Multiple Connexins Colocalize in Canine Ventricular Myocyte Gap Junctions

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We have recently shown that adult canine ventricular myocytes express three distinct gap junction channel proteins, connexin40 (Cx40), connexin43 (Cx43), and connexin45 (Cx45). These proteins have unique cytoplasmic domains that likely confer connexin-specific physiological properties. To determine whether the three distinct channel proteins are distributed in identical or different populations of gap junctions, we performed double-label immunofluorescence on disaggregated canine ventricular myocytes incubated simultaneously with a mouse monoclonal anti-Cx43 and affinity-purified polyclonal rabbit antibodies against Cx40 or Cx45. Analysis of double-labeled cardiac myocytes using laser scanning confocal microscopy revealed virtually identical patterns of immunoreactivity for both the Cx43/Cx40 and Cx43/Cx45 pairs. Double-label immunoelectron microscopy confirmed that ultrastructurally identical cardiac myocyte gap junctions contain multiple channel proteins. Thus, three channel proteins colocalize in canine cardiac myocyte gap junctions. The presence of multiple functionally distinct connexins suggests complex possibilities regarding the composition of individual channels and the regulation of intercellular coupling. (Circulation Research 1993;73:344-350)

Key Words: gap junction channel proteins • immunocytochemistry • electrical conduction • myocardium • connexins

Electrical conduction in the heart requires the intercellular transfer of current at gap junctions, which are specialized membrane regions containing densely packed intercellular channels.1 Cardiac myocytes are extensively interconnected by gap junctions.1-3 Gap junction channels are formed by members of a family of proteins called connexins.4 These proteins are characterized by conserved membrane-spanning and extracellular domains but distinct cytoplasmic domains. The unique cytoplasmic regions apparently confer connexin-specific physiological properties.5-7 Recently, we have shown that adult canine cardiac myocytes express three distinct gap junction proteins, connexin40 (Cx40), connexin43 (Cx43), and connexin45 (Cx45), which differ markedly in their putative regulatory cytoplasmic domains.8 The chick homologues of these mammalian cardiac gap junction proteins form channels with different sensitivities to transjunctional voltage and distinct unitary conductances.9

The purpose of the present study was to characterize the distribution of Cx43, Cx40, and Cx45 in cardiac myocyte gap junctions and to determine whether individual junctions contain one- or multiple-channel proteins. We used antibodies specific for Cx40, Cx43, and Cx45 and double-label immunocytochemical techniques to determine whether multiple-channel proteins colocalize to the same gap junction plaques of canine ventricular myocytes.

Materials and Methods

Anticonnexin Antibodies

Cx43 was detected immunocytochemically with a mouse immunoglobulin (Ig) G1 monoclonal antibody raised against amino acids 252-270 of rat Cx43 and obtained from Chemicon International Inc, Temecula, Calif, or Zymed Laboratories Inc, South San Francisco, Calif. Preparation and characterization of connexin-specific rabbit antisera against Cx40, Cx43, and Cx45 have been described previously.8,10 Synthetic peptide immunogens composed of residues 252-271 of rat Cx43 (GPLSPKSQCGPKYAFNGC), residues 316-329 of dog Cx40 (CYAQKPEVPGASPG), and residues 285-298 of dog Cx45 (CSAPPGYINAVKDQ) were used to raise rabbit polyclonal antisera against Cx43, Cx40, and Cx45, respectively.

The anti-Cx40 and anti-Cx45 antibodies were affinity-purified by chromatography on peptide-agarose columns (Sulforlink coupling gel, Pierce Chemical Co, Rockford, Ill) prepared according to the manufacturer's directions and eluted with 0.1 M glycine.

In Vitro Translation and Immunoprecipitation of Connexins

cDNAs for human Cx45, rat Cx43, and rat Cx40 were subcloned into plasmid vectors containing bacteriophage T7 promoter sequences pGEM7Zf (Promega Corp, Madison, Wis) or pBluescript KS+ (Stratagene Inc, La Jolla, Calif). [35S]Methionine–labeled connexin translation products were synthesized in vitro using a Promega TnT T7–coupled transcription-translation system according to the manufacturer's directions. The translation products were incubated with 1 μg affinity-purified rabbit polyclonal antibody against Cx45 or
Cx40 or with 10 μL polyclonal rabbit antiserum against Cx43, 20 μL protein A-IPA 300 affinity resin (Repligen Corp., Cambridge, Mass.), and 1 mL RIPA buffer (150 mM NaCl, 100 mM NaPO4 buffer [pH 7.5], 1% Triton X-100, 0.6% sodium dodecyl sulfate, and 1000 U apro
tinin/mL) for 2 hours at 4°C. The tubes were centri
guged briefly at 14,000 rpm, and the supernatant was removed. The pellets were washed four times with 1 mL
RIPA buffer, and the immunoprecipitated material was electrophoresed on 12.5% sodium dodecyl sulfate–poly
acrylamide gels and identified with fluorography.

Cell Cultures and Connexin cDNA Transfection

Cells of the RIN rat insulinoma cell line11 were grown in minimal essential medium (GIBCO/BRL) supplemented
with 10% heat-inactivated (56°C for 30 minutes) fetal calf serum (JRH Biosciences), 1x nonessential amino acids
(GIBCO/BRL), 2 mM l-glutamine, 100 U/mL penicillin,
and 100 μg/mL streptomycin (GIBCO/BRL). The cDNA for rat Cx4312 was cloned into the EcoRI site
of the eukaryotic expression vector pSFFV-neo.13 RIN cells in 60-mm dishes were transfe
ccted with 20 μg linearized plasmid using the lipofectin reagent
(GIBCO/BRL) according to the manufacturer’s direc
tions, and stable neomycin-resistant colonies were se
elected in 0.5 mg/mL G418 (GIBCO/BRL) (as in Refer
cence 9). Connexin expression was verified by Northern blotting of total RNA prepared from selected clones.

Immunofluorescence Staining of Cultured Cells

RIN cells were grown on glass microscope slides
(Nunc, Naperville, Ill). Before immunostaining, cells were
exposed to phosphate-buffered saline (PBS), fixed in
50% methanol/50% acetone for 2 minutes at room
wax, washed three times in PBS, permeabili
duced by incubating in PBS with 1% Triton X-100,
and blocked by incubating with PBS containing 2% normal
goat serum and 1% Triton X-100. Cells were incubated
for 45 minutes with primary rabbit polyclonal or mouse
monoclonal anti-connexin antiserum, washed three times,
reacted with 1:1000 fluorescein isothiocyanate–conju
gated goat anti-rabbit or anti-mouse IgG (Boehringer
Mannheim Corp., Indianapolis, Ind) for 45 minutes,
washed three times, and then observed with a fluores
cence microscope. All antibody incubations and washes
were performed with PBS containing 2% normal goat
serum and 1% Triton X-100.

Double-Label Immunofluorescence Microscopy
of Ventricular Myocytes

Immunofluorescence microscopy was performed on
disaggregated canine ventricular myocytes that were
preparad as previously described.8 Myocytes were fixed
in 1% paraformaldehyde for 15 minutes and washed
in PBS before being immunostained. Cells were precu
bated with 3% normal goat serum in PBS and then
incubated with primary antibodies for 1 hour at room
temperature or overnight at 4°C. Double-label experi
ments were performed by coinoculating myocytes with
mouse monoclonal antibody (diluted 100-fold in PBS)
and either affinity-purified anti-Cx40 antibodies (dilut
ed 50- or 100-fold) or affinity-purified anti-Cx45 anti
bodies (diluted 100-fold). Normal (nonimmune) mouse
sodium dodecyl sulfate–poly

Results

Characterization of Monoclonal Anti-Cx43

Before being used in double-label immunocytochemi
cal studies, the mouse monoclonal anti-Cx43 antibody
was characterized with immunofluorescence and immu
noelectron microscopy using disaggregated canine car
diac myocytes. Fig 1, A, demonstrates that this mono
clonal antibody specifically stained isolated canine
myocytes in a pattern consistent with the known dis	ubution of gap junctions in intercalated disks. An
identical pattern of staining has been observed using
rabbit polyclonal antisera against Cx43.9,10 To confirm
that the mouse monoclonal antibody was bound to ultrastruturally recognizable gap junctions, we per
formed immunoelectron microscopy. Fig 1, B, shows
gold-labeled anti-mouse IgG decorating a gap junction
in a myocyte incubated with the mouse monoclonal
anti-Cx43 antibody.

Verification of Antibody Specificity

To test the specificity of the different anti-connexin reagents, we performed immunoprecipitation experi
ments using [35S]-labeled polypeptides that had been translated in vitro from cloned Cx40, Cx43, and Cx45
(Fig 2). These studies confirmed that the polyclonal

Double-Label Immunoelectron Microscopy

After incubating isolated myocytes with both mouse
and rabbit primary antibodies as described above, cells
were coinoculated with gold-conjugated secondary antibi
dies, washed, embedded, and processed for electron
microscopy as described.14 Secondary antibodies used
were goat anti-mouse IgG conjugated to 10-nm gold
particles (EY Laboratories) and goat anti-rabbit IgG
conjugated to 20-nm gold particles (EY Laboratories).
The size difference of the electron-dense gold particles
permits localization of each connexin at the ultra
structural level of resolution. In preliminary experimen
tss to characterize the specificity of the commercial
anti-Cx43 monoclonal antibody, the distribution of
Cx43 was characterized with immunoelectron micro
scopy in disaggregated canine ventricular myocytes.

coincubated with goat anti-mouse antibodies conjuga
ted to Texas red (Fisher) and goat anti-rabbit IgG
conjugated to fluorescein isothiocyanate (Boehringer
Mannheim) (each diluted 200-fold) for 1 hour at room
temperature. Double-labeled myocytes were examined
by conventional fluorescence microscopy or with a laser
scanning confocal microscope (model MRC 500, Bio
Rad Laboratories, Richmond, Calif) with a filter set
appropriate for double-label immunofluorescence. For
confocal microscopy, an argon laser—emitting light with
a wavelength of 514 nm was used to excite the fluoro
tines. After several preliminary filtering steps, fluo
rescein emission spectra were recorded after passage
through a band-pass filter of 540 nm, and Texas red
emission spectra were recorded after passage through a
long-pass filter of 600 nm. This series of filters pre
vented virtually any overlap of emission spectra re
corded in separate photomultipliers for fluorescein and
Texas red. The resulting fluorescence signals were re
corded, processed, and displayed simultaneously on a
computer monitor.

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rabbit antibodies to Cx40, Cx43, and Cx45 would precipitate only the corresponding connexin sequence to which they were directed but did not react with the other two connexins.

To exclude cross-reactivity of the anti-Cx40 and anti-Cx45 antisera with the Cx43 expressed by cardiac myocytes, we performed control immunofluorescence experiments using these reagents in cultured Cx43-transfected RIN cells, which express only Cx43. The parent RIN cell line showed no detectable gap junction channels when screened by dye transfer or double whole-cell patch-clamp recordings; it showed no detectable connexin expression as assayed by Northern blotting with probes for the following connexins: Cx26, Cx31, Cx32, Cx37, Cx40, Cx42, Cx43, Cx45, Cx46, and Cx56; and it showed no reactivity by immunofluorescence with the anti-Cx43 antibodies (data not shown). We stably transfected this communication-deficient cell

![Image](https://example.com/image1)

FIG 1. Immunohistochemical (A) and immunoelectron microscopic (B) analysis of the distribution of connexin43 in canine cardiac myocytes identified with the mouse immunoglobulin G monoclonal antibody. Bar=0.2 μm.

![Image](https://example.com/image2)

FIG 2. Immunoprecipitation of in vitro translated connexin polypeptides by anti-connexin antisera. Connexin45 (Cx45, a, d, and g), connexin43 (Cx43, b, e, and h), and connexin40 (Cx40, d, f, and i) polypeptides labeled with [35S]methionine were translated in vitro from the cloned sequences and precipitated with anti-connexin antibodies as described in “Materials and Methods.” Lanes a through c were immunoprecipitated with the affinity-purified anti-Cx45 antibodies; lanes d through f were immunoprecipitated with the anti-Cx43 serum; and lanes g through i were immunoprecipitated with the affinity-purified anti-Cx40 antibodies. Immunoprecipitation of Cx40 and Cx43 translation products each resulted in a single immunoreactive band. Immunoprecipitation of Cx45 yielded a major band at ≈45 kD and a smaller band, not seen in all preparations, which likely represents proteolysis. Migration of molecular weight standards (97 400, 66 200, 55 000, 42 700, 40 000, 32 000, and 21 500) is indicated by arrowheads to the left of figure.
or Cx43. This monoclonal antibody was directed against a peptide immunogen (amino acids 252-270 of rat Cx43) that included 19 of the 20 amino acids used to produce the rabbit polyclonal anti-Cx43 (residues 252-271), which we have shown is monospecific and does not recognize Cx40 or Cx45. The peptide used to generate both the mouse monoclonal and the rabbit polyclonal anti-Cx43 reagents is unique to Cx43. No homologous sequences exist in either dog Cx40 or Cx45. We showed that the monoclonal antibody stained transfected RIN cells, known to express only Cx43 (Fig 3). Furthermore, identical patterns of immunostaining were observed in disaggregated canine ventricular myocytes incubated with the mouse monoclonal and rabbit polyclonal antibodies against Cx43. Thus, taken together, these observations support the assumption that the mouse monoclonal anti-Cx43 did not recognize canine Cx40 or Cx45.

Double-Label Immunofluorescence Microscopy

Conventional fluorescence microscopy of disaggregated myocytes stained simultaneously with mouse monoclonal antibody against Cx43 and rabbit polyclonal antisera against either Cx40 or Cx45 revealed similar patterns of staining by each pair of anti-connexin antibodies. Photographs created by double exposures demonstrated overlap of fluorescein and Texas red-stained structures and absence of single color staining (data not shown). To assess more definitively the spatial distribution of pairs of gap junction proteins, we analyzed double-labeled myocytes with laser scanning confocal microscopy. Fig 4 shows high-resolution confocal images of a representative myocyte stained with antibodies against Cx43 and Cx40 (Fig 5). Double-label immunofluorescence with an irrelevant mouse monoclonal antibody and preimmune rabbit sera showed no specific staining.

Double-Label Electron Microscopy

To confirm that each pair of anti-connexin antibodies localized connexins in the same individual ultrastructurally identified gap junction, we performed double-label immunoelectron microscopy. Small (10-nm) gold particles conjugated to anti-mouse antibodies demonstrated the presence of Cx43, and large (20-nm) particles conjugated to the anti-rabbit antibodies identified either Cx40 or Cx45. Fig 6 shows representative junctions of myocytes stained with anti-Cx40 and anti-Cx43 antibodies or with anti-Cx45 and anti-Cx43 antibodies. Both large and small gold particles are seen decorating the same ultrastructurally identified gap junction.

Discussion

In the present study, we have demonstrated using confocal microscopy and double-label immunofluorescence that three connexins, Cx40, Cx43, and Cx45, colocalize in a pattern consistent with the known distribution of gap junctions in canine cardiac myocytes. Using immunoelectron microscopy, we have shown that pairs of these proteins localize to the same ultrastructurally identified gap junctions. These immunostaining experiments do not provide quantitative information about the amounts of the three connexins present.
FIG 4. Confocal microscopic images of cardiac myocytes double-labeled with mouse anti-connexin43 and affinity-purified rabbit anti-connexin45 antibodies. Single focal plane confocal images show the distribution of anti-connexin43 (left) and anti-connexin45 (right) immunoreactive sites. The spatial distribution of fluorescent signal is very similar in both images.

However, the immunoprecipitation and immunofluorescence control experiments demonstrated that the anti-Cx40 and Cx45 antibodies do not cross-react with Cx43. As described in the previous section, several lines of evidence suggest that the monoclonal antibody is highly specific for Cx43 and does not recognize Cx40 or Cx45. Even if the monoclonal antibody cross-reacted weakly with Cx40 or Cx45, the results of the double-label experiments still indicate that Cx43/Cx40 and Cx43/Cx45 colocalize. Therefore, our observations indicate that individual canine ventricular myocyte gap junctions contain all three connexins.

Traub et al. have previously shown that multiple gap junction proteins may be found within the same tissue. Two distinct connexins, Cx26 and Cx32, were found to be expressed in the liver, although in somewhat different histological distributions. Double-label immuno-electron microscopy has demonstrated that both liver connexins are identifiable within the same gap junction. Multiple connexins including Cx43, Cx46, and Cx50 have also been found in the eye lens.16,17 However, the locations and distributions of these connexins within the lens, assessed immunocytochemically, may not be identical.

Veenstra et al. have shown that when the chick homologues of these mammalian cardiac gap junction proteins were transfected into gap junction-deficient cell lines, each connexin formed channels with different sensitivities to transjunctional voltage and distinct uni-

FIG 5. Confocal microscopic images of a cardiac myocyte double-labeled with mouse anti-connexin43 and affinity-purified rabbit anti-connexin40 antibodies. Single focal plane confocal images show the similar distribution of anti-connexin43 (right) and anti-connexin40 (left) immunoreactive sites. The minute particulate signal represents nonspecific binding of secondary antibodies, and the occasional larger fluorescent signals in the fluorescein channel (left) that do not appear in the Texas red channel are due to lipofuscin granules.
tary conductances. Furthermore, changes in the voltage dependence of junctional coupling in the developing chick heart were found to correlate with the developmentally regulated expression of these connexins.\textsuperscript{18,19} These observations in developing chick hearts and transfected cell lines suggest that different levels of expression of multiple cardiac gap junction proteins could be a mechanism regulating the overall electrophysiological properties of the mammalian heart.

Although each chick connexin can form channels with unique physiological properties in transfected cells, channel composition may not be homogeneous in cardiac myocytes that express multiple gap junction proteins. There are two potential mechanisms by which multiple connexins might mix to form a single channel with mixed or novel properties. A single hemichannel could contain multiple connexins, or hemichannels might join with other hemichannels of different connexin composition. The protein composition of a single hemichannel has not been described. However, it has been demonstrated that hemichannels of distinct connexin composition can pair to form functional channels.\textsuperscript{20,21} For example, by injecting mRNAs for distinct connexins into each cell of \textit{Xenopus} oocyte pairs, Swenson et al\textsuperscript{20} and Werner et al\textsuperscript{21} have shown that Cx32/Cx43 pairs were functional, whereas some other connexin pairs were not. Cx43 paired with an endogenously expressed \textit{Xenopus} connexin formed channels with asymmetric voltage dependence. Thus, hybrid channels may have unique physiological properties determined by the hemichannel composition.

In summary, we have shown that multiple connexins colocalize to the same gap junctions connecting canine ventricular myocytes. Based on analysis of homologous chick cardiac connexins, it seems likely that these mammalian connexins have distinct electrophysiological properties. Thus, regulation of intercellular coupling in the heart might be controlled at multiple levels, including differential expression of connexins in individual gap junctions.

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