Remodeling of Gap Junctional Channel Function in Epicardial Border Zone of Healing Canine Infarcts

Jian-An Yao, Wajid Hussain, Pravina Patel, Nicholas S. Peters, Penelope A. Boyden, Andrew L. Wit

Abstract—The epicardial border zone (EBZ) of canine infarcts has increased anisotropy because of transverse conduction slowing. It remains unknown whether changes in gap junctional conductance ($G_j$) accompany the increased anisotropy. Ventricular cell pairs were isolated from EBZ and normal hearts (NZ). Dual patch clamp was used to quantify $G_j$. At a transjunctional voltage ($V_j$) of $+10$ mV, side-to-side $G_j$ of EBZ pairs (9.2±3.4 nS, n=16) was reduced compared with NZ side-to-side $G_j$ (109.4±23.6 nS, n=14, $P<0.001$). $G_j$ of end-to-end coupled cells was not reduced in EBZ. Steady-state $G_j$ of both NZ and EBZ showed voltage dependence, described by a two-way Boltzmann function. Half-maximal activation voltage in EBZ was shifted to higher $V_j$ in positive and negative directions. Immunofluorescence planimetry and quantification showed no change in connexin43 per unit cell volume or surface area in EBZ. Decreased side-to-side coupling occurs in EBZ myocytes, independent of reduced connexin43 expression, and is hypothesized to contribute to increased anisotropy and reentrant arrhythmias. (Circ Res. 2003;92:437-443.)

Key Words: gap junction • myocardial infarction • arrhythmias

After coronary occlusion, a border zone of myocytes survives on the epicardial surface of healing canine infarcts, the epicardial border zone (EBZ).1,2 The EBZ is characterized by reduced conduction velocity and increased anisotropy1,2 associated with the occurrence of reentrant circuits and ventricular tachycardia.1 Reduced sodium current3,4 in EBZ myocytes may contribute to decreased conduction velocity. We studied gap junctional conductance in pairs of EBZ myocytes to determine if alterations occur and, therefore, might also contribute to changes in conduction and anisotropy. Connexin43 (Cx43) was quantified to determine if conductance changes were related to alterations in quantity of this gap junctional protein.

Materials and Methods

Preparation of Myocyte Pairs

Cell pairs were obtained from EBZ of infarcted canine left ventricle, 5 days after coronary occlusion. Surgical methods for occlusion1,2 and enzymatic techniques for cell disaggregation4 have been described. EBZ tissue was removed from a region between the LAD and first circumflex branch that was visibly identified as infarct by its pale appearance (Figure 1), similar to the region sampled in previous studies of EBZ cells.3,4 Because tachycardia was not induced nor reentry mapped, tissues did not come specifically from the central common pathway of reentrant circuits where we previously described redistribution of Cx43 gap junctions6 (Figure 1). For normal pairs (NZ), tissue from a similar region in noninfarcted hearts was used.

Studies were performed on both end-to-end- and side-to-side-coupled EBZ and NZ myocyte pairs, 2 to 8 hours after isolation. The morphological criterion for side-to-side coupling was greater than 50% contact of cell lengths. The criterion for end-to-end coupling was contact of more than 60% of the end-to-end surfaces between each of two paired cells and less than 10% contact of side-to-side cell surfaces.6 According to these criteria, about 60% of all cell pairs isolated were end-to-end coupled, and 40% were side-to-side coupled. End-to-end coupled pairs were easily identified under the optical microscope, because intercalated disks between the two cells could be seen clearly. It was sometimes difficult to identify a side-to-side coupled pair under the microscope because, on occasion, either a single large myocyte, or three myocytes coupled with each other without clear borders, resembled a cell pair. To verify that currents were recorded from a cell pair, we required that the macroscopic transjunctional conductance between paired cells ($G_j$) be blocked by halothane7 particularly if there was a question concerning the presence of a cell pair. In 17 experiments, $G_j$ was reversibly reduced by 91.7±3.1% at a transjunctional voltage ($V_j$) of +40 mV with 10 mmol/L halothane. Also, after experiments were completed, the supposed myocyte pairs were mechanically separated by moving the recording pipettes apart to confirm that there had been two coupled myocytes.

Electrophysiological Recordings

An aliquot of resuspension solution containing myocytes was placed on a poly-L-lysine–coated coverslip at the bottom of a 1-mL superfusion chamber on the stage of a Nikon inverted microscope. A Nikon Coolpix 990 digital camera recorded morphology of studied cell pairs. Transjunctional currents were recorded with a double whole cell patch clamp method8–10 using two independent patch clamp amplifiers (Axopatch 1D and 1C, Axon Instruments). Voltage-clamp protocol generation and data acquisition were controlled by computers equipped with A/D-D/A interfaces (Digidata 1320, Axon Instruments) and Pclamp8 software (Axon Instruments). Currents were...
To minimize errors of conductance measurement, we used large-tipped (4 to 5 μm), low resistance (0.5 to 0.9 MΩ) patch pipettes to reduce Rg and minimize the difference between the applied and the actual Vj. To reduce the effects of sarcolemmal membrane resistance on Vj, bath and pipette solutions were designed to minimize currents through nonjunctional ion channels (potassium, calcium, Na+–Ca2+ exchanger currents). Sodium current (INa) and residual L-type calcium current (ICaL) were inactivated by holding membrane voltage at 0 mV. Bath solution was Ca2+- and K+-free and composed of (in mmol/L) NaCl 146, MgCl2 0.5, NiCl2 6, BaCl2 1, and CsCl 2, HEPES 5, dextrose 5.5, pH 7.3 adjusted with NaOH at 23±2°C. Pipette solution contained (in mmol/L) Cs-Aspartate 115, TEA-CL 20, EGTA 10, HEPES 10, ATP (Mg salt) 5, pH adjusted to 7.3 with CsOH.

For comparison between groups, Gj obtained at V1=±10 mV, at which Gj is close to its maximal level (see Results), was used so that voltage clamp errors caused by large currents through electrodes were minimized. Corrected Vj was 8.97±0.01 mV (n=66) at a V1 of ±10 mV.

To determine voltage dependence of Gj, cell pairs with maximal Gj<50 nS and uncompensated Rs<1.6 MΩ were selected. As a result, the difference between V1 and the corrected Vj was less than 10% at V1=±100 mV. Steady-state conductance (Gj,ss) was measured at the end of each voltage step and normalized to the instantaneous conductance (Gj,in) measured at the beginning of the voltage step. The Gj,ss-Vj relationship was described using the two-way Boltzmann function:

\[ G_{j,ss} = \frac{(G_{max} - G_{min})}{1 + \exp\left[A(V_j - V_0)\right]} + G_{min} \]

where Gmax is the maximum conductance, Gmin is the sustained conductance at the end of voltage steps (also called voltage-insensitive residual conductance), V0 is the transjunctional voltage at the halfway between Gmax and Gmin (Vj at which Gj,ss=(Gmax−Gmin)/2), and A is a constant that defines the voltage sensitivity (see Table).

Data sampling rate was reduced to 200 Hz by a digital filter in pClamp8 before analysis. Peakfit (SPSS Science) was used for fitting data with the Boltzmann function. Excel (Microsoft) and SigmaStat (SPSS Science) were used for mathematical and statistical analysis (unpaired t test, P<0.05 was considered significant). Summarized data are presented as mean±SE.

Immunocytochemistry

Cx43 immunolabel was quantified in single myocytes from the same aliquot of cells from which cell pairs were obtained for electrophysiological studies. The immunolabeling protocol has been previously described. It was adapted to give optimal labeling of cells to ensure maximal signal-to-noise ratio. Cells were fixed in 100% methanol and washed in phosphate-buffered saline before blocking in 1% bovine serum albumin (Sigma). The primary antibody was a mouse anti Cx43 IgG1 raised against a synthetic peptide corresponding to positions 252 to 270 of rat Cx43 (Chemicon International). A secondary antibody tagged with CY3 fluorescent marker (Chemicon) was used. Cell pellets

![Diagram of epicardial surface](base)

**Figure 1.** Diagrammatic representation of the epicardial surface of the anterior wall of the canine left ventricle (EBZ) that is infarcted by occlusion of the left anterior descending coronary artery (LAD). Margins of the infarct are labeled between LAD, base, and lateral left ventricle (LAT). Regions enclosed by solid, dashed, and dotted lines indicate the locations of the central common pathway of three reentrant circuits that were mapped in the experiments of Peters et al. where connexin43 was redistributed around the entire margins of the myocytes throughout the full thickness of the EBZ (50 to 200 cell layers). Shaded region is where tissue was obtained for this study. Scale bar=6 mm.

Boltzmann Parameters for Gap Junctions in Normal and Infarct Hearts

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NZ (n=12)</th>
<th></th>
<th>EBZ (n=11)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A, V⁻¹</td>
<td>0.085±0.005</td>
<td>−0.076±0.006</td>
<td>0.076±0.006</td>
<td>0.068±0.009</td>
</tr>
<tr>
<td>V₀, mV</td>
<td>66.6±2.0</td>
<td>68.6±2.2</td>
<td>79.2±2.1***</td>
<td>82.4±3.1***</td>
</tr>
<tr>
<td>G_{max}</td>
<td>0.258±0.032</td>
<td>0.299±0.026</td>
<td>0.367±0.042*</td>
<td>0.374±0.033</td>
</tr>
<tr>
<td>G_{min}</td>
<td>0.999±0.017</td>
<td>0.994±0.019</td>
<td>1.008±0.013</td>
<td>1.060±0.017</td>
</tr>
</tbody>
</table>

Parameters were obtained by fitting G_{j,ss}-Vj relationships of NZ and EBZ cell pairs with the Boltzmann function (see Materials and Methods). Data are presented as mean±SE.

G_{max} indicates maximum conductance; G_{min}, voltage-insensitive residual conductance; A, voltage sensitivity; V₀, Vj at the halfway between G_{max} and G_{min}.

*p<0.05, **p<0.001 vs NZ.
EBZ myocytes have rough “bumpy” membranes with blurred striations and small, dark, granules (lipid inclusions). In Figure 2A, representative current traces recorded from a side-to-side–coupled NZ cell pair (shown in Figure 2C). The \( I_v \) (recorded in cell 2) was generated by voltage steps of +10 mV (thick trace) and −10 mV (thin trace) applied to cell 1. In this example, \( I_v \) was 0.9 nA and \( G_j \) after correction of \( R_s \) was 101 nS. Figure 2B shows \( I_v \) recorded in a side-to-side–coupled EBZ cell pair (shown in Figure 2D) in response to the same voltage clamp protocol. \( I_v \) amplitude is decreased to 0.3 nA (with a \( G_j \) of 32 nS) at both positive and negative directions.

Figure 3 summarizes \( G_j \) obtained at +10 mV (near maximal conductance; see next section) for all groups. For NZ myocyte pairs, \( G_j \) was 109.4 ± 23.6 nS (mean ± SE, range of 24 to 338 nS) for side-to-side–coupled pairs, and 92.7 ± 17.4 nS (16 to 297 nS) for end-to-end–coupled pairs (not significantly different from each other; \( P > 0.05 \)). In contrast, \( G_j \) of side-to-side–coupled EBZ myocytes (9.2 ± 3.4 nS, range 0 to 63 nS) was significantly lower than \( G_j \) of side-to-end–coupled EBZ myocytes (82.6 ± 20.5 nS, range 9 to 285 nS; \( P < 0.01 \)). \( G_j \) of end-to-side–coupled EBZ myocytes was also significantly lower than \( G_j \) of side to side NZ myocytes (\( P < 0.001 \)). \( G_j \) of end-to-end–coupled EBZ myocytes was not different from NZ end-to-end–coupled myocytes (\( P > 0.05 \)).

Voltage Dependence of Gap Junctions in EBZ and NZ

Voltage-dependent properties of gap junctions in cell pairs with \( G_j \) of <50 nS at +10 mV were determined. \( I_v \) was recorded from cell 2, whereas voltage steps from −100 to +100 mV were applied to cell 1 in 10-mV increments (Figure 4A). In both NZ (left) and EBZ (right), \( I_v \) generated by voltages > +40 mV or < −40 mV showed symmetrical decay phases, and current decay was faster as \( V_j \) became larger. These characteristics suggest that the gating processes of gap junction channels in canine ventricular myocytes are time- and voltage-dependent. The \( I_v \)-\( V_j \) relationships in side-to-side coupled myocytes are summarized in Figure 4B. In both NZ (left) and EBZ (right) pairs, instantaneous currents (\( I_v \),
Data obtained from side-to-side coupled pairs. B, I-V relationships for side-to-side coupled NZ (n=5) and EBZ (n=6) pairs. Instantaneous currents (I_{in}, open circles) measured at beginning of voltage steps, and steady-state currents (I_{ss}, filled circles) measured at end. Symbols and vertical bars denote mean values and standard errors, respectively. C, I-V relationships for end-to-end coupled NZ (n=8) and EBZ (n=6) cell pairs. *P<0.05, **P<0.01 vs NZ.

Figure 5A. Voltage dependence of G_{ss} was normalized to G_{ss} and plotted against V_j. Data summarized from 12 NZ pairs (open circles, 5 side-to-side and 7 end-to-end) and 11 EBZ pairs (filled circles, 5 side-to-side and 6 end-to-end). Symbols and bars denote mean±SE. Smooth curves superimposed on data points are calculated based on a two-way Boltzmann function with best-fit results. See Table for a summary of parameters. *P<0.05, **P<0.01 vs NZ. B. Voltage dependence of time constants of current decay. Time courses of I_j decay fit with double exponential function (inset). Symbols stand for mean values of fast (τ_f) and slow (τ_s) time constants for NZ and EBZ pairs, respectively. Vertical bars represent SE. Numbers of observations are in parentheses. *P<0.05 vs NZ.

Because voltage-dependent properties of side-to-side and end-to-end gap junctions are similar in both EBZ and NZ pairs, data for the G_{ss}-V_j relationships were combined from both side-to-side- and end-to-end-coupled pairs within each group (Figure 5A). The G_{ss}-V_j relationships are bell-shaped for both NZ and EBZ pairs such that normalized G_{ss} reaches its maximum at V_j close to 0 mV (±10 mV, where G_{ss}=G_{max}), and then decreases symmetrically as V_j changes in either a positive or negative direction. Minimum conductance (maximum rectification) occurs at V_j near ±100 mV. The G_{ss}-V_j relationship could be described by a two-way Boltzmann function with the best-fit parameters (see Table). The half maximal activation voltage (V_m) in EBZ pairs was +79.2±2.1 mV as compared with +66.6±2.2 mV for the Nz group (P<0.001) in positive polarity, and −82.4±3.1 mV (EBZ) as compared with −68.7±2.2 (NZ) (P<0.001) in the negative polarity. A reduction in voltage sensitivity (A) in EBZ relative to NZ did not reach statistical significance (P>0.05). Voltage-insensitive residual conductance (G_{min}) in EBZ pairs was significantly increased compared with NZ (P<0.01).
In NZ cell pairs, the time course of current decay at Vj > ±70 mV was best described by a double exponential function. The fast and slow time constants (τ1 and τ2) became smaller as Vj increased (Figure 5B). For example, τ1 and τ2 at a Vj of −70 mV were 0.46±0.09 and 2.85±0.69 seconds (n=9), respectively. The τf and τs at a Vj of −100 mV were 0.15±0.04 (P<0.01 versus −70 mV) and 1.00±0.12 seconds (n=9, P<0.05 versus −70 mV), respectively. In EBZ pairs, the time course of current decay was prolonged although the voltage dependence of the time constants was similar to those in NZ cells. For example, τf and τs in EBZ pairs were 0.98±0.25 and 4.95±1.06 seconds (n=11) at −70 mV, and 0.32±0.18 (P<0.05 versus −70 mV) and 1.36±0.20 seconds (n=11, P<0.01 versus −70 mV) at −100 mV (P<0.05 for both τf and τs, compared with NZ). These results indicate that the function of gap junctions in EBZ is modified in terms of voltage dependence as well as current inactivation kinetics.

Connexin43 in EBZ and NZ Myocytes

Images of typical Cx43 immunolabeled myocytes from EBZ and NZ are shown in Figure 6. Planimetry of 180 cells from 9 EBZ preparations and 200 cells from 10 NZ preparations showed that cell volume of EBZ cells (36887±2504 μm3) was not significantly different from NZ cells (39570±2296 μm3; P<0.44). EBZ cells displayed a reduction in Cx43 immunolabel per cell (2454±382 μm2 versus 3811±436 μm2; P<0.04). When corrected for cell volume, Cx43 immunolabel was not significantly reduced (NZ 0.104±0.01 μm2/μm3 versus EBZ 0.07±0.01 μm2/μm3; P<0.07). There was also no significant difference in cell surface area (NZ 5549±288 μm2 versus EBZ 5142±271 μm2; P<0.34) or in Cx43 area per unit cell surface area (NZ 0.75±0.09 μm2/μm2 versus EBZ 0.48±0.08 μm2/μm2; P<0.06).

The ratio of Cx43 immunolabel located at the ends and sides of myocytes (longitudinal/transverse Cx43 label distribution) was also not significantly different in EBZ cells compared with NZ cells (0.45±0.058 versus 0.47±0.055; P=0.86). However, despite the lack of quantitative change, the Cx43 along the sides of 18% of EBZ cells was redistributed from normal small transversely oriented clusters (Figure 6A), to longitudinally arrayed streaks of Cx43 labeling (Figure 6B), as previously shown in cells immediately abutting the necrotic infarct and constituting the central common pathway.5

Discussion

Pathological Alterations in Gap Junctions

Information on pathological alterations of gap junctional electrophysiology as a cause of conduction abnormalities and arrhythmias is limited. Increased longitudinal resistance related to slowing of conduction, partly a consequence of decreased cell coupling, has been shown in papillary muscle during acute ischemia.13 Also, decreased conduction in multicellular preparations from hypertrophied hearts results from increased junctional resistance.14 Methods used in those studies did not permit determination of Gj nor resolution of increased Rj to side-to-side or end-to-end connections. Gj decreased in cell pairs from hearts after 30 minutes of coronary occlusion and was related to increased intracellular Ca2+ and decreased intracellular pH.15 Alterations in Gj associated with other pathologies have not been described.

Electrical Properties of Canine NZ and EBZ Gap Junctions

To determine if Gj was altered in EBZ, we studied adult canine ventricular myocyte pairs, a preparation that had not previously been investigated. To minimize errors of conductance measurement inherent in investigations of adult myocyte pairs, we used large-tipped patch pipettes to reduce Rs. However, errors in Gj measurements, particularly of high conductance junctions, cannot be totally eliminated due to current-passing limits of the recording pipettes.10

Macroscopic conductance of canine NZ gap junctions was 24 to 338 nS for side-to-side–coupled cells and 16 to 297 nS for end-to-end–coupled cells at a Vj of 10 mV (maximal values). Gj reported for mammalian adult ventricular myocytes has varied from 26 to 3073 nS, depending on species and recording methodology.9,15,16–18 Our data are similar to rabbit ventricular myocyte pairs, studied using similar methods and experimental conditions.17

We demonstrated that adult canine myocyte gap junctions from normal hearts have voltage-dependent properties, not described in earlier studies on adult myocyte pairs.9,16,18–20 Gj had the characteristic bell-shaped voltage dependence, similar to that of expressed connexin43 channels.21,22 Failure to show voltage dependence in previous studies may have been secondary to inaccurate control of Vj because of uncompensated Rj.

In EBZ, there was a significant decrease in coupling conductance in the side-to-side direction compared with NZ. No difference in coupling conductance of end-to-end connections was found between EBZ and NZ pairs. However, because end-to-end Gj was relatively high in both groups, differences between the groups may have been obscured by...
errors in the methodology described above.\textsuperscript{10} \(G_j\) in EBZ also had bell-shaped voltage dependence. However, EBZ showed weaker responses to changes in voltage.

**Relationship of Changes in \(G_j\) to Changes in Cx43**

Redistribution of Cx43 around the cell perimeter occurs in myocardies abutting necrotic fibrin and constituting the reentrant circuit central common pathway in EBZ.\textsuperscript{5} We did not induce reentrant tachycardia to locate the central common pathway in this study and did not, therefore, subselect a myocyte population for Cx43 redistribution. A majority (82\%) of myocytes that we studied did not have redistribution of Cx43 characteristic of the central common pathway because they likely came from outside of this region (see Figure 1), nor did they show a reduction in amount of Cx43 as previously described for more prolonged ischemia.\textsuperscript{12}

Although there was no quantitative alteration in end-to-side ratio of Cx43 distribution, indicating that reduction of transverse \(G_j\) occurred independently of a decrease in Cx43 immunolabel, distribution of Cx43 label along transverse membranes was altered in 18\% of myocytes, changing from normal small transversely oriented clusters to longitudinally arrayed streaks. Any relationship between this redistribution and altered conductance is unknown, but a relationship between the pattern of this redistribution across EBZ and reentry has been shown.\textsuperscript{5} It is also possible that some gap junctions on the lateral sides of EBZ myocytes are not functional.\textsuperscript{23}

Changes in other connexins may also be related to altered EBZ conductance. Connexin45 has been detected in ventricular myocardium.\textsuperscript{24} Immunolabeling for Cx45 (Cx45 antibody, Chemicon International) in frozen tissue sections of NZ and EBZ have shown indistinguishably scant or absent labeling (unpublished data, 2002), suggesting that significant upregulation of this connxin does not occur in EBZ.

**Relationship to Increased Anisotropy and Reentry in the EBZ**

In EBZ of healing canine infarcts, lines of functional block that bound the central common pathway of reentrant circuits causing tachycardia develop in regions of high anisotropy caused by slow transverse propagation at the interface between myocytes with structurally remodeled gap junctions (see previous section) and normally distributed gap junctions.\textsuperscript{1,5} Slowing of transverse impulse propagation in regions of reentrant circuits outside the central common pathway (outer pathways), attributed to increased anisotropy, also facilitates the occurrence of reentry.\textsuperscript{1} The decrease in transverse \(G_j\) that we have shown provides a possible electrophysiological mechanism for increased anisotropy and slowing of transverse conduction. However, we did not directly assess the influence of reduced gap junction conductance on conduction slowing or enhanced anisotropy. A more direct demonstration of such a role for decreased transverse \(G_j\) might be obtained from measuring cell-to-cell coupling in the in situ heart.\textsuperscript{25}

Other important factors are also likely to change anisotropy, such as arrangement of myocardial fiber bundles and properties of the extracellular environment.\textsuperscript{26} An increased extracellular resistance accompanies conduction slowing during acute ischemia.\textsuperscript{13} It is uncertain how changes in extracellular space of EBZ at 5 days affect extracellular resistance and anisotropy. Although new collagen deposition is not yet significant,\textsuperscript{5} swelling and edema occur.\textsuperscript{2} Extracellular resistance and conduction velocity are sensitive to changes in volume of the interstitial space.\textsuperscript{27} Cell size also influences anisotropic properties,\textsuperscript{28} but myocytes of the EBZ have similar cell size as normal.\textsuperscript{4}

The pathophysiological role of altered EBZ gap junction voltage dependence is uncertain. When myocytes are depolarized in pathological conditions, voltage sensitivity may play a protective role by closing junctional channels, isolating the damaged region electrically from healthy tissue.\textsuperscript{29} Because gap junctions in EBZ were less sensitive to transjunctional voltage, such isolation would be less severe and permit continuation of cell communication.

**Acknowledgments**

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**References**


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