show that the colchicine effects are partially reversible.

We previously reported that adenyl cyclase (AC) is involved in the colchicine-induced alteration of Ca\textsuperscript{2+} signaling. The resulting PKA activation induces phosphorylation of the L-type Ca\textsuperscript{2+} channel. This would account for the increase in the whole-cell \(I_{\text{Ca}}\) and global [Ca\textsuperscript{2+}], transient.\textsuperscript{18} PKA has also been shown to phosphorylate RyRs,\textsuperscript{14} which dissociates them from FKBP12.6\textsuperscript{35} and sorcin.\textsuperscript{36} Furthermore, dissociation of FKBP12.6 from RyR channels incorporated into planar bilayers results in subconductance states because of uncoupling of the RyRs that will not open synchronously.\textsuperscript{35} The decrease in Ca\textsuperscript{2+} spark amplitude found in cardiomyocytes after microtubule disruption may well reflect these effects.

During the 30-minute waiting period, the cell was perfused by a colchicine-free (hatched bar, \(n=5\)) or colchicine-containing (1 \(\mu\)mol/L, black bar, \(n=4\)) solution. All data were obtained in a bathing solution containing 1.8 mmol/L Ca\textsuperscript{2+} and no colchicine. \(P<0.05\) compared with the same cells at the beginning of the experiments.

**Figure 5.** Effects of colchicine on the microtubule network and [Ca\textsuperscript{2+}], transient are reversible on washout. A, 3D reconstruction of confocal images taken along the z-axis after immunolabeling with anti-\(\beta\)-tubulin in rat cardiomyocytes maintained in control solution (top), after 3-hour incubation with 1 \(\mu\)mol/L colchicine (middle), and after 30-minute colchicine washout (bottom). B, Bar graph showing averaged immunofluorescence in control (white bar, \(n=15\)) and colchicine-treated cells before (black bar, \(n=5\)) and after (hatched bar, \(n=24\)) 30-minute washout. C, Top, Examples of [Ca\textsuperscript{2+}]\textsubscript{i} transients in a colchicine-treated myocyte elicited by field stimulation at 1 Hz before (a\textsubscript{1}) and after (a\textsubscript{2}) 30-minute colchicine washout. b\textsubscript{1} and b\textsubscript{2} show the same experimental protocol as in a\textsubscript{1} and a\textsubscript{2}, except the cell was continuously perfused by a colchicine-containing solution. Bottom, Bar graph showing averaged amplitude of fluorescence [Ca\textsuperscript{2+}] transient (\(F/F_0\) measured as in Figure 4) at the end of the 30-minute period (\(F/F_{0\text{end}}\)) normalized to the [Ca\textsuperscript{2+}] transient amplitude recorded in the same cell before the 30-minute period (\(F/F_{0\text{begin}}\)). During the 30-minute waiting period, the cell was perfused by a colchicine-free (hatched bar, \(n=5\)) or colchicine-containing (1 \(\mu\)mol/L, black bar, \(n=4\)) solution. All data were obtained in a bathing solution containing 1.8 mmol/L Ca\textsuperscript{2+} and no colchicine. \(P<0.05\) compared with the same cells at the beginning of the experiments.

Despite the difficulty, or impossibility, of accurately measuring \(I_{\text{Ca}}\) in rat ventricular myocytes without blocking K\textsuperscript{+}

Figure 6. Proposed mechanism of EC coupling modulation after microtubule disruption. Colchicine treatment induces an increase in the free \(\alpha\)\(\beta\)-tubulin that activates G protein and AC. PKA will be activated by the consequent elevation in cAMP and phosphorylate: Ca\textsuperscript{1+} channel (DHPR [dihydropyridine receptor]), increasing Ca\textsuperscript{2+} influx (\(I_{\text{Ca}}\)), and triggered Ca\textsuperscript{2+} release; phospholamban (PLN), accelerating Ca\textsuperscript{2+} uptake by SERCA; and RyRs, altering the characteristics of Ca\textsuperscript{2+} sparks.

At first glance, our results in field-stimulated myocytes (Figure 4) are inconsistent with the data presented by Calaghan et al.\textsuperscript{20} Our two sets of results differ in two aspects: the maintenance or nonmaintenance of the full \(\beta\)-adrenergic response and the similarity or difference of the [Ca\textsuperscript{2+}], transient amplitude in control and colchicine-treated cells. As shown in their Table, Calaghan et al\textsuperscript{20} observed that colchicine, at 10 \(\mu\)mol/L, induced a significant acceleration of the [Ca\textsuperscript{2+}], transient decay in intact myocytes, as we did. Also, from the 30 cells presented in their Table, Calaghan et al studied the isoproterenol response in a subset of 9 cells. This subpopulation exhibited [Ca\textsuperscript{2+}], transient characteristics, such as the decay time constant, that were significantly different from those of the whole population (compare Table and Figure 3 in Calaghan et al\textsuperscript{20}). Their conclusion should thus be taken with caution. Furthermore, the bathing solution used by Calaghan et al\textsuperscript{20} contained insulin, which is known to increase \(I_{\text{Ca}}\) in rat cardiac myocytes.\textsuperscript{37} Insulin effects on rat ventricular myocytes are only seen in cells with an intact microtubule network.\textsuperscript{38} It is thus possible that [Ca\textsuperscript{2+}], transients were similar in control and colchicine-treated cells because \(I_{\text{Ca}}\) was increased by insulin only in the control myocytes, whereas it was increased by microtubule disruption in the colchicine-treated cells. An alternative explanation would be the low-stimulation frequency used by Calaghan et al\textsuperscript{20} twice as slow as the stimulation frequency they used in another study on the decrease in [Ca\textsuperscript{2+}], transient after microtubule stabilization.\textsuperscript{19}
currents, Calaghan et al\textsuperscript{20} studied $I_{Ca}$ under two variations of the same technique: conventional whole-cell patch-clamp and perforated patch-clamp. These investigators have obtained different results with these two techniques and suggested two major mechanisms to account for the different results: (1) the loss of cellular tubulin activates AC and (2) microtubule disruption renders cells more susceptible to mechanical stimulation.

The first suggestion is weakened by the following observations: (1) If free tubulin acts by binding and activating G\textsubscript{i} protein in cardiac myocytes, after microtubule stabilization with taxol less tubulin will be free to activate G\textsubscript{i}. Thus, no change or increase in [Ca\textsuperscript{2+}]\textsubscript{i} transient would be expected after taxol application to intact cells. However, the opposite was demonstrated by Howarth et al.\textsuperscript{19} (2) Perforated patch-clamp was initially developed to reduce dialysis in small and spherical cells.\textsuperscript{39} Given the cardiac myocyte shape and the molecular weight of the complex $\alpha$\textbeta-tubulin–G\textsubscript{i} protein, it is unlikely that free tubulin is rapidly lost. Indeed, we recorded an increased $I_{Ca}$ in colchicine-treated ventricular myocytes even just after rupture of the patch (see Figure 7 in Reference 18) that argues against the dialysis dependence of microtubule-disruption effects. (3) It is widely accepted that carbachol activates G\textsubscript{i} protein and thus inactivates AC and decreases $I_{Ca}$, only if this pathway has been previously stimulated by PKA activation.\textsuperscript{40} After microtubule disruption, muscarinic stimulation effectively decreases $I_{Ca}$ on whole-cell patch-clamped myocytes (Gallo MP, Malan D, Bendeti I, Biasin C, Alloatti G, Levi R, unpublished data, 2000) and isoproterenol recovers its activity (data not shown). This demonstrates both that the lack of isoproterenol effect is not due to intracellular dialysis and that G\textsubscript{i} protein is not lost by dialysis. The G\textsubscript{i} protein pathway is predominant in adult cardiac myocytes, whereas the G\textsubscript{i} protein is predominant in neonatal myocytes.\textsuperscript{41} The entire set of data presented in this report and observed in intact cells argues against this hypothesis.

The finding by Calaghan et al\textsuperscript{20} that after 13 minutes of ruptured-patch achievement colchicine-treated myocytes are shrunk—compared with what occurs in control myocytes after 10 minutes—is surprising assuming no osmolarity differences between solutions and no negative pressure inside the pipette. Nevertheless, the suggestion that AC is activated as a result of mechanical stimulation, if any, in patch-clamp does not hold because the family of AC that is activated by cell deformation is not present in heart. Cardiac myocytes contain mainly type V and VI of AC, which are colocalized with the L-type Ca\textsuperscript{2+} channel\textsuperscript{42} and inhibited by low [Ca\textsuperscript{2+}]\textsubscript{i}.\textsuperscript{43–46} The results presented in the present report were performed in myocytes with intact sarcolemma (neither ruptured nor perforated) and were consistent with the activation of AC after microtubule disruption we reported earlier. We observed that a 30-minute washout period in a colchicine-free solution allows for rescue of the microtubule network in colchicine-treated myocytes (Figure 5). Furthermore, together with tubulin repolymerization, [Ca\textsuperscript{2+}]\textsubscript{i} transient elicited in field-stimulated myocytes is decreased. Other authors have also reported tubulin repolymerization on cardiac myocytes\textsuperscript{10} and isolated rat hearts.\textsuperscript{47}

The discordance between the results of Calaghan et al\textsuperscript{20} obtained under two variations of the patch-clamp technique is unexpected. The strength of their argument is, however, weakened first by the technical difficulties related to the large series resistance in the perforated mode and, more importantly, by the maze of steps required to measure $I_{Ca}$ in rat ventricular myocytes without effectively blocking K\textsuperscript{+} currents. Rat cardiocytes exhibit several outward K\textsuperscript{+} currents, including a large transient one ($I_{k}$), in a range of potentials overlapping with the one inducing activation of the inward $I_{Ca}$.\textsuperscript{48,49} Furthermore, the data of Calaghan et al in intact cells is not internally consistent (compare Table and Figures 3D and 2C in Calaghan et al\textsuperscript{20} under the same conditions). They do find an effect of colchicine in intact cells: an increase in [Ca\textsuperscript{2+}], ($P<0.01$) and an acceleration of [Ca\textsuperscript{2+}] transient decay ($P<0.001$). This last effect is characteristic of AC activation. Colchicine, in their hands, blocks some response to isoproterenol (their Figure 3C). The presence of insulin in their bathing solution and the selection of cells to analyze \beta-adrenergic response (see previous discussion) may account for the apparent discrepancy with our results. In any case, they do observe some effects of colchicine on intact cardiomyocytes that would instead suggest that the perforated patch introduces some pitfalls, leading to a masking of colchicine effects.

Taken together, the present results demonstrate that microtubule disruption by colchicine does modulate Ca\textsuperscript{2+} handling in intact ventricular myocytes. Both Ca\textsuperscript{2+} spark characteristics in quiescent myocytes and Ca\textsuperscript{2+} transients in field-stimulated cardiomyocytes are altered by microtubule disruption. Microtubules in heart may thus have important implications in physiological and pathological conditions in which the level of tubulin polymerization is altered.

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