Brief Definitive Communication

Distinct Patterns of Connexin Expression in Canine Purkinje Fibers and Ventricular Muscle

H. Lee Kanter, James G. Laing, Scott L. Beau, Eric C. Beyer, and Jeffrey E. Saffitz

Electrical conduction is more rapid in Purkinje fibers than in ventricular muscle, which are distinct cardiac tissues that have different active and passive electrophysiological properties. We have recently demonstrated that canine myocardium contains multiple gap junction proteins or connexins that form channels with unique electrophysiological properties. To determine whether differences in connexin expression may account, in part, for the characteristic conduction properties of Purkinje fibers and ventricular muscle, we assessed the amounts of mRNA for two connexins, Cx40 and Cx43, in these tissues obtained from canine hearts by Northern blot analysis and in situ hybridization. We also characterized the distribution and relative abundance of these two connexins in gap junctions with immunocytochemistry. A significantly greater amount of Cx40 mRNA was observed in Purkinje fibers compared with ventricular muscle, a difference that was at least threefold according to quantitative in situ hybridization (p<0.001) and densitometric analysis of Northern blots. Purkinje fibers also demonstrated greater immunostaining intensity when incubated with anti-Cx40 antibodies than did ventricular muscle. In contrast, Cx43 mRNA and protein appeared to be abundant in both tissues. Quantitative in situ hybridization demonstrated a modest but not statistically significant increase in Cx43 mRNA in Purkinje fibers compared with ventricular myocardium. These results indicate that Purkinje fibers and ventricular muscle express distinct patterns of connexins. This tissue-specific pattern of connexin expression could contribute to differences in the conduction properties of Purkinje fibers and ventricular muscle. (Circulation Research 1993;72:1124–1131)

KEY WORDS • gap junctions • myocardial conduction • intercellular coupling • in situ hybridization • immunofluorescence

Intercellular transfer of current in cardiac myocytes occurs at gap junctions, specialized membrane regions containing densely packed channels that directly connect the cytoplasm of adjacent cells.1 We have recently shown that adult canine ventricular myocytes express three distinct gap junction proteins or connexins: Cx40, Cx43, and Cx45.2 These proteins have unique amino acid sequences in regions of the molecule predicted to reside intracellularly.3 In fact, channels formed between cells transfected with chick homologues of the three mammalian cardiac connexins exhibit unique unitary conductances and voltage sensitivities.4 Chick (Cx42), the homologue of dog Cx40, forms channels with large unitary conductances (predominantly 121 or 158 pS), whereas channels made by chick Cx43 have a predominant unitary conductance of 44 pS.4 The unique intracellular sequences may, therefore, determine the specific physiological properties of gap junctions composed of different connexins.5–7 Furthermore, characteristic changes in myocardial coupling observed in the developing chick heart are temporally related to developmental switches in the pattern of connexin mRNA expression, suggesting that the connexin composition of gap junctions may determine conduction properties of the tissue.5,9

Gap junctions are a major source of internal resistance to current flow. Hence, the number, size, and spatial distribution of intercellular junctions are important determinants of passive electrophysiological properties and likely account for the anisotropic conduction velocities characteristic of ventricular muscle.10,11 The rapid longitudinal conduction velocity in Purkinje fibers may be attributable to both active and passive properties of this unique cardiac tissue.12 With the recent discovery of multiple physiologically distinct cardiac connexins, one possible mechanism contributing to differences in the conduction properties between Purkinje fibers and ventricular muscle is expression of tissue-specific patterns of connexins. Accordingly, we performed the present study to determine whether ventricular muscle and Purkinje fibers may exhibit different patterns of connexin expression.

Materials and Methods

Northern Blot Analysis

Total cellular RNA was extracted from the large extramural right ventricular Purkinje fiber bundles and
a sample of left ventricular myocardium from each of three dogs by use of the guanidinium thiocyanate–phenol–chloroform procedure as previously described.\textsuperscript{13} RNA (10 \( \mu \)g of each sample) was electrophoresed on formaldehyde/agarose gels, transferred to nylon membranes, and cross-linked with ultraviolet light as previously described.\textsuperscript{14} Specific \( ^{32} \)P]dCTP-labeled DNA probes for dog Cx40, dog Cx45, and rat Cx43 were synthesized using the random primer technique as previously described.\textsuperscript{2,14} In some experiments, a probe encoding a mouse 18S ribosomal RNA was used as a control. The blots were hybridized for approximately 18 hours at 65°C, washed stringently, and analyzed by autoradiography. Bands were quantified by densitometry.

**Synthesis of Specific Complementary RNA Probes for In Situ Hybridization**

Specific carboxyl-terminal fragments of dog Cx40 and rat Cx43 were subcloned into the plasmid Bluescript (Stratagene Inc., San Diego, Calif.) for use as templates in the synthesis of complementary RNA probes. The Cx40 template extended from the \( \text{Pst} \) I site at nucleotide 460 of the dog Cx40 coding sequence to the carboxyl terminus and was 616 nucleotides in length.\textsuperscript{2} The Cx43 template started at the \( \text{Sac} \) I site at nucleotide 680 of the rat Cx43 coding sequence and extended through the 3′ end of clone G2,\textsuperscript{15} 30 nucleotides after the termination codon. This sequence was 511 nucleotides in length. The maximum degree of sequence identity observed when the nucleotide sequences of the two RNA probes were aligned was 34%. Plasmids were linearized with appropriate restriction endonucleases, and sense and antisense RNA probes were synthesized using T3 or T7 RNA polymerases (Stratagene) and \([\text{35}^S]\)UTP (Amersham, Arlington Heights, Ill.). After digestion with RNase-free DNase, RNA probes were isolated using the RNaid technique (Bio 101, La Jolla, Calif.).

**Connexin-Transfected Cells**

In some control experiments, in situ hybridization analysis was performed on neuro2A (N2A) cells or RIN cells\textsuperscript{15} transfected with either Cx40 or Cx43. Untransfected N2A cells are communication deficient. They contain no gap junctional channels detectable by double whole-cell patch-clamp analysis and do not express detectable mRNA for any known connexin.\textsuperscript{4} Untransfected RIN cells rarely show intercellular coupling, and although some clones express low levels of Cx43, no other connexin mRNAs can be detected.\textsuperscript{16,17} We have previously produced N2A cells stably transfected with the dog Cx40 DNA.\textsuperscript{18} We produced Cx43-expressing cells by stably transfecting RIN cells with rat Cx43 cDNA clone G2 from Reference 15 using the pSFFV-neo vector\textsuperscript{19} as described.\textsuperscript{4} Northern blot analysis demonstrated that these transfected N2A cells express large amounts of only the expected single-connexin mRNA. For control in situ hybridization experiments, connexin-transfected cells were scraped from culture dishes, fixed in 2% paraformaldehyde, pelleted at 13,000 rpm for 5 minutes, and then frozen. Cryostat sections of cell pellets were mounted on slides and processed for in situ hybridization in the same manner as tissue sections.

**In Situ Hybridization**

Freshly dissected extramural canine Purkinje fibers and canine ventricular myocardium from three separate canine hearts were fixed in 4% paraformaldehyde in phosphate-buffered saline for 2–4 hours. Fixed tissue was frozen, and cryosections 12 \( \mu \)m in thickness were mounted on polylysine-coated slides and postfixed in 4% paraformaldehyde for 20 minutes. In situ hybridization was performed as described by Simmons et al\textsuperscript{20} with minor modifications. A total of seven separate experiments using sections from three hearts were performed. Mounted tissue sections were pretreated with 0.001% proteinase K, acetylated, and dehydrated through graded ethanol. Hybridization solution containing 5 \( \times \)10\textsuperscript{6} cpm/ml Cx43 or Cx40 antisense or sense probe was layered on coverslips, which were then applied to the slides, sealed with DPX mountant (BDH Ltd., Poole, UK), and incubated for 18 hours at 59°C. After rinsing in 4× standard saline citrate (150 mM NaCl and 15 mM trisodium citrate, pH 7.0) to remove coverslips, unhybridized single-stranded RNA probe was digested with RNase A. Nonspecific hybridization was further reduced with a high-temperature (75°C) low-salt (0.1× standard saline citrate) wash for 30 minutes. Sections were dehydrated through graded ethanol, and autoradiography was performed using emulsion-coated (Kodak NTB-2) coverslips, as previously described.\textsuperscript{21} Autoradiographic grain densities and the percentage of section area occupied by myocytes in ventricular muscle and Purkinje fibers were measured as previously described.\textsuperscript{21} Specific grain density values, expressed as mean±SD, were calculated by subtracting mean grain density measurements observed in sections incubated with sense probes from mean values determined in sections labeled with antisense probes. These mean grain density values were based on at least 20 separate measurements for each probe in each tissue in seven separate experiments.

Statistical analyses of autoradiographic grain density values were performed with analysis of variance of repeated measures.\textsuperscript{22} We tested the statistical significance of grain densities measured in separate in situ hybridization slides within individual experiments as well as the grain densities of the two different tissues incubated with each probe. Differences were considered significant at \( p<0.05 \).

**Anti-Connexin Antibodies**

A rabbit antiserum to a synthetic peptide in Cx43 (amino acids 252–271) was produced previously and has been extensively characterized.\textsuperscript{23,24} A commercial mouse immunoglobulin (Ig) G1 monoclonal antibody raised against a similar sequence (amino acids 252–270 of rat Cx43) was obtained from Chemicon, Temecula, Calif. This antibody showed similar reactivity to the polyclonal anti-Cx43 in labeling cardiac myocytes in the known distribution of gap junctions. A rabbit polyclonal antiserum directed against Cx40 was raised as previously described\textsuperscript{23} by immunizing rabbits with a synthetic peptide representing amino acids 316–329 of dog Cx40 (YAQKPEVPNGASPG)\textsuperscript{25} conjugated to keyhole limpet hemocyanin via a cysteine residue added to the amino terminal end. The anti-Cx40 antibody was affinity-purified by chromatography on a peptide-agarose col-

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Cx43 protein, but not Cx40; conversely, the affinity-purified anti-Cx40 antibody precipitated only the Cx40 protein, but not Cx43.

**Immunohistochemical Analysis of Connexins**

Extramural canine Purkinje fibers and adjacent fragments of ventricular muscle from an additional dog heart were fixed in 2% paraformaldehyde, cryoprotected with 30% sucrose in phosphate-buffered saline, frozen rapidly, and sectioned at a thickness of 12 µm with a cryostat. Sections were incubated with primary antibodies (diluted 100-fold in phosphate-buffered saline) for 16 hours at 4°C and, after being washed, were incubated with 200-fold dilutions of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, Calif.) or rhodamine-conjugated goat anti-mouse IgG (Boehringer Mannheim Corp., Indianapolis, Ind.) for 3 hours at 22°C. Sections were rinsed, mounted with coverslips, and examined with a fluorescence microscope.

**Results**

To compare the relative abundance of connexin mRNA in canine left ventricular muscle and Purkinje fibers, we performed Northern blot analysis. Extramural Purkinje fibers were carefully dissected to ensure that no ventricular muscle was included in the sample. Total cellular RNA was extracted from these extramural Purkinje fibers and from a transmural sample of left ventricle from each of the dogs. Figure 1 shows a representative Northern blot using [³²P]DNA probes specific for Cx40 and Cx43. Purkinje fibers contained approximately three times more Cx40 mRNA than did ventricular muscle. The amounts of Cx43 mRNA were approximately the same in Purkinje fibers and ventricular muscle. Low, but similar, levels of Cx45 were seen in both tissues (data not shown). The approximately equal signal intensity seen in Purkinje fibers and ventricular muscle after hybridization with a probe that recognized 18S ribosomal RNA indicated that equal amounts of sample RNA were loaded on the gels.

The specific Cx40 probe hybridized to Purkinje fiber RNA bands of three different sizes, raising the possibility of multiple species. With longer exposure, the ventricular muscle sample also showed evidence of such lower mobility forms. A similar pattern of multiple Cx40

**Figure 1.** Representative Northern blot of total RNA extracted from canine Purkinje fibers (PF) and left ventricular myocardium (V) and hybridized with [³²P]DNA probes for Cx40, Cx43, and 18S ribosomal RNA. Autoradiography exposure times were 3 days for Cx40, 1 day for Cx43, and 5 minutes for 18S RNA.

**Figure 2.** Low-power section of an extramural Purkinje fiber (PF) and the adjacent ventricular muscle (V). The spatial separation of these two tissues facilitated in situ hybridization analysis of specific grain densities in each component.
FIGURE 3. In situ hybridization of Purkinje fibers and ventricular muscle incubated with antisense and sense RNA probes for Cx40 and Cx43. Panels show grain densities of tissues incubated with antisense (left) and sense (right) probes. Panel A: Ventricular muscle incubated with Cx40 probes. Panel B: Purkinje fibers incubated with Cx40 probes. Panel C: Ventricular muscle incubated with Cx3 probes. Panel D: Purkinje fibers incubated with Cx43 probes.
hybridizing bands has been observed in RNA prepared from canine aorta and other regions of the canine heart. Quantitation of these bands by densitometry demonstrated that the major (=2.6 kb) band consistently accounted for at least 50% of the signal in each sample. Because of the stringent hybridization conditions used, these multiple Cx40 bands must represent very closely related species (perhaps alternatively processed Cx40 mRNAs). For example, under these conditions, the Cx40-transfected cells gave only a single band, and RNA from cells transfected with Cx43 or Cx45 showed no hybridization. It seems extremely unlikely that an mRNA unrelated to the connexin family would show such a high degree of similarity.

To confirm results of Northern blot analysis and to measure more directly the relative amounts of connexin mRNA in myocytes of Purkinje fibers and ventricular muscle, we performed in situ hybridization using connexin-specific RNA probes. Because of the low expression of Cx43 on Northern blots, in situ hybridization analysis was focused on the relative distributions of Cx40 and Cx43 mRNAs. We first sought to validate the specificity of hybridization under the experimental conditions by performing in situ hybridization with cells transfected with Cx40 and Cx43 sequences. Quantitative analysis of multiple slides in four separate experiments revealed that cells transfected with Cx40 had a specific grain density of 4.3±1.7 grains per cell profile when hybridized with the Cx40 antisense probe but exhibited background grain densities when hybridized with Cx40 antisense probe (1.1±0.4 grains per cell) or Cx40 sense probe (0.9±0.3 grains per cell) (both p<0.05 compared with Cx40 antisense). Cells transfected with Cx43 showed a high specific grain density of 12.1±9.1 grains per cell when hybridized with the Cx43 antisense probe but only background levels when hybridized with the Cx40 antisense probe (0.7±0.3 grains per cell) or Cx43 sense probe (1.1±0.2 grains per cell) (both p<0.01 compared with Cx43 antisense). These results indicate that these carboxyl-terminal RNA probes do not cross-react.

Figure 2 shows a low-power micrograph of a typical section containing an extramural Purkinje fiber bundle and adjacent ventricular muscle. The two tissues are spatially distinct, thereby permitting unequivocal quantification of grain densities reflecting mRNA levels present within myocytes of each tissue. Figure 3 shows representative in situ hybridization preparations illustrating relative grain densities of ventricular muscle and Purkinje fibers incubated with Cx40 and Cx43 antisense and sense riboprobes. Results of quantitative grain density measurements are shown in Figure 4. In seven separate in situ hybridization preparations, consistent differences in specific grain densities were observed between Purkinje fibers and ventricular muscle for both Cx40 and Cx43. In sections incubated with Cx40 antisense probe, the mean specific grain density overlying Purkinje fibers was 2.9 times greater than the specific labeling observed over areas of closely packed ventricular myocytes (p<0.001). The mean specific grain density in sections hybridized with the Cx43 antisense probe was 1.4 times greater in Purkinje fibers than in ventricular muscle. This difference did not achieve statistical significance (p=0.056). No significant differences were found between specific grain density measurements in separate slides of individual experiments.

Purkinje fibers are normally separated by fibrous tissue into groups of fibers. The processing required for in situ hybridization caused additional separation of Purkinje fiber myocytes such that the proportion of total section area occupied by Purkinje cell profiles was artifically reduced. The degree of myocyte separation was greater in the Purkinje fibers than in ventricular muscle. The emission energy of ³²S resulted in a relatively uniform distribution of grains over the Purkinje regions despite the separation of the individual cells. Accordingly, the specific grain density values originally determined as grains per 10² μm² of section area were adjusted to reflect labeling per unit myocyte area in sections of both Purkinje fibers and ventricular muscle. Morphometric analysis indicated that myocyte profiles occupied 94.8±2.0% of section area in ventricular muscle regions but only 53.1±6.9% in Purkinje fibers. If it is assumed that most, if not all, of the specific grains originated in cardiac myocytes, then the adjusted relative specific grain density for Cx40 was 5.2 times greater in Purkinje fibers than in ventricular muscle and for Cx43 was 2.5 times greater in Purkinje fibers than in ventricular muscle.

To confirm that both Cx40 and Cx43 proteins occur in gap junctions connecting Purkinje fiber and ventricular myocytes and to assess their relative abundance in these tissues, we incubated frozen sections with specific anti-connexin antibodies and fluorescently labeled secondary antibodies. Figure 5 shows high-intensity fluorescence signals in a discrete linear pattern between Purkinje fiber myocytes stained with affinity-purified rabbit polyclonal anti-Cx40 antibodies (Figure 5A) and mouse monoclonal anti-Cx43 antibodies (Figure 5C).
An identical pattern of staining was observed in Purkinje fibers stained with an extensively characterized\textsuperscript{23,24} rabbit polyclonal antiserum against rat Cx43. The immunostaining pattern observed in Purkinje fibers was identical to the distribution of the large straight intercalated disks characteristic of Purkinje fibers and indicates the abundance of both Cx40 and Cx43 in gap junctions. The staining intensity seen in ventricular muscle incubated with anti-Cx43 antibodies was roughly equivalent to that seen in Purkinje fibers stained with this antibody (Figure 5D). The pattern of specific immunostaining observed in ventricular muscle incubated with mouse monoclonal anti-Cx43 antibodies was identical to that observed in previous studies\textsuperscript{23,24} and demonstrated the complex size variation and spatial distribution of intercellular junctions characteristic of ventricular myocytes. A similar pattern was discernable in ventricular muscle stained with anti-Cx40 antibodies (Figure 5B), but the staining intensity was considerably less than that seen in Purkinje fibers incubated with anti-Cx40 antibodies, indicating a lower abundance of Cx40 in ventricular myocytes. No specific staining was observed in sections incubated with nonimmune sera (Figure 5E).

**Discussion**

The results of this study indicate that canine Purkinje fibers express a pattern of gap junction channel proteins...
distinct from that of ventricular muscle. Northern blot analysis showed an approximately threefold greater content of Cx40 mRNA in total tissue RNA isolated from extramural Purkinje fibers compared with an equivalent amount of tissue RNA from ventricular muscle. In contrast, abundant and roughly equivalent amounts of Cx43 mRNA and low but roughly equivalent levels of Cx45 mRNA were observed in the two tissues. The relative abundance of Cx40 and Cx43 mRNAs seen in Northern blots was confirmed with in situ hybridization studies in which specific signals overlying tissue areas composed of Purkinje fiber myocytes and ventricular myocytes were quantified with light microscopic resolution. The specificities of the antisense RNA probes and hybridization conditions used were validated by cells transfected with either Cx40 and Cx43 sequences and shown with Northern blot analysis to express only a single connexin species.

Immunofluorescence analysis revealed a marked disparity in staining intensity between Purkinje fibers and ventricular muscle incubated with anti-Cx40 antibodies, indicating greater abundance of this protein in Purkinje fiber gap junctions, a finding consistent with the difference in Cx40 mRNA observed. Cx43 was readily identified immunocytochemically in both Purkinje fibers and ventricular muscle. In contrast to our findings, van Kempen et al.25 detected no Cx43 immunostaining in the proximal Purkinje system of the adult rat heart. However, Gourdie et al.26 found significant immunostaining of distal Purkinje fibers by an anti-Cx43 antibody. The basis for this apparent discrepancy is unknown. Perhaps differences in connexin expression occur in distal and proximal sections of the Purkinje system.

Potential differences in Cx40 and Cx43 RNA probe specific radioactivity, length, and hybridization efficiency preclude precise quantitative determination of the Cx40/Cx43 mRNA ratio in Purkinje fibers and ventricular muscle. Differences in specific binding characteristics of primary and secondary antibodies limit this comparison in immunostained preparations. However, the present studies do provide direct comparisons of the amount of specific mRNA for Cx40 or for Cx43 in Purkinje fibers versus ventricular muscle. Our results indicate a different pattern in the two tissues. Several differences in expression of proteins of multigene families have been observed between Purkinje fibers and ventricular muscle, including contractile proteins, ion channels, enzymes, and cytoskeletal proteins.27–30 Thus, connexin expression would appear to follow a general pattern of molecular specialization that distinguishes these two tissues of the heart.

Propagation of action potentials in the direction parallel to the long myocyte axis is much more rapid in Purkinje fibers (2–3 m/sec) than in ventricular muscle (0.3–0.4 m/sec).12 This marked difference is undoubtedly due to many differences in the active and passive properties that distinguish these tissues. For example, the resting membrane potential and V_{max} of phase 0 of the action potential are both greater in Purkinje fibers,12 features that would contribute to more rapid propagation of local circuit currents. Important differences in passive properties reflected by different space constants (≈2 mm for Purkinje fibers versus 0.8 mm for ventricular muscle) are probably related to structural differences that contribute to the lower internal axial resistance to current flow in Purkinje fibers.31 These include the increased diameter of Purkinje fibers and the greater density of gap junctions in intercalated disks of Purkinje fiber myocytes in comparison with ventricular muscle.32,33

The results of the present study have identified a potential new mechanism (differential connexin expression) that might contribute to the distinct conduction properties of Purkinje fibers. Electrophysiological studies of single-channel events in communication-deficient cells transfected with individual chick cardiac connexins that are closely homologous to the mammalian connexins have demonstrated that channels formed by the chick homologue of Cx40 have a greater unitary conductance than Cx43 channels.4 It seems plausible to suggest that gap junctions enriched in Cx40 would offer less resistance to current transfer than gap junctions containing more Cx43. Gap junctional conductance is regulated by multiple mechanisms involving acidosis, lipophilic agents, and protein phosphorylation,5–7,34 which may alter channel open probability or channel density. The different connexin composition of Purkinje fiber gap junctions might, therefore, confer different properties on the cellular coupling of these cells and might permit specific pharmacological modulation of these individual tissues. However, further work is required to test these hypotheses.

Acknowledgments

The authors thank Karen Green, Eileen Westphal, and Tim Tolley for technical assistance and Mary Creasy for typing the manuscript.

References

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Circ Res. 1993;72:1124-1131
doi: 10.1161/01.RES.72.5.1124

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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