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Circulation. 2008;118:2330-2337; originally published online November 17, 2008;
doi: 10.1161/CIRCULATIONAHA.108.789149

Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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Electrophysiological Consequences of Acute Regional Ischemia/Reperfusion in Neonatal Rat Ventricular Myocyte Monolayers

Carlos de Diego, MD*; Rakesh K. Pai, MD*; Fuhua Chen, MD; Lai-Hua Xie, PhD; Jan De Leeuw, PhD; James N. Weiss, MD; Miguel Valderrábano, MD

Background—Electrophysiological changes promoting arrhythmias during acute regional ischemia/reperfusion are challenging to study in intact cardiac tissue because of complex 3-dimensional myocardial and vascular geometry. We characterized electrophysiological alterations and arrhythmias during regional ischemia/reperfusion in a simpler 2-dimensional geometry of cultured neonatal rat ventricular myocyte monolayers.

Methods and Results—Optical mapping of intracellular Ca (Ca_i) and voltage was performed with the use of Rhod 2-AM and Rh-237, respectively. Regional ischemia was mimicked by covering the central portion of monolayer with a glass coverslip, and reperfusion was mimicked by removing the coverslip. Monolayers were stained with fluorescent antibodies to detect total and dephosphorylated connexin-43 at various time points. During coverslip ischemia, action potential duration shortened, Ca_i transient duration was prolonged, and local conduction velocity (CV) slowed progressively, with loss of excitability after 10.6 ± 3.6 minutes. CV slowing was accompanied by connexin-43 dephosphorylation. During ischemia, spontaneous reentry occurred in 5 of 11 monolayers, initiated by extrasystoles arising from the border zone or unidirectional conduction block of paced beats. On reperfusion, excitability recovered within 1.0 ± 0.8 minutes, but CV remained depressed for 9.0 ± 3.0 minutes, promoting reentry in the reperfused zone. As connexin-43 phosphorylation recovered in the reperfused zone, CV normalized, and arrhythmias resolved.

Conclusions—Acute regional ischemia/reperfusion in neonatal rat ventricular myocyte monolayers recapitulates electrophysiological alterations and arrhythmias similar to those observed during acute coronary occlusion/reperfusion in intact hearts. During early reperfusion, slow recovery from connexin-43 dephosphorylation leads to persistent CV slowing, creating a highly arrhythmogenic substrate. (*Circulation*. 2008;118:2330-2337.)

Key Words: arrhythmia ■ fibrillation ■ optical mapping ■ reentry ■ ischemia ■ reperfusion

Ventricular arrhythmias during acute regional myocardial ischemia after coronary occlusion are a major cause of sudden cardiac death.¹ Although the electrophysiological consequences of global ischemia have been characterized extensively,^{2–5} the heterogeneous electrophysiological alterations during regional ischemia are more challenging to study because of the complex 3-dimensional geometry of the myocardium and its vascular supply, particularly at the interface between normal and ischemic tissue.^{3,6} Recently, Pitts and Toombs⁷ described a “coverslip” model of regional ischemia/reperfusion (IR) in cultured neonatal rat ventricular myocyte (NRVM) monolayers that reproduced metabolic and ultrastructural changes leading to IR injury in intact cardiac muscle. In the present study, we used the

coverslip technique to characterize the effects of acute regional IR on electrophysiological alterations and arrhythmias in NRVM monolayers that lack complex 3-dimensional myocardial and vascular structures. We find that regional IR in this preparation recapitulates many of the electrophysiological alterations previously described in intact tissue, including conduction slowing, action potential duration (APD) shortening, impaired intracellular Ca (Ca_i) handling, spontaneous extrasystoles, and reentrant arrhythmias, with the border zone playing a critical role in the generation of arrhythmias. Connexin-43 (Cx43) dephosphorylation perpetuates slow conduction and increases susceptibility to reentrant arrhythmias during early reperfusion.

Received April 29, 2008; accepted September 19, 2008.

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The online-only Data Supplement is available with this article at <http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.108.789149/DC1>.

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Circulation is available at <http://circ.ahajournals.org>

DOI: 10.1161/CIRCULATIONAHA.108.789149

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Methods

Cultured NRVM Monolayers

NRVM monolayers were isolated by standard methods⁸ and plated on 22×22-mm polyvinyl chloride coverslips (PGC Scientifics, Frederick, Md). Briefly, the hearts harvested from 2- to 3-day-old neonatal Sprague-Dawley rats were digested with collagenase (0.02%; Worthington Biochemical Corp, Lakewood, NJ) and pancreatin (0.06%; Sigma-Aldrich, St Louis, Mo). Myocytes were isolated with the use of a Percoll (Pharmacia Biotech AB, Uppsala, Sweden) gradient and plated at a density of 10⁶ cells/mm³ per coverslip. In most experiments (n=27), monolayers were cultured for 4 to 5 days to ensure full confluence (by phase contrast microscopy) and homogeneous electrical propagation before experimental use.

Specimens were superfused with oxygenated Tyrode's solution at 37°C. The monolayer was stimulated at 2 Hz (unipolar stimuli at 10 V at the edge of the coverslip) with a Grass stimulator (Astro-Med Inc, West Warwick, RI).

Regional IR Model

We adapted the method described by Pitts and Toombs,⁷ who mimicked regional IR in NRVM monolayers by covering the central region of the monolayer with a glass coverslip, creating a diffusion barrier that restricted the access of underlying cardiomyocytes to nutrients and oxygen, while the adjacent nonischemic regions remained unaffected. They validated that the model replicated metabolic and ultrastructural hallmarks of ischemia, resulting from restricted diffusion rather than mechanical effects of the coverslip. After obtaining baseline optical recordings, we lowered an 18-mm-diameter glass coverslip onto the central area of the 22×22-mm monolayer to create a central ischemic zone, surrounded by a nonischemic zone (Figure 1A). The rim of tissue under the coverslip within 1 mm of the nonischemic zone was defined as the border zone. Optical Ca_i and/or voltage maps were obtained every 1 to 2 minutes before and during ischemia until the ischemic zone became unexcitable. In most experiments, the coverslip was then lifted to begin the reperfusion period, and optical maps were obtained every 1 to 2 minutes.

To ascertain that the observed effects were due to ischemia rather than mechanical effects of the coverslip, 6 monolayers were cultured on top of semipermeable matrigel membranes (Becton-Dickinson,

Franklin Lakes, NJ) that permit diffusion of oxygen and substrates from underneath, as described previously.⁷

Optical Mapping

NRVM monolayers were stained by immersion into oxygenated Tyrode's solution (in mmol/L: 136 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.33 NaH₂PO₄, 1 MgCl₂, 10 HEPES, and 10 glucose; pH 7.3) containing the fluorescent Ca dye Rhod-2AM (5 μmol/L for 40 minutes) plus 0.016% (wt/wt) Pluronic (Molecular Probes, Eugene, Ore) and/or the voltage dye RH-237 (5 μmol/L for 5 minutes) at 37°C.⁹ Fluorescence was excited by 2 light sources (each with 4 light-emitting diodes; Luxeon, Ontario, Canada) filtered at 540±20 nm. The emitted fluorescence was separated with the use of a dichroic mirror (at 630 nm), directed to 2 separate charge-coupled device cameras with their corresponding emission filters (715 nm for RH-237 and 585 nm for Rhod-2, respectively). Simultaneous voltage and Ca mapping were performed in 11 specimens. We used electron-multiplying, back-illuminated, cooled charge-coupled device cameras (Photometrics Cascade 128+; 128×128 pixels), acquiring at 0.6 to 5 ms per frame. Signals were digitized with 16 bits of precision and processed offline as described previously.⁹ Briefly, charge-coupled device recordings were subjected to (1) spatial filter (2×2 binning); (2) 5-point median temporal filter; (3) polynomial curve fitting to eliminate baseline drift caused by photobleaching; and (4) range normalization. After processing, this yielded a final spatial resolution of 64×64, corresponding to pixel size of 340×340 μm, with temporal resolution of 15 to 25 ms (3 to 5 ms per frame ×5). In some experiments (n=6), temporal resolution was optimized by applying only spatial filtering (4×4 binning). This yielded a post-processing spatial resolution of 32×32, corresponding to a pixel size of 680×680 μm, with a maximal temporal resolution of 0.6 ms per frame. Both methods yielded comparable results.

Isochronal maps and conduction velocity (CV) were obtained as described previously.^{9,10} Wavelength was measured as the distance from wavefront to waveback of the action potential at 80% repolarization.

Cx43 Immunostaining

Anti-Cx43 antibodies were used to detect changes in total and dephosphorylated Cx43 during IR. A rabbit polyclonal anti-Cx43 (Zymed Laboratories Inc, South San Francisco, Calif), directed against the amino acid sequence of 252 to 270 that recognizes both the phosphorylated and the dephosphorylated forms of Cx43, was used to identify the total amount of Cx43.¹¹ A mouse monoclonal antibody (clone CX-1B1) generated against amino acids 360 to 376 (Zymed Laboratories Inc, South San Francisco, Calif) was used to

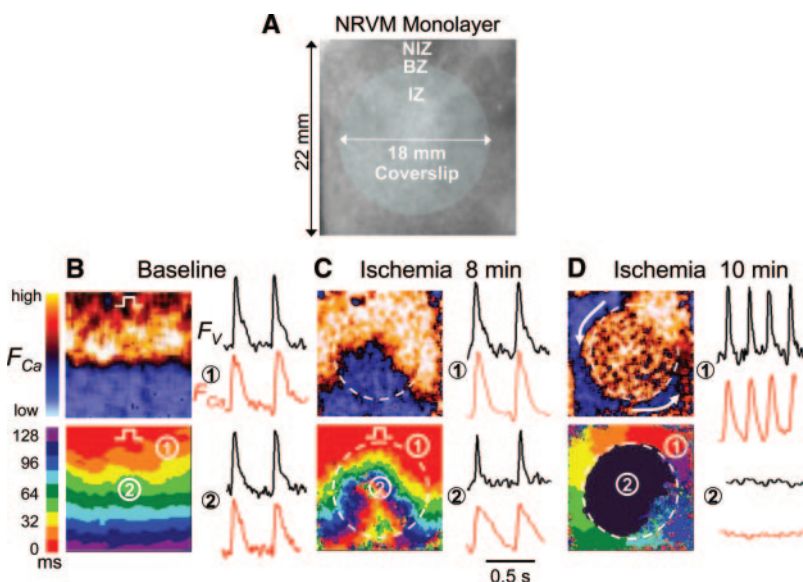


Figure 1. Effects of regional ischemia on action potentials and Ca transients in NRVM monolayers and typical activation patterns during ischemia. A, Photograph of an NRVM monolayer (22×22 mm) with the 18-mm coverslip in place. NIZ indicates nonischemic zone; BZ, border zone; and IZ, ischemic zone. B through D, Snapshot of Ca_i fluorescence (F_{Ca}; top) and the isochronal activation map (bottom) during pacing from the upper middle region, at baseline and after 8 and 10 minutes of coverslip ischemia. The action potential (F_V; black) and Ca_i transient (F_{Ca}; red) tracings at sites 1 and 2 on the isochronal map are shown to the right of each map. Propagation was uniform at baseline but by 8 minutes had slowed progressively only in the ischemic zone, which became inexcitable by 10 minutes, with macroreentry circulating counter-clockwise around it.

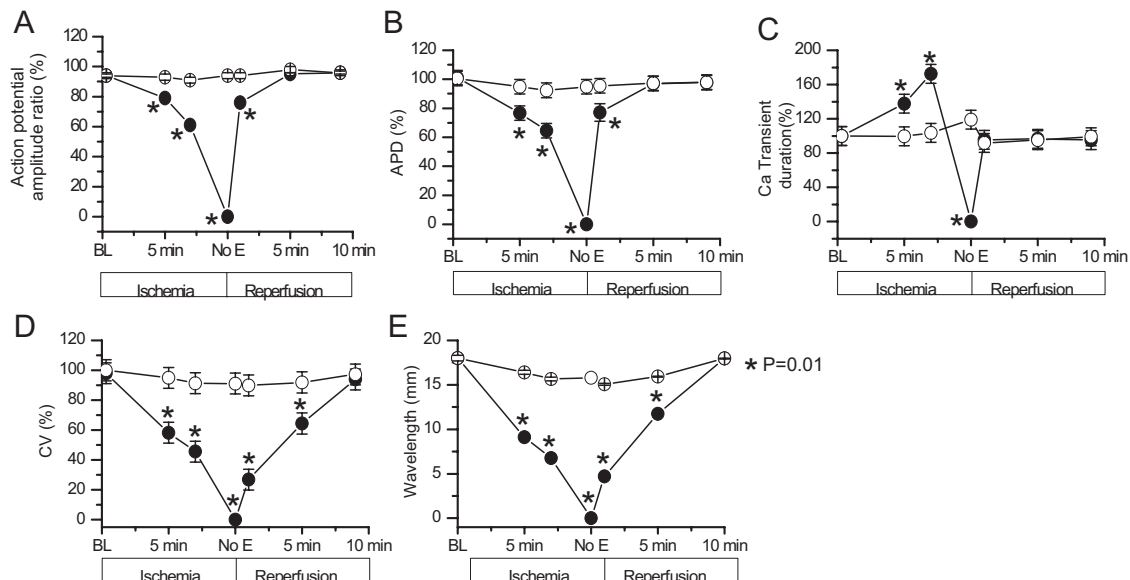


Figure 2. Summary of electrophysiological changes during regional coverslip IR in NRVM monolayers. Average changes in action potential amplitude ratio (A), APD (B), Ca_i transient duration (C), CV (D), and wavelength (E) in the ischemic zone (solid circles) vs the nonischemic zone (open circles) at various time points during coverslip IR are shown. No E refers to the time point at which the ischemic zone became inexcitable (average, 10.6 ± 3.6 minutes). Values are mean \pm 2 SD.

identify dephosphorylated Cx43.^{12,13} Immunohistochemical studies using one or the other antibody were performed at baseline and at different stages of IR (n=30) so that propagation patterns could be correlated with Cx43 phosphorylation state. After optical mapping, monolayers were removed from the bath apparatus and fixed with 2% formaldehyde for 10 minutes in 0.1 mmol/L Ca^{2+} Tyrode's solution. Monolayers were incubated with protein block solution and then exposed to either monoclonal (against dephosphorylated Cx43) or polyclonal (against total Cx43) primary antibodies for 1 hour and with secondary antibodies for 45 minutes. Finally, monolayers were treated with DAPI (Sigma, St Louis, Mo) to stain nuclei for 2 minutes and then mounted on slides. All slides were viewed on an epifluorescence microscope and digitally photographed for later analysis. To quantify changes in Cx43 fluorescence at different times during IR, the ratio of Cx43 fluorescence intensity in the ischemic zone to nonischemic zone was measured. Quantitative results are presented as the average ratio of ischemic zone to nonischemic zone of dephosphorylated or total Cx43 fluorescence at appropriate time points.

Data Analysis

The authors had full access to and take responsibility for the integrity of the data. All the authors have read and agreed to the manuscript as written. Data are presented as mean \pm 2 SD. The repeated-measures test was used to compare mean values of serial measurements over time. Briefly, we used a simple repeated-measures model that assumed a constant intraclass correlation between the different time points, or, equivalently, we assumed a random coefficient regression model in which the ischemic and nonischemic curves have random intercepts. Parameters and their SEs are estimated by maximum likelihood methods. In each of the curves, we have drawn CIs around the points, extending from 2 SD above the mean to 2 SD below the mean. The differences between the means are significant at the 0.01 level.

Results

Electrophysiological Changes During IR

At baseline, mature monolayers exhibited uniform propagation during pacing at 2 Hz (n=11). APD_{80} averaged 117 ± 12 ms, Ca_i transient duration 160 ± 36 ms, CV 0.16 ± 0.02 m/s, and wavelength 18 ± 0.3 mm. When the glass coverslip was

lowered over the central region, APD and the ratio of the action potential amplitude in the ischemic zone to nonischemic zone progressively decreased, and the Ca_i transient was prolonged in the ischemic zone but not in the nonischemic zone (Figures 1 and 2). CV also progressively decreased in the ischemic zone, which eventually became unexcitable after an average of 10.6 ± 3.6 minutes (Figure 1). On average, CV in the ischemic zone decreased to $58 \pm 12\%$ of the preischemic value ($P < 0.05$) at 5 minutes of ischemia, to $46 \pm 16\%$ at 7 minutes ($P < 0.05$), and to 0% after 10.6 ± 3.6 minutes. In contrast, normalized CV in the nonischemic zone did not change significantly during ischemia (by $-5 \pm 7\%$ at 5 minutes of ischemia, $-9 \pm 7\%$ at 7 minutes, and $-9 \pm 7\%$ at the time of loss of tissue excitability in the ischemic zone; $P = NS$). Thus, a CV gradient between the ischemic zone and nonischemic zone progressively developed during ischemia (Figure 2D). Wavelength also shortened dramatically in the ischemic zone because of the combined decrease in CV and APD (Figure 2E).

Reperfusion was initiated by removing the coverslip after the ischemic zone became inexcitable (n=11). In 9 monolayers, the ischemic zone recovered excitability after reperfusion, with the AP amplitude ratio, APD_{80} , and Ca_i transient duration returning to near basal values within minutes of reperfusion (Figure 2A and 2B). However, recovery of CV and wavelength was delayed, requiring an average of 9.0 ± 3.0 minutes to achieve $>90\%$ of the preischemic value in 7 of 9 monolayers (Figure 2C and 2D). In 2 monolayers, CV recovered only partially (to $44 \pm 4\%$).

Seven monolayers were exposed to a longer duration of ischemia. In 4 monolayers in which the coverslip was removed after an average of 17 ± 1 minutes (range, 15 to 20 minutes), 3 recovered excitability in the ischemic zone after reperfusion. However, in 3 monolayers reperfused after 25 ± 6 minutes of coverslip ischemia, none recovered excitability.

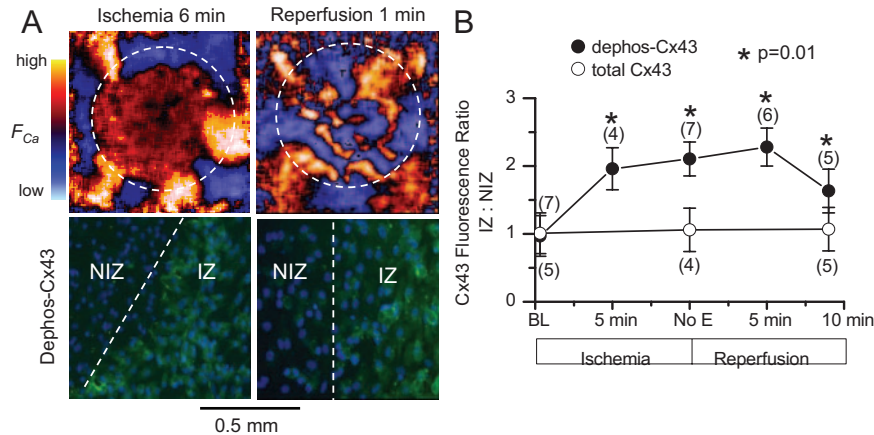


Figure 3. Cx43 fluorescence during coverslip IR. A, Snapshot of Ca_i fluorescence (F_{Ca}) after 6 minutes of coverslip ischemia, showing multiple wave macroreentry around the inexcitable ischemic zone (left panel) and during early reperfusion (right panel), showing reentry in the border zone and fibrillatory conduction block in the ischemic zone. Dashed white circles demarcate the ischemic zone (IZ) and nonischemic zone (NIZ). Lower panels show dephosphorylated Cx43 (Dephos-Cx43) immunofluorescence (green) in a magnified image across the border zone region, demonstrating increased fluorescence in the ischemic zone compared with the nonischemic zone. Blue fluorescence indicates nuclei staining with DAPI. B, Average ratio of Cx43 immunofluorescence in the ischemic zone to nonischemic zone for dephosphorylated Cx43 (solid bars) and total Cx43 (open bars) at various times during coverslip IR. The dephosphorylated Cx43 fluorescence ratio increased during ischemia and recovered partly by 10 minutes of reperfusion, whereas the total Cx43 ratio did not change. Values are mean \pm 2 SD for the number of monolayers indicated.

Three monolayers were exposed to a preconditioning ischemic episode in which the coverslip glass was removed after 2:1 conduction block developed (average of 6 ± 1.7 minutes) but before complete loss of excitability. After a reperfusion period sufficient to allow recovery of 1:1 conduction recovery (averaging 7 ± 2 minutes), the coverslip was reapplied. During the second episode of coverslip ischemia, monolayers maintained 1:1 conduction in the ischemic zone for 16 ± 3 minutes, with complete loss of excitability at 18 ± 2 minutes (compared with 10.6 ± 3.6 minutes for ischemia without preconditioning; $P < 0.05$).

To establish that these electrophysiological alterations were due to ischemia rather than mechanical effects of the coverslip, 6 monolayers were cultured on a semipermeable membrane to permit diffusion of oxygen and other substrates from underneath ($n = 6$).⁷ Under these conditions, the presence of the coverslip caused no significant changes in APD, Ca_i transient duration, or CV over a 30-minute period (Figure I in the online-only Data Supplement), consistent with the previous findings of Pitts and Toombs.⁷

Cx43 Dephosphorylation During IR

Two major determinants of CV are Na current amplitude and gap junction conductance. During ischemia, Na current availability decreases because of membrane depolarization, and gap junction conductance also decreases in parallel with dephosphorylation of Cx43.¹³ During reperfusion, we found that APD recovered rapidly (as early as 1 minute). To examine whether persistent Cx43 dephosphorylation might contribute to the delayed recovery of CV during early reperfusion, we immunostained monolayers for dephosphorylated and total Cx43 at various time points during IR (Figure 3A). During ischemia, dephosphorylated Cx43 fluorescence increased markedly in the ischemic zone relative to the nonischemic zone, and this difference persisted during early reperfusion. Figure 3B summarizes the ratio of dephosphory-

lated Cx43 fluorescence in the ischemic zone to nonischemic zone for each monolayer at various time points. The ratio increased within the first 5 minutes of ischemia and remained elevated during early reperfusion, eventually recovering by 10 minutes of reperfusion. The ratio of total Cx43 in the ischemic zone to the nonischemic zone did not change significantly after 10 minutes of ischemia or after 10 minutes of reperfusion (Figure 3B). Thus, delayed recovery of CV during reperfusion paralleled the recovery from Cx43 dephosphorylation.

Arrhythmias During Ischemia

During ischemia, spontaneous rotors occurred in 5 of 11 monolayers after an average of 6.0 ± 3.7 minutes. The rotors originated in the border zone in 4 monolayers and in the ischemic zone in 1 monolayer. With progressive ischemia, propagation of rotors into the ischemic zone exhibited prominent Ca_i transient alternans before 2:1 conduction block (Figure 4 and Movie I in the online-only Data Supplement). During late ischemia, after the ischemic zone became unexcitable, rotors converted to stable macroreentry, with single (Figure 1D) or multiple waves (Figure 3A) circulating around the inexcitable ischemic zone (Movies II and III in the online-only Data Supplement).

In 3 monolayers, the onset of reentry was fortuitously captured during optical recording. In 2 monolayers, reentry was initiated by a spontaneous extrasystole arising from the border zone, as illustrated in Figure 5. The extrasystole, which originated at site 1, was unable to propagate into the ischemic zone but successfully propagated outward and clockwise around the nonischemic zone. Note that at site 1, the APD was short, and the Ca_i transient was dramatically prolonged, a combination that has been proposed to cause triggered activity in atrial myocardium.¹⁴ The snapshot of Ca_i just before the extrasystole also shows that Ca_i remained persistently high in the region from which the extrasystole originated (Figure 5C). Spontaneous extrasystoles were fre-

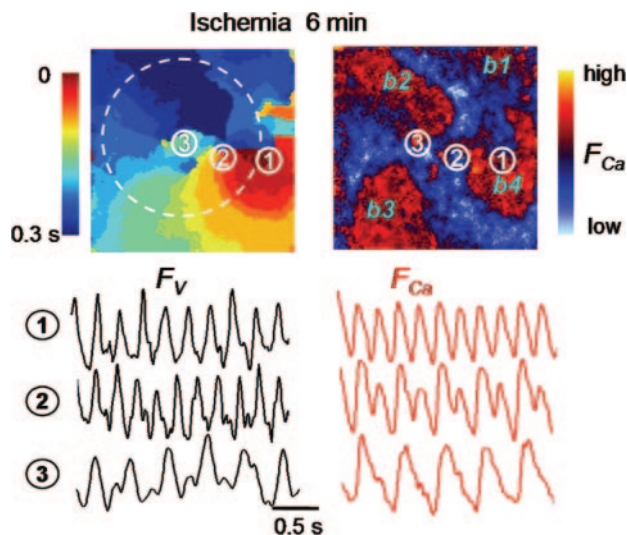


Figure 4. Spontaneous reentry during ischemia. Right and left panels show a snapshot of Ca_i fluorescence (F_{Ca}) and the isochronal activation map, respectively, in a monolayer after 6 minutes of coverslip ischemia. A rotor anchored in the border zone near 4 o'clock (see isochrone map) generated wavefronts circulating around the nonischemic zone (b1 to b4). Representative optical action potential (F_v) and Ca_i transient (F_{Ca}) tracings below show reentry at site 1 and Ca_i transient alternans (but not resolvable APD alternans) at site 2 near the border zone, with 2:1 conduction block at site 3 near the center of the ischemic zone.

quent during ischemia, and their site of origin is summarized in Figure 5D. Extrasystoles predominantly originated from the border zone, although during early ischemia, they occasionally arose from the ischemic zone when it was still excitable.

In the remaining monolayer, reentry was initiated by a paced beat that developed unidirectional conduction block while propagating into the ischemic zone (Figure 6). The paced beat successfully propagated part way around the nonischemic zone and then reentered the border zone to initiate reentry.

Arrhythmias During Reperfusion

The action potential and Ca_i transient duration in the ischemic zone recovered rapidly on reperfusion after an average of 1.0 ± 0.8 minutes (Figure 2). However, CV remained depressed for ≈ 10 minutes after reperfusion, and the combination of recovered excitability and persistent slow CV created a highly arrhythmogenic substrate. Propagation during early reperfusion was fibrillation-like in 45% of the cases ($n=5/11$), characterized by rotors anchored in the border zone, propagating with 1:1 conduction into the nonischemic zone but with fibrillatory conduction block into the reperfused ischemic zone (Figure 7 and Movie IV in the online-only Data Supplement). This mother rotor fibrillation pattern^{15,16} typically developed within 1 minute of reperfusion, consistent with rapid recovery of excitability (Figure 7A and Movie IV in the online-only Data Supplement). A rotor anchored at the border zone propagated with a long wavelength around the nonischemic zone and a very short wavelength into the reperfused ischemic zone. After 5 minutes of reperfusion, reentry became organized, with the wavelength increasing in the reperfused ischemic zone because of further recovery of APD and partial recovery of CV. By 10 minutes of reperfusion, reentry had self-terminated, and propagation of paced beats through the reperfused ischemic zone and nonischemic zone was indistinguishable.

Discussion

In the present study, we used dual voltage and Ca mapping in cultured NRVM monolayers to characterize electrophysiological alterations and arrhythmias during regional coverslip IR. We show that coverslip IR recapitulates with a similar time course many of the electrophysiological changes (APD shortening, Ca_i transient broadening, slowed conduction) and arrhythmogenic consequences (premature extrasystoles, Ca_i alternans, conduction block, reentry) previously observed in

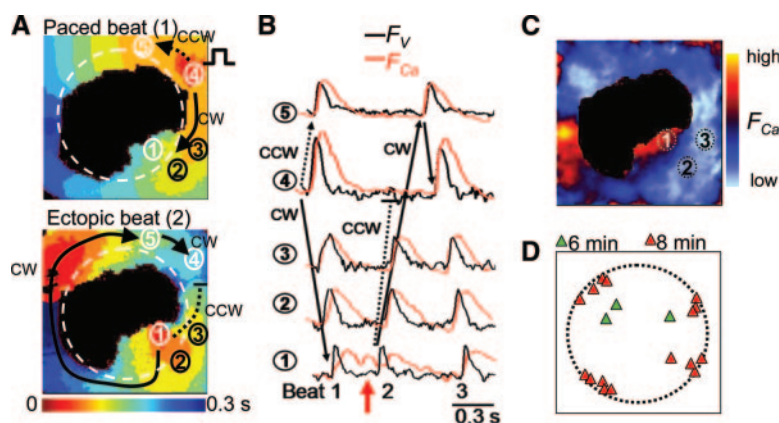


Figure 5. Initiation of reentry by an extrasystole during coverslip ischemia. A, Isochronal activation maps of a paced beat (top panel, beat 1) followed by an ectopic beat arising from the border zone (beat 2) in a monolayer after 8 minutes of ischemia. Note that the central ischemic zone is inexcitable (black). Beat 1 at the pacing site (site 4) propagated counterclockwise (CCW) to site 5 (dashed arrow) and clockwise (CW) to sites 3 to 1 (solid arrow). Then the ectopic beat 2 originating from site 1 propagated in the counterclockwise (CCW) direction to sites 2 and 3 but blocked (dashed arrow). Meanwhile, the same beat propagated clockwise (solid arrow) to reach sites 5 and 4 for the opposite direction and then continued (through the site of previous block of the counterclockwise impulse) to activate sites 3 and 2 initiating reentrant beat 3. B, Optical action potential (F_v) and Ca_i transient (F_{Ca}) tracings at sites 1 to 5, illustrating the earliest activation of the paced beat at site 4 and the origin for the extrasystole from site 1. Note the short APD and prolonged Ca_i transient at site 1. Arrows indicate propagation sequence. C, Ca snapshot immediately before ectopic beat 2 (corresponding to red arrow in B), demonstrating the persistently elevated Ca_i in the area where the ectopic beat is about to emerge. Note that beat 1 is still propagating clockwise through the nonischemic zone (left side) at this time point. D, Diagram showing the site of origin of ectopic beats during ischemia in different monolayers, demonstrating a predilection for the ischemic zone at 6 minutes and for the border zone at 8 minutes.

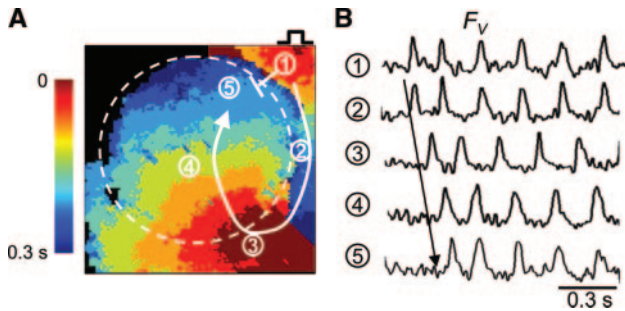


Figure 6. Spontaneous reentry initiated by a paced beat during coverslip ischemia. A, Isochronal activation map of a paced beat initiating reentry during ischemia. The paced beat at site 1 blocked in the ischemic zone but conducted clockwise partway around the nonischemic zone, invading the ischemic zone between sites 3 and 4 to initiate reentry. B, Optical action potential tracings (F_v) at sites 1 to 5 at the initiation of reentry. Arrow indicates the activation sequence.

3-dimensional cardiac tissue subjected to acute regional IR via coronary occlusion. We also found that a preconditioning coverslip ischemic episode delayed the progression of electrophysiological changes. This model may therefore be useful for studying arrhythmogenesis and testing antiarrhythmic and cardioprotective interventions during acute regional IR, especially because the border zone is well demarcated and less anatomically complex than in intact cardiac tissue.

Time Course of Electrophysiological Changes During IR

In developing the coverslip IR model in NRVM monolayers, Pitts and Toombs⁷ focused primarily on characterizing the time course of cellular injury, and most of their observations pertain to ischemic time points beyond 15 minutes. However, they observed that contraction decreased or ceased in the myocytes under the coverslip within 15 minutes, providing evidence that the early ischemic consequences are comparably severe and rapid to intact heart. In our study, the time courses of APD shortening, Ca_i transient broadening, and CV slowing were also rapid in onset and culminated in the central ischemic zone becoming inexcitable after slightly >10 minutes, within the range typically observed in intact cardiac muscle after coronary occlusion^{17–19} and severe hypoxia.²⁰ In the latter cases, changes in APD preceded intercellular uncoupling (reflecting decreased gap junction conductance), which began to decrease after 15 to 20 minutes of ischemia⁴ or anoxia.²⁰ Although we did not measure changes in electrical coupling directly, we documented increased dephosphorylation of Cx43 within the first 5 to 10 minutes of coverslip ischemia, which is linked to electrical uncoupling during ischemia.¹³ This suggests that Cx43 dephosphorylation contributes to early CV slowing and conduction block in the ischemic zone in NRVM monolayers.

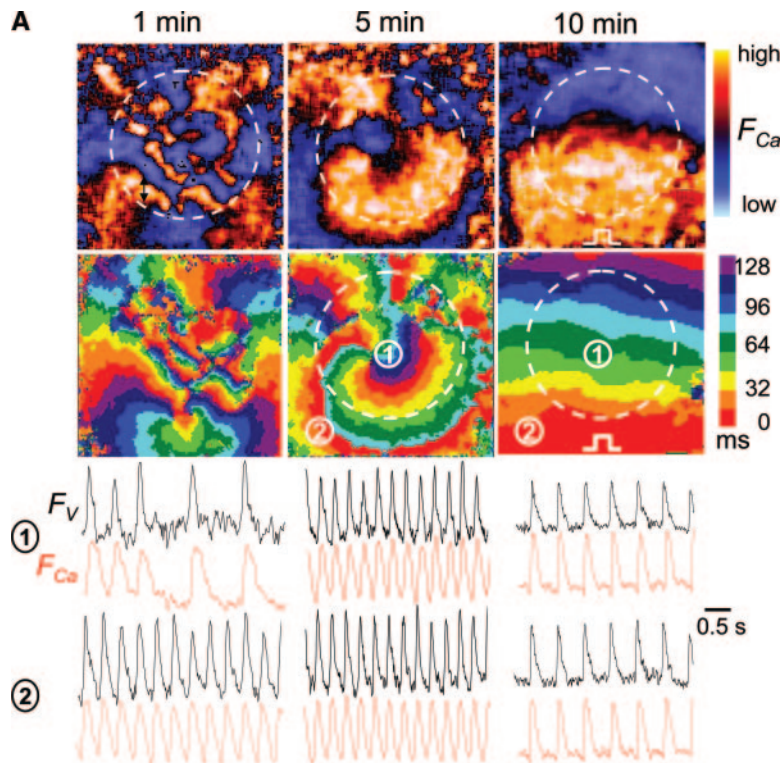


Figure 7. Reperfusion arrhythmias. A, Snapshots of Ca fluorescence (F_{Ca} , top panels), isochronal maps (middle panels), and representative action potential (F_v) and Ca_i transient (F_{Ca}) tracings recorded from site 1 (reperfused ischemic zone) and site 2 (nonischemic zone) at 1, 5, and 10 minutes after reperfusion. At 1 minute, a figure-8 rotor anchored at the border zone (6 o'clock) propagated normally through the nonischemic zone but with slow conduction, short wavelength, and fibrillatory conduction block in the ischemic zone, resembling mother rotor fibrillation. At 5 minutes, a single rotor was present in the repurfused ischemic zone and propagated everywhere without conduction block, resembling ventricular tachycardia. At 10 minutes, reentry had spontaneously terminated, and propagation was uniform from the pacing site.

Spontaneous Premature Extrasystoles

Premature extrasystoles triggering ventricular arrhythmias are common after coronary occlusion in intact cardiac muscle. Experimentally, the high incidence of ventricular arrhythmias during the first 15 to 30 minutes^{3,21} of acute regional ischemia has been attributed to membrane depolarization by diastolic injury currents flowing across the border zone^{22,23} into the nonischemic zone or subendocardial Purkinje fibers, triggering extrasystoles.^{6,24} During coverslip ischemia in monolayers, we found that extrasystoles originated mostly from the border zone and less frequently from the ischemic zone while it remained excitable. Whether extrasystoles were caused by automaticity, triggered activity, or microreentry cannot be discerned from our data. In the example in Figure 5, however, the observation that Ca_i remained persistently elevated after repolarization at the border zone site from which the extrasystole originated may favor triggered activity, as postulated previously in atrium¹⁴ and subacutely infarcted ventricle.²⁵ Unfortunately, the limited spatial resolution (340 μ m) precluded imaging Ca_i waves in individual myocytes or detection of small microreentry circuits.

Arrhythmias During Ischemia

Consistent with intact cardiac muscle,^{3,6} we found that extrasystoles could initiate reentry as a result of unidirectional conduction block in the border zone and ischemic zone. In addition, paced beats also developed unidirectional conduction block during propagation across the border zone into the ischemic zone region, directly initiating reentry. During reentry, impulses propagating into the ischemic zone commonly showed Ca_i transient alternans, a common observation during ischemia in intact hearts.^{26,27} We did not detect APD alternans accompanying Ca_i transient alternans, possibly because the APD was shorter during ischemia and the beat-to-beat APD variation was below our detection limit. However, Ca_i alternans was usually followed shortly thereafter by conduction block, reflecting the bidirectional coupling between APD and Ca_i cycling.²⁸ Ca_i transient alternans in the setting of acute ischemia is likely to be driven primarily by altered Ca_i cycling dynamics (particularly impaired sarcoplasmic-endoplasmic reticulum Ca-ATPase activity)^{28,29} because APD restitution slope tends to become flatter during acute ischemia.³⁰ However, we did not measure APD restitution in NVRM monolayers during ischemia. Finally, the role of elevated diastolic Ca_i during IR could not be assessed directly in our experiments because baseline drift due to photobleaching made calibration of absolute Ca_i concentration unreliable over time.

Overall, our findings are generally consistent with experimental observations in intact heart on arrhythmias during acute regional ischemia.³ In 3-dimensional tissue in which only the surface was mapped, however, it cannot be excluded that unmapped subsurface events played a role in wavebreak and maintenance of reentry. Our findings show that the border zone plays a critical role in anchoring reentry, whereas the ischemic zone was the most frequent site of wavebreak. Anchoring of reentry in areas of spatial electrophysiological heterogeneity is well described.^{31,32} In the intact heart, wavebreak occurred frequently in the ischemic zone,^{33,34} although

also in the border zone.³⁵ Consistent with observations in intact heart,³⁶ this created a mother rotor fibrillation pattern until the central ischemic zone became inexcitable, after which fibrillation converted to macroreentry around the inexcitable ischemic zone.

Reperfusion Arrhythmias and Cx43

Reperfusion arrhythmias in intact heart have been associated with both focal activation patterns and reentry.³⁷ Reperfusion arrhythmias in the coverslip IR model were associated with delayed recovery of CV relative to recovery of excitability. Ca_i overload is an important factor in IR arrhythmias,² promoting triggered activity and decreasing intercellular uncoupling.³⁸ Although we could not directly assess the contribution of Ca_i overload or determine whether focal activations initiated reentry during reperfusion, the reperfused tissue substrate was highly arrhythmogenic, promoting a fibrillation-like state in monolayers during early reperfusion. Moreover, when CV recovered fully to the preischemic value, this fibrillation-like state spontaneously resolved, paralleling the time course of the recovery of CV and Cx43 phosphorylation. To our knowledge, the time course of recovery of Cx43 phosphorylation during reperfusion of intact cardiac muscle has not been characterized in detail. Thus, our findings raise the novel possibility that the delay in Cx43 rephosphorylation may be a significant factor contributing to reperfusion arrhythmias by perpetuating slow conduction after recovery of membrane excitability.

Limitations

Although the NVRM monolayers subjected to regional coverslip IR share many similarities with intact cardiac muscle after coronary occlusion, caution should be exercised in extrapolating the findings to IR in larger mammals, including humans. Neonatal myocytes are immature, with different electrophysiological and Ca_i cycling features than adult ventricular myocytes.⁸ Energetics, metabolism, and Cx43 turnover may also be different. Acute coronary occlusion in intact heart is also associated with variable degrees of residual blood flow from collateral blood vessels, which is not present in the coverslip ischemia model.³⁹

Acknowledgments

The authors are indebted to Tony Mottino for excellent technical advice and assistance with immunofluorescence experiments. We would like to thank Alan Garfinkel and Nicolas Lellouche for statistical assistance.

Sources of Funding

This study was supported by National Institutes of Health/National Heart, Lung, and Blood Institute grant P01 HL078931, the American Heart Association (0625048Y to Dr de Diego; 0365133Y and 0565149Y to Dr Valderrábano), and the Laubisch and Kawata Endowments (to Dr Weiss).

Disclosures

None.

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CLINICAL PERSPECTIVE

The present study shows that acute ischemia/reperfusion, created by placing a glass coverslip over a portion of a cultured neonatal rat ventricular myocyte monolayer, exhibits the major electrophysiological hallmarks of acute ischemia/reperfusion, including spontaneous arrhythmias that are observed in intact 3-dimensional hearts subjected to coronary occlusion. Because of the much simpler geometry of the ischemic zone, border zone, and nonischemic zone, as well as the ability to image cellular events at high resolution, this 2-dimensional model will be useful for studying the cellular basis of electrophysiological alterations and arrhythmias during acute ischemia/reperfusion. In addition, it may prove to be useful for evaluating antiarrhythmic and cardioprotective interventions.

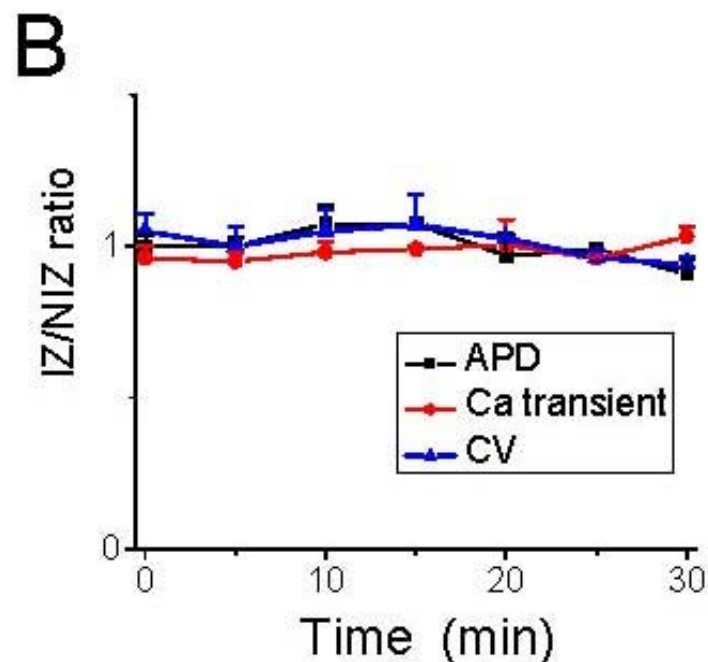
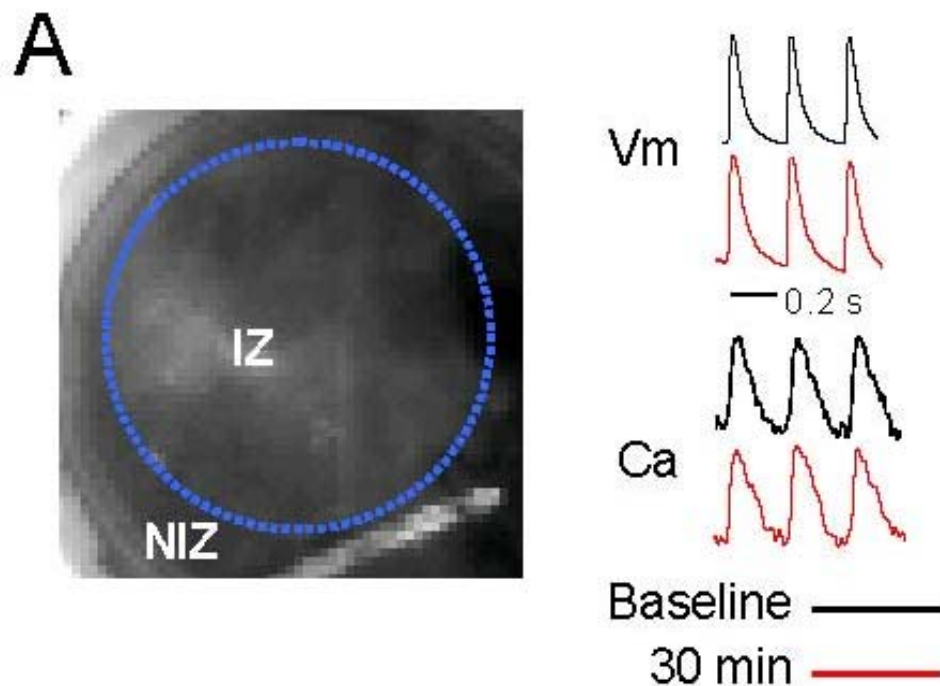
Supplemental Figure 1: Electrophysiological effects of the coverslip ischemia method in semipermeable membrane inserts.

Movie 1: borderzone reentry during ischemia with alternans and wavebreak in the ischemic zone.

Movie 2: borderzone reentry during ischemia without propagation in the ischemic zone.

Movie 3: macrorrenty around ischemic zone.

Movie 4: Fibrillation-like activation in early reperfusion.



Supplemental Figure 1. Panel A shows the semipermeable insert after the glass application. Voltage and calcium traces did not change after 30 min of ischemia compared to baseline recordings. Values of conduction velocity, calcium transient duration and action potential duration were measured from the area under the glass (IZ) and around the glass (NIZ) and then normalized as shown in Panel B.