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Effect of Antipeptide Antibodies Directed against Three Domains of Connexin43 on the Gap Junctional Permeability of Cultured Heart Cells

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Abstract. Cell-to-cell communication can be blocked by intracellular injections of antibodies raised against gap junction proteins, but the mechanism of channel obstruction is unknown. Binding to connexins could lead to a conformational change, interfere with regulatory domains or cause a steric hindrance. To address these questions, the effects on cell-to-cell communication of affinity purified polyclonal antibodies raised against peptides reproducing the intracellular sequences 5–17, 314–322 and 363–382 of rat connexin43 were investigated in cultured rat ventricular cells. The antibodies against sequence 363–382 were characterized by immunoblotting and immunocytochemistry. Characterization of antibodies 5–17 and 314–322 has been previously reported. In a first series of experiments, the effect on gap junctional communication was assessed by injecting a junction-permeant fluorescent dye into cells adjacent to one cell previously microinjected with antibodies. In a second series, junctional permeability was quantitatively determined on records of fluorescence recovery after the photobleaching of 6-carboxyfluorescein-loaded cells. Antibodies 5–17 marked a 43 kDa band on immunoblots, but did not immunolabel gap junctions and had no functional effect. Antibodies 314–322 recognized the 43 kDa protein and labeled the intercalated disks, but failed to interfere with junctional permeability. Antibodies to the nearby sequence 363–382, for which all immunospecific tests had been positive, caused a delayed diffusional uncoupling in 50% of the microinjected cells. It is suggested that the blocking of junctional communication by antibodies results from interference with a regulatory domain of the connexin.

Key words: Cardiac myocytes — Gap junction — Intercellular communication — Connexin43 — Antipeptide antibodies — Cell-to-cell dye transfer

Introduction

The cell-to-cell membrane channels in the gap junctions establish electrical and diffusional communication in most tissues. These channels are formed by the association of two similar elements, the connexons, comprising six protein subunits inserted in the membranes of two adjacent cells and protruding into the narrow extracellular space where they tightly assemble (Makowski et al., 1977). Combined protein and gene analysis has made it possible to establish the primary sequence of a 32 kDa gap junction protein from mammalian livers (Kumar & Gilula, 1986; Paul, 1986), then of a 43 kDa junctional protein from the rat heart (Beyer, Paul & Goodenough, 1987). A series of homologous proteins, the connexins, encoded by a multigene family (Beyer, Paul & Goodenough, 1990; Bennett et al., 1991; Willecke et al., 1991; Beyer, 1993), has now been characterized and localized in the gap junctions of various tissues.

Immunoelectron microscopic visualization of antibodies directed against synthetic peptides corresponding to defined domains of gap junction proteins has shown that both the amino- and the carboxy-termini of connexins are cytoplasmic, and that the four transmembrane segments are linked by one cytoplasmic loop and two

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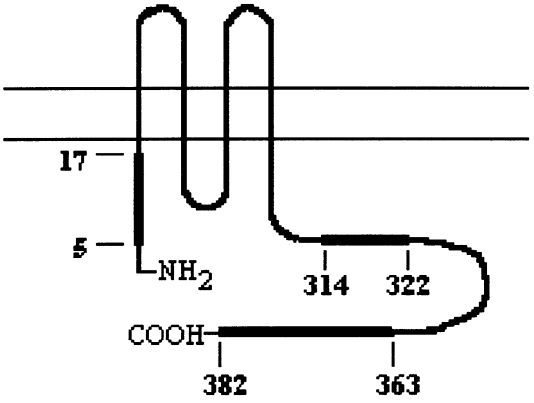

Fig. 1. Schematic representation of the membrane insertion of the gap junction protein connexin43, according to Laird and Revel (1990). The horizontal parallels represent the lipid bilayer, with extracellular side upwards. Bold segments indicate the position of the three peptides that have been used to raise the antibodies assayed in the present study.

extracellular loops (Beyer et al., 1987; Milks et al., 1988; Yancey et al., 1989; Laird & Revel, 1990; Yeager & Gilula, 1992).

The most conserved domains in the connexin family are the NH_2-terminus, the third transmembrane segment and the two extracellular loops that combine with similar elements belonging to the opposite cell. The more variable intracellular loop and COOH-terminus appear to be instrumental in the different channel conductances and their diverse sensitivities to regulating factors in several cell types expressing the same or different connexins.

Intracellular injections of anti-connexin antibodies have been shown to affect cell-to-cell communication through gap junctions. Sheep antibodies raised against gap junctions isolated from rat liver blocked diffusional and electrical coupling in cell pairs dissociated from rat liver or heart (Hertzberg, Spray & Bennett, 1985). Rabbit antibodies raised against the whole proteins eluted from rat liver gap junctions have been used to investigate the effect of interfering with gap junctional communication in the 8-cell stage of the *Xenopus* (Warner, Guthrie & Gilula, 1984) and mice embryos (Lee, Gilula & Warner, 1987) on the subsequent development.

Like these antibodies against the whole proteins eluted from cardiac gap junctions, site-directed antibodies against connexins may affect the functioning of the cell-to-cell channels. It can be surmised that their binding to specified amino acid sequences could provide information on the functional and regulatory properties of different domains of this channel protein. In the present study, antipeptide antibodies raised against three sequences of connexin43, the main gap junction protein that has been detected in the rat working myocardium (Beyer et al., 1989; Yancey et al., 1989; van Kempen et al., 1991), were microinjected into cultured heart cells isolated from newborn rats. Control microinjections of antibodies raised against the whole protein electroluted from isolated gap junctions, prepared and characterized by Yancey et al. (1989) and kindly provided by Dr. J.-P. Revel's laboratory (Caltech, Pasadena, CA), were also performed. In previous work, the effects of antibodies on cell-to-cell communication have generally been estimated by observing the diffusion of fluorescent dyes from one microinjected cell into its neighbor cells (Yancey et al., 1989; Lal, Laird & Revel, 1993; Becker et al., 1995). In the present research, after an effect had been detected by this method, a more precise quantitative measurement of cell-to-cell dye transfer was obtained by analyzing records of fluorescence recovery after the photobleaching of cells previously loaded with 6-carboxyfluorescein (gap-FRAP). The antibodies tested were directed against the amino acid sequence 5–17 of the NH_2-terminus, and against the sequences 314–322 and 363–382 of the COOH-terminus of Cx43. The first two antibodies did not interfere with cell-to-cell communication, whereas the third blocked diffusional coupling in about 50% of the microinjected cells, an effect comparable to that of the antibodies against the whole gap junction protein. These results indicate that the functional effect of an antibody on gap junctional communication critically depends on the particular amino acid sequence to which it can bind, and that the COOH-terminus region of Cx43, which comprises several potential phosphorylation sites, is a functionally important domain of the gap junctional channel. Similarities and differences between the functional effects of several antibodies raised against peptide sequences of Cx43 (Yancey et al., 1989; Lal et al., 1993; Becker et al., 1995) are discussed. Part of these results have been published in preliminary form (Bastide et al., 1993, 1995).

Materials and Methods

Characterization of Anti-Cx43 Antibodies Directed Against Residues 363–382

The preparation and characterization of rabbit polyclonal antibodies directed to synthetic oligopeptides corresponding to the amino acid sequences 5–17 (NH_2-terminus, antibodies 5–17) and 314–322 (60 amino acids away from the carboxy-terminus, antibodies 314–322) of rat Cx43 (Beyer et al., 1987) have been previously reported (antibodies 5–17: Dupont et al., 1988; antibodies 314–322: El Aoumari et al., 1990; Giaume et al., 1991) (Fig. 1). A third site-directed antibody, corresponding to residues 363–382 (the COOH-terminus, antibodies 363–382) of the same connexin, was prepared by immunizing rabbits with the peptide YPSSRASSRASSRPRPDDLEI, synthesized according to Merrifield (1963) and coupled to keyhole limpet hemocyanin (KLH, Sigma) by means of bis-diazobenzidine (Briand, Müller & Van Regenmortel, 1986). The immunization of rabbits, collection of sera, purification of antibodies by affinity chromatography, and the preparation of the preimmune fractions used in control experiments were performed as described elsewhere (Dupont et al., 1988; Gros et al., 1994).

For the characterization of antibodies 363–382, rat heart gap junc-

tions were isolated using the procedure described by Manjunath, Goings and Page (1984), in the presence of phenylmethylsulfonyl fluoride (1 mMol/l). The analysis by electrophoresis of isolated gap junctions and of freeze-dried myocardium, the characterization of gap junction proteins by immunoblotting and the control tests were carried out as previously reported (El Aoumari et al., 1990; Gros et al., 1994). Some samples fractionated by electrophoresis were stained with Coomassie Brilliant Blue R-250. Other samples were electrotransferred onto nitrocellulose membranes and incubated overnight with rabbit anti-Cx43 antibodies as previously described (Gros et al., 1994). The primary antibodies were revealed on immunoblots either by means of ^{125}I-labeled protein A or with biotinylated secondary antibodies and peroxidase-labeled streptavidin.

Detection of Cx43 by immunohistochemistry with antibodies 363–382 was performed on cryosections of paraformaldehyde-fixed rat ventricles. After quenching the endogenous peroxidase activity with methanol containing 0.3% H_2O_2 for ten min, the sections were incubated overnight with the antipeptide antibodies. The primary antibodies were detected by a peroxidase reaction, after their sequential combination with biotinylated goat antirabbit antibodies, then with a biotin/avidin/horseradish-peroxidase complex (Vectastain, Vector Laboratories, Burlingame, CA). The preparatory procedures for immuno-electron microscopy have been reported in detail previously (Gros et al., 1994). The primary antibodies were detected by means of gold-labeled protein A (BioCell Research, Cardiff, UK). The specificity of labeling was controlled with preimmune rabbit sera and with primary antibodies preincubated with the immunogenic peptide (50 to 100 μg/ml).

The polyclonal affinity-purified antipeptide antibodies used in microinjection experiments were extensively dialyzed against a solution containing 8 mM/l of either K^+-citrate or KCL and concentrated to 1 mg/ml. They were stored at 4°C without preservative and were used within a few days after purification.

Preparation and Culture of Cardiac Myocytes

Minced ventricles from 1–2 day old rats were subjected to enzymatic dissociation, under gentle stirring at 37°C, in a Ca^{2+} and Mg^{2+}-free saline medium (Spinner) containing 0.02% crude trypsin (Boehringer, Mannheim). The dissociated cells were collected 5 times at intervals of 10 to 15 min. The first sample was discarded and the last four samples were centrifuged and resuspended in Ham's culture medium. Preplating in 100 mm culture dishes (Nunc, Roskilde, Denmark) for 60 min allowed removal of most fibroblasts by attachment to the plastic surface. Cells were then seeded at a density of about 5×10^5 per 35 mm plastic culture dish, in a Ham's F10 medium supplemented with 10% fetal calf serum, 10% heat inactivated horse serum, penicillin G (40 IU/ml) and streptomycin sulfate (17 μM/l). The dishes were maintained at 37°C in an atmosphere of 5% CO_2 and 95% air.

Microinjection of Dyes and Antibodies

On the second or third day of culture, the dishes with attached neonatal rat heart cells were transferred onto the stage of a fluorescence microscope and the culture medium was replaced by a Tyrode's solution containing (in mM/l): NaCl 144, KCl 5.4, $MgCl_2$ 1, $CaCl_2$ 2.5, NaH_2PO_4 0.3, glucose 5.6 and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) 5, pH 7.4. All experiments were performed at room temperature (22–24°C). The micropipettes used for microinjections had an electrical resistance of 15–20 MΩ when filled with 3 M KCL. They were backfilled with the fluid containing the affinity-purified and dialyzed antibodies (1 mg/ml), to which an aqueous solution containing LiCl and HEPES was added to reach final concentrations of 0.8 mg/ml for the antibodies and of 130, 8 and 10 mM/l for, respectively, LiCl, KCl and HEPES. In some control experiments, a phosphate buffered saline (PBS) was used instead for dilution. No difference was noted between the two procedures. To help monitor the injection process, a fluorescent dye (Fluorescein or Lucifer Yellow-CH, 55 mM/l) was added to the pipette solution.

The microinjections were given to one cell inside a group of cardiac myocytes, which could be easily distinguished from the few remaining fibroblasts by their spontaneous contractile activity, under epifluorescence observation at a magnification of 400×. An apparatus (Socolar & Loewenstein, 1979) designed to apply air pulses at slowly increasing pressures to the micropipettes was used. The injection proceeded for a few seconds after fluorescence began to appear inside the injected cell and was interrupted when the cell became uniformly fluorescent. Although the injected volume was not estimated, this procedure served to monitor the injection process, and significant variations of the amount injected could thus be avoided. The eventual blocking effect of the antibodies on the gap junction channels was always delayed, as demonstrated by the rapid (within seconds) spread of fluorescence from the injected cell into its adjacent neighbors that was observed in all successful injections. Obviously, the smaller fluorescent molecules had diffused past the gap junctions before the antibodies had time to reach the connexons and to combine with their epitopes on Cx43. This rapid cell-to-cell spread of fluorescence, before any effect of the antibodies on intercellular dye-transfer, had the advantage of providing a means of checking the initial permeability of the junctions and their functional state after completion of the injection process. In this way, any accidental uncoupling that might have occurred due to mechanical injury could be detected and rejected from the data. All trials in which morphological alterations were apparent at the time an uncoupling effect was noticed were also rejected. It is felt that by means of these tests, all fortuitous uncoupling events could be discarded.

Probing the Permeability of the Gap Junctions

Double Injection Experiments

The effect of antibodies on cell-to-cell diffusional communication through gap junctions was assayed by two different methods ten min after the intracellular injection. In a first series of experiments, test injections of Lissamine Rhodamine (Gurr, UK), another gap junction permeating molecule (MW 580.6), were given to myocardial cells contacting the antibodies-injected cell. The different wavelengths of fluorescent emission of Lucifer Yellow and of Lissamine Rhodamine allowed the microscopic images of cell-to-cell diffusion given by the two dyes to be viewed and photographed separately, by the use of appropriate filters. An abrupt interruption of the spread of Lissamine Rhodamine fluorescence at the boundary of the antibodies-injected cells was interpreted as an impairment of gap junctional permeability secondary to the antibody injection.

Quantitative Measurement of Cell-to-Cell Dye Transfer by Fluorescence Recovery after Photobleaching

In another series of assays, the permeability of the gap junctions was quantified in control and in antibodies-injected cultured myocytes by analyzing the fluorescence recovery after photobleaching of cells containing 6-carboxyfluorescein (gap-FRAP method, Wade, Trosko & Schindler, 1986). The practical application of this technique by means of the ACAS 570 apparatus (Meridian Instruments, Okemos, MI) has

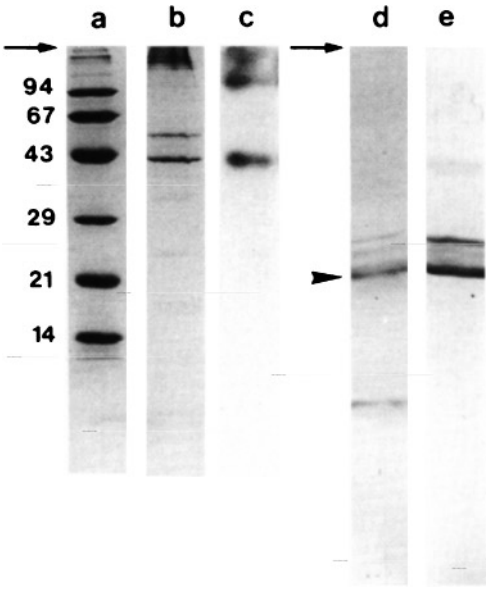

Fig. 2. Characterization by immunoblotting of the affinity-purified rabbit antipeptide antibodies directed to residues 363–382 (COOH-terminus) of rat Cx43. Lane *a:* Electrophoretic separation of molecular weight standards (indicated in kDa) used to determine the relative weights of proteins detected in the gels and the immunoreplicas shown in lanes *b* and *c*, respectively. Lane *b:* Electrophoretic analysis of gap junction-enriched fraction extracted from rat heart. Two major proteins of 43 kDa and of about 50 kDa were detected with Coomassie-Blue staining. The slower migrating band, above 100 kDa, results from aggregation. Lane *c:* Immunoblot analysis of the proteins sorted in (*b*). The antibodies 363–382 (3 μg/ml), detected with ^{125}I-tagged protein A, recognize the 43 kD band and the heavier aggregate. Lanes *d* and *e:* Replicas of total extracts from rat heart probed with antibodies 363–382 and antibodies 314–322, respectively. Three bands were detected in both cases, at 40–41 kDa (arrowhead), 43 and 45 kDa. The relative intensity of the bands varied from one experiment to the other. The experiments illustrated in lanes *d* and *e* were carried out independently of those shown in lanes *b* and *c*, which accounts for the difference in Cx43 mobility. In *d* and *e*, the primary antibodies were detected by means of biotinylated secondary antibodies and peroxidase-labeled streptavidin (revealed with chloronaphtol).

been described in detail (Pluciennik, Joffre & Délèze, 1994). Briefly, the cells in a tissue culture dish were loaded with the junction-permeant fluorescent molecule 6-carboxyfluorescein (6-CF) in the form of its diacetate ester (Sigma), which becomes hydrolyzed by intracellular esterases (fluorochromasia; Rotman & Papermaster, 1966). Photobleaching was performed by directing strong light pulses from an argon laser tuned at 488 nm onto selected cells. The fluorescent emission, stimulated by scanning the field at preset time intervals with laser pulses of low intensity focused to a diameter of about 1 μm by the microscope lens, was collected by the same lens, separated from the laser light by dichroic mirrors and filters and recorded together with the *x*,*y* coordinates. These data were used for computer reconstruction of the fluorescence intensities in a false color scale (e.g., Fig. 6) and for offline analysis of fluorescence recovery by integrating the levels of fluorescent light in selected cells.

The rise of fluorescence intensity in the photobleached cells, which reflects the flux of 6-CF molecules diffusing from unbleached adjacent cells across permeable gap junctions, initially follows a closely exponential time course (e.g., Fig. 7*A*). For the duration of this first-order process, the bleached cell and the set of its adjacent neighbors can therefore be considered as a diffusion system comprising two compartments separated by a rate-limiting concentration boundary located at the gap junctional membranes. Under these conditions, the rate constant k (min^{-1}) of the initial recovery curve provides a quantitative index of cell-to-cell dye transfer, which is related to the permeability coefficient P of the junctional membrane by a geometrical factor ($k = Pa/v$), where a is the area of the gap junctions and v the volume of the bleached cell, considered constant within the duration of an experiment.

In a thin layer of solution (100 μm) and at low concentrations (range for fluorescein: 10^{-8} to 10^{-3} M), the fluorescent intensities vary linearly with the dye concentrations (Barrows et al., 1984). Therefore the rate constant k of fluorescence recovery in the bleached cells can be obtained from the equation:

$$(F_i - F_t)/(F_i - F_o) = e^{-kt} \quad (1)$$

where F_i, F_o and F_t are the integrated fluorescence intensities in the bleached cells, respectively before, immediately after and at time t after photobleaching. Control measurements of the gap junctional permeability were performed before the microinjections of antibodies into one cell. The efficiency of the injection procedure was monitored by the presence of fluorescein in the injected solution (60 mM), which, if the injection was successful, spread uniformly in the cell cluster before the permeability of the gap junctions became blocked.

Results

Specificity of Antibodies Directed to Residues 363–382 of Cx43

Figure 2 summarizes the characterization of antibodies 363–382 by immunoblotting. When stained with Coomassie Blue, the electrophoresis of partially purified rat heart gap junctions shows a well-defined band centered at 43 kDa (Fig. 2, lane b). A band of similar electrophoretic mobility was labeled with antibodies 363–382 on the immunoreplicas of the same samples (Fig. 2, lane *c*). Analysis of replicas of total extracts from rat hearts probed with antibodies 363–382 revealed a major band at 40-41 kDa associated with two other bands at 43 and 45 kDa (Fig. 2, lane *d*). Bands of similar mobility were also seen in replicas probed with the previously characterized antibodies 314–322 (Fig. 2, lane *e*). These bands correspond to the various states of phosphorylation of Cx43, as shown for example by Laird, Puranam and Revel (1991) and Lau, Hatchpigott and Crow (1991). A number of controls were carried out to ascertain the specificity of the immunoreactions. No labeling was detected in immunoreplicas when the antibodies were omitted or replaced by a preimmune fraction or when the antibodies had been blocked by overnight preincubation at 4°C with the immunogenic peptide at concentrations ranging from 50 to 100 μg/ml.

In frozen sections of paraformaldehyde-fixed rat ventricle incubated with antibodies 363–382, immuno-

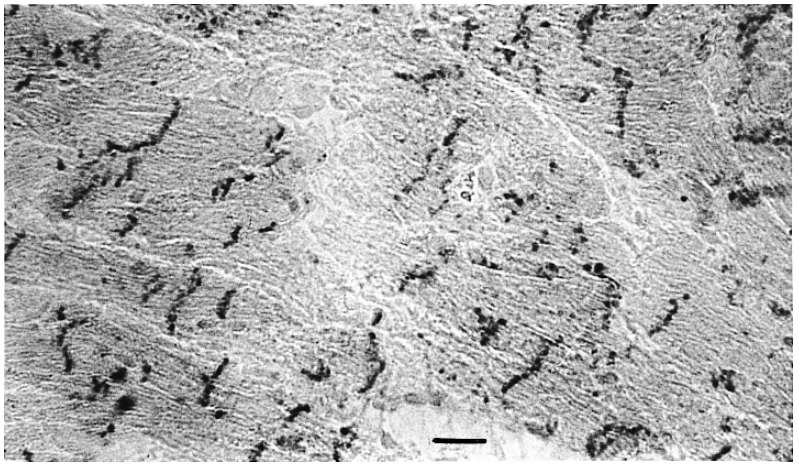

Fig. 3. Immunodetection of Cx43 by a peroxidase reaction in a 4 μm frozen section of a paraformaldehyde-fixed rat ventricle. Antibodies 363–382 were labeled with biotinylated goat antirabbit antibodies, which were then combined into a biotin/avidin/horseradish-peroxidase macromolecular complex. The localization of the antibodies coincides with the typical aspect of intercalated disks, which contain numerous gap junctions connecting the heart myocytes. Bars: 50 μm.

detection by means of a peroxidase reaction showed a pattern of labeling consistent with the distribution of intercalated disks (Fig. 3).

Examination of ultrathin sections from rat ventricle prepared for immunoelectron microscopy shows the colloidal gold particles associated exclusively with gap junctions (Fig. 4). No labeling was seen in control samples incubated either with a preimmune serum fraction or with antibodies preincubated with the immunogenic peptide (50 μg/ml).

As a whole, these results demonstrate that antibodies 363–382 specifically recognize Cx43.

Effect of the Microinjected Antibodies on Diffusional Communication

Antibodies Directed against Residues 5–17

Ten minutes after microinjecting these antibodies into cultured cardiac cells, the gap junctional channels remained permeable to the fluorescent molecule Lissamine Rhodamine, as shown by the typical experiment depicted in Fig. 5*A–C*. No case of uncoupling was observed in 13 similar double injection experiments.

Antibodies Directed against Residues 314–322

Antibodies prepared against this sequence, located 60 amino acids away from the carboxyl-terminus of Cx43, bind a 43 kDa protein from rat heart gap junctions, label the intercalated disks on frozen sections and mark the intracellular side of isolated gap junctions in immunoelectron microscopic preparations (El Aoumari et al., 1990). Despite this specific binding to Cx43 *in situ*, these antibodies were also ineffective in blocking junctional communication in vivo (one uncoupling was observed in eleven double injection experiments).

Antibodies Directed against Residues 363–382

A comparison of cell-to-cell dye transfer in control cells and in cells injected with antibodies 363–382, raised against the polypeptide corresponding to the last twenty residues of the carboxy-terminus of Cx43, was performed by means of the gap-FRAP method. The experiment represented in Fig. 6*A–C*, and the corresponding quantitative assessment (Fig. 7*A*) show that, in control conditions, fluorescence recovered in photobleached cardiac cells in contact with unbleached neighbors, but not in isolated cells. Furthermore, in a cell pair, the exponential recovery curve of the photobleached cell was closely symmetrical to the exponential decay curve of the companion cell (e.g., curves *1* and *2* in Fig. 7*A*). These observations allow us to conclude that the fluorescence recovery recorded in the photobleached cells is caused by diffusion from unbleached adjacent cells. That this cell-to-cell dye transfer takes the route of gap junctional channels, as opposed to cytoplasmic bridges, has been demonstrated in cultured cardiac myocytes by the reversible blocking of fluorescence recovery under the action of the aliphatic alcohol heptanol (Bastide et al., 1995).

Figure 6*D–F* and the corresponding analysis in Fig. 7*B* show an example of complete suppression of dye diffusion through gap junctions in a cardiac cell that had been injected with antibodies 363–382 10 min before photobleaching.

The rate constants k obtained from the recovery curves (Fig. 7), which are proportional to the permeability coefficient of the gap junctions, are represented in Fig. 8. Control values measured after injections of PBS ($n = 7$, Fig. 8*A*) are compared to those obtained after injections of unspecific proteins (bovine serum albumin, 1mg/ml, or human γ-globulins, 2mg/ml, $n = 9$, Fig. 8*B*), and after injections of antibodies 363–382 (Fig. 8*C*). Complete ($k = 0$) or partial ($k < 0.1$ min^{-1}) blocking of

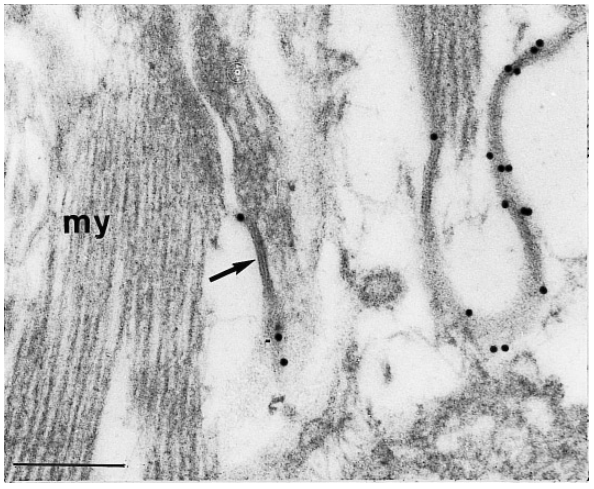

Fig. 4. Immunogold detection of Cx43 in ultrathin sections of rat ventricle. Antibodies 363–382 were revealed with protein A labeled with 10 nm gold particles. Arrows indicate double-membrane profiles which identify gap junctions. No labeling is seen outside gap junctions. my: myofilaments. Bar: 0.25 μm.

dye diffusion was observed in about 50% of the cells microinjected with these antibodies.

Antibodies Directed against the Whole Gap Junction Protein

In a series of control experiments, the functional effect of introducing an antibody raised against the whole protein electroluted from isolated gap junctions (Yancey et al., 1989) into cultured heart cells was assayed by successive microinjections of the antibodies, then of a secondary fluorescent dye into neighboring cells. A delayed inhibition of cell-to-cell dye transfer occurred in 8 out of 16 successful injections (Fig. 5*D–F*). Considering that this antibody was used after a storage for several months and had been accidentally thawed during transportation, these results are comparable to the 24 diffusional blocks previously reported by Yancey et al. (1989) in a series of 30 injection experiments.

Discussion

Delayed Effect of Antibodies on Junctional Communication

In our experiments, Lucifer Yellow or Fluorescein injected simultaneously with the antibodies always had time to diffuse into neighbor cells before gap junctional communication became impaired, which indicates that diffusion of the antibodies and combination with their epitopes on the connexons are relatively slow events. As stated in Materials and Methods, this initial cell-to-cell diffusion provided a means to check the junctional permeability, and thus to detect accidental uncouplings during the injection process.

A delay of several minutes has also been noted between antibody injection and blocking effect in the experiments of Yancey et al. (1989), but an immediate blocking of cell-to-cell dye transfer was obtained in myocytes pairs of adult rat hearts injected with highly concentrated (40–100 μg/ml) sheep antibodies against the 27-kDa protein extracted from liver gap junctions (Hertzberg et al., 1985). The delay in the present experiments can be accounted for by the much higher dilution of the antibodies (the usual concentration in the injected fluid was 0.8 μg/ml).

Antibodies Directed Against Residues 5–17

These antibodies against a sequence of the *N*-terminal region of Cx43 have previously been shown to specifically recognize a 43 kDa protein on immunoblots from rat heart extracts and from isolated intercalated disks and gap junctions (Dupont et al., 1988), but all attempts to label intercalated disks by immunohistology of cardiac tissues, including several recently performed tests, have been unsuccessful. Gap junctional communication was not altered when these antibodies were microinjected into cultured rat heart cells (Fig. 5*A–C*). It may be argued that this lack of effect was to be expected as a direct consequence of the inability to bind the epitopes, suggested by the negative attempts at immunostaining. But the conditions for antigen-antibody interactions are quite different in living cells, where the antigens are in their native form, and in immunohistological experiments. Indeed, all preparatory procedures for immunostaining result in denatured proteins, and the absence of immunoreaction on histological sections does not necessarily preclude the possibility of in vivo binding with consequent functional effects.

It has recently been reported that antibodies raised against the sequence 1–16 of rat Cx43 do not impede dye transfer when introduced into one cell of the 8- to 16-cell mouse embryo (Becker et al., 1995). These antibodies also do not immunostain gap junctions in the mouse heart or embryo, though they recognize Cx43 on Western blot analysis (Becker et al., 1995). Similarly, antibodies raised against residues 7–21 of Cx32 did not significantly label gap junctions. (Zimmer et al., 1987). The lack of a functional effect of these antibodies, and of the antibodies 5–17 tested in this report, contrasts with other observations of a very high proportion of junctional blocks when antibodies directed against the amino acids 1–20 of Cx43 were injected into cultured heart cells (Yancey et al., 1989; Lal et al., 1993). Comparison of this polypeptide with sequences 5–17 (this report) and 1–16 (Becker et al., 1995) indicates that the blocking

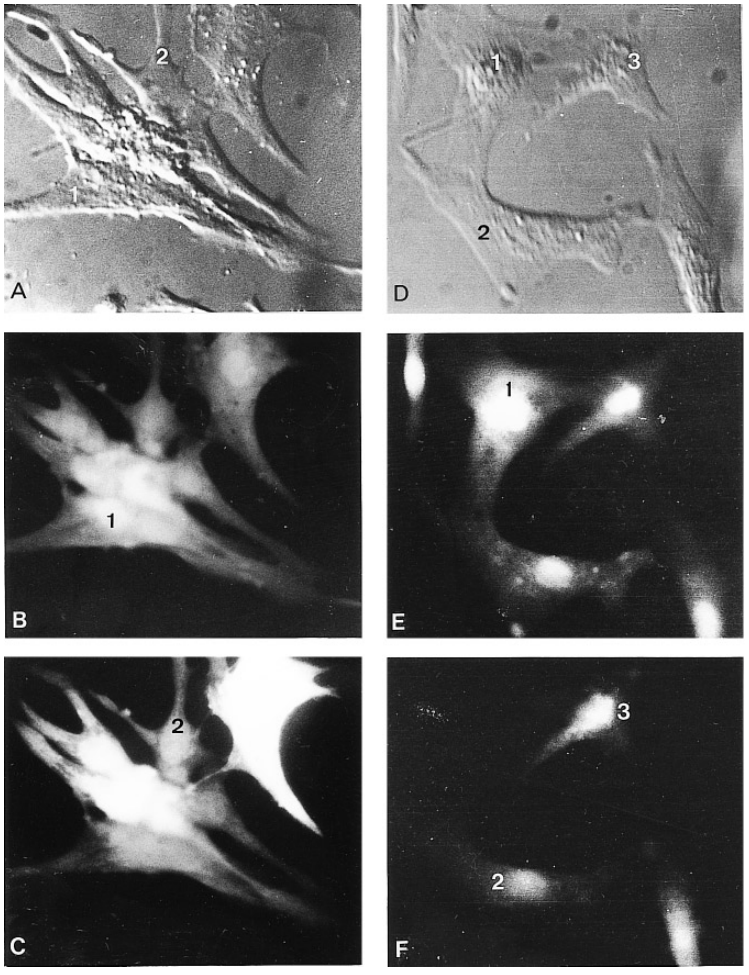

Fig. 5. Probing the effect of antibodies on cell-to-cell dye transfer (diffusional communication by gap junctions) by double injection experiments. Antibodies were microinjected together with the fluorescent dye Lucifer Yellow (LY) into one cell. One or two test injections of Lissamine Rhodamine (LR), another gap junction permeating molecule, were performed 10 min later into one or two adjacent cells. Both dyes emit at different wavelengths and can be viewed separately. *A–C:* Diffusional coupling is maintained after injection of antibodies 5–17. (*A*) The group of cultured cardiac cells under investigation is viewed by Nomarsky interference contrast optics. The antibodies are injected into cell (1). (*B*) Fluorescence picture of the same field obtained a few seconds later, showing the diffusion of LY from the injected cell into the four cells of this cluster. (*C*) When an isotonic solution containing LR is injected into another cell (2) 10 min later, the fluorescence picture shows that dye diffusion reaches all cells in the cluster, including cell (1) previously injected with the antibodies. (*D–F*) Diffusional coupling is interrupted after injection of antibodies against proteins extracted from whole gap junctions. (*D*) Nomarsky interference contrast of a group of cultured heart cells. The antibody is injected into cell (1) together with LY. (*E*) Fluorescence picture of the same field obtained a few seconds later, showing LY diffusion from the injected cell (1) into the four other cells of this cluster. (*F*) The effect of the antibodies on junctional permeability is probed 10 min later by injecting an isotonic solution containing LR into the adjacent cells (2, 3). The picture of the LR fluorescent emission shows that dye diffusion reaches all cells in the cluster, except cell (1) previously injected with the antibodies, and the cell in the upper left corner, which is connected to the cluster only through cell (1).

effect of the antibodies of Yancey et al. (1989) involves an epitope comprising the last four amino acids of sequence 1–20.

Antibodies Directed against Residues 314–322

Though all immunospecificity tests performed with these antibodies were positive (El Aoumari et al., 1990), they did not affect junctional communication when microinjected into cultured heart cells. This result shows that the binding of antibodies to connexons is not a sufficient condition to cause closure of the gap junctional channels. This lack of a functional effect of antibodies 314–322 is at variance with observations by Becker et al. (1995) of a weak but significant block of dye transfer in cells of the mouse embryo injected with antibodies raised against a peptide reproducing a slightly more extended region of Cx43 (amino acids 314–325). A hypothetical explanation could be provided by possible differences in the post-translational processing of Cx43 and in the regulation of the junctional channels in the mouse embryo and in the rat cardiac myocyte.

Antibodies Directed Against Residues 363–382

Antibodies 363–382 blocked or significantly decreased diffusional communication in cultured neonatal rat heart cells in about 50% of the trials. The blocking efficiency of these antibodies is comparable to that of the antibodies raised by Yancey et al. (1989) against the whole Cx43 protein extracted from rat heart gap junctions. The failure to observe an effect in a higher proportion of assays can be accounted for by the difficulty of controlling the volume of microinjected fluid. The presence of junctional channels made up of other cardiac connexins, such as Cx40, which differs from Cx43 in the carboxyl-terminal sequence (Haefliger et al., 1992), could provide another explanation. Cx40 and its messenger RNAs

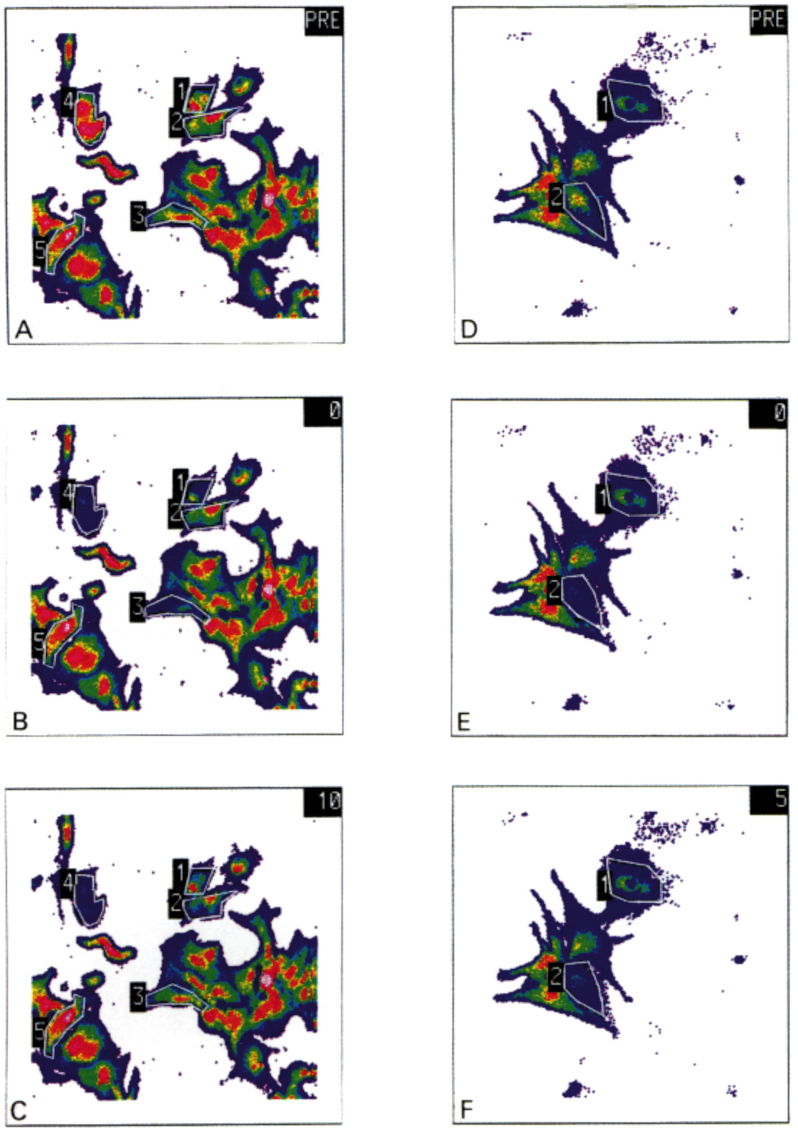

Fig. 6. Fluorescence Redistribution After Photobleaching as applied to the measurement of cell-to-cell communication by gap junctions (gap-FRAP method). The fluorescent emission of 6-carboxyfluorescein loaded into cultured heart cells is stimulated at preset time intervals, by moving the sample with a scanning stage in front of a pulsed laser beam of low intensity, focused to a diameter of about 1 μm by the microscope lens. Automatized recording of the *x,y* coordinates and of the corresponding fluorescent intensities collected by the same lens (separated from the laser light by dichroic mirrors and filters) allows computer reconstruction of the fluorescence intensities in a color-coded scale (the intensities decrease from red to violet). (*A–C*) Principle of the method and control experiment on normal rat heart cells. (*A*) Fluorescent emission at the beginning of the assay. Cells delineated by polygons labeled (1, 3, 4) are selected for subsequent photobleaching by intense laser pulses. The unbleached cell (5) serves as a control for spontaneous fluorescent decay. (*B*) The same field is scanned immediately after photobleaching. The fluorescent emission is markedly reduced in the bleached cells. (*C*) The fluorescent emission recorded in the same field 10 min later shows fluorescence recovery in the bleached cells (1) and (3), that are in contact with unbleached neighbors, but not in the bleached isolated cell (4). *D–F:* Diffusional uncoupling by the polyclonal antipeptide antibodies directed to residues 363–382 (COOH-terminus) of rat Cx43. (*D*) Fluorescent emission of a group of cultured heart cells loaded with 6-carboxyfluorescein, taken 10 min after injection of the antibodies into cell (2). The unbleached cell (1) serves as a control for spontaneous fluorescent decay. (*E*) Same field immediately after photobleaching cell (2). Note the color shift from yellow-green to deep blue, corresponding to the decreased fluorescent emission. (*F*) Five minutes later there is no change in the fluorescence intensity recorded in cell (2).

have been detected in the rat heart during embryonic development, but their expression decreases at the time of birth and disappears in the adult ventricular contractile myocytes (Van Kempen et al., 1995). Cx40 protein has not been detected in cultured myocytes from neonatal rat hearts, although its mRNA is present (Darrow et al., 1995), and it is therefore unlikely that channels constructed with this connexin could account for a detectable dye transfer if the Cx43 channels were blocked. A more likely possibility is the presence of a third connexin, Cx45, which has been recently immunolocalized in a punctate pattern along the apposed membranes of cultured myocytes from neonatal rat ventricules (Darrow et al., 1995). The primary sequence of Cx45 (Kanter, Saffitz & Beyer, 1992) markedly differs from Cx43 in the carboxy-terminal region and does not contain an amino acid sequence resembling peptide 363–382 of Cx43.

Possible Mechanisms of Junctional Blocking by Anti-connexin Antibodies

The operation of the gap junctional channels is characterized by random transitions between open and closed states. The conditions that antibodies raised against partial sequences of a connexin must fulfill to affect the permeability of the cell-to-cell channels are not known. Binding of these antibodies to one or several constituent proteins might obstruct the channel by steric hindrance. This simple mechanism does not seem to apply to blocking by antibodies 363–382, since antibodies 314–322 against nearby epitopes are ineffective.

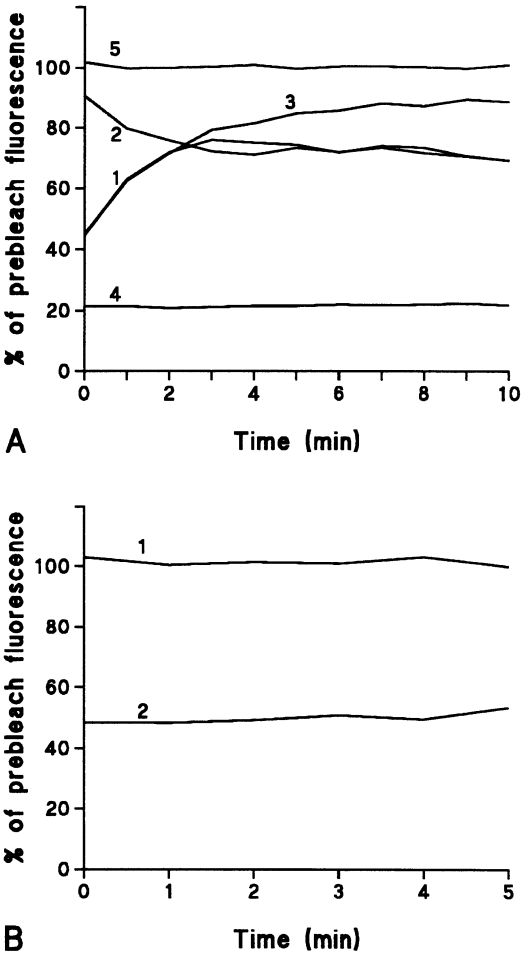

Fig. 7. Fluorescence recovery curves corresponding to the gap-FRAP experiments depicted in Fig. 6, obtained by integrating, in the polygons drawn around the selected cells, the levels of fluorescent light recorded after photobleaching at 1-min intervals. (*A*) Data from the control experiment of Fig. 6*A–C*. As expected from diffusion kinetics applied to compartments separated by a permeable membrane (here, the gap junctional membrane), the initial part of the recovery curves in the bleached cells (1, 3) connected to unbleached neighbors follows an exponential time course. That fluorescence recovery takes place by dye diffusion through gap junctions is shown by two characteristics: fluorescence does not increase in an isolated bleached cell (e.g., cell 4), and when one cell of a pair is bleached (cell 1), the exponential recovery curve of the sink (bleached) cell is closely symmetrical to the exponential decay curve simultaneously recorded in the source (unbleached) cell (2). The very slow background fluorescence decay measured in the unbleached control cell (5) shows that the nonjunctional membrane is much less permeant for 6-CF than is the gap junctional membrane. (*B*) Data from the experiment of Fig. 6*D–F*, in which antibodies 363–382 were injected into cell (2) 10 min before photobleaching. Recovery curves are shown for the bleached cell and for a control cell (1). The persisting low post-bleach fluorescence value in cell (2) shows that cell-to-cell diffusion of 6-carboxyfluorescein is blocked (diffusional uncoupling).

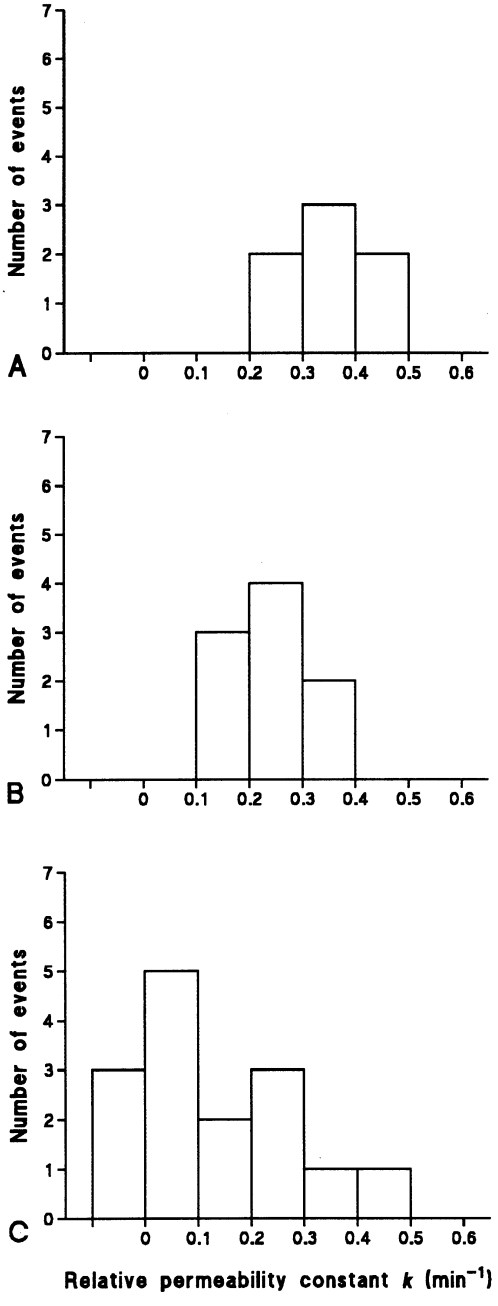

Fig. 8. Frequency histograms of the relative permeability constants k (the inverse values of the time constant of fluorescence recovery) of the gap junctional membranes obtained in cultured heart cells by the photobleaching (gap-FRAP) method, in two control conditions and after microinjections of antipeptide antibodies 363–382. (*A*) Control values of k in cells injected with PBS prior to photobleaching. (*B*) Pooled control values of k measured 10 min after microinjections of either Bovine Serum Albumin or human γ-globulins into the tested cell. (*C*) A partial ($k < 0.1$ min^{-1}) or complete ($k = 0$) diffusional uncoupling is observed with antibodies 363–382 in about 50% of the trials.

The functioning of the gap junction channels might also become impaired by alterations in the conformation of the connexins, which could restrict the opening or prevent the gating mechanism from operating, for instance by interfering with regulatory domains that control the opening probability of the channels. Cx43 is phosphorylated in the neonatal rat heart (Laird et al., 1991; Lau et al., 1991), and protein kinase activators have been shown to modify the gap junctional conductance in several cell types (*see* Sáez et al., 1993). Examination of the primary sequence of Cx43 (Beyer et al., 1987) shows that the COOH-terminal sequence 363–382, recognized by the antibodies that block the cell-to-cell dye transfer, comprises three pairs of serine bracketed by arginine residues, each one being a typical motif frequently found in substrates phosphorylated by protein kinase C and by cAMP-dependent protein kinases (Kennelly & Krebs, 1991). A regulatory role of the COOH-terminus of Cx43 is furthermore suggested by recent experiments showing that a synthetic peptide, corresponding to sequence 360–375, is phosphorylated by PKC on the serine residues 368 and 372 (Sáez et al., 1993). Comparison with sequences 5–17 and 314–322, which do not contain consensus sequences for kinases, allows the tentative suggestion that the efficiency of antibodies 364–382 is related to their interference with a regulatory domain of Cx43.

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