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Role of Catenins in the Development of Gap Junctions in Rat Cardiomyocytes

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Abstract Gap junctions are intercellular communicating channels responsible for the synchronized activity of cardiomyocytes. Recent studies have shown that the membrane-associated guanylate kinase protein, zonula occludens-1 (ZO-1) can bind to catenins in epithelial cells and act as an adapter for the transport of the connexin isotype, Cx43 during gap junction formation. The significance of catenins in the development of gap junctions and whether complexes between catenins and ZO-1 are formed in cardiomyocytes are not clear. In this study, immunofluorescence and confocal microscopy showed sequential redistribution of α-catenin, β-catenin, ZO-1, and Cx43 to the plasma membrane when rat cardiomyocytes were cultured in low Ca^{2+} (<5 μM) medium, then shifted to 1.8 mM Ca^{2+} medium (Ca^{2+} switch). Diffuse cytoplasmic staining of α-catenin, β-catenin, ZO-1, and Cx43 was seen in the cytoplasm when cardiomyocytes were cultured in low Ca^{2+} medium. Staining of α-catenin, β-catenin, and ZO-1 was detected at the plasma membrane of cell–cell contact sites 10 min after Ca^{2+} switch, whereas Cx43 staining was first detected, colocalized with ZO-1 at the plasma membrane, 30 min after Ca^{2+} switch. Distinct junctional and extensive cytoplasmic staining of α-catenin, β-catenin, ZO-1, and Cx43 was seen 2 h after Ca^{2+} switch. Immunoprecipitation of Triton X-100 cardiomyocyte extracts using anti-β-catenin antibodies showed that β-catenin was associated with α-catenin, ZO-1, and Cx43 at 2 h after Ca^{2+} switch. Intracellular application of antisera against α-catenin, β-catenin, or ZO-1 by electroporation of cardiomyocytes cultured in low Ca^{2+} medium inhibited the redistribution of Cx43 to the plasma membrane following Ca^{2+} switch. These results suggest the formation of a catenin–ZO-1–Cx43 complex in rat cardiomyocytes and that binding of catenins to ZO-1 is required for Cx43 transport to the plasma membrane during the assembly of gap junctions. J. Cell. Biochem. 88: 823–835, 2003.

Key words: α-catenin; β-catenin; ZO-1; Cx43; gap junction; cardiomyocyte

INTRODUCTION

Intercalated discs, prominent features of cardiac tissue, are present at the termini of neighboring cardiomyocytes in an end-to-end arrangement (for review, see Severs, 1995). Gap junctions, one of the major junctional components in intercalated discs, are intercellular communicating channels playing important roles in coordination, differentiation, and growth of the cells, and are responsible for the synchronized electrical activation and contraction of cardiomyocytes. Each gap junction channel consists of a pair of hemichannels, also known as connexon, on the opposing plasma membranes. Connexon, a hexameric structure, is composed of six connexin (Cx) molecules. Cxs are integral membrane proteins which contain four transmembrane domains and in which both the amino (N)- and carboxyl (C)-termini face the cytoplasm (for review, see Goodenough et al., 1996). At least three main Cx isotypes, Cx37, Cx43, and Cx45, are found in heart tissue; in mammalian cardiomyocytes, the principal Cx molecule expressed is Cx43 [Severs et al., 2001].

Another type of cell junction found in intercalated discs is the adherens junction. The transmembrane protein of the adherens junction in intercalated discs is N-cadherin (A-CAM),

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which is responsible for adherens junction assembly and the attachment of contractile filaments in cardiomyocytes [Volk and Geiger, 1984, 1986]. The extracellular domain of cadherin mediates its cell–cell adhesive function through a Ca^{2+}-dependent mechanism, while the intracellular portion is associated with a group of cytoplasmic anchor proteins named catenins, which include α-catenin, β-catenin, and plakoglobin (γ-catenin) [Ozawa et al., 1989; Peifer et al., 1992; Wheelock and Knudsen, 1991]. α-Catenin is a vinculin homologue and is associated with β-catenin and the actin cytoskeleton. Actin association is mediated either by a direct linkage between α-catenin and actin or by its indirect association with other actin-binding proteins, such as vinculin and α-actinin [Herrenknecht et al., 1991; Nagafuchi et al., 1991; Knudsen et al., 1995; Weiss et al., 1998]. β-Catenin, which shares ~70% sequence identity with the *Drosophila* armadillo gene product, binds directly to both α-catenin and the cytoplasmic domain of cadherin [McCrea and Gumbiner, 1991]. Formation of the cadherin–catenin complex is required for maintenance of the adhesive function of the cadherins [Nagafuchi and Takeichi, 1989; Ozawa et al., 1990]. In addition to their role in adherens junction formation, cadherins are also involved in the formation and/or function of gap junctions. Treatment of teratocarcinoma PCC3 cells with anti-E-cadherin antibodies by inclusion of the antibodies in culture medium results in uncoupling of the cells and inhibits cell-to-cell dye transfer [Kanno et al., 1984]. Microinjection of anti-N-cadherin antibodies inhibits dye transfer between Novikoff hepatoma cell pairs and the assembly of gap junctions [Meyer et al., 1992]. Incubation of embryonic chick lens cells with anti-N-cadherin antibodies also inhibits dye transfer between cell clusters [Frenzel and Johnson, 1996]. The expression of an extracellular domain-deleted N-cadherin mutant in adult rat cardiomyocytes results in disruption of cell–cell contacts and disassembly of gap junctions [Hertig et al., 1996b].

Zonula occludens-1 (ZO-1), a 225 kDa polypeptide, is a member of the membrane-associated guanylate kinase family and was originally identified as a tight junction-associated, peripheral membrane protein [Stevenson et al., 1986; Kim, 1995]. Although immunoprecipitation analyses showed that ZO-1 binds to tight junction proteins, including ZO-2 [Jesaitis and Goodenough, 1994] and occludin [Furuse et al., 1994], it has also been shown to bind to catenins (adherens junction proteins) in Madin-Darby canine kidney (MDCK) epithelial cells [Rajasekaran et al., 1996]. In addition to being present in epithelial and endothelial cells, ZO-1 is detected in cells lacking tight junctions. Immunoblot analyses have demonstrated ZO-1 expression in a variety of non-epithelial cells, including fibroblasts, Schwann cells, astrocytes, and myeloma, sarcoma, and glioma cells [Howarth et al., 1992]. Immunofluorescence and immunoelectron microscopic studies of rat and chick cardiac muscle also demonstrated localization of ZO-1 in the intercalated discs, where it is concentrated at the adherens junction [Itoh et al., 1991; Itoh et al., 1993]. A study in which cDNAs encoding various lengths of ZO-1 were introduced into mouse L fibroblasts demonstrated direct binding of ZO-1 to α-catenin [Itoh et al., 1997], whereas co-expression of epitope-tagged Cx43 and ZO-1 in human embryonic kidney HEK293 cells demonstrated a direct association of the C-terminal of Cx43 with the N-terminal of ZO-1 [Toyofuku et al., 1998].

Although localization of ZO-1 to the adherens junction and evidence for the role of cadherins in gap junction formation have been demonstrated, a specific interaction between catenins and ZO-1 and the role of catenins in the assembly of gap junctions in cardiomyocytes have not been established. In the present study, immunofluorescence and confocal microscopy were performed to explore the relationship of α- and β-catenins to ZO-1 and Cx43 during their redistribution from the cytoplasm to cell–cell contact sites using a Ca^{2+} switch assay in cultured neonatal rat cardiomyocytes. Immunoprecipitation and immunoblot analyses were used to demonstrate the association of catenins with ZO-1 and Cx43. The results show colocalization and close association of catenins with ZO-1 and Cx43 during gap junction assembly. Functional analyses were performed by electroporation of antibodies against α- and β-catenin into cardiomyocytes cultured in a low Ca^{2+}-containing medium. When the antibody-incorporated cardiomyocytes were placed in normal Ca^{2+}-containing medium, Cx43 was not targeted to the plasma membrane and formation of gap junction plaques was inhibited, suggesting a role for catenins in the development of gap junctions.

MATERIALS AND METHODS

Culture of Neonatal Rat Cardiomyocytes

Rat cardiomyocyte cultures were prepared from 3-day-old rat pups as described previously [Spector et al., 1997] with minor modifications. Briefly, ventricular tissue was isolated and washed with Ca^{2+}-/Mg^{2+}-free Hank's balanced saline solution (HBSS, Sigma) to remove excess blood, then minced and incubated for 8 min at 37°C with 0.125% trypsin–EDTA and 0.083% collagenase type II. The dissociated cells were collected, mixed with ice-cold nutrient medium [10% fetal bovine serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine in minimal essential medium (MEM) containing Earle's salts; GIBCO, Grand Island, NY], and centrifuged at 160*g* for 10 min. They were then resuspended in 10 ml of nutrient medium, preplated on a 10 cm Petri dish, and maintained in a 5% CO_2 incubator at 37°C for 1 h. After attachment of fibroblasts, the suspended cells were collected, diluted with nutrient medium, and grown on collagen-coated 35 mm Petri dishes or coverslips at a density of $2.0–3.5 \times 10^5$ cells/dish at 37°C. On the day after plating, the nutrient medium was replaced with growth medium (10% calf serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine in MEM), which was replaced every other day. For the Ca^{2+} switch assay, cardiomyocytes were plated in low Ca^{2+} medium (10% calf serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, 5 μM $CaCl_2$, and 2 mM glutamine in Ca^{2+}-free MEM), which was then replaced on the day after plating with 5 μM $CaCl_2$ growth medium (10% fetal bovine serum, 100 IU/ml of penicillin, 100 μg/ml of streptomycin, and 2 mM glutamine in Ca^{2+}-free MEM).

Ca^{2+} Switch Assay

For the Ca^{2+} switch assay, cardiomyocytes, cultured in low Ca^{2+} (5 μM) medium for two days, were transferred to normal growth medium (1.8 mM Ca^{2+}; see cardiomyocyte culture above), maintained in a 5% CO_2 incubator at 37°C for 0, 10, 30, 60, 120 min, or 24 h, then processed for immunofluorescence microscopy, immunoprecipitation, or immunoblot analyses.

Antibodies

Mouse monoclonal antibody against ZO-1 (MAB1520) and mouse monoclonal antibody against Cx43 (MAB3067) were purchased from Chemicon (Temecula, CA). Mouse monoclonal antibody against β-catenin (C19220) was purchased from Transduction Laboratories (Lexington, KY). Rabbit anti-Cx43 antiserum (71-0700) and rabbit anti-ZO-1 antiserum (61-7300) were purchased from Zymed Laboratories Inc (San Francisco, CA). Rabbit anti-α-catenin antiserum (C-2081), anti-β-catenin antiserum (C-2206), and normal rabbit serum (R-9133) were purchased from Sigma Chemical Co (St. Louis, MO).

Immunofluorescence and Confocal Microscopy

Cardiomyocyte cultures were fixed for 5 min in cold acetone at −20°C. After rehydration with phosphate-buffered saline (PBS), cultures were blocked for 15 min at room temperature with 5% nonfat dry milk in PBS, then incubated for 1 h at 37°C with a 1:100 dilution of mouse hybridoma supernatant [anti-Cx43 (13-8300) or anti-ZO-1 (MAB1520)] or a 1:200 dilution of rabbit antiserum against α-catenin (C-2081), β-catenin (C-2206), or Cx43 (71-0700). Bound primary antibodies were detected by incubation for 1 h at 37°C with a 1:100 dilution of Texas red-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rabbit IgG (Vector, Burlingame, CA), as appropriate. Double-labeling was performed using a mixture of mouse monoclonal antibody and rabbit polyclonal antiserum, followed by a mixture of the secondary antibodies. After washing in PBS, cultures were mounted in a mixture of 2% n-propyl gallate and 60% glycerol in 0.1 M PBS (pH 8.0), sealed with nail polish and examined under a Zeiss Axiophot microscope (Carl Zeiss, Oberkocheu, Germany) equipped with epifluorescence, images being captured and digitized using a Nikon DIX digital camera (Nikon, Tokyo, Japan). For confocal microscopy, immunofluorescence-labeled cardiomyocytes were analyzed on a Leica TCS SP2 laser scanning microscope (Leica, Heidelberg, Germany) as described previously [Wu et al., 1999, 2002]. Optical sections of the cells were assessed using a 63X, 1.32 numerical aperture oil immersion objective, an agron ion/HeNe laser, and a beam splitter. About 35 horizontal (X–Y) confocal images were obtained at intervals of 0.1 μm from the cell-substratum contact (ventral) to the dorsal cell surface.

Immunoprecipitation

Immunoprecipitation was performed as described previously [Lou et al., 1999]. Cardio-

myocytes were washed three times with ice-cold PBS, then extracted for 30 min at 0°C with 1 ml of 50 mM Tris-HCl, pH 7.5, 1% Triton X-100 (TX-100), 1 mM EDTA, and 1 mM PMSF, sonicated for 30 s, and insoluble material removed by centrifugation at 12,000*g* for 30 min at 4°C. The supernatant was transferred to a new microtube and mixed for 1 h at 4°C with constant rocking with 2 μg of monoclonal anti-β-catenin antibody (C19220), then 100 μl of protein G-Sepharose bead slurry (50 μl of packed beads; Pharmacia, Uppsala, Sweden) was added, mixing continued for another 30 min at 4°C, and the Sepharose-bound immune complexes were spun down by centrifugation at 12,000*g* for 1 min at 4°C. After washing the beads five times with 50 mM Tris-HCl, pH 7.5, 1% TX-100, 1 mM EDTA, and 1 mM PMSF, the pellets were collected and heated for 5 min at 95°C in 500 μl of Laemmli sample buffer, and the supernatant collected and used for SDS–PAGE.

Immunoblot Analysis

Proteins in immunoprecipitates were separated on a 7.5% SDS–polyacrylamide gel and transferred electrophoretically to PROTRANE BA83 nitrocellulose membranes (Schleicher & Schuell Inc., Keene, NH) as described previously [Wu et al., 2002]. The nitrocellulose membranes were blocked for 1 h at room temperature using 5% skimmed milk and 0.1% Tween 20 in PBS, pH 7.4, then incubated overnight at 4°C with a 1:1,000 dilution of rabbit antiserum against ZO-1 (61-7300), α-catenin (C-2081), β-catenin (C-2206), or Cx43 (71-0700), then for 2 h at room temperature with a 1:15,000 dilution of alkaline phosphatase-conjugated, goat anti-rabbit IgG antiserum (Promega Corporation, Madison, WI). Detection of immunoreactive bands was performed by substrate development using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in 100 mM NaCl, 100 mM Tris-base, 5 mM $MgCl_2$, pH 9.5.

Electroporation Experiments

Electroporation was performed as described previously [Wu et al., 1999] with minor modifications. Cardiomyocytes cultured on coverslips in low Ca^{2+} (5 μM) medium were washed twice with Hepes-buffered saline (HBS; 21 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM glucose, pH 7.5), then the attached cardiomyocytes and coverslip were transferred to an electroporation cuvette containing 700 μl of HBS or a 1/10 dilution in HBS of normal rabbit serum or rabbit polyclonal antiserum against α-catenin, β-catenin, or ZO-1. After incubation for 5 min on ice, the cardiomyocytes were electroporated at room temperature in a Cell Porator (Gibco BRL, Gathersburg, MD) using an electrode distance of 0.4 cm, a field strength of 750 V/cm, and a duration of 0.7–0.9 ms. The settings used were 300 V initial voltage, low resistance, and 60-μF capacitance. After electroporation, the cardiomyocytes were left on ice for 15 min in the electroporation cuvette, then, after a brief wash with HBS to remove excess antibody, the cultures were transferred to a 35 mm Petri dish containing normal growth medium (1.8 mM Ca^{2+}) and incubated in a 5% CO_2 incubator at 37°C for another 2 h before processing for immunofluorescence microscopy. Antibody-incorporated cardiomyocytes were double-labeled for incorporated rabbit IgG using a FITC-conjugated goat anti-rabbit IgG secondary antibody and for gap junctions using mouse anti-Cx43 antibodies and Texas red-conjugated goat anti-mouse IgG secondary antibody.

RESULTS

Effects of Low Ca^{2+} Medium and Ca^{2+} Switch on the Localization of α-Catenin, β-Catenin, ZO-1, and Cx43 in Cardiomyocytes

In untreated day 2 cultures of cardiomyocytes, colocalization of α-catenin (Fig. 1A) and Cx43 (Fig. 1B) and of β-catenin (Fig. 1C) and ZO-1 (Fig. 1D) was seen both at cell–cell contact sites corresponding to the junctional regions and in the cytoplasm. Since cadherin-mediated cell–cell interactions and junction assembly are prevented when MDCK cells are cultured in low calcium medium, but take place when the cells are transferred back to culture medium containing normal Ca^{2+} levels [Gumbiner et al., 1988], we used a Ca^{2+} switch assay to localize α-catenin, β-catenin, ZO-1, and Cx43 during early stages in the development of cell junctions. In cardiomyocytes cultured in low Ca^{2+} medium (5 μM), diffuse staining for α-catenin (Fig. 2A), β-catenin (Fig. 2B), ZO-1 (Fig. 3A), and Cx43 (Fig. 3B) was detected in the cytoplasm. Ten minutes after the cells were transferred back to normal Ca^{2+}-containing medium (1.8 mM), staining for α-catenin (Fig. 2C), β-catenin (Fig. 2D), and ZO-1 (Fig. 3C), but not for Cx43 (Fig. 3D), was detected at cell–cell contact sites and in the cytoplasm (arrowheads in Fig. 2C,D and Fig. 3C).

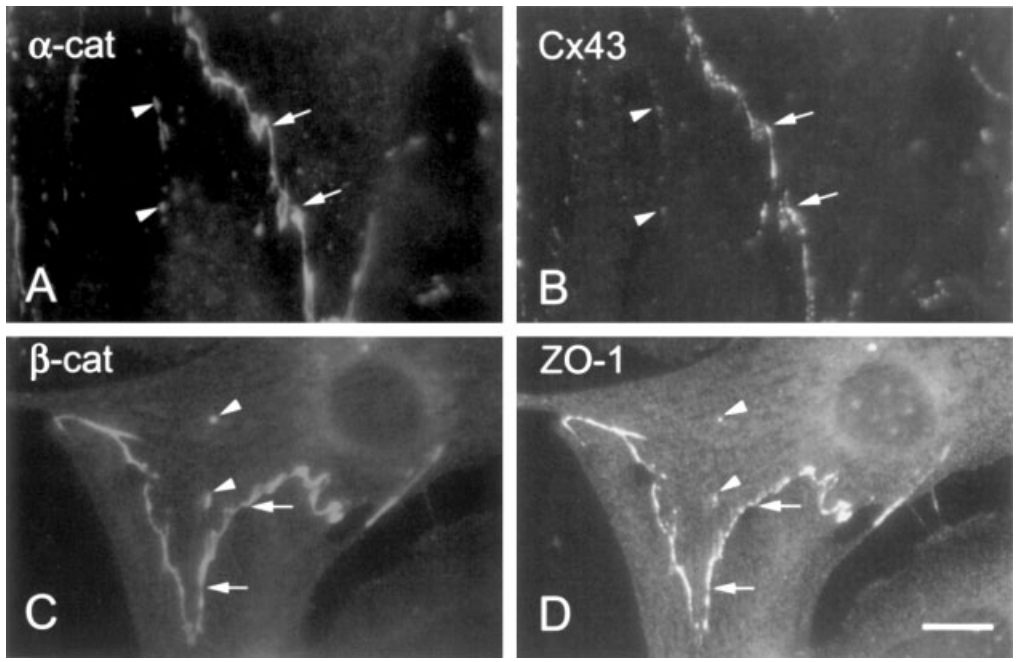

Fig. 1. Immunofluorescence microscopy showing α-catenin, β-catenin, Cx43, and zonula occludens-1 (ZO-1) staining in cardiomyocytes. Day 2 cardiomyocytes were double-stained for α-catenin (**A**) and Cx43 (**B**) or β-catenin (**C**) and ZO-1 (**D**). The arrows indicate the junctional distribution, and the arrowheads the cytoplasmic distribution, of α-catenin, β-catenin, Cx43, and ZO-1. Bar = 10 μm.

Although clear staining of α-catenin (Fig. 2E), β-catenin (Fig. 2F), and ZO-1 (Fig. 3E) was seen at cell–cell contact sites 10 min after Ca^{2+} switch, Cx43 (Fig. 3F) was first detected at these sites at 30 min after Ca^{2+} switch, where it was colocalized with ZO-1 (Fig. 3E). At 120 min after Ca^{2+}switch, distinct junctional and extensive cytoplasmic staining for α-catenin (Fig. 2G), β-catenin (Fig. 2H), ZO-1 (Fig. 3G), and Cx43 (Fig. 3H) was seen. Twenty-four hours after Ca^{2+}switch, the staining pattern of the junctional components was similar to those in cardiomyocytes cultured throughout in normal medium (data not shown). In order to further evaluate changes in the distribution of junctional components following Ca^{2+} switch, cells were double-labeled for ZO-1 and Cx43 or β-catenin and the results examined by laser scan confocal microscopy. In cardiomyocytes cultured in normal medium, optical sectioning along the Z-axis of the cells showed that Cx43 and β-catenin both colocalized with ZO-1 and were concentrated in the central region of the plasma membrane at cell–cell contact sites (Fig. 4A,B). Although extensive broader distribution of ZO-1, Cx43, and β-catenin along the contact site plasma membrane was seen 1 h after Ca^{2+} switch (Fig. 4C,D), these proteins became concentrated in the central region of the contiguous plasma membrane 24 h after Ca^{2+} switch (Fig. 4E,F).

Association Between α-Catenin, β-Catenin, Cx43, and ZO-1 in Cardiomyocytes Following Ca^{2+} Switch

Since the non-ionic detergent, TX-100, extracts free cytoplasmic cadherins and catenins in MDCK cells, but not the cytoskeleton-bound cadherin/catenin complex [Pasdar and Nelson, 1988], an immunoprecipitation study was performed to evaluate binding between the cytoplasmic catenins, ZO-1, and Cx43 during gap junction development in cardiomyocytes. Immunoprecipitation using anti-β-catenin antibodies and subsequent immunoblot analyses showed that α-catenin, ZO-1, and CX43 coprecipitated with β-catenin from TX-100 extracts of cardiomyocytes 2 h after Ca^{2+} switch (Fig. 5).

Electroporation of α-Catenin, β-Catenin, and ZO-1 Antibodies Interferes With Gap Junction Assembly

In previous studies, we and others have successfully used electroporation to introduce antibodies into cells in order to study major cellular events, including myofibrillogenesis

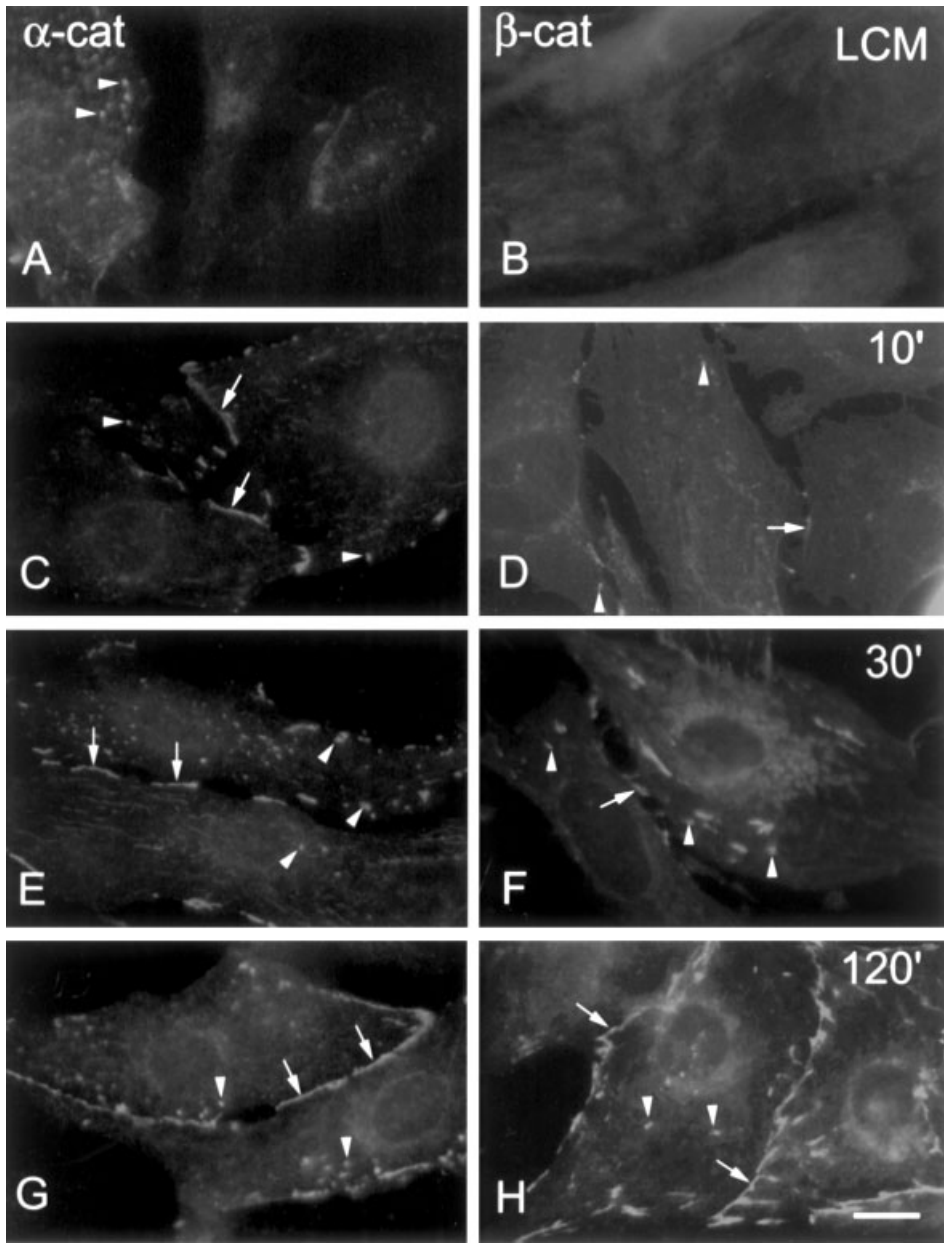

Fig. 2. Redistribution of α- and β-catenin in cardiomyocytes after Ca^{2+} switch. Cardiomyocytes cultured in low Ca^{2+} medium (LCM) for 2 days (**A**, **B**), were transferred to culture medium containing normal Ca^{2+}levels for 10 (**C**, **D**), 30 (**E**, **F**), or 120 (**G**, **H**) min, then subjected to immunofluorescence staining for α-catenin (A, C, E, G) or β-catenin (B, D, F, H). Both α- and β-catenin redistributed to the cell surface 10 min after Ca^{2+} switch. At 120 min after Ca^{2+} switch, extensive α- and β-catenin staining was observed at cell–cell contact sites. The arrowheads indicate the cytoplasmic distribution, and the arrows the junctional distribution, of α- and β-catenin. Bar = 10 μm.

[Chakrabarti et al., 1989; Wang et al., 1997; Wu et al., 1999]. To further determine the role of catenins and ZO-1 in gap junction assembly, antisera against α-catenin, β-catenin, or ZO-1 were incorporated into cardiomyocytes by electroporation. Electroporation with buffer alone had no effect on the junctional distribution of α-catenin, β-catenin, ZO-1, and Cx43 in cardiomyocytes cultured in normal medium (data not shown). In cardiomyocytes cultured in low Ca^{2+} medium, electroporation with buffer containing normal rabbit serum had no effect on the redistribution of α-catenin, β-catenin, ZO-1, and Cx43 at contiguous plasma membranes after Ca^{2+} switch (Fig. 6); however, when antiserum against α-catenin (Fig. 7A), β-catenin (Fig. 7C),

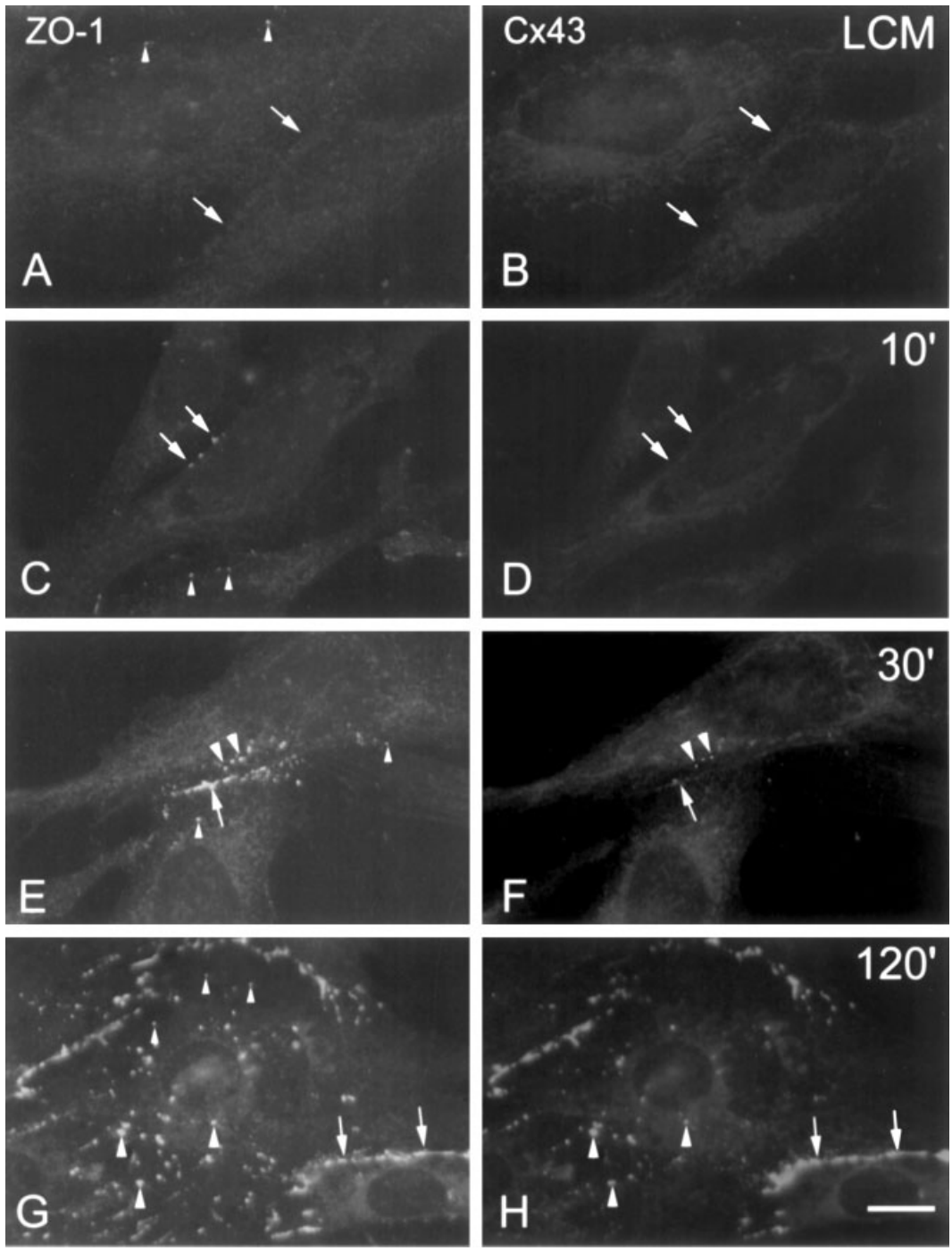

Fig. 3. Redistribution of ZO-1 and Cx43 in cardiomyocytes after Ca^{2+} switch. As in Figure 2 but staining for ZO-1 (**A**, **C**, **E**, **G**) and Cx43 (**B**, **D**, **F**, **H**). Redistribution of ZO-1 or Cx43 to the cell surface was observed, respectively, 10 (C) or 30 (F) min after Ca^{2+} switch. The small arrowheads in A, C, E, and G show cytoplasmic ZO-1 without codistribution of Cx43. The large arrowheads in E and G indicate the same positions in the cytoplasm as the large arrowheads in F and H, respectively. The arrows in C, E, and G indicate the same positions on the cell–cell contact sites as the arrows in D, F, and H, respectively. Bar = 10 μm.

or ZO-1 (Fig. 7E) was used for electroporation, Cx43 failed to assemble into gap junction plaques in the plasma membrane after Ca^{2+} switch and diffuse Cx43 staining was seen in the cytoplasm (Fig. 7B,D,F).

DISCUSSION

In this study, immunofluorescence microscopy was used to explore the spatiotemporal distribution of α-catenin, β-catenin, ZO-1, and

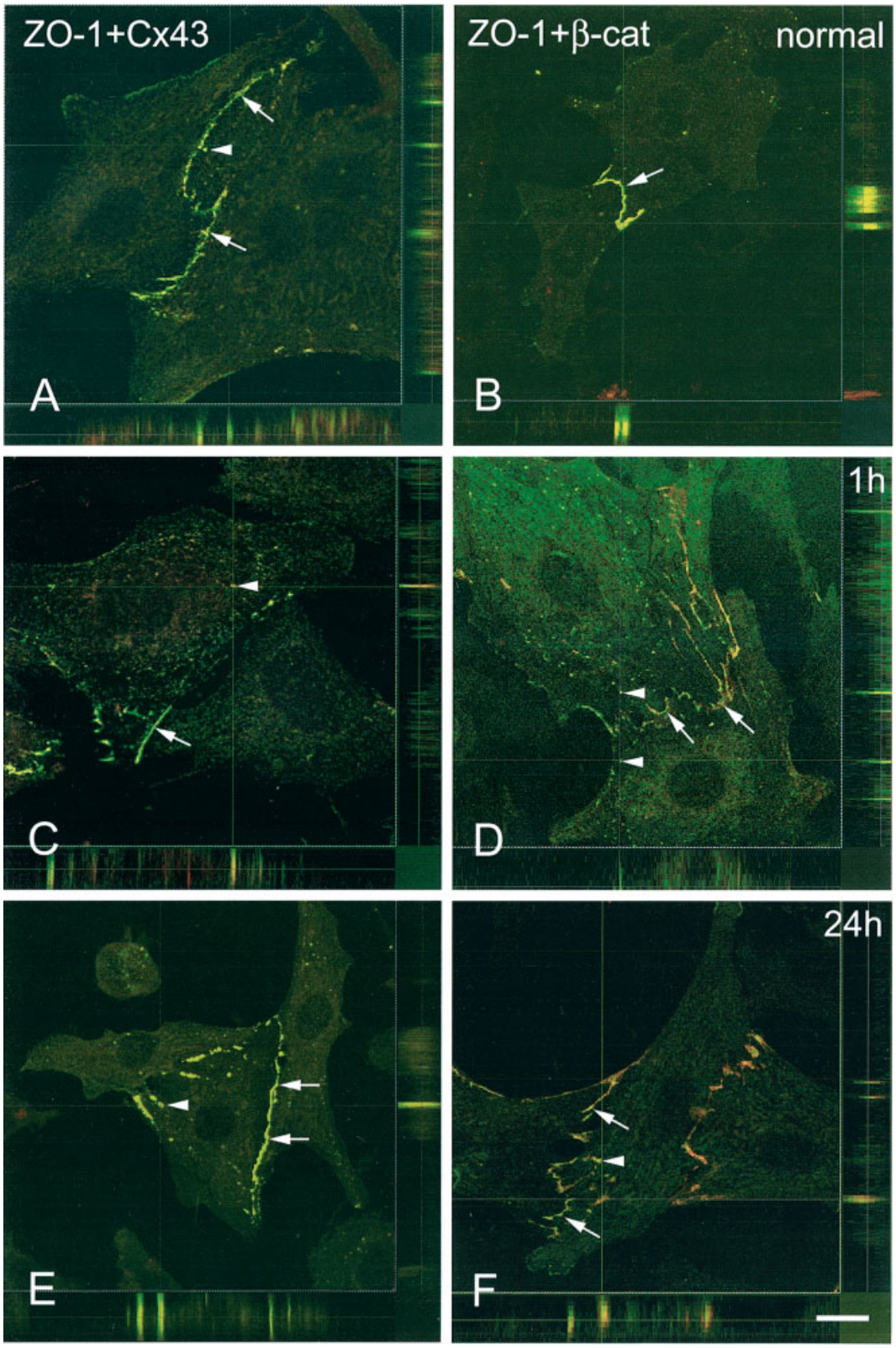

Fig. 4. Confocal micrographs showing ZO-1, Cx43, and β-catenin localization in cardiomyocytes. Cardiomyocytes cultured for 2 days in normal (**A**, **B**) or in low Ca^{2+} medium were subjected to Ca^{2+} switch for a further 1 (**C**, **D**) or 24 (**E**, **F**) h. A, C, and E are representative images of cardiomyocytes double-labeled for ZO-1 (green) and Cx43 (red), while B, D, and F are representative images of cardiomyocytes double-labeled for ZO-1 (green) and β-catenin (red). The optical sections were scanned through the Z-axis from the ventral to the dorsal cell surface at intervals of 0.1 μm. Areas in which Cx43 or β-catenin staining and ZO-1 staining are superimposed are seen as yellow, indicating precise colocalization of ZO-1 and Cx43 (A, C, E), or ZO-1 and β-catenin (B, D, F). The arrowheads indicate cytoplasmic, and the arrows junctional, staining. Bar = 10 μm.

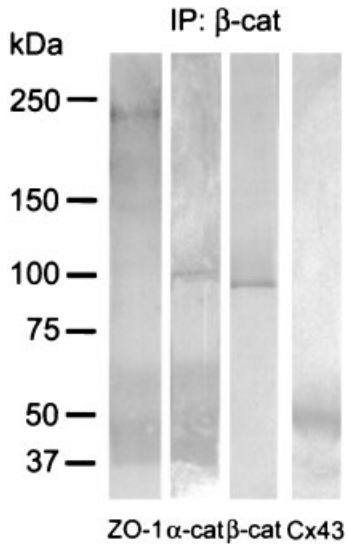

Fig. 5. ZO-1 and Cx43 coprecipitate with the catenin complex in cardiomyocytes. Cardiomyocytes cultured in low Ca^{2+} medium for two days were subjected to Ca^{2+} switch for a further 2 h, extracted with 1% TX-100, and the TX-100-soluble fraction immunoprecipitated (IP) with anti-β-catenin antibodies. After taking up the samples in Laemmli buffer, equal volumes of each were resolved by 7.5% SDS–PAGE and the proteins transferred electrophoretically to a nitrocellulose membrane and probed with antisera specific for ZO-1 (ZO-1), α-catenin (α-cat), β-catenin (β-cat), or Cx43 (Cx43). The numbers on the left indicate the positions of molecular mass markers in kilodaltons (kDa).

Cx43 during the early stages of junction formation in cardiomyocytes. Although a sequential redistribution of catenins, ZO-1, and Cx43 to the plasma membrane was seen after Ca^{2+} switch (see below), confocal microscopy showed colocalization of these proteins in the cytoplasm, suggesting that they form a close association before transport to the plasma membrane during gap junction formation. Optical sectioning along the Z-axis demonstrated that both β-catenin and Cx43 colocalized with ZO-1 at the central region of cell–cell contact sites (Fig. 4). In a study of tight junction development in MDCK cells, ZO-1 was found to colocalize with E-cadherin at the cell surface 2 h after Ca^{2+} switch, whereas, at 48 h after Ca^{2+} switch, an apical, tight junction-associated ZO-1 ring was seen separate from the lateral E-cadherin staining [Rajasekaran et al., 1996]. A similar segregation was reported in an immunofluorescence microscopy study of rat WIF-B9 hepatocytes, in which double-labeling showed that ZO-1 colocalized with E-cadherin at cell–cell contact sites in doublet cells; as the cells became polarized, ZO-1 colocalized with occludin at the cell apex, while E-cadherin and catenins were localized to cell–cell contact sites at the lateral pole [Decaens and Cassio, 2001]. Guerrier et al., 1995 reported that Cx43, but not Cx32, is colocalized with ZO-1 at the lateral plasma membrane in epithelial cells of pig thyroid cryostat sections, and, based on this finding and other biochemical evidence, inferred that the distribution of Cx43 to the plasma membrane of tight junctions was related to the establishment of cell polarity. In contrast to epithelial cells, cardiomyocytes do not contain tight junctions and do not establish an epithelial-like polarity, but develop polarity by forming intercalated discs at cell termini. In the present study, although extensive distribution of catenins, ZO-1, and Cx43 along the cell–cell contact site was detected in cardiomyocytes 1 h after Ca^{2+} switch (Fig. 4C,D), no separation of ZO-1 from catenins or Cx43 was detected after a later time (24 h) after Ca^{2+} switch (Fig. 4E,F). Since the adherens junction is found in both cardiomyocytes and epithelial cells, targeting of Cx43 to the plasma membrane of cardiomyocytes may be regulated by complex formation with ZO-1 and catenins.

Immunoprecipitation analyses provided biochemical evidence for the association of catenins with ZO-1 and Cx43 in cardiomyocytes following Ca^{2+} switch, and the formation of a catenin–ZO-1–Cx43 complex following Ca^{2+} switch also implied that catenins are involved in the assembly of gap junctions in cardiomyocytes. Rajasekaran et al., 1996 demonstrated that, in addition to α- and γ-catenin, ZO-1 can be coprecipitated with β-catenin from detergent extracts of MDCK cells at 0 and 2 h after Ca^{2+} switch, indicating association of ZO-1 with the catenin complex during the early stage of junction assembly. A study in which cDNAs encoding full-length ZO-1 or the N-terminal or C-terminal half of the molecule were incorporated into mouse L fibroblasts demonstrated direct binding of the C-terminal half of ZO-1 to α-catenin, while the N-terminal half colocalized with actin stress fibers [Itoh et al., 1997]. In a study of gap junction formation, coexpression of epitope-tagged Cx43 and ZO-1 in human embryonic kidney HEK293 cells demonstrated a direct association between the C-terminal of Cx43 and the N-terminal of ZO-1 [Toyofuku et al., 1998]. Taken together, these results suggest the formation of a catenin–ZO-1–Cx43

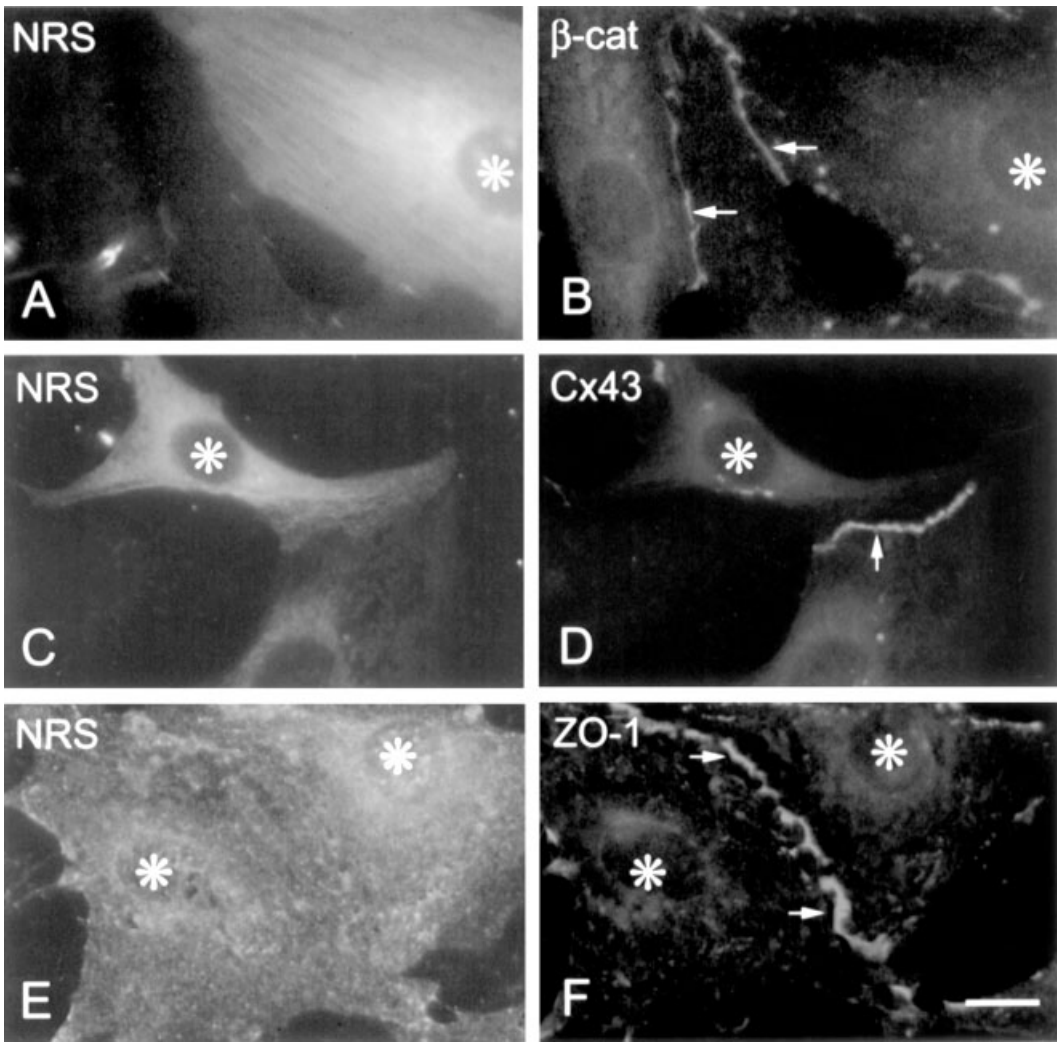

Fig. 6. Electroporation control of normal rabbit serum incorporated into cardiomyocytes. Cardiomyocytes cultured in low Ca^{2+} medium for two days were electroporated with HBS containing normal rabbit serum (NRS) subjected to Ca^{2+} switch for another 2 h, then double-labeled for incorporated rabbit IgG using an FITC-conjugated goat anti-rabbit IgG secondary antibody (**A**, **C**, **E**) and for cell junctions using mouse primary antibodies specific for β-catenin (**B**), Cx43 (**D**), or ZO-1 (**F**) and Texas red-conjugated goat anti-mouse IgG secondary antibody. All cardiomyocytes showed a normal staining pattern for cell junctions. Bar = 10 μm.

complex during the process of protein redistribution to the plasma membrane during junction formation. This suggestion was further supported by the precipitation of ZO-1 and Cx43 from TX-100 extracts of cardiomyocytes using anti-β-catenin antibodies.

In our Ca^{2+} switch assays, immunofluorescence microscopy showed that α-catenin, β-catenin, and ZO-1 redistributed to the plasma membrane at an earlier time (10 min) than Cx43 (30 min), implying a temporal sequence of the assembly of junctional components in cardiomyocytes. A previous time-course study of the appearance and distribution of junctional proteins in cultured adult rat cardiomyocytes indicated that adherens junction proteins, including N-cadherin, α-catenin, and β-catenin, were found at cell–cell contacts at an earlier time than Cx43 during the reassembly of intercalated discs [Hertig et al., 1996a; Kostin et al., 1999]. Quantitative confocal microscopy also revealed a progressive increase in Cx43 fluorescence intensity in intercalated discs after the establishment of an organized adherens junction [Kostin et al., 1999]. The formation of adherens junctions is suggested to be a prerequisite for the subsequent progressive formation of gap junctions in cardiomyocytes [Hertig

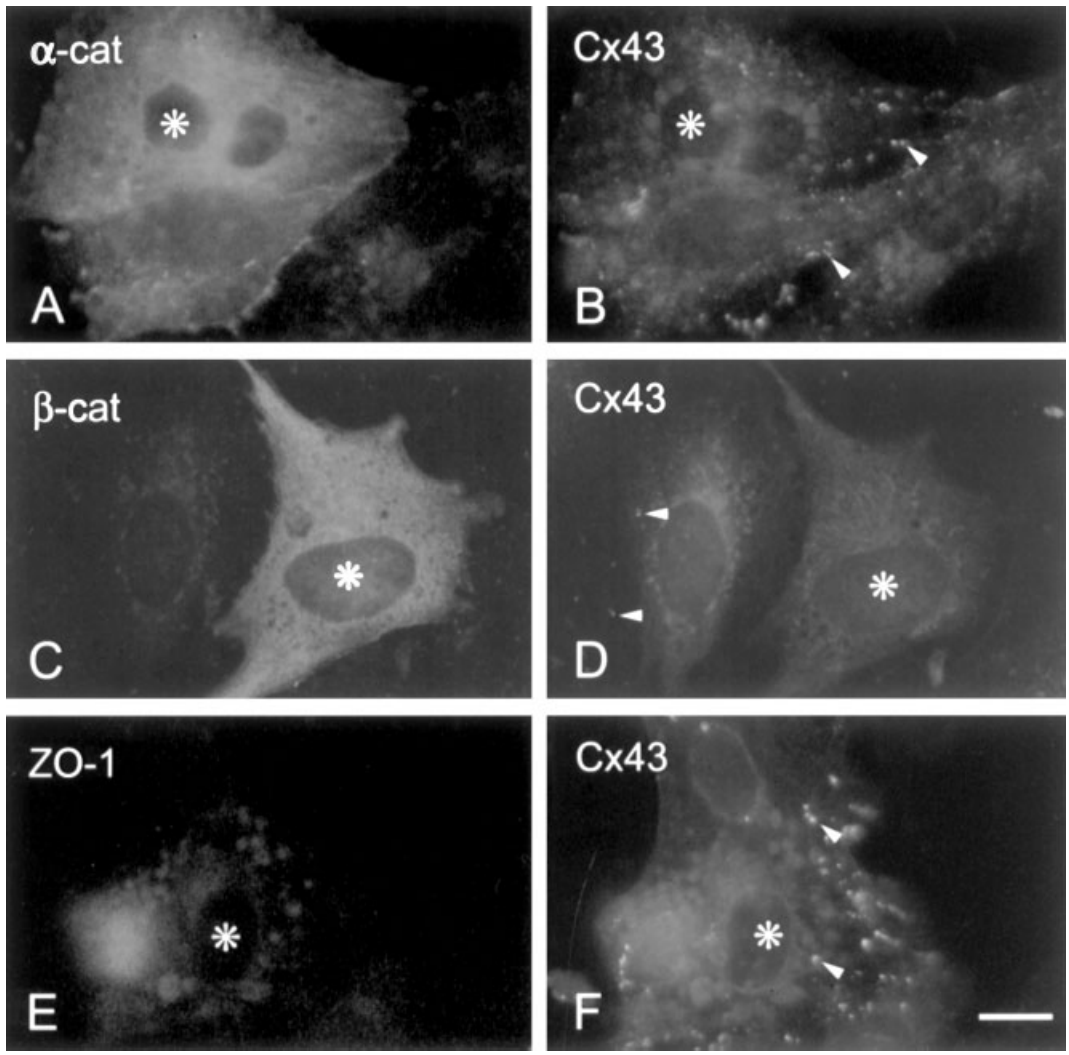

Fig. 7. Incorporation of antisera against α-catenin, β-catenin, and ZO-1 into cardiomyocytes by electroporation. Cardiomyocytes cultured in low Ca^{2+} medium for two days were electroporated with HBS containing rabbit antiserum against α-catenin (**A**), β-catenin (**C**), or ZO-1 (**E**), subjected to Ca^{2+} switch for another 2 h, then fixed with cold acetone and double-stained for rabbit antibodies and Cx43 (**B**, **D**, **F**). The arrowheads indicate Cx43 staining spots in the cytoplasm. The asterisks indicate the nuclei of cardiomyocytes containing rabbit antiserum. Bar = 10 μm.

et al., 1996a; Kostin et al., 1999]. This proposed prerequisite for gap junction formation is in good agreement with the functional analyses in the present study, which showed that electroporation of antisera against α-catenin, β-catenin, and ZO-1 inhibited the formation of adherens junctions and the assembly of gap junctions in cardiomyocytes. Itoh et al., 1997 proposed that ZO-1 functions as a cross-linker between the cadherin/catenin complex and the actin-based cytoskeleton. In the present study, the incorporated antibodies may interfere with the formation of functional N-cadherin/catenin complexes and hinder their binding to the actin cytoskeleton, thus reducing N-cadherin/N-cadherin adhesiveness and the subsequent formation of adherens junctions, thus inhibiting the assembly of gap junctions [Hertig et al., 1996a; Kostin et al., 1999]. Alternatively, the catenins may function synergistically with ZO-1 as an adapter for Cx43 transport during gap junction formation in cardiomyocytes [Toyofuku et al., 1998]. Intracellular application of antibodies to cardiomyocytes cultured in low Ca^{2+} medium interfered with the association of catenins with ZO-1 and perturbed the formation of the catenin–ZO-1–Cx43 complex. When the antibody-incorporated cardiomyocytes were placed in normal Ca^{2+}-containing medium, Cx43 transport to the contiguous plasma membrane

was inhibited, and the assembly of the gap junction plaque was blocked, as shown by the diffuse Cx43 staining in the cytoplasm (Fig. 7). In conclusion, we have demonstrated an association between α-catenin, β-catenin, ZO-1, and Cx43 in rat cardiomyocytes and that this association is required for the development of gap junctions. The regulatory role of catenins and ZO-1 in gap junction formation merits further investigation.

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