Connexin45 Expression Is Preferentially Associated With the Ventricular Conduction System in Mouse and Rat Heart

Steven R. Coppen, Emmanuel Dupont, Stephen Rothery, Nicholas J. Severs

Abstract—Cardiac myocytes are electrically coupled by gap junctions, clusters of low-resistance intercellular channels composed of connexins. Variations in the quantities and spatial distribution of different connexin types have been implicated in regional differentiation of electrophysiological properties in the heart. Although independent studies have demonstrated that connexin43 is abundant in working ventricular myocardium and that connexin40 is preferentially expressed in the atrioventricular conduction system of a number of species, information on the spatial distribution of connexin45 in the heart is limited to data obtained using an antibody raised to a single peptide sequence. In the present study, we report on the production and characterization of a new anti-connexin45 antibody and its application to the investigation of connexin45 expression in mouse and rat myocardium. The affinity-purified antiserum, raised in guinea pig to residues 354 to 367 of human connexin45, recognized a single 45-kD band on Western blots of HeLa cells transfected to express connexin45 and gave punctate immunolabeling at the cell borders, demonstrated by freeze-fracture cytochemistry to represent gap junctions. Only low levels of connexin45 mRNA were detected on Northern blots of mouse and rat cardiac tissues, and connexin45 protein levels were below the limit of detection on Western blots. Confocal microscopy of immunolabeled ventricular tissue revealed that the major part of the working myocardium was immunonegative for connexin45. A clearly defined zone containing connexin45-expressing cells was, however, localized to the endocardial surface, overlapping with connexin40-expressing myocytes of the conduction system. As these results contrast with the prevailing view that connexin45 is widely distributed in working ventricular myocytes, we compared the immunolabeling pattern obtained with a commercially supplied anti-connexin45 antiserum raised against the same peptide that was used in previous studies. The commercial connexin45 antiserum gave widespread labeling throughout the ventricular myocardium, but this labeling was inhibited by a six–amino acid peptide matching part of the connexin43 sequence, indicating cross-reaction of the commercial connexin45 antiserum with connexin43 in the tissue. Further evidence for such cross-reactivity came from observations on connexin43-transfected cells, which gave positive immunolabeling with the commercial anti-connexin45 antiserum. Our demonstration of a specific association of connexin45 with connexin40-expressing myocytes in rat and mouse ventricle raises the possibility that connexin45 contributes to the modulation of electrophysiological properties in the ventricular conduction system and highlights the need for reappraisal of the distribution and role of connexin45 in other species. (Circ Res. 1998;82:232-243.)

Key Words: gap junction ▪ connexin45 ▪ intercellular conduction ▪ confocal microscopy

Gap junctions are clusters of transmembrane channels that directly link the cytoplasmic compartments of neighboring cells. In all multicellular systems, these junctions serve as pathways for the direct cell-to-cell transfer of ions and small molecules, a key function in tissue homeostasis, development, and differentiation.1-3 In excitable tissues like the heart, gap junctions provide sites for low-resistance electrical coupling between myocytes, mediating the orderly spread of action potentials that governs sequential contraction of the cardiac chambers.4,5 The constituent proteins of gap junction channels, connexins, form a multigene family of closely related proteins.1,6,7 A total of 13 different connexins have been identified in mammals to date. Electrophysiological studies on transfected cells indicate that gap junction channels made from different connexin types have distinctive conductance, voltage dependence, and molecular permeability properties.8-11 Most tissues, including those of the heart, express multiple connexins. The diversity and differential expression of connexins is thought to permit functional modulation of gap junctions within distinct specialized zones of organs and tissues, especially during development and adaptive physiological processes.

The major connexin of mammalian cardiomyocytes is connexin43, but in addition, connexin40 and connexin45 are...
also expressed (for reviews see References 4 and 5). The relative quantities of these connexins are reported to vary in defined regions of the heart that are characterized by distinctive electrophysiological properties.12,21 Although there are many common features in the patterns of cardiac connexin expression across mammalian species, some interspecies variation is also apparent.22,23 However, connexin43 is found in large quantities in the ventricles and atria of all mammalian species studied. In the rat, mouse, dog, and human heart, connexin40 is abundant in the atrioventricular conduction system, ie, the His bundle, bundle branches, and Purkinje fibers, where it is implicated in facilitating fast conduction.13,15,17,21,22,24 Connexin45 is reported to have a widespread distribution in the dog and human heart,16,17,19,25–27 although information on the distribution of this connexin in the heart of other species is more limited. Furthermore, although connexin43 and connexin40 have been studied using multiple antibodies produced in different laboratories, information involving connexin45 in the mammalian heart has to date depended on an antibody raised against a single peptide sequence that is 100% conserved in dog, mouse, and human.15,17,19,25–27 This antibody has also been widely used in other studies on noncardiac tissues and cells to verify the expression of connexin45.24–27 While alternative well-characterized antibodies have been produced to study connexin45 in noncardiac systems,33,34 their application in the investigation of connexin45 distribution in the heart has yet to be reported.

In the present study, we have raised and characterized a new antibody to connexin45. When applied in immunofluorescence labeling, this antibody, rather than showing the expected widespread distribution throughout the ventricular myocardium, revealed that connexin45 expression is highly restricted in the rat and mouse heart. By contrast, a commercially supplied anti-connexin45 antisera, raised to the same peptide as that used in the previously published studies on connexin45, gave widespread labeling throughout the ventricular myocardium. We show that this apparent widespread distribution is due to cross-reaction of the commercial antisera with connexin43. Our findings raise the possibility that specific patterns of connexin45 expression may contribute to the modulation of electrophysiological properties in the atrioventricular conduction system in rat and mouse and suggest the need for a more general reappraisal of the distribution and functional role of this connexin in the mammalian heart.

Materials and Methods

Animals and Tissue Collection

Fifteen NF1 mice and 10 Sprague-Dawley rats were used. Hearts from both species were either snap-frozen whole in isopentane cooled by liquid nitrogen or dissected to give separate atria, ventricles, and interventricular septa before freezing. The former samples were used for cryosectioning and immunofluorescent microscopy, and the latter were pulverized for Northern and Western blot analysis. For blotting studies, samples of lung and of human heart were also processed as controls. Animal procedures were conducted according to the Animals (Scientific Procedures) Act, 1986. Human heart material comprised ventricular myocardial samples from explanted hearts of transplant patients and from normal hearts intended for transplantation that were not used for technical reasons and was obtained following procedures approved by the local ethics committee.

Cells and Culture Conditions

Nontransfected (wild-type) human cervix carcinoma HeLa cells and transfected HeLa cells expressing mouse connexin37, connexin40, connexin43, or connexin45 were provided by Professor Klaus Willecke (Institute für Genetik, Bonn, Germany).10 The nontransfected cells were cultured in DMEM (GIBCO-BRL) supplemented with 10% FBS (GIBCO-BRL), 100 U/mL penicillin, and 100 mg/mL streptomycin (GIBCO-BRL). The transfected cells were cultured in the same medium but with the addition of 0.5 μg/mL puromycin (Sigma). Cell cultures were maintained at 37°C in a moist atmosphere of 5% CO2.

Northern Blot Analysis

Total RNA, isolated from tissues and cultured cells using the guanidinium isothiocyanate/acid phenol extraction method,18 was denatured by heating in formamide and formaldehyde/RNA buffer. Equal amounts (5 μg) of each sample were run in formaldehyde agarose gels and capillary-transfered onto nylon membrane (Hybond N, Amersham). The RNA was cross-linked on the membrane by ultraviolet light. The resulting membrane was then hybridized at high stringency (5× SSC, 65°C) with a random-primed probe generated from 25 ng of purified human connexin45 DNA fragment (radioactivity incorporation is routinely 70%). The membrane was washed at high stringency (0.5× SSC, 65°C) and exposed to Kodak X-OMAT film at −70°C using an intensifying screen.

Antibodies

Peptides corresponding to residues 354 to 367 of human connexin45 and to residues 255 to 270 of rat connexin40 were synthesized as immunogens for the production of polyclonal antibodies in guinea pigs and rabbits, respectively, using a customized service (Research Genetics Inc). The anti-connexin45 antisera, designated Q14E(GP42), and the anti-connexin40 antisera, designated S15C(R83), are hereafter referred to as GP42 and R83, respectively. A further polyclonal antisera, Q15N(R402), was generated in rabbit against a peptide corresponding to residues 300 to 314 of human connexin45. The resulting sera were affinity-purified against the peptide coupled to an activated chromatography matrix. A 50:50 mixture of diamidopropylamine-activated and carboxy-activated Ultralink gels (Pierce & Warner Ltd, Chester, UK) was used for the sera to connexin45, and pure diamidopropylamine-activated gel was used for the anti-connexin40 sera. The antibodies were eluted from the column using 0.1 mol/L glycine/2% acetic acid, pH 2.9, and immediately neutralized with 1 mol/L Tris, pH 12. The eluted antibodies were then passed down a 10 mg desalting column (Bio-Rad) to remove the glycine. Glycerol was added to 50% and azide to 0.02%, and the resulting antibody solutions were stored at −20°C. The anti-connexin45 antibody (GP42) was used at 1:30 dilution for both Western blot analysis and immunofluorescence, and the anti-connexin40 antibody (R83) was used at 1:100 dilution for Western blots and at 1:1000 dilution for immunofluorescence.

The results obtained with our GP42 custom-made anti-connexin45 antibody were compared not only with the results obtained with the alternative anti-connexin45 antibody, R402, but also with those obtained using a commercially available polyclonal anti-connexin45 antibody (purchased from Chemicon). This commercial antibody was raised against a peptide corresponding to residues 285 to 298 of mouse connexin45.30 This peptide was the same sequence that was used to generate the anti-connexin45 sera in the studies of connexin45 published by other laboratories.17,19,25,30,36

For comparative studies on connexin43, a monoclonal antibody against residues 252 to 270 of rat connexin43 (Chemicon) was used at 1:1000 dilution for immunofluorescence.

Alkaline phosphatase–conjugated secondary antibodies to guinea pig IgGs (used at 1:1000 dilution) were purchased from Zymed; all the fluorophore-conjugated (CY3 and CY5) secondary antibodies were purchased from Chemicon (used at 1:500 dilution). All the fluorophore-conjugated antibodies were adsorbed against IgGs from a range of species to enable their use for double-labeling as well as single-labeling experiments.
Sample Preparation and Western Blotting
For Western blotting, total cell homogenates were prepared by lysing washed cell monolayers in SB20 (20% SDS and 0.15 mol/L Tris, pH 6.8). An aliquot was removed for protein estimation (Bio-Rad DC protein assay), and 2-mercaptoethanol was added to the remainder to a final concentration of 2.5%. Total tissue homogenates were prepared similarly by lysing powdered frozen tissue in SB20, removing an aliquot for protein estimation, and adding 2-mercaptoethanol to the remainder.

To increase the concentration of gap junction proteins and thereby enhance their detection, alkaline membrane preparations were prepared by scraping the cells into protein buffer (0.5 mL/100 cm²), 10 mmol/L Tris, pH 7.5, 10 mmol/L iodoacetamide, 10 mmol/L tetrasodium pyrophosphate, 10 mmol/L EDTA, 2 mmol/L sodium orthovanadate, 2 mmol/L phenylmethylsulfonyl fluoride, 1 mg/mL leupeptin, and 1 mg/mL pepstatin A. The cell suspension was then made alkaline by the addition of 0.7 mL of 40 mmol/L NaOH and put on ice. The suspension was sonicated for 30 seconds at power 3 (W-380 sonicator from Heat Systems-Ultrasonics, Inc) and centrifuged at 23°C for 30 minutes. Ten micrograms of total protein per lane was run on 12.5% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane at constant voltage overnight. Transfer was assessed by Ponceau S staining, and the membrane was blocked with TBS/0.1% Tween 20 with 5% dried milk powder. The membrane was incubated with primary antibody (diluted in TBS/0.1% Tween/1% BSA) for 1 hour at room temperature, washed, and incubated with alkaline phosphatase–conjugated secondary antibody (diluted in TBS/0.1% Tween/10% normal goat serum) for 1 hour. The membrane was washed, and the enzyme activity was revealed using a freshly prepared NBT/BCIP substrate solution (Pierce and Warriner Ltd).

Immunofluorescent Labeling
For immunolabeling, cells were plated onto 13-mm-diameter glass coverslips in 24-well plates and grown for 2 days until ~75% confluent. The cells were washed twice with PBS, fixed with methanol at −20°C, and washed a further 3 times with PBS. After blocking with 1% BSA in PBS for 1 hour, the coverslips were placed cell-side down on 50 µL drops of primary antibody (diluted in PBS/1% BSA) and incubated in a moist atmosphere for 2 hours at room temperature. The cells were washed 5 times with PBS over 30 minutes by placing the coverslips on a series of 100 µL drops and were then incubated with secondary antibody solution for 1 hour. After washing 5 times with PBS over 30 minutes, the coverslips were mounted on slides using Citifluor mounting medium (Agar) and sealed with nail varnish.

For the tissues, frozen sections (10 µm) were cut from the frozen samples and mounted on poly-l-lysine–coated glass slides, which were then stored at −20°C until use (normally 24 hours). The sections were fixed by immersing the slides in methanol at −20°C for 5 minutes and were then washed 3 times with PBS. Blocking was carried out for 1 hour with 1% BSA in PBS before incubating with
primary antibody (diluted in 1% BSA in PBS) for 2 hours (GP42, R402, and R83) or 1 hour (monoclonal anti-connexin43). After washing 5 times with PBS over 30 minutes, the sections were incubated with CY3-conjugated secondary antibodies (diluted in 1% BSA in PBS) for 1 hour. The slides were washed a further 5 times with PBS over 30 minutes and mounted with Citifluor, and the coverslips were sealed with clear nail varnish.

For double labeling of connexin43 and connexin45 and of connexin40 and connexin45, the sections were incubated with the primary antibodies sequentially. The order of the primary antibodies had no effect on the result. The secondary antibodies (CY3-conjugated anti–guinea pig IgG and CY5-conjugated anti-mouse or anti-rabbit IgG) were also applied sequentially.

For peptide inhibition experiments, the antibodies were incubated with 50 μg/mL peptide at room temperature for 1 hour before application to the tissue sections or Western blots.

Controls for the immunofluorescent labeling experiments were (1) omission of the primary antibody (one or both in the case of double labeling) and (2) using the inappropriate secondary antibody for each individual primary antibody.

Immunolabeled sections were examined by confocal laser scanning microscopy using a Leica TCS 4D system, which was equipped with an argon/krypton laser and fitted with the appropriate filter blocks for the detection of CY3 and CY5 fluorescence. The images recorded were projections of five consecutive single optical sections taken at 0.5-μm intervals. For the double-immunolabeled sections, simultaneous dual scanning was performed.

**Freeze-Fracture Replica Labeling**

SDS-digested freeze-fracture replicas were prepared and labeled by a method modified from that of Fujimoto. In this freeze-fracture cytochemical technique, conventional freeze-fracture replicas are first prepared; the biological material is then digested using SDS. The SDS removes the bulk of the biological material, leaving a fine layer of proteins adherent to the replica, which may then be localized in the plane of the membrane using immunogold labeling. HeLa cell lines grown on Thermomax coverslips were washed with PBS, and the coverslips were cut into small squares (5 mm square). The squares were immersed in 20% glycerol for 5 minutes, mounted on gold holders with polyvinyl alcohol, and rapidly frozen in liquid nitrogen slush. The frozen specimens were fractured by flipping off the coverslip, and platinum/carbon replicas were prepared at –120°C and a vacuum of better than 10⁻⁶ mbar in a Balzers BAF 400T freeze-fracture unit. The replicas were then floated off their holders in PBS and transferred to 2 mL of 2.5% SDS (Sigma Chemical Co) containing 10 mmol/L Tris-HCl and 30 mmol/L sucrose, pH 8.3. SDS digestion was carried out overnight at room temperature. The replicas were washed for 1 hour with four changes of PBS and, before labeling, were blocked with 1% BSA at room temperature for 30 minutes. Labeling with primary antibody was then carried out overnight. The specimens were then washed 3 times in PBS, followed by a 1-hour incubation with goat anti–guinea pig or anti-rabbit antibodies conjugated with 10 nm gold. Thorough rinsing (five washes in PBS) was followed by a 3-minute postfixation in 1% glutaraldehyde. The labeled replicas were

![A) Coomassie Blue Gel](image1)

![B) Connexin45](image2)

![C) Connexin45 + Peptide](image3)

**Figure 3.** A, Coomassie blue–stained gel equivalent to those transferred to polyvinylidene fluoride membrane for probing with the connexin45 (Cx45) antibody, GP42. The gel was loaded with equal amounts of protein from membrane preparations of mouse and rat right atrium, left atrium, septum, right ventricle, and left ventricle. Membrane preparations from the Cx45-expressing cells and nontransfected cells were also run. Wt indicates wild-type. B, Western blot, equivalent to the gel shown in panel A, probed with GP42. Cx45 was only detected in the transfected cell line. C, Western probed with GP42 preincubated with the peptide against which it was raised. The labeling of Cx45 was inhibited, but the band at 31 kD that was present in all the tissue samples was not inhibited by the peptide, indicating that this was nonspecific binding by the antibody.
Connexin45 in Mouse and Rat Heart

4A: Cx45 Cells
Cx45 Ab

4B: Cx45 Cells
Cx45 Ab + Peptide

4C: Wild-type Cells
Cx45 Ab

4D: Cx40 Cells
Cx40 Ab

4E: Cx40 Cells
Cx40 Ab + Peptide

4F: Wild-type Cells
Cx40 Ab
finally floated on distilled water and picked up on copper 460-mesh grids for electron microscopic examination.

Results

Expression of Connexin45 mRNA

Northern blot analysis revealed the presence of connexin45 mRNA in ventricles, atria, and septa from the mouse and rat hearts (Fig 1). Total RNA from lungs of both species served as positive controls, since adult mouse lungs are reported to express connexin45.39 In addition to the rodent connexin45, a larger transcript was also detected in the HeLa cell lines which was the same molecular weight as the major band labeled in samples of human ventricle (Fig 1, arrow). This major band at 8 kb is similar to the mobility observed by Kanter et al.,40 indicating that specific splicing of this mRNA occurs. From these results, it can be seen that the cell lines (derived from humans) express trace amounts of human connexin45 but that only the connexin45-transfected cell lines express mouse connexin45. For optimal presentation of these data using the same gel, 2.5-fold more total RNA was loaded from the tissues than from the cell lines, and the blot was exposed for 10 days. These conditions reflect the overall low quantity of connexin45 present in all cardiac samples. For comparison, a blot giving similar signal intensity for connexin43 would only require 5 to 10 hours of exposure.

Detection of Connexins by Western Blotting

Western blots of the transfected and nontransfected cells confirmed the specificity of the affinity-purified anti-connexin45 and anti-connexin40 antibodies (Fig 2A and 2B, respectively). Our connexin45 antibody (GP42) labeled a single band at 45 kD specifically in the connexin45-transfected cells; this band was more prominent for the membrane preparations than for the whole-cell homogenates (Fig 2A). No connexin45 was detectable in the Western blot of nontransfected cells despite the presence of low levels of human connexin45 mRNA transcripts. For connexin40, Western blots of the membrane preparations of connexin40-transfected cells showed conspicuous bands at \( \approx 40 \) kD (Fig 2B). Labeling of these bands was inhibited by preincubation of each antibody with the peptide to which it was raised, and there was no equivalent labeling of the nontransfected (wild-type) cells, as illustrated in Fig 2B. In addition to the 40-kD band, a 67-kD band was also present in both the transfected and wild-type cells. The identity of this protein is unknown, but since there was no labeling of the wild-type cells by immunofluorescence, the 67-kD band was attributed to exposure of a site by the SDS-PAGE/Western blot procedure that was unrelated to connexins. That the R83 anti-connexin40 antibody specifically labels gap junctions when used for immunolabeling has previously been demonstrated by immunogold labeling of morphologically identified endothelial gap junctions.41

Immunocytochemical Analysis

Immunconfocal microscopy of connexin45-transfected cells labeled with our connexin45 antibody (GP42) revealed a conspicuous punctate signal (Fig 4A). The labeling was completely inhibited by preincubation of the antibody with the peptide against which it was raised (B). For the most part there was no labeling of the nontransfected cells (C), but on closer examination, some faint labeling could be observed (arrows). R83 gave punctate labeling of the connexin40 (Cx40)–transfected cells (D) that was inhibited by preincubation with peptide (E). There was no labeling of the nontransfected cells by R83 (F). Cx40 Ab indicates anti-Cx40 antibody. Bar=25 \( \mu \)m.
very low levels of labeling in the form of small infrequent spots were observed at the cell borders (Fig 4C), consistent with the endogenous expression of human connexin45 in these non-transfected cells.

Our connexin40 antibody (R83) similarly gave prominent punctate labeling at the cell borders of the HeLa cells transfected with mouse connexin40 (Fig 4D). The labeling was inhibited by preincubation with the peptide, and there was no labeling of the wild-type cells (Fig 4E and 4F).

That the fluorescent spots observed in the connexin45-transfected cells with our antibody GP42 do indeed represent gap junctions was demonstrated by the SDS freeze-fracture replica labeling technique (Fig 5). These preparations revealed gold label specifically associated with morphologically defined gap junctions, with negligible labeling of surrounding non-junctional membrane. Corresponding results were obtained on the connexin40-transfected cells with antibody R83.

In sections of mouse and rat ventricle examined by immunofluorescence microscopy, anti-connexin45 (GP42) gave punctate labeling at clearly resolved intercalated disks, but over limited regions of the tissue, with extensive intervening regions remaining immunonegative (Fig 6A and 6B). A similar pattern of labeling was observed when the alternative connexin45-specific antiserum (R402) was applied. The positive connexin45 zones were consistently restricted to the endocardial surface, where components of the conduction system are localized. As myocytes of the conduction system, in contrast to those of the working myocardium, characteristically express connexin40, double labeling was carried out with the anti-connexin40 (GP42) and anti-connexin40 (R83) antibodies to determine the spatial relationship in the distribution of these two connexins. These experiments demonstrated that connexin40 and connexin45 commonly colocalize to the same intercalated disks in both rat and mouse hearts (Fig 6C and 6D). However, connexin45 was not completely restricted to regions expressing connexin40; connexin45 tended to occur also for a limited distance (one to two cells) beyond the region of coexpression (Fig 6C). Further confirmation that the punctate labeling observed with our connexin45 antibody (GP42) truly represents gap junctions was obtained by double labeling for connexin45 and connexin43. Where connexin45 spots occurred, they consistently colocalized with connexin43 spots, although the connexin45 signal was highly restricted in its distribution compared with the connexin43 signal (Fig 6E and 6F).

In marked contrast to the result obtained using our connexin45 antibody (GP42), the commercially available anti-connexin45 antibody (GP42) did not result in specific labeling of the transfected cells expressing connexin45 (Fig 7B). The similarity of labeling pattern was so striking that we were prompted to check the specificity of the commercially available anti-connexin45. On checking the peptide sequence to which this anti-connexin45 was raised, it was clear that a 4-amino acid sequence within this peptide is common to both connexin45 and connexin43. The sequence, PPGY, corresponds to residues 287 to 290 of human connexin45 and residues 283 to 286 of human connexin43 and is also present in mouse, rat, cow, and chick connexin43. The presence of this common sequence raised the possibility that the extensive labeling observed with the commercially available anti-connexin45 antibody was due to cross-reaction with connexin43. To establish whether this was so, a synthetic peptide, PPGYK, corresponding to the connexin43 sequence and encompassing the common four amino acids was used for competitive inhibition experiments. This peptide was indeed found to inhibit gap junction labeling by the commercial anti-connexin45 in the rat and mouse ventricular tissues (Fig 7C). The peptide did not inhibit the labeling of connexin45 by GP42 or connexin43 by the monoclonal anti-connexin43 (results not shown). Importantly, preincubation of the commercial connexin45 antibody with the peptide did not inhibit labeling of the transfected cells expressing connexin45 (Fig 7D). However, the labeling of the cells by the commercial antibody alone (Fig 7E) was not as extensive as that obtained using our anti-connexin45 antibody, GP42 (Fig 7F). When tested against HeLa cells transfected with connexin43, the commercial anti-connexin45 antibody gave positive labeling (Fig 8A), which was inhibited by inclusion of the peptide SPPGYK (Fig 8B), further demonstrating the cross-reactivity of this antibody. Our anti-connexin45 antibody (GP42) gave negligible labeling of the connexin43-transfected cells (Fig 8C), whereas the monoclonal anti-connexin43 antibody gave positive labeling confirming the expression of connexin43 in these cells (Fig 8D).

Discussion
With the aid of a carefully characterized anti-connexin45 antibody, we report in the present study new findings on the spatial expression of connexin45 in the mouse and rat ventricular myocardium. Our Northern and Western blot data, in accordance with earlier studies,42 all point to the presence of very low levels of connexin45 in the adult ventricular myocardium. However, our immunofluorescence results demonstrate that rather than being distributed ubiquitously, these low quantities are concentrated in the vicinity of conduction myocytes and are undetectable in the major portion of the working ventricular myocardium in the species investigated.

Cardiac myocytes are reported to express connexin43, connexin40, and connexin45.12,16,19,23,27,40,43,44 Although the

Figure 6. Confocal micrographs showing labeling by GP42 (anti-connexin45 antibody [Cx45 Ab]) of gap junctions in mouse interventricular septum (A) and rat left ventricular myocardium (B) close to the cavity. Connexin45 is localized toward the endocardial surface of the working myocardium in both the rat and mouse hearts, where it is found to colocalize with connexin40 (C and D). Connexin45 (green) does not always colocalize with connexin40 (red) (C, arrows). Where yellow is shown, there is colocalization of two connexins. Cx45/40 Ab indicates double labeling with anti-connexin45 and anti-connexin40 antibodies. Panels E and F show double labeling of connexin45 (green) and connexin43 (red) of the same tissues. Again, where yellow is seen there is colocalization of the two connexins, but connexin43 has a much more widespread distribution than connexin45. Cx45/43 Ab indicates double labeling with anti-connexin45 and connexin43 antibodies. Bar=25 μm.
abundant expression of connexin43 throughout the working myocardium has been repeatedly confirmed and several independent laboratories have verified that connexin40 is preferentially expressed by myocytes of the atrioventricular system, existing data on connexin45 are more limited. In a series of studies on canine and human hearts, connexin45 is reported to be distributed in a pattern similar to that for connexin43, being readily apparent throughout the ventricular myocardium and also present in parts of the atrioventricular conduction system. Using the same antibody, connexin45 is also reported to be abundant in rabbit atrial and ventricular myocardium. Previous data on connexin45 in the rat heart are confined to cultured neonatal rat ventricular myocytes and corresponding intact tissue, where a staining pattern similar to that given for connexin43 was reported. Taken together, these findings have led to the general impression that connexin45, though less abundant than connexin43, is typically widely distributed throughout the mammalian working myocardium. Our present findings stand in clear contrast to this general conclusion, demonstrating that connexin45 in rat and mouse has a restricted distribution, associated with but not identical to that of connexin40.

The validity of this conclusion is critically dependent on our use of a new connexin45 antibody (GP42) of carefully documented specificity. In contrast to the results obtained with this antibody, we found that the commercially available anti-connexin45 antibody, produced against the same peptide that was used in previously published studies on connexin45, gave widespread labeling of gap junctions in the working ventricular myocardium of both rat and mouse, which was similar to that reported in dog and human. Clues to the explanation for this apparent discrepancy come from the presence of an amino

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**Figure 7.** Confocal micrographs of rat left ventricle labeled using the Chemicon anti-connexin45 (Chemicon anti-Cx45 Ab) (A) and for comparison anti-connexin43 antibody (anti-Cx43 Ab) (B). When the Chemicon anti-Cx45 Ab was preincubated with the peptide SPPGYK, corresponding to part of the Cx43 sequence, all gap junction labeling was abolished (C). Similar results were obtained for mouse ventricular tissue. Cx45-transfected cells labeled with the Chemicon anti-Cx45 Ab preincubated with the peptide SPPGYK (D) show only slightly reduced labeling compared with the Chemicon antibody alone (E). The labeling of the cells by the Chemicon anti-Cx45 Ab was not as intense or clear as that obtained with GP42 (F). Bar=35 μm.

**Figure 8.** Confocal micrographs showing the labeling of HeLa cells transfected with connexin43 (Cx43 cells). The commercial anti-connexin45 antibody (Chemicon anti-Cx45 Ab) gave positive labeling of the cells (A), which was inhibited when the antibody was preincubated with the peptide SPPGYK (B). GP42 gave negligible labeling of the cells (C), whereas the monoclonal anti-Cx43 antibody gave positive labeling (D). Bar=35 μm.
Channels composed of connexin40 have a higher unitary conductance than those composed of connexin43 (connexin40, 150 to 200 pS; connexin43, 60 pS), and the presence of large quantities of connexin40 in the terminal parts of the atrioventricular conduction system is hypothesized to contribute to the rapid conduction of the impulse throughout the working myocardium. The additional presence of connexin45 potentially adds a further level of complexity in the regulation of gap-junctional channels at these sites. Chick and human connexin45 channels are reported to have low conductance values (26 and 32 pS, respectively) compared with connexin40 or connexin43 channels, but the possibility exists that mouse and rat connexin45 channels have quite different conductances.

In vitro expression systems, gap junction channels composed of connexin43 do not form functional channels with those composed of connexin40, probably because of failure to dock. However, connexin45 channels are compatible with both connexin43 and connexin40, and connexin45 has been shown to alter channel properties when transfected into a cell line that endogenously expresses connexin43. The possibility exists that connexin45 may facilitate continuity of functional linkage of gap-junctional channels in zones that express the otherwise incompatible connexin43 and connexin40, but the precise effects on function cannot be predicted in the absence of knowledge of the molecular organization at the level of the individual gap junction and channel, in particular, the organization of the three connexins with respect to possible heterotypic or heteromeric configurations. A clearer idea of the functional significance of connexin45 in the heart may in the future emerge from studies on connexin45 knockout mice.

In conclusion, apart from novel findings on the spatial distribution of connexin45, our findings highlight the potential pitfalls of inadequately characterized antibody probes and suggest the need for a more general reappraisal of the distribution and functional role of connexin45 in the mammalian heart. Further studies are currently under way to map in precise detail the distribution of connexin45 in relation to connexin40 and connexin43 in the atrioventricular conduction system.

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34. Coppen et al. 243
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