Propagation of Cardiomyocyte Hypercontracture by Passage of Na⁺ Through Gap Junctions

Marisol Ruiz-Meana, David Garcia-Dorado, Bettina Hofstaetter, H. Michael Piper, Jordi Soler-Soler

Abstract—Prolonged ischemia increases cytosolic Ca²⁺ concentration in cardiomyocytes. Cells with severely elevated cytosolic Ca²⁺ may respond to reperfusion, developing hypercontracture, sarcolemmal disruption, and death. Cardiomyocytes are efficiently connected through gap junctions (GJs) to form a functional syncytium, and it has been shown that hypercontracture can be propagated to adjacent myocytes through a GJ-mediated mechanism. This study investigated the mechanism of propagation of cell injury associated with sarcolemmal rupture in end-to-end connected pairs of isolated rat cardiomyocytes. Microinjection of extracellular medium into one of the cells to simulate sarcolemmal disruption induced a marked increase in cytosolic Ca²⁺ (fura-2) and Na⁺ (SBFI) in the adjacent cell and its hypercontracture in <30 seconds (22 of 22 cell pairs). This process was not modified when Ca²⁺ release from the sarcoplasmic reticulum was blocked with 10 μmol/L ryanodine (5 of 5 cell pairs), but it was fully dependent on the presence of Ca²⁺ in the extracellular buffer. Blockade of L-type Ca²⁺ channels with 10 μmol/L nifedipine did not alter propagation of hypercontracture. However, the presence of 15 to 20 μmol/L KB-R7943, a highly selective blocker of reverse Na⁺/Ca²⁺ exchange, prevented propagation of hypercontracture in 16 of 20 cell pairs (P<0.01) without interfering with GJ permeability, as assessed by the Lucifer Yellow transfer method. Addition of the Ca²⁺ chelator EGTA (2 mmol/L) to the injection solution prevented hypercontracture in the injected cell but not in the adjacent one (n=5). These results indicate that passage of Na⁺ through GJ from hypercontracting myocytes with ruptured sarcolemma to adjacent cells, and secondary entry of [Ca²⁺], via reverse Na⁺/Ca²⁺ exchange, can contribute to cell-to-cell propagation of hypercontracture. This previously unrecognized mechanism could increase myocardial necrosis during ischemia-reperfusion in vivo and be the target of new treatments aimed to limit it. (Circ Res. 1999;85:280-287.)

Key Words: hypercontracture • gap junction • propagation • ischemia • reperfusion

Gap junctions (GJs) play an essential role in the normal function of the cardiovascular system; they mediate the spread of electrical impulses that stimulate synchronized contraction of the cardiac muscle and coordinate the function of endothelial and smooth muscle cells in arterial walls.1,2 GJ-mediated communication is also essential for normal embryonic development as well as for the function of many other adult organs, including the lungs, retina, lens, liver, and central nervous system.3–5 Recent studies have demonstrated that neuron GJs remain open during brain ischemia, therefore linking dying cells with potentially viable ones, and that cerebral infarcts may expand over time by a GJ-mediated mechanism.6 The extent of ischemic injury can be significantly decreased when GJ permeability is reduced with octanol.7

However, the role of GJs in chemical communication of cardiac muscle cells, and in particular their possible involvement in cell death secondary to ischemia-reperfusion, is poorly understood. After an ischemic episode, cardiomyocytes may respond to reenergization by developing an abrupt and extreme distortion of their architecture, resulting from development of excessive contractile force.8 This phenomenon, named hypercontracture, causes extreme cell shortening and may occur during the first minutes of reflow as the consequence of ATP availability in the presence of abnormally high [Ca²⁺] intraocular pressure and cytoskeletal fragility caused by ischemia.9,10 Reperfused myocardial infarcts consist almost exclusively of areas of contraction band necrosis formed by hypercontracted dead myocytes.11 A striking feature of these areas of necrosis is that they usually contain hypercontracted cardiomyocytes in compact clusters, with highly indented and irregular borders or even a patchy appearance, whereas hypercontracted cardiomyocytes in scattered distribution are virtually absent.12

Received December 7, 1998; accepted May 28, 1999.
From the Department of Cardiology (M.R-M., D.G-D., J.S-S.), Hospital General Universitario Vall d’Hebron, Barcelona, Spain, and Justus-Liebig-Universität (B.H., H.M.P.), Physiologisches Institut, Giessen, Germany.
Correspondence to David García-Dorado, MD, PhD, Department of Cardiology, Hospital General Universitario Vall d’Hebron, Pg. Vall d’Hebron 119-129, Barcelona 08035, Spain. E-mail dgdorado@hge.hvhebron.es
© 1999 American Heart Association, Inc.
Circulation Research is available at http://www.circresaha.org

280
necrosis but cannot explain the continuity of these areas and the absence of scattered hypercontracted myocytes. Studies using computer simulation of infarct morphology demonstrate that some kind of cell-to-cell interaction must be taken into account to reproduce these features of infarct geometry. In a recent study, we found that the GJ blocker heptanol, applied only at the time of reenergization, was able to reduce the final extent of myocardial necrosis and to alter infarct geometry in intact rat and pig hearts. At the cellular level, we observed that hypercontracture of a myocyte was consistently propagated to the adjacent cell and that chemical uncoupling with heptanol prevented this spreading of cell injury. Moreover, these effects of heptanol were observed at concentrations that did not afford any protection against reoxygenation-induced hypercontracture in single cells. These studies strongly support the hypothesis that the fate of a cardiomyocyte after an ischemic period is not only determined by its own biochemical derangements but may also be influenced by the survival or death of adjacent cells. There is ample evidence that, in contrast to what happens in isolated cardiomyocytes, in myocardial tissue hypercontracture is associated with sarcotermal rupture and enzyme release. Sarcolemmal disruption causes dramatic changes in cytosolic composition that can be propagated to adjacent cells through GJs and result in cell-to-cell propagation of hypercontracture. The present study was aimed at identifying the cytosolic composition that can be propagated to adjacent cells by replacing Ca\(^{2+}\) by 2 mmol/L EGTA. To assess GJ permeability, 2% Lucifer Yellow was added to the microinjection solution in a series of experiments. All experiments were performed at 37°C (Digital Warm Stage Controller, Linkam) on the stage of an inverted microscope (Olympus IMT-2). Cell images were continuously video-recorded at ×400 magnifications. Relative changes in cell length were measured on the videorecorded images. Measurements were performed in both cells every 2 seconds during the first 30 seconds after microinjection, except when indicated otherwise. Transfer dye through GJs was monitored under fluorescent microscopy using a 420-nm excitation light with a bandwidth of 15 nm.

### Materials and Methods

#### Cardiomyocyte Isolation Procedure

Animals were handled according to the Declaration of Helsinki, and the experimental procedures were approved by the Research Commission of the Hospital Vall d’Hebron. Ventricular heart muscle cells were isolated from adult male Sprague-Dawley rats (300 g) as previously described. Briefly, whole hearts were retrogradely perfused in a Langendorff system with a modified Krebs buffer (in mmol/L, NaCl 110, KCl 2.6, KH\(_2\)PO\(_4\) 1.2, MgSO\(_4\) 1.2, NaHCO\(_3\) 25, and glucose 11) containing 45 mmol/L CaCl\(_2\) to simulate sarcolemmal disruption, and its propagation between end-to-end connected cell pairs was analyzed under different interventions.

#### Microinjection and Experimental Conditions

One of the cells of each pair was microinjected with extracellular medium to simulate sarcolemmal disruption occurring during reoxygenation-induced hypercontracture. A hydraulic micromanipulator with digital control (digital micromanipulator model 5171 and transector model 5246, Eppendorf) was used. Microinjection was performed through a 0.2- to 0.5-μm-tip sterile micropipette (Sterile Femtotips, Eppendorf) by a pulse pressure of 400 hPa of 0.1-second duration. The axial and depth limits were set to allow penetration of the micropipette 3 to 5 μm below cell surface at an angle of 45°.

### Results

#### Role of [Ca\(^{2+}\)], in the Propagation of Hypercontracture

In control conditions (extracellular and microinjected solutions composed of HEPES buffer with ionic composition...
mimicking physiological extracellular media), microinjection of extracellular buffer consistently induced hypercontracture of the injected myocyte, and the rapid rise in cytosolic Ca$^{2+}$ concentration in the microinjected cell was followed within a few seconds by a similar rise in the adjacent cell and by its hypercontracture (Figure 1). However, when Ca$^{2+}$ was replaced in the extracellular medium by the Ca$^{2+}$ chelator EGTA, but not in the injected buffer, the rise in Ca$^{2+}$ concentration and hypercontracture observed in the injected myocyte were not propagated to the adjacent cell in 16 of 18 cell pairs (Figure 2). Failure of propagation of hypercontracture in the absence of [Ca$^{2+}$], was not due to reduced GJ permeability, because transfer of the GJ-permeant dye Lucifer Yellow from the microinjected to the adjacent cell was not modified in the absence of [Ca$^{2+}$], (Figure 2).

On the basis of the observed role of [Ca$^{2+}$], in propagation of hypercontracture, we investigated L-type Ca$^{2+}$ channels as a likely route of Ca$^{2+}$ entry into the adjacent cell. However, this possibility was ruled out by repeating the microinjection in the presence of 10 μmol/L of the channel blocker nifedipine. Blockade of L-type Ca$^{2+}$ channels did not modify either Ca$^{2+}$ rise in the adjacent cell or propagation of hypercontracture (Figure 2).

Ca$^{2+}$ release from the sarcoplasmic reticulum (SR) had no effect on cell propagation of hypercontracture. Addition of ryanodine to the extracellular medium (10 μmol/L for 15 minutes) did not prevent Ca$^{2+}$ rise or hypercontracture in the adjacent cell (Figure 2).

**Passage of Na$^{+}$ and Role of Reverse Na$^{+}$/Ca$^{2+}$ Exchange**

We used ratiofluorescence imaging of cells loaded with the Na$^{+}$ indicator SBFI to demonstrate a rapid and almost simultaneous increase in cytosolic Na$^{+}$ concentration in the microinjected and the adjacent cell (Figure 3). To determine the role of reverse Na$^{+}$/Ca$^{2+}$ exchange in the increase of [Ca$^{2+}$], concentration and hypercontracture of the adjacent cell, microinjection of extracellular medium was performed in the presence of the novel isothiourea derivative KB-R7943, a highly selective blocker of Na$^{+}$/Ca$^{2+}$ exchange in its reverse.
Addition of 20 μmol/L of KB-R7943 to the extracellular medium prevented propagation of hypercontracture in 12 of 15 cell pairs and markedly delayed it in the remaining 3 (Figure 3). A similar effect was observed at 15 μmol/L (propagation was observed only in 1 of 5 cell pairs). However, at 10 μmol/L of KB-R7943, propagation was observed in 12 of 13 cell pairs, although it was significantly delayed (after 30 seconds of microinjection, hypercontracture was propagated in only 6 of 13 cell pairs). KB-R7943 at 15 μmol/L had no influence on either cell shortening (10.3±0.4% and 10.0±0.5%, respectively, in the presence and the absence of the drug) or Ca²⁺ transients induced by field stimulation (Figure 4). Despite the absence of propagation of hypercontracture, KB-R7943 at 20 μmol/L did not alter GJ permeability, as assessed by cell-to-cell diffusion of Lucifer Yellow (Figure 3).

The role of passage of Na⁺ through GJ and subsequent exchange with [Ca²⁺], in cell-to-cell propagation of hypercontracture was further tested in a series of experiments in which Ca²⁺ was replaced by 2 mmol/L EGTA in the microinjection buffer. This consistently resulted in hypercontracture of the adjacent cell despite the absence of any change in length in the microinjected cell (Figure 3) and clearly ruled out the mechanical distortion of GJs as the main cause of propagation of hypercontracture. The fact that the hypercontracture in the adjacent cell is delayed in these experiments may indicate that passage of Ca²⁺ from the primarily injured cell can contribute to hypercontracture of the adjacent cell.
Figure 3. Role of passage of Na⁺ through GJs in propagation of hypercontracture. A, Sequential SBFI ratiofluorescence images from a pair of myocytes obtained after microinjection of one of them (arrow), demonstrating a rapid increase in intracellular Na⁺ concentration in both cells. The last image (bottom right) is separated from the first (top left) by 60 seconds. *Position of intercalated disc; arrow indicates micropipette. The end of a third cell, very close to the injected cell but without physical contact with it, can also be observed (broken line). B, Average changes in SBFI ratio in the microinjected cell (○) and the adjacent one (●) in 6 experiments performed under control conditions. Change in ratio (Δ) at a given time is defined as the value measured at that time divided by the initial value before microinjection. C, Effect of blockade of reverse Na⁺/Ca²⁺ exchange on propagation of hypercontracture in the presence of KB-R7943 in the extracellular medium, immediately before (left) and 240 seconds after microinjection (right). *Position of the intercalated disc. Note that despite chemical coupling (passage of Lucifer Yellow from microinjected to adjacent cell), blockade of reverse Na⁺/Ca²⁺ exchange prevented propagation of hypercontracture. D, Average changes in cell length in 15 experiments performed in the presence of KB-R7943. In 12 of 15 cases, blockade of reverse Na⁺/Ca²⁺ exchange prevented propagation of hypercontracture. In the other 3 cell pairs, hypercontracture was propagated, although with some delay. E, Microinjection of Ca²⁺-free buffer containing 2 mmol/L EGTA.
Ca\textsuperscript{2+} myocytes.\textsuperscript{21–25} It could be expected that direct passage of cell-to-cell signaling in many cell types, including cardiomyocytes, does not represent an additive route of Ca\textsuperscript{2+} entry from the extracellular space or Ca\textsuperscript{2+} release from intracellular stores. Our results demonstrate that propagation of microinjection-induced hypercontracture to adjacent cells is fully dependent on [Ca\textsuperscript{2+}], and is caused by the passage of Na\textsuperscript{+} through GJs that, in turn, is able to induce Ca\textsuperscript{2+} entry by the reverse mode of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. L-type Ca\textsuperscript{2+} channels do not represent an additive route of Ca\textsuperscript{2+} entry from the extracellular medium to the adjacent cell, given that blockade of the channels with 10 \textmu mol/L of nifedipine did not modify Ca\textsuperscript{2+} rise in the adjacent cell nor propagation of hypercontracture (Figure 2). Similar results were obtained with the inorganic Ca\textsuperscript{2+} channel blocker Ni\textsuperscript{2+} (data not shown). The potential role of Ca\textsuperscript{2+} release from the SR in the rise in [Ca\textsuperscript{2+}], concentration and hypercontracture of the adjacent cell was ruled out in experiments in which hypercontracture was induced in control extracellular buffer containing 10 \textmu mol/L ryanodine. This blocker of Ca\textsuperscript{2+} release from the SR had no effect on Ca\textsuperscript{2+} rise or hypercontracture (Figure 2). These results also demonstrate that rapid and important increases in cytosolic Ca\textsuperscript{2+} concentration associated with sarcolemmal rupture do not result in GJ closure rapid enough to prevent propagation of cell injury.

Transmission of cAMP, IP\textsubscript{3}, or Ca\textsuperscript{2+} has been involved in cell-to-cell signaling in many cell types, including cardiomyocytes.\textsuperscript{21–25} It could be expected that direct passage of Ca\textsuperscript{2+} through GJs without the need of Ca\textsuperscript{2+} entry from the extracellular space could suffice for cell-to-cell propagation of hypercontracture. Our results demonstrate that this is not the case. A possible explanation is that GJ permeability is closely regulated by [Ca\textsuperscript{2+}], concentration.\textsuperscript{26} Previous studies have shown that GJs have a low ability to transmit Cu\textsuperscript{2+},\textsuperscript{27,28} and GJ closure caused by exposure to extracellular levels of Ca\textsuperscript{2+} could prevent passage of sufficient Ca\textsuperscript{2+} ions as to induce hypercontracture in the adjacent cell. Transfer of Na\textsuperscript{+} ions, because of the large gradient in cytosolic Na\textsuperscript{+} concentration between the microinjected and the adjacent cell, may allow Ca\textsuperscript{2+} influx into the second cell through the reverse Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange after GJ closure. GJs have been shown to allow efficient equalization of cytosolic Na\textsuperscript{+} concentration in other cell types.\textsuperscript{29}

The observation that passage of Na\textsuperscript{+} through GJs can propagate hypercontracture to adjacent cardiomyocytes may have important implications. There is strong evidence that during myocardial reperfusion, hypercontracture is often associated with sarcolemmal disruption\textsuperscript{6} because of the effect of excessive mechanical stress on a membrane with reduced mechanical resistance.\textsuperscript{16} Membrane disruption allows influx of extracellular medium with high Na\textsuperscript{+} concentration into the hypercontracting cell, a situation mimicked by microinjection of extracellular medium. Although GJs may be closed during ischemia, they are expected to reopen rapidly in response to oxidative metabolism. The proposed mechanism of cell-to-cell propagation of hypercontracture may be thus operative during myocardial reperfusion in vivo. The fact that cell-to-cell propagation of hypercontracture depends on sarcolemmal rupture could impose a limit and explain why it does not progress to the whole heart. Cells exposed to less severe ischemia, as those receiving collateral flow, could withstand with normal [Ca\textsuperscript{2+}]\textsubscript{o} concentration (1 mmol/L). Images of a cell pair obtained before microinjection (left) and 60 seconds after microinjection (right) demonstrate hypercontracture of the adjacent cell but not of the microinjected one. F. Average changes in cell length in 5 cell pairs microinjected with extracellular buffer in which Ca\textsuperscript{2+} had been replaced by EGTA. In all cases, ● indicates injected myocyte; ○, adjacent cell. Data are mean±SEM.
hypercontracture without developing sarcolemmal rupture and Na⁺ overload. If the amount of Na⁺ is not enough to promote exchange in the reverse mode, propagation would be interrupted.

The sarcolemmal Na⁺/Ca²⁺ exchanger is a major regulator of [Ca²⁺], homeostasis in excitable cells. In most mammalian cardiac muscle cells, the exchanger works mainly in the forward mode, i.e., as rapid extruder for Ca²⁺ that has entered the cardiomyocyte via the sarcolemmal L-type Ca²⁺ channels to trigger the release of Ca²⁺ from the SR. Available evidence suggests that in heart, the exchanger competes for Ca²⁺ with the SR Ca-ATPase to bring about relaxation to a degree dependent on species, developmental stage, and/or physiological state of the heart. However, under pathological conditions such as ischemia-reperfusion injury, the direction of its electrogenic exchange can be changed, and the exchanger is thought to cause Ca²⁺ overload because of an ischemia-dependent increase in Na⁺. Ca²⁺ influx through reverse Na⁺/Ca²⁺ exchange may suffice to induce contraction when intentionally driven into that mode of action. In our study, addition of the reverse Na⁺/Ca²⁺ exchange inhibitor KB-R7943 prevented propagation of hypercontracture in most cases at concentrations of 15 μmol/L or greater. KB-R7943 has been shown to act as a potent blocker of reverse Na⁺/Ca²⁺ exchange in rat cardiomyocytes (90% inhibition at 10 μmol/L) without significant effects on other ion transporters or channels at concentrations <30 μmol/L. At 30 μmol/L, KB-R7943 inhibited the dihydropyridine-sensitive Ca²⁺ uptake by 35% and forward Na⁺/Ca²⁺ exchange by 38% without significant influence on Na⁺/H⁺ exchange, sarcolemmal or SR Ca²⁺ ATPase, or Na⁺/K⁺ ATPase activities.

In our study, the concentration of KB-R7943 that effectively prevented propagation of hypercontracture had no significant effect on Ca²⁺ transients or systolic cell shortening of isolated myocytes submitted to field stimulation. KB-R7943 also slowed hypercontracture of the microinjected cell, which suggests that the reverse mode of Na⁺/Ca²⁺ exchange represents one of the routes of Ca²⁺ entry in myocytes primarily injured with microinjection.

The role of passage of Na⁺ through GJs and subsequent exchange with [Ca²⁺], in cell-to-cell propagation of hypercontracture was further confirmed when Ca²⁺ was replaced by EGTA in the microinjection buffer. This consistently resulted in hypercontracture of the adjacent cell, at a slower rate than in control conditions, despite the absence of any change in length in the microinjected cell (Figure 3). This result constitutes direct evidence that propagation of hypercontracture may occur independently of any physical distortion of GJs secondary to mechanical forces imposed by hypercontracture on intercalated discs. However, a possible role of mechanical interaction in sarcolemmal disruption occurring during hypercontracture cannot be excluded, as was previously suggested. In previous studies injection of EGTA in one cell has not been found to reduce cytosolic Ca²⁺ concentration in adjacent, GJ-connected cells, which indicates that EGTA does not significantly diffuse through GJs. The slower rate of hypercontracture in the second cell in these experiments could suggest that direct passage of Ca²⁺ through GJs, although not sufficient to induce hypercontracture of the second cell, may contribute to its development.

The results presented here encourage the study of other potential deleterious effects of GJ-mediated chemical communication in other multicellular systems. Harmful chemical exchange in tissues with well-developed cell-to-cell communication, such as liver or central nervous system, could represent a more generalized mechanism associated with ischemia or hypoxia. In myocardial tissue, cell-to-cell propagation of hypercontracture due to the passage of Na⁺ through GJs could represent a previously unrecognized mechanism of cell death secondary to ischemia-reperfusion. This mechanism could allow propagation of hypercontracture of severely injured cells to other cells with milder ischemic injury that could have otherwise survived. Considering that myocytes are connected to multiple cells through GJs, even a small probability of cell-to-cell progression of hypercontracture could have a major impact in infarct size. Under experimental conditions, drugs decreasing GJ permeability, such as heptanol or octanol, have successfully reduced transmission of potentially harmful metabolic signals in different cell types. Their clinical application is, however, limited by their effects on myocardial contractile function and their arrhythmogenic properties. This study describes a mechanism of cell injury propagation through GJs that could be the target of a very selective intervention without major effects on GJ permeability or ion homeostasis in normal cells.

Acknowledgments

This work was supported by the PL-951254 BIOMED-2 program of the European Union and by Grant 97/0948 from the Fondo de Investigaciones Sanitarias (Spain). We thank Y. Puigfel for her technical assistance and A. Garcia-Dorado, J. Inserte, L. Agulló, J. Sagristà, and J. Cinca for their helpful discussions and suggestions.

References


Propagation of Cardiomyocyte Hypercontracture by Passage of Na⁺ Through Gap Junctions
Marisol Ruiz-Meana, David Garcia-Dorado, Bettina Hofstaetter, H. Michael Piper and Jordi Soler-Soler

Circ Res. 1999;85:280-287
doi: 10.1161/01.RES.85.3.280

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/85/3/280

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at: http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at: http://circres.ahajournals.org/subscriptions/