Persistence of gap junction communication during myocardial ischemia

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Cardiac myocytes are tightly interconnected by means of highly specialized regions of the plasma membrane known as gap junctions (GJ), which are composed of multiple intercellular channels. These channels are the physical mediators of electrical coupling in the heart and allow myocardium to behave as a functional syncytium. In ventricular myocytes, GJ channels are mainly formed by connexin43 (Cx43) (11, 42). There is strong evidence that impaired electrical coupling due to alterations in the number, spatial distribution, or function of myocardial GJ has a causative role in the genesis of arrhythmias associated with different conditions, including acute ischemia and chronic myocardial infarction (6, 22, 35). However, although GJ allow the exchange of small molecules and ions (20, 26), little is known about the consequences of metabolic coupling between adult cardiac myocytes under normal or pathologic conditions such as myocardial ischemia-reperfusion. There is increasing evidence that chemical communication through GJ may allow spread of various types of cell injury in different tissues (7, 32, 33), and it has been recently proposed that it may allow propagation of cell injury during myocardial reperfusion (16). It is generally admitted that cardiomyocytes damaged by ischemia close their GJ and isolate themselves from surrounding cells. This phenomenon known as “healing over” has been teleologically explained as a defensive mechanism aimed to limit cell-to-cell diffusion of cytosolic derangements. The main evidence of this phenomenon in myocardium are the changes in its electrical behavior induced by ischemia. These changes have been related, at least in part, to a reduced ability of GJ to allow propagation of the action potential (4, 10, 24, 45), and their appearance shows a close temporal association with the development of rigor contracture (4, 5, 10, 24). Rigor contracture is the result of the formation of stable, low-energy, Ca2+-independent cross bridges between actin and myosin as a consequence of low ATP availability. It is manifested by an abrupt shortening in isolated cardiomyocytes and by an increase in rest tension in in situ myocardium, which occurs when cytosolic ATP concentration is reduced to a critical threshold level (1). Rigor contracture contributes itself to the aggravation of ischemic injury by accelerating ATP depletion and promoting cytosolic Ca2+ rise (1), and studies in isolated cardiomyocytes demonstrate that the time elapsed between rigor development and restoration of oxygen supply predicts survival or death of cardiomyocytes upon reenergization (44). Thus once ischemic rigor develops, cytosolic Ca2+ starts to increase progressively until it reaches a plateau, and shortly after the initiation of Ca2+ rise, changes in the electrical behavior of the myocardium appear and progress slowly during the following minutes (5, 10).

The mechanism by which ischemia alters electrical coupling has not been completely elucidated, but increased cytosolic Ca2+ concentration is thought to be...
the most important factor (10, 40). Because at the time when rigor develops, Ca$^{2+}$ overload and signs of electrical uncoupling are absent or negligible, the question arises whether GJ communication may allow synchronization of the onset of rigor contracture across ischemic myocardium.

Furthermore, the time course of the alterations in GJ permeability occurring during ischemia and the time point at which GJ-mediated intercellular communication is totally abolished have not been established. This question cannot be easily answered from the analysis of electrical behavior, mainly due to the complex relationship between changes in the electrical properties of ischemic myocardium and changes in GJ conductance (39). On the other hand, direct measurement of GJ conductance in isolated cell pairs by the patch-clamp technique cannot be performed under true ischemic conditions (43).

The purpose of our study was to investigate whether GJ communication may allow synchronization or cell-to-cell propagation of rigor contracture of cardiomyocytes during energy deprivation and to analyze whether biologically significant GJ-mediated chemical communication may persist in ischemic myocardium after rigor onset and in isolated rat hearts submitted to global ischemia. The results indicate that communication through GJ allows propagation of rigor contracture from one cell to the adjacent one and that GJs remain permeable after the development of rigor.

METHODS

Handling of animals conformed with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (National Institutes of Health Publication 85–23, Revised 1996) and the experimental procedures were approved by the Research Commission on Ethics of the Hospital Vall d’Hebron.

Isolated Cardiac Myocytes

Ventricular heart muscle cells were isolated from adult male Sprague-Dawley rats as previously described (16). Simulated ischemia was performed by exposure to either 2 NaCN mM/20 mM 2-deoxyglucose, or dinitrophenol (DNP) 20 μM in glucose-free HEPES buffer at pH 6.4. Onset of rigor contracture, GJ permeability, and changes in cytosolic intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) under metabolic inhibition (MI) were investigated in end-to-end connected myocytes resulting from incomplete dissociation and were compared with those observed in pairs of randomly selected nonconnected cells within the same optical field. When a cell presented a deteriorated appearance, it was excluded from the comparison. Forty micromoles of the GJ blocker 18α-glycyrrhetinic acid (18α-GA) was added to the MI buffer to analyze the effect of GJ on rigor propagation and subsequent Ca$^{2+}$ rise. This concentration has been shown to efficiently block dye transfer in noncardiac cells expressing Cx43 (18). We have observed that perfusion of isolated rat hearts with 40 μM 18α-GA induces a rapid reduction in impulse propagation velocity, assessed by analysis of transmembrane action potentials, followed by ventricular fibrillation (unpublished results). To ensure that the effects of 18α-GA were dependent on its uncoupling properties, two additional series of experiments were performed in which GJ were blocked with the chemically unrelated uncouplers heptanol (2 mM) or oleic acid (50 μM).

Cell morphology, microinjection of Lucifer yellow, and [Ca$^{2+}$]$_i$ measurement. Cell images were continuously video recorded at ×200 magnification on the stage of an inverted microscope (Olympus IX70) at 37°C. Changes in cell length were measured on the video-recorded images in both cells of a pair every 1 s, and the first image in which cell length changed (i.e., became shorter than the precedent one) was labeled and used to identify rigor onset. Measurements were expressed as the relative changes in length before MI. To assess GJ permeability, one of the cells of each pair was microinjected with 2% Lucifer yellow (LY) 10 min after rigor development (16, 41). Transfer of the dye through GJ was monitored under fluorescence microscopy by using a 420-nm excitation light. [Ca$^{2+}$]$_i$, was monitored throughout MI with a ratio fluorescence imaging system (QuantiCell 900, Visitech) (41). For this purpose, myocytes were microinjected with 200 μM at 37°C with 2.5 μM of the fura 2-acetoxyethyl ester ( Molecular Probes) in medium 199, washed twice, and postincubated for 10 min in medium 199. After the addition of the metabolic inhibitor, cells were alternatively excited at 340 and 380 nm by means of a fast-speed monochromator. Emitted light was collected by an air-cooled intensified digital camera with a resolution of 160 × 160 pixels. 340/380 ratios were calculated for each pixel at 500-ms intervals from background-subtracted signal intensities in pairs of images consecutively obtained at the two wavelengths, and color-coded 340/380 ratio images were generated. The average ratio was calculated for regions of interest defined in cell images, and changes in these average ratio values through time were analyzed. The onset of [Ca$^{2+}$]$_i$ rise was identified visually from a curve connecting the lowest values in the 340/380 ratio curve.

Isolated Perfused Rat Heart

Hearts from adult male Sprague-Dawley rats (300–350 g, n = 20) were perfused with a Krebs-Henseleit bicarbonate buffer at 37°C at 60 mmHg (19). Left ventricular (LV) pressure was monitored by means of a water-filled latex balloon placed in the left ventricle and connected to a pressure transducer (19). After 15 min of equilibration, hearts were submitted to nonflow ischemia at 37°C or to 45 min of normoxic perfusion at pH 7.4 (control) or at pH 6.4 (acidosis). In an additional series of experiments, 18α-GA at either 10 or 40 μM was added during 30 min to the normoxic perfusion buffer.

Loading of LY and rhodamine dextran in the whole heart. Assessment of GJ permeability in myocardial tissue was performed by a modification of a previously described method (13, 14). Two fluorescent dyes, rhodamine-conjugated dextrans (RD) and LY, were used to quantify diffusion through GJ from previously marked cells in which dye loading had been performed by a direct exposure after mechanical damage. Because of its high molecular weight (10,000), RD cannot diffuse through GJ and remains circumscribed to cells with broken sarcolemma, whereas LY (molecular weight, 457) acts as a tracer for diffusion through GJ channels. In a series of experiments, 30 min after the onset of rigor (~45 min of ischemia) the whole hearts were placed in phosphate-buffered saline (PBS) containing 2.5 mg/ml RD and 2.5 mg/ml LY at 37°C and continuously gassed with N$_2$. A deep incision in the LV wall was made in each heart from base to apex with...
a surgical blade, and direct exposure to the anoxic dye containing buffer was allowed for 10 min. After dye loading, hearts were washed in PBS and fixed in 25% Karnovsky’s solution for 4 h. In other series of ischemic hearts, the excision and dye loading were performed after 2 h of ischemia. For normoxic hearts, the N2 used to gas solutions was replaced by O2.

Tissue processing and laser confocal microscopy. Fixed tissue was frozen by immersion in hexane cooled with liquid N2 and stored at ~80°C. Sixty-micrometer transversal sections including the whole right and left ventricles were obtained with a cryostat (Leica CM1325) and mounted on glass. Tissue sections were examined by using a confocal laser scanning microscopy (DMRIBE Leica, software TCS-NT) equipped with an argon-krypton gas laser. FITC and tetramethylrhodamine isothiocyanate excitation filters were selected to discriminate between LY (Ex488/515) and RD (Ex566/590) signals, and two separated 1,024 × 1,024 digital images per slice were obtained at ×10. The difference between the area of distribution of LY fluorescence and the area of distribution of RD fluorescence was quantified with commercially available software (Olympus Microimage) and expressed as a fraction of the area of RD distribution as an index of GJ permeability.

Statistics

Statistical analysis was performed with the aid of commercially available software (SPSS for Windows). Comparisons between groups were performed by ANOVA and the Student-Newman-Keuls test. A critical P value of 0.05 was used in all comparisons. Data are expressed as means ± SE.

RESULTS

Cell-to-Cell Propagation of Rigor Contracture

The average time at which rigor appeared was not different in connected cells with respect to single cells (1,097.5 ± 70.8 vs. 938.5 ± 66.8 s, P = not significant).

In end-to-end connected cell pairs submitted to MI, development of rigor contracture in one cell was consistently followed by near-simultaneous development of rigor contracture in the adjacent cell (Fig. 1). This temporal association between rigor onset in end-to-end connected cells was observed independently of the inhibitor used (NaCN or DNP), although rigor appeared earlier in cells exposed to DNP compared with those exposed to NaCN. The average difference in time between the onset of rigor in two cells forming a connected pair during MI was 3.70 ± 0.75 s (n = 30). In contrast, in nonconnected cells randomly selected within the same optical field this interval was 71 ± 11.7 s (n = 14). The involvement of GJ in the temporal association of rigor onset in connected cells was investigated by adding the GJ blocker 18α-GA to the MI buffer. The presence of 18α-GA significantly increased the time elapsed between both cells in the development of rigor contracture (Fig. 2) without affecting the average time at which rigor appeared (648.6 ± 163.8 s in the absence of 18α-GA vs. 568.8 ± 90 s in its presence, P = not significant). Similar results were obtained when inhibition of GJ communication was performed with either heptanol or oleic acid (interval between rigor onset in connected cell 11.5 ± 3.4 s, n = 17, and 11.3 ± 2.7 s, n = 3, respectively).

To investigate whether the temporal association in rigor onset was the result of the synchronization in the progression of injury or reflected cell-to-cell propagation of rigor, we examined the degree of shortening in the cell that initiates rigor and in the adjacent cell. It was assumed that if rigor developed synchronously but independently in two connected cells, the magnitude of shortening should be equal in both cells. On the contrary, a significant association between the magnitude of shortening and the order of rigor onset would indicate directionality, i.e., propagation. This was indeed the case: when cells were connected, the degree of
shortening of the adjacent cell was less pronounced than that observed in the cell initiating the rigor. As expected, there was no association between the order of initiation of rigor and the magnitude of shortening in the case of nonconnected cell pairs (Fig. 3). In connected pairs, 18α-GA had a clear influence on the shortening of the cell developing rigor in the second place. However, the direction of this influence was toward a greater difference in the percentage of shortening compared with control cell pairs. The same result was obtained with heptanol and oleic acid (Fig. 3).

Equilibration of $[Ca^{2+}]_i$, Rise and LY Diffusion in Connected Cell Pairs

During MI, $[Ca^{2+}]_i$ remained unchanged until the onset of rigor contracture. Rigor was associated to a marked increase in $[Ca^{2+}]_i$. In connected cell pairs, the time course of $[Ca^{2+}]_i$ was virtually identical in both cells, with an excellent correlation between the time to the onset of $[Ca^{2+}]_i$ rise in both cells ($r^2 = 0.99$, $n = 18$). In contrast, in nonconnected cells within the same optical field the magnitude and time-course of $[Ca^{2+}]_i$ rise showed a great variability ($r^2 = 0.49$, $n = 14$) (Fig. 4). In the presence of 18α-GA, the curves of $[Ca^{2+}]_i$ in connected cells tended to diverge as the time of MI progressed, and the difference between ratios in the two cells was significantly higher in the presence of 18α-GA ($0.293 \pm 0.08$ vs. $0.064 \pm 0.01$, $P = 0.022$, Fig. 5). These results suggest that GJ communication persists after the onset of rigor contracture. After micro-injection of 2% LY in one of the cells of the pair 10 min after rigor development, there was dye transfer in six of seven pairs (85.7%) of end-to-end connected myocytes, confirming chemical coupling after rigor development (Fig. 6).

Persistence of GJ Permeability in Ischemic Rat Hearts

LY diffusion was assessed in isolated rat hearts submitted to nonflow ischemia at 37°C. The abrupt increase in LV end-diastolic pressure occurring after 16.8 \pm 1.1 min was considered the in vivo marker of the development of rigor contracture. Thirty minutes after rigor onset (~45 min of global ischemia), the difference between the extent of LY diffusion in each slice (from the incision made for dye exposure to the adjacent myocardium) and the RD diffusion (circumscribed to myocytes with broken sarcolemma) was 2.97 \pm 1.27 ($n = 8$). This value was similar to that obtained in normoxic hearts (3.58 \pm 1.33, $n = 4$), demonstrating diffusion of LY to cells located far away from those with disrupted sarcolemma under ischemic conditions (Fig. 7). However, when hearts were submitted to 2 h of global ischemia, the area of LY diffusion was coincident with the area of RD diffusion (the difference in the area of diffusion between both dyes was $0.10 \pm 0.01$, $n = 2$). Acidotic perfusion without ischemia resulted in a marked reduction of GJ permeability (the difference in the area of LY diffusion and RD diffusion was $0.91 \pm 0.37$, $n = 2$) (Fig. 8). Addition of 18α-GA at 10 or 40 \(\mu\)M during normoxic perfusion did not abolish LY transmission, although it resulted in a reduction in its diffusion (the differences between LY diffusion and RD diffusion were $1.5 \pm 1.25$ and $0.88 \pm 0.28$, respectively, $P < 0.05$ in respect to controls, $n = 4$).

DISCUSSION

The results of this study show that in end-to-end connected cardiac myocytes submitted to MI, the development of rigor contracture in one of the cells is systematically followed within a few seconds by the development of rigor contracture in the adjacent cell. This close temporal association between onset of rigor contrasted with the large variability in the time of rigor onset observed in nonconnected cells within the same optical field and was sensitive to the GJ uncoupler 18α-GA. The curves of $[Ca^{2+}]_i$ rise observed after rigor onset varied from cell to cell, but were virtually superimposable in the two cells connected forming a pair, suggesting that GJ communication allows equalization of ionic cytosolic concentrations after the development of rigor contracture. Microinjection of 2% LY in one of the two connected cells 10 min after rigor onset demonstrated persistent dye coupling. LY transfer was also documented 30 min after the onset of rigor contracture in isolated rat hearts submitted to nonflow global ischemia. Altogether, these results are in agreement with the hypothesis that GJ may allow cell-to-cell propagation of rigor contracture and equalization of $Ca^{2+}$ overload in ischemic cardiomyocytes.
Propagation of Rigor Contracture

Contracture appears during energy depletion as the consequence of the formation of low energy Ca\(^{2+}\)-independent bonds between actin and myosin when decreasing ATP concentration reaches a critical value. Studies in rabbit papillary muscle and in situ porcine myocardium have shown that in ischemic myocardial tissue, signs of electrical uncoupling such as slowing of impulse propagation and increased intracellular resistance are absent during the first minutes of ischemia and appear only after development of rigor contracture and [Ca\(^{2+}\)]\(_i\) rise (4, 10). Because GJ are permeable to most physiologically relevant ions, intracellular messengers, and high-energy compounds including ATP, it is not surprising that continuous equilibration of cytosolic composition between adjacent ischemic myocytes results in uniform progression of ischemic injury. Synchronization of the onset of rigor contracture may thus reflect the uniform progression of injury, but can also be the consequence of cell-to-cell propagation of rigor contracture through GJ-mediated communication. Attenuated magnitude of rigor shortening in the cell that develops rigor in the second place within a cell pair.
suggests that this is indeed the case. The present findings do not provide information on the messenger responsible for propagation of rigor. There is a lack of candidates to play this role, because rigor is not due to accumulation of any substance, but to ATP depletion. However, ATP consumption by rigor bonds in one cell should be expected to generate an intercellular gradient in cytosolic ATP concentration. It can be speculated that this gradient drives the passage of ATP from the adjacent cell, therefore reducing its cytosolic ATP concentration to the rigor threshold. However, the attenuated magnitude of rigor shortening induced by uncouplers in the adjacent cell is difficult to explain within this hypothesis. One possibility is that the rate of ATP depletion may influence the ATP level at which rigor starts. Uncouplers would reduce the ATP transfer between cells and the rate of ATP depletion in the second cell induced by development of rigor in the first one.

GJ-mediated Communication After Rigor Onset

The present observation that GJ-mediated intercellular communication may persist after the development of ischemic rigor contracture and $[\text{Ca}^{2+}]$ rise in cardiomyocytes challenges the notion that healing over prevents cell-to-cell communication among severely ischemic cardiomyocytes. This notion is based on several lines of evidence. In the first place, detailed cable analysis of myocardial preparations (4, 10, 24, 45) and in vivo studies (5) show that development of ischemic rigor contracture is consistently associated to the appearance of progressive abnormalities in electrical tissue resistance and impulse propagation (4, 5, 10, 24). These abnormalities progress slowly during the minutes after the development of rigor. In the isolated papillary muscle, conduction block and maximal tissue resistance are reached only after 10–20 min of rigor contracture (4, 10). In in situ ischemic porcine myocardium, electrical impedance (phase angle shift) rises steadily during the 90-120 min after rigor onset (5). The slow progression of electrical abnormalities could reflect, in part, a progressive decrease of GJ conductiv-

![Fig. 6](image-url)  
**Fig. 6.** Sequences of images obtained after microinjection of Lucifer yellow (LY) in a pair of connected cardiomyocytes during MI. LY was microinjected in cell 1 10 min after rigor onset and diffused to the adjacent cell (cell 2) in less than 30 s, demonstrating that the GJ remained permeable. All arrows indicate the ID. The graph shows the increase in fluorescence intensity in both cells after microinjection. au, Arbitrary units.
available information is compatible with persistent chemical communication through GJ in severely ischemic, rigor-contractured myocardium. The mechanism by which GJ may remain permeable in ischemic myocardium despite acidosis, Ca$^{2+}$ overload, ATP depletion, and accumulation of amphipathic catabolites cannot be elucidated by this study. It can be speculated that the modification of the phosphorylation status of Cx43 caused by ischemia impairs its regulation by these stimuli. Ischemia rapidly reduces the phosphorylation status of different important proteins. Inhibition of phosphatases has been found to be protective against different manifestations of ischemic injury (2, 30, 46), whereas activation of protein kinases seems an essential step in the protective effect of ischemic preconditioning (47). There is strong evidence that GJ gating may be regulated by changes in connexin phosphorylation. Also, there is compelling evidence that phosphorylation of Cx43 by different protein kinases, including mitogen-activated protein kinase and p34 kinase, reduces GJ communication (21, 23, 27, 31), whereas dephosphorylation increases it (8). It has been recently described (12) that phosphorylation of Cx43 in cardiomyocytes depends on the epsilon subtype of protein kinase C (PKC-ε). The inhibitory effects of Cx43 phosphorylation on GJ communication could be involved in the protective effect of interventions such as ischemic preconditioning, a beneficial effect that appears to be mediated by activation of protein kinases, particularly the PKC-ε isoform. These observations suggest that phosphorylation of Cx43 at specific sites induces conformational changes that affect its gating properties. The present observation that exposure to acidic perfusion reduces GJ permeability in intact rat myocardium during normoxic conditions, whereas ischemia of the same duration, expected to result in
pronounced acidosis, does not reduce GJ permeability, supports this hypothesis.

The results of the present study are in agreement with recent observations demonstrating the persistence of GJ communication during ischemic conditions in tissues other than myocardium. Several studies have shown that GJ coupling during cerebral ischemia can result in an amplification of cell injury (7, 32, 36). Using a model of cultured astrocytes, Cotrina et al. (7) found that coupling was reduced but never abolished after 2 h of MI and that chemical communication through GJ occurred up to the terminal loss of membrane integrity. Interestingly, lowering pH to 6.0 caused no detectable decrease in GJ permeability. Chemical coupling in astrocytes was corroborated in hippocampal slices during ischemic conditions. Similar results (32) were obtained in neocortices of rats after applying the technique of fluorescent recovery after photo bleaching. Although a decrease in fluorescence recovery (reflecting a decreased diffusion through GJ) was observed, Lin et al. (32) found that astrocytic GJ remains open in the anoxic brain. The contribution of GJ to the expansion of injury is an emerging field. It has been described that GJ coupling may result in amplification of injury due to causes other than ischemia. There is evidence that cell-to-cell communication can mediate the death of tumor cells adjacent to those directly targeted by different antineoplastic treatments (bystander effect) (17, 34, 37). Previous studies (16, 41) had shown that GJ-mediated communication allows spreading of cell injury during myocardial reperfusion.

**Study Limitations**

In the present study, GJ uncouplers failed to completely abolish dye transfer and delayed, but did not prevent, propagation of rigor contracture. This limitation is difficult to circumvent because there is a lack of substances proven to be able to completely block dye coupling in end-to-end connected cardiomyocytes or in myocardium. Another potential limitation is that metabolic inhibition or ischemia could open undocked hemichannels and allow LY loading from the extracellular space (25). This possibility seems extremely unlikely in our study because in previous observations cardiomyocytes submitted to prolonged metabolic inhibition (60 min) failed to become loaded with LY present in the extracellular medium.

The present results demonstrate that, contrary to expectations, cardiomyocytes can communicate with their counterparts also during ischemia and this communication persists after the onset of rigor contracture, a critical period when survival or death response to subsequent reperfusion is defined. These results add to previous observations showing that during reperfusion, transjunctional movements of Na⁺ may result in propagation of cardiomyocyte hypercontracture (41) and that inhibition of GJ communication during initial reperfusion may limit infarct size (16). Altogether these studies indicate myocardium may respond to a large extent as a functional syncytium to ischemia-reperfusion. This previously neglected cellular interaction might represent a more generalized mechanism of spreading cell injury in tissues with well-developed cell-to-cell communication. Understanding the biochemical basis of such interaction should provide insight into the role of GJ in the progression of ischemic injury and open the possibility of new therapeutic approaches.

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