Heterogeneous gap junction remodeling in reentrant circuits in the epicardial border zone of the healing canine infarct

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Abstract

Background: The epicardial border zone (EBZ) of surviving myocytes in the healing, 4- to 5-day-old canine infarct is an arrhythmogenic substrate characterized by both structural and functional remodeling of Cx43. Unknown is whether the remodeling of gap junction conductance is heterogeneous in the EBZ like that of sarcolemmal ion channel remodeling and how remodeling of the gap junction influences conduction and anisotropy.

Methods and results: Ventricular tachycardia was initiated by programmed stimulation in healing canine infarcted hearts. Reentrant circuits were mapped and the central common pathway (CCP) and outer pathway (OP) regions localized. Epimyocardium removed from the CCP was disaggregated to generate myocyte pairs for conductance measurements. Cx43 distribution was determined by immunofluorescent confocal microscopy. While transverse coupling (gap junction conductance) was markedly decreased in OP cells, CCP cells with lateralized Cx43 gap junctions showed normal conductance. Longitudinal coupling in both OP and CCP was no different than normal. Consistent with conductance measurements, the anisotropic ratio in the CCP was similar to that of normal tissue. In the OP it was increased. Despite normal longitudinal and transverse conductance and anisotropic ratio, longitudinal and transverse conduction velocities were decreased in the CCP with respect to normal epicardium, possibly as a result of the remodeling of sarcolemmal ion channels in this region.

Conclusions: Gap junction conductance and distribution is heterogeneous in different regions of reentrant circuits. Lateralization of Cx43 gap junctions in CCP of reentrant circuits is associated with normal transverse conductance between cell pairs. In contrast, absence of lateralization in OP is associated with reduced transverse conductance. Despite normal anisotropic ratio, conduction velocity in CCP region remains slower than normal. This suggests that the effects of Cx43 remodeling in the infarcted heart should be interpreted in conjunction with other types of remodeling occurring in the EBZ (i.e. sarcolemmal ion channels).

Keywords: Infarction; Remodeling; Arrhythmia
can be structural as exemplified by infiltration of connective tissue between myocardial fiber bundles during aging or infarct healing [1]. Structural remodeling may lead to functional changes; in the above examples, conversion of conduction properties from uniform to non uniform anisotropy occurs [2,3]. Remodeling can also be functional occurring without obvious structural changes such as changes in ion channel function that accompany ischemia [4].

The epicardial border zone (EBZ) of surviving myocytes in the healing, 4- to 5-day-old canine infarct is an arrhythmogenic substrate characterized by both structural and functional remodeling [5]. Structural remodeling is exemplified by reorganization of Cx43 gap junctional protein so that it is predominantly located along lateral sarcolemmal membranes [6]. Functional remodeling is exemplified by changes in ion channel function and gap junctional conductances [7,8]. Some of these remodelings are heterogeneous. For example, structural remodeling of Cx43 occurs in cells of the central common pathway of reentrant circuits causing ventricular tachycardia and not in those of the outer pathways [6]. Characteristics of functional remodeling of sarcolemmal ion channels are different in cells from the central common and outer pathways of reentrant circuits [9].

Despite this, several problems remain to be elucidated in determining the role of remodeling in EBZ arrhythmogenesis. For example, it is not known whether the functional remodeling of gap junction conductance is heterogeneous like that of sarcolemmal ion channel remodeling [9]. A reduction in conductance has been described for outer pathway myocytes without Cx43 lateralization, but the conductance of central common pathway myocytes with lateralization has not been determined and is a focus of this study. In addition, the influence of these heterogeneities of structural and functional remodeling of gap junctions on conduction has not been elucidated and thus this is the second purpose of these experiments.

2. Methods

We mapped reentrant circuits in the epicardial border zone of 5-day-old infarcted dog hearts during sustained ventricular tachycardia (>30 s) induced by programmed stimulation as before [5]. Reentrant circuits consisted of two counter rotating waves that traveled around two lines of block in a figure-8 pattern. The locations of the lines of block, which determine the location of the central common pathway (CCP) and the outer pathway (OP) of the reentrant circuit (Fig. 1), were marked on the surface of the heart using a method previously described in detail [9]. The mapping studies were performed at 37 °C. The sampling rate of the mapping system was 1 ms. Our experiments conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Fig. 1. At the top is a lead II ECG recorded during sustained ventricular tachycardia induced by programmed stimulation in a dog with a 5-day healing myocardial infarction. Below is an isochronal map of one reentrant cycle associated with the tachycardia. Activation begins at the 0–10 ms isochrone and follows the sequence shown by the arrows. The thick vertically oriented black lines represent functional lines of block. The central common pathway (CCP) of the circuit is between the 2 lines of block, the outer pathways (OP) are to the left and right, respectively, of the left and right lines of block. The regions of the circuit delineated by the dashed rectangles show the position of the small high density electrode array in the central and outer pathways.

2.1. Cx43 gap junction conductance

To determine gap junction conductance in cells that form the substrate of the CCP, slices of epicardium about 10 × 20 mm² with a thickness of 2–3 mm, were removed from the CCP of reentrant circuits in 8 dogs with sustained tachycardia. Enzymatic techniques were used to generate CCP cell pairs for conductance measurements as previously described [7]. Studies were performed on both end-to-end and side-to-side coupled myocytes, as defined in our previous publication (see Fig. 1C in Yao et al. [7]). Determination of transjunctional macroscopic currents and gap junction conductance was accomplished using a dual whole cell patch clamp method [7]. The command voltages for cell 1 (or electrode 1), $V_1$, and cell 2 (or electrode 2), $V_2$, were initially held at 0 mV. Then $V_1$ was stepped to the test voltages in 10-mV increments. The test pulse duration was 5 s with an interpulse interval of 10–15 s. The transjunctional current ($I_j$) was recorded at cell 2. $I_j$ was expressed as $I_j = I_2(1 + r_2/R_2)$, where $I_2$ is the current through electrode 2, $r_2$ the series resistance of electrode 2, and $R_2$ the non-junctional membrane resistance. Since the
membrane potential of cell 2 remained unchanged, the transjunctional current \( (I_j) \) is approximated as \( I_2 \) when electrode 2 has a tight seal with the membrane of cell 2 \( (r_2/R_2 \text{ approaches } 0) \) [10,11]. Gap junctional conductance was calculated as \( I_j/V_j \), where \( V_j \) is transjunctional voltage. \( V_j \) is influenced by the series resistance of both electrodes \( (r_1 \text{ and } r_2) \) and currents through them \( (I_1 \text{ and } I_2) \). Errors in measurements of transjunctional conductance caused by series resistance were minimized as previously described [12,13]. For comparison between cell groups, gap junctional conductance obtained at a low test voltage \( (V_t, +10 \text{ mV}) \) was used such that voltage clamp errors caused by large currents through electrodes were minimized. The \( V_j \) of +10 mV was also chosen because conductance generated at this voltage is close to its maximal level [7].

2.2. Cx43 gap junction cellular distribution

In some experiments (3 NZ, 8 CCP, 8 OP cells), Cx43 immunolabeling was quantified in single myocytes dispersed from tissues from the central and outer pathways of reentrant circuits localized as above. Myocyte isolation procedures are as before [7,14]. Freshly isolated cells were seeded onto laminin-coated (Invitrogen) Lab-Tek chamber slides (Nalge Nunc International) fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, followed by multiple PBS washes). The cells were then permeabilized with 0.4% Triton X-100 in PBS for 20 min, and blocked with 1% bovine serum albumin (Sigma) for 30 min. Cells were incubated overnight at 4 °C with a monoclonal anti Cx43 IgG1 against amino acid positions from 252 to 270 of rat Cx43 (Chemicon International, MAB3067) (1:1000). Following rinse, the cells were incubated at room temperature for 2 h with secondary CY3-conjugated goat anti-mouse IgG antibody (Chemicon International, AP181C) (1:1000). Following rinse, the cells were mounted with Gel/Mount medium (Biomeda Corp.). Myocytes were then examined using a Zeiss LSM 510 META laser scanning confocal microscope (Zeiss Inc.). For each cell, serial confocal sections at 1 μm intervals were recorded, giving a confluent series of 1 μm slices through the z-axis of the entire cell. The planimetry was performed for 30 randomly selected cells from each sample. ImageJ 1.30v program (NIH, Bethesda, MD) was used to measure total immunolabeled Cx43 gap junctional area at the intercalated disk regions as well as at lateral membranes and cell surfaces as the number of pixels/μm². The dense lateral regions are scored as small intercalated disks. Fiber direction was used to determine longitudinal vs. transverse lateral membrane.

2.3. Statistics

All results were presented as mean±S.E. and were compared using Student’s t test; significance is reported at the \( p<0.05 \) level.

2.4. Tissue studies conduction velocity

In 4 dogs with sustained tachycardia, an electrode array (24×19 mm²) consisting of 312 bipolar electrodes with spacing between bipolar pairs of 1.25 mm, mounted on a micromanipulator that had controls for positioning in the x, y and z axes, was positioned first in the region of the central common pathway and then in the outer pathway location, after tachycardia terminated (during sinus rhythm) (dotted lines in Fig. 1). Electrograms were recorded during pacing from the center and the periphery of the array at each location, at different cycle lengths ranging from 3 to 5 Hz and activation maps generated. Local conduction velocities in areas of 1.25×1.25 mm² were calculated from the activation times in 4 adjacent electrodes that delimit the local area [15,16]. Each local vector velocity was characterized by a magnitude and a direction (of propagation). Local vector velocities were binned by direction of propagation every 30° and their magnitudes averaged to obtain a global value of conduction velocity in the area of the array (24×19 mm²) in that direction of propagation. The direction in which the global value of the velocity was the fastest was defined as the longitudinal conduction velocity. The direction in which the global value of the conduction velocity was the slowest was defined as the transverse conduction velocity [2,5,6]. The anisotropic ratio (ANR) was calculated as the ratio of the longitudinal and transverse conduction velocity.

After conduction velocity measurements, epicardial slices of tissue (1×1 cm²) were removed from both the central and outer pathways of the reentrant circuits to determine myocyte Cx43 distribution using immunohistochemical techniques that have been previously described [6].

3. Results

3.1. Gap junction conductance in myocyte pairs of the central common pathway (CCP) of reentrant circuits

Gap junction conductance was determined for myocyte pairs dispersed from central common pathway (CCP) of mapped reentrant circuits and compared to the conductance in border zone myocytes without structurally remodeled Cx43 that are typical of outer pathway myocytes (OP), and to pairs from normal epicardium (NZ) from our previous study [7]. Fig. 2A shows transjunctional currents in response to a +10 mV test pulse in side-to-side cell pairs from NZ, OP and CCP. It is apparent that the current in the CCP pair is larger than that of the OP pair and similar to NZ pair. Data from 12 CCP pairs show a mean gap junction conductance in the side-to-side direction of 117±35 nS which is not significantly different from that of NZ side-to-side conductance (123±23 nS, 19 pairs) (Fig. 3). In the end-to-end direction, CCP conductance (121±27 nS, 8 pairs) is also not significantly different than normal (95±16 nS, 25 pairs) (Fig. 3). However, when compared to conductance of OP myocytes (Fig. 3) without structural Cx43 remodeling (see
next section), CCP conductance in the side-to-side direction was significantly greater (117±35 nS, 12 CCP pairs vs. 13±4 nS, 21 OP pairs) while that in the end to end direction was similar (121±27 nS, 8 CCP pairs vs. 91±19 nS, 24 OP pairs).

3.2. Structural remodeling of Cx43 gap junctions

Cx43 distribution was quantified in single myocytes isolated from the central and outer pathways of mapped reentrant circuits and compared to that of NZs. Fig. 4A shows the normal localization of Cx43 in a NZ at the longitudinal ends (intercalated disk region) of the cell. Fig. 4B quantifies Cx43 staining density (in fluorescent pixels/μm) at the intercalated disk region and the lateral membranes for NZs. Note that little Cx43 is found on the lateral membranes of NZs. A myocyte from the CCP shows cell surface lateralization of Cx43 (Fig. 4C) in addition to Cx43 at the intercalated disk regions (Fig. 4D). This is in contrast to what is seen in the OP cells (Figs. 4E and F) where the location of Cx43 is similar to that of NZs. A comparison of the Cx43 staining density by cell region (intercalated disk (Fig. 4G) and lateral membrane (Fig. 4H)) shows that there is a significant increase in the Cx43 immunosignal localized on lateral membranes only in cells from the CCP (Fig. 4H).

In sum, Cx43 distribution in cells dispersed from the OP is similar to that of normal cells and Cx43 distribution in cells dispersed from the CCP is abnormal (lateralized), consistent with our previous findings in tissues isolated from the OP and CCP [6]. Interestingly, while cells from the CCP have an abnormal Cx43 distribution, they have an average gap junctional conductance which is not different from normal cells. OP cells have normal Cx43 distribution but abnormal (side-to-side) gap junctional conductance.

3.3. In vivo mapping of conduction in central and outer pathways of reentrant circuits

To further understand how the interplay of Cx43 distribution and gap junctional conductance affects propagation in the CCP and OP, we measured conduction velocities in both regions of the mapped reentrant circuits.
Fig. 5 shows representative isochronal maps and the corresponding conduction velocity measurements (below) during stimulation from a point source (pulse symbol) at the center of the electrode array, in normal epicardium (panel A), outer pathway (panel B) and central pathway (panel C) epicardium of mapped circuits. Elliptical isochrones characteristic of anisotropic myocardium occurred in all regions. Note that owing to the small size of the high resolution mapping electrode, part of the concentric ellipses occurred outside the mapped region. The long axis of the elliptical activation indicates the rapid longitudinal direction of conduction and the short axis shows the slow transverse direction. The small arrows on the map in Panel A show the gradient of activation times (and the direction of propagation and the vector velocity) at each local area, calculated from the activation times (time resolution 1 ms) at the 4 electrode sites that delimit the local area (1.25 × 1.25 mm²). From the local conduction velocities, the global velocities were calculated (see Methods). In the bottom panels in Fig. 5, global velocities for each of the 30° sectors are plotted. The thick black arrows indicate the most rapid and the slowest propagation velocities indicating the direction of...

Fig. 4. Connexin43 redistribution in single cells from NZ, CCP and OP regions. In normal myocytes Cx43 is found primarily at the intercalated disk regions (A) in significantly higher levels than that found on the lateral membranes (B). In contrast, cells from the CCP show Cx43 staining at the intercalated disk region as well as along the lateral membranes of the cells (C). There is no difference between the levels of Cx43 on the lateral membranes as compared with the intercalated disk region (D). OP Cells resemble NZs in their Cx43 localization (E) with a significantly higher level of Cx43 found at the intercalated disk region as compared to the lateral membranes (F). Comparisons of Cx43 distribution by region for all three cell groups are shown in (G, H). The scale in Fig. 4A also applies to Figs. 4C and E.
longitudinal (L) and transverse (T) conduction velocities. Fig. 6A summarizes the results of the measurements of conduction velocities. Longitudinal conduction velocity in the outer pathway (37±4 cm/s) was reduced compared to normal NZ LV (45±7 cm/s), but did not reach statistical significance. However, longitudinal velocity was significantly reduced in the central common pathway compared to that of both normal and outer pathways (29±5 cm/s) (Fig. 6A, left). Transverse velocities in the outer and central pathway were both significantly reduced (17±3 and 18±5 cm/s, respectively) compared to normal NZ LV (28±5 cm/s) (Fig. 6A, center) and did not differ from each other. As a consequence of these changes in velocity, anisotropic ratio in the outer pathway (2.1±0.2) (normal longitudinal velocity/decreased transverse velocity) was greater than that in normal LV (1.6±0.3) while the anisotropic ratio in the central pathway (1.6±0.3) (decreased longitudinal velocity/decreased transverse velocity) did not differ from normal (Fig. 6A, right).

Tissue distribution of Cx43 was determined in slices of epicardial tissues from each of the mapped regions (center and outer pathways and normal LV) in which these conduction velocities were determined. Confirming our previously published data [6], Cx43 was redistributed along the lateral membrane in the central but not in the outer pathway or in normal LV (Fig. 6B).

4. Discussion

We have shown here that just as for sarcolemmal ion channels [9] there are also heterogeneous gap junctional functional changes in cell pairs from the CCP versus those from OP of mapped reentrant circuits. Interestingly, the marked functional changes in gap junctional conductance in OP pairs [7] are NOT accompanied by Cx43 lateralization along cell membranes (structural remodeling). Rather and in contrast to CCP cells and tissues where prominent Cx43 structural remodeling has occurred, functional changes in gap conductance have not.

4.1. Remodeling

Both sarcolemmal ($I_{Na}$, $I_{Ca_{L}}$, $I_{to}$, $I_{K}$) [8] and gap junction channels [7] undergo functional remodeling in the EBZ that
alters the characteristics of membrane currents, action potentials and intercellular impulse transmission. Recently we determined that remodeling of sarcolemmal ion channel function is heterogeneous, with different properties of remodeled Na and Ca currents occurring in the reentrant circuit central pathway vs. the outer pathway [9]. We showed that these heterogeneous changes were important for formation of stable functional reentrant circuits. Cx43 gap junction remodeling also may play an important role in arrhythmogenesis in the EBZ [7]. We have shown that structural remodeling of Cx43, characterized by cell surface lateralization, is heterogeneous, being located primarily in the cells of the CCP of reentrant circuits and not in those of the OP. Nevertheless, while functional remodeling of gap junctions occurs in the OP cells, we found no evidence of Cx43 structural remodeling in either cells or tissues [6,7].

4.2. Heterogeneous gap junction functional remodeling; gap junctional conductance in central common pathway vs. outer pathway type myocytes

For this study we used our method to isolate myocyte pairs from the CCP of mapped circuits for dual patch clamp determination of coupling conductance [9]. Recognizing that Cx43 turnover may be increased in isolated cells, we dispersed cells from normal ventricle, the OP, and the CCP using identical methodology to determine whether differences in Cx43 localization and expression were due to preparation artifact. Immunofluorescent studies verified that the myocytes of the CCP had localized Cx43 while those from normal and OP regions did not (Fig. 4). Further, comparison of cell data with data from sections of rapidly frozen tissue indicates that CCP cell Cx43 lateralization is similar in the single cell and the intact CCP tissue (Fig. 6B). Thus isolated cells can serve as an appropriate model for examining Cx43 localization and gap junction conductance following infarction.

In our previous study we showed that transverse coupling conductance was reduced in EBZ myocyte pairs without Cx43 lateralization, characteristic of outer pathway myocytes. Longitudinal conductance was no different from normal [7]. Here we show that both longitudinal and transverse coupling conductance are no different from normal values in pairs of central common pathway myocytes. Thus, the primary functional difference between myocytes with Cx43 lateralization and those without lateralization is an increase in transverse coupling conductance. If we assume that a reduction in transverse cell-to-cell coupling is a basic pathological response of gap junctions to ischemia and infarction as exemplified by the response to acute ischemia.

Fig. 6. (A) Conduction velocities (ordinates) in normal (NZ), central common pathway (CCP) and outer pathway (OP) (abscissa). Longitudinal velocities are at the left, transverse velocities in the center and anisotropic ratios at the right. Numbers in bars indicate number of experiments in each group. (B) Immunofluorescent images of epicardial border zone of the CCP and OP of a mapped reentrant circuit. Location of Cx43 shown by red fluorescence. In the central pathway significant amounts of Cx43 are located along lateral membranes while also maintaining a presence at the ends of the myocytes. In the normal and outer pathway tissues fluorescence is only seen at the intercalated disk regions of the myocytes. Scale bars: 25 μm.
[17] as well as chronic ischemia in outer pathway myocytes [7], then it is possible that lateralization of Cx43 protein may be a compensatory cellular response to restore the reduced coupling conductance. Alternatively, it is possible that transverse conductance in CCP cells is never reduced during the acute ischemic event and thus there was no adaptive response of these cells to acute ischemia. Rather, the Cx43 redistribution (structural remodeling) seen here only in CCP cells results from other factors that occur during myocardial infarction; for example, regional stretch. Furthermore, it is also possible that Cx43 protein we found at the CCP lateral membranes does NOT form functional gap junctions. In this case, the lateralization that occurs in CCP cells would not be expected to have any effect on transverse conductance.

4.3. Effects of heterogeneous structural and functional remodeling of Cx43 on conduction

In normal ventricular myocardium, Cx43 gap junction protein is primarily located in the plicate and interplicate segments of the intercalated disks and little or no Cx43 occurs on non-disk lateral membranes [18,19]. Thus intercellular current flow occurs primarily at the cell termini although propagation can occur both longitudinally and transversely [19,20]. Although it has been shown in both experimental and clinical cardiac pathology, that increased Cx43 can become prominent along lateral membranes of myocytes (so-called structural remodeling) [21,22], the functional significance had not been determined. Influences of Cx43 lateralization on propagation should be dependent on how much of this Cx43 actually forms functional gap junction channels and the functional characteristics (conductance) of these channels. In addition, other features of remodeling, such as changes in cell size and shape and alterations of sarcolemmal channels are predicted to also have important influences [23,24].

In prior studies, conduction properties of the EBZ were characterized globally, that is, conduction over the whole region was studied without subdividing it into regions with and without Cx43 structural remodeling [5]. In this study we determined conduction properties in the two regions by first mapping to localize the reentrant circuits and then mapped utilizing a high density electrode array that could be positioned for example, regional stretch. Furthermore, it is also possible that Cx43 protein we found at the CCP lateral membranes does NOT form functional gap junctions. In this case, the lateralization that occurs in CCP cells would not be expected to have any effect on transverse conductance.

4.4. Limitations

In summary we have found functional and structural heterogeneities in Cx43 between cells of the CCP and OP of reentrant circuits which translate at the tissue level into a heterogeneity in anisotropic ratio. Although measurements

![Fig. 7. A summary of properties of central common pathway (CCP), outer pathway (OP) and normal myocardium (NZ) are shown in each column. CV is conduction velocity in transverse (arrows to right) and longitudinal directions (downward arrows). Circled numbers are anisotropic ratios. GJC represents gap junction conductance in transverse (horizontal lines) and longitudinal directions (vertical lines). Downward arrows next to $I_{Na}$ at bottom show relative alteration of kinetics of this current with it being greater in CCP than OP. The reduction in conduction velocity (CV) in longitudinal and transverse directions in CCP despite lateralized Cx43 and normal coupling conductance is hypothesized to be a result of the alterations in $I_{Na}$.

Fig. 7. A summary of properties of central common pathway (CCP), outer pathway (OP) and normal myocardium (NZ) are shown in each column. CV is conduction velocity in transverse (arrows to right) and longitudinal directions (downward arrows). Circled numbers are anisotropic ratios. GJC represents gap junction conductance in transverse (horizontal lines) and longitudinal directions (vertical lines). Downward arrows next to $I_{Na}$ at bottom show relative alteration of kinetics of this current with it being greater in CCP than OP. The reduction in conduction velocity (CV) in longitudinal and transverse directions in CCP despite lateralized Cx43 and normal coupling conductance is hypothesized to be a result of the alterations in $I_{Na}$.
of gap junctional conductance suggest that lateralization of Cx43 improves lateral coupling, we do not provide direct evidence that Cx43 in the lateral membrane actually forms these junctions. Furthermore, additional studies are needed to determine how functional and structural heterogeneities in Cx43 and anisotropy contribute to the stability of reentrant circuits in the epicardial border zone of infarcted hearts.

References


