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Limited Association of Connexin43 and ZO-1 in the Intercalated Disks of Adult Rat Ventricular Myocardium

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Abstract: Recent biochemical studies have suggested that zonula occludens-1 (ZO-1), a PDZ domain-containing protein may play an important role in targeting the major gap junction protein, Cx43 to the intercalated disk region of cardiac muscle. Information for *in vivo* is, however, still limited. We investigated the distribution of Cx43 and ZO-1 proteins in the ventricular myocardium of adult rats using high-resolution analysis with a confocal microscopy. Immunolabeling spots for Cx43 and ZO-1 were largely confined to the intercalated disk regions of ventricular muscles. ZO-1 was also localized mainly in the intercalated disk region. These spots were a marked difference in the positions, indicating low levels of colocalization of Cx43 and ZO-1 immunolabled spots in the intercalated disks. These results suggest that ZO-1 does not play an important role in intercalated disk-targeting of Cx43 molecules in the normal ventricular myocardium.

Key words: gap junction, Cx43, ZO-1

It is well established that Cx43 gap junctions are almost entirely confined in the intercalated disks at the cell termini of adult ventricular cells, and this type of distinct spatial distributions may contribute to physiological anisotropic conduction properties of the ventricular myocardium (Kumar and Gilula, 1996). However, it is not much known about the molecular mechanisms underlying the localization of Cx43 proteins in the specific subcellular domains of cell-to-cell junctions. Recent studies have suggested that ZO-1, a PDZ domain-containing protein localized at the intercalated disks, may mediate anchorage of Cx43 proteins to the actin cytoskelton. ZO-1 is a member of the membrane-associated guanylate kinase (MAGUK) family, which is known to function in protein targeting, signal transduction and determination of cell polarity in various types of cells (Toyofuku et al, 1998). Biochemical studies including yeast two-hybrid assays, immunoprecipitation and transfection experiments have provided evidences of direct interactions between Cx43 and ZO-1 molecules *in vitro* (Giepmans and Moolenaar, 1998), but only limited data is available for *in vivo* localization of these proteins in the myocardium except low-resolution immunostaing in cultured cardiomyocytes (Toyofuku et al, 2001).

The aim of this study is to investigate exact localization of Cx43 and ZO-1 proteins in the ventricular myocardium of adult rats using high-resolution analysis with a confocal microscopy.

Materials and Methods

1. Immunohistochemistry

In this study, 9-week-old Wistar rats were used. Tissue samples were fixed with 4% paraformaldehide and labeled with a mouse monoclonal anti-Cx43 antibody (Chemicon) and a rabbit polyclonal anti-Zo-1 antibody (Zymed) by methods similar to those described previously (Beardslee et al, 2000; Nagy et al, 1997). Primary antibody-bound Cx43 complexes were detected by FITC-conjugated anti-mouse IgG, and Zo-1 were detected by biotinylated anti-rabbit IgG and Texas Red-conjugated streptavidin. The labeled samples were examined using a confocal microscope (Zeiss LSM-510). In addition to single plane evaluation, series of single optical sections were taken at an interval of 0.5 μm throughout the whole thickness of the cells, and these images were projected on a single plane to construct a composite projection images (ie, en face) (Uzzaman et al, 2000).

Results and Discussion

Figure 1 shows a representative example of single confocal optical slices through longitudinally sectioned right ventricular myocardium double-immunolabeled for Cx43 and ZO-1. The single channel image of FITC fluorescence showing the distribution of Cx43 is illustrated in Fig. 1A and that of Texas Red for ZO-1 is in Fig. 1B. Immunolabeling spots for

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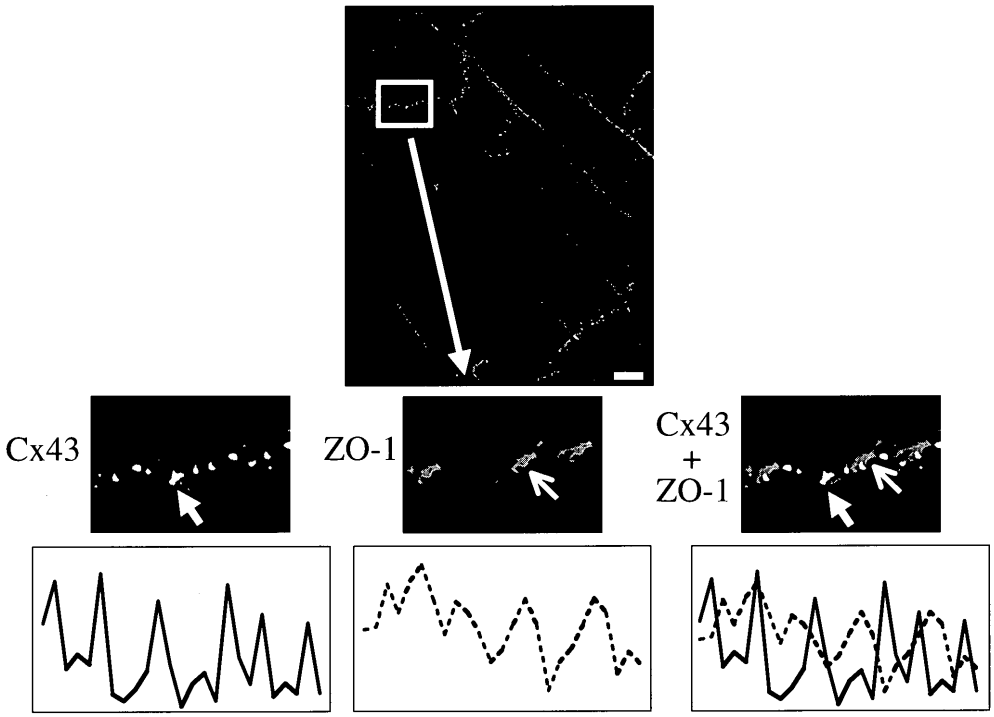

Fig. 1 Immunoconforcal images immunolabeled with anti-Cx43 antibody (A) and anti-Zo-1 antibody (B) and both antibodies (C).

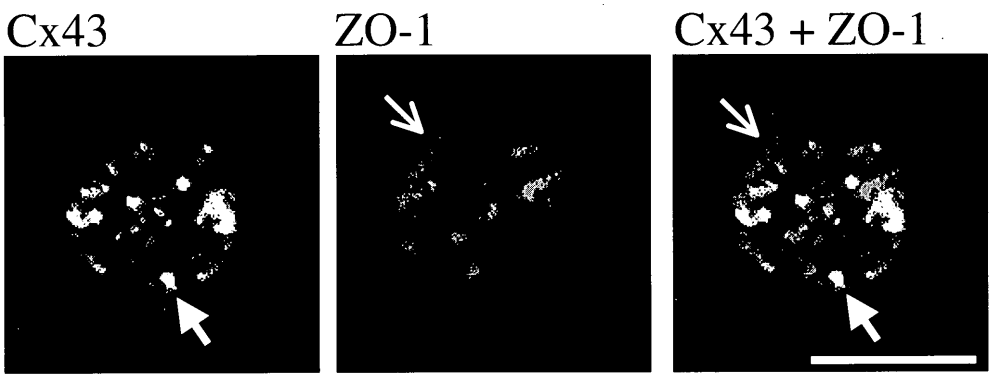

Fig. 2 The en face images immunolabeled with anti-Cx43 antibody (A) and anti-Zo-1 antibody (B) and both antibodies (C).

Cx43 were largely confined to the intercalated disk regions of ventricular muscles. Similar immunolocalization to the intercalated disk regions was observed in the image for ZO-1. These results are consistent with previous findings reported by other investigators. Fig. 1C shows the superimposed image of FITC and Texas Red fluorescence of the same sample. Close inspection of these images revealed that there was relatively low degree point-by-point overlap of immunolabeled spots for Cx43 and ZO-1 in the intercalated disks. Bottom graphs show profiles of fluorescence intensity of FITC and/or Texas Red along the line shown in each image. There was a marked difference in the positions with high intensity signals for FITC and Texas Red, indicating low levels of colocalization of Cx43 and ZO-1 immunolabled spots in the intercalated disks.

Limited colocalization of Cx43 and ZO-1 was confirmed in the images of the intercalated disks seen en face. Fig. 2 shows en-face views of the intercalated disks double-immunolabeled for Cx43 and ZO-1 in the right ventricular myocardium sectioned transversely. Series of confocal optical slices were taken at an interval of 0.5 μm through the full thickness of the intercalated disks and their perspectives were constructed by the projection of each image to a single plane. The image of FITC fluorescence for Cx43 is shown in Fig. 2A and that of Texas Red for ZO-1 is in Fig. 2B. There was limited overlap of immunolabeling spots for Cx43 and those for ZO-1 in the intercalated disks. Their superimposed image re-

vealed that the most of Cx43-negative area in the intercalated disks was occupied by ZO-1 (Fig. 2C). Essentially similar results were obtained from tissue sections of the left ventricular myocardium (not shown).

Our results showing limited colocalization of Cx43 and ZO-1 immunolabeling in the intercalated disks of the normal ventricular muscles are inconsistent with the hypothesis that ZO-1 is an important factor in the intercalated disk-targeting and functioning of Cx43 gap junctions. Barker et al. have recently reported that increased ZO-1 is coimmunoprecipitated with Cx43 in freshly isolated ventricular myocytes compared with intact ventricle, and they concluded that molecular interaction between Cx43 and ZO-1 may be particularly increased under specific circumstances such as gap junction internalization and formation of annular gap junctions associated with enzymatic dissociation (Barker et al, 2002). It is interesting that similar increases in Cx43-ZO-1 association was observed during redistribution of Cx43 gap junctions from the membrane to the cytoplasmic perinuclear region in Sertoli cells (Defamie et al, 2001).

Nevertheless, further studies will be needed to clarify the functional roles of Cx43-ZO-1 association in the heart.

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