Phosphorylation of serine 262 in the gap junction protein connexin-43 regulates DNA synthesis in cell-cell contact forming cardiomyocytes

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Summary

Mitogenic stimulation of cardiomyocytes is associated with decreased gap junction coupling and protein kinase C (PKC)-mediated phosphorylation of the gap junction protein connexin43 (Cx43). Identification of and interference with the amino acid(s) that becomes phosphorylated in response to stimulation are important steps towards defining the relationship between Cx43 phosphorylation and cell cycle. Using immunoblotting and phosphospecific antibodies we were able to show that serine-262 (S262) on Cx43 becomes phosphorylated in response to growth factor or PKC stimulation of cardiomyocytes. To examine the effect of Cx43, S262 phosphorylation and cell-cell contact (and/or coupling) on DNA synthesis, we overexpressed wild-type (wt) or mutant Cx43, carrying a S262-to-alanine (S262A, simulating the unphosphorylated state) or a S262-to-aspartate (S262D, simulating constitutive phosphorylation) substitutions in cultures of cell-cell contact forming or isolated cardiomyocytes. Overexpression of wt-Cx43 caused a significant decrease in DNA synthesis irrespective of the presence of cell-cell contact. In cell-cell contact forming cultures, the S262D mutation reversed while the S262A mutation increased the inhibitory effect of Cx43. In the absence of cell-cell contact, the S262-Cx43 mutations had no significant effect on Cx43 inhibition of DNA synthesis. Dye-coupling, evaluated by scrape-loading, indicated increased gap junction permeability in S262A (compared to wt or S262D) overexpressing myocytes. We conclude that Cx43 inhibits cardiomyocyte DNA synthesis irrespectively of cell-cell contact or coupling. Cell-cell contact, and possibly gap junction-mediated communication is required, however, in order to reverse Cx43 inhibition of DNA synthesis by S262 phosphorylation.

Key words: Connexin43, Phosphorylation mutants, DNA synthesis, Cell-cell contact, Cardiomyocytes

Introduction

Connexins form plasma membrane intercellular communication channels termed gap junctions (GJs) (Goodenough et al., 1996). GJs mediate electrical and metabolic coupling and ensure coordinated activity in organs such as the heart. In addition, extensive studies conducted mostly on neoplastic cells have pointed to a tumour suppressor role for connexins (Yamasaki et al., 1999). Mitogens, tumour promoters and activated oncogenes generally decrease connexin expression and GJ-mediated communication. (Yamasaki et al., 1999). Overexpression of connexins such as connexin43 (Cx43), a widely expressed connexin isoform, in cancer cell lines, has been found to reduce growth and oncogenicity to varying degrees (King et al., 2000; Mehta et al., 1991; Mesnil et al., 1995; Zhu et al., 1991).

Cx43 is the main constituent of the GJ in working cardiomyocytes, and has been the subject of numerous investigations in the context of adult cardiac function and maintenance of proper rhythm (Dhein, 1998; Kanno and Saffitz, 2001). It has never been examined in the context of cardiomyocyte hyperplastic growth. Our previous studies provided evidence for a link between mitogenic stimulation of neonatal cardiomyocytes and effects on Cx43. Fibroblast growth factor 2, FGF2, stimulates cardiomyocyte proliferation (Kardami, 1990; Pasumarthi et al., 1996) as well as protein kinase C (PKC; ε subtype)-dependent additional serine phosphorylation of Cx43 (Doble et al., 1996; Doble et al., 2000b). In contrast, transforming growth factor β, a factor that cancels out the mitogenic effect of FGF2 on cardiomyocytes (Kardami, 1990), prevented the FGF2-induced Cx43 phosphorylation (Kardami, 1998). These data suggested that the phosphorylation status and pattern of Cx43 and/or coupling between cells, may be important in cardiomyocyte cell cycle regulation.

Cx43 exists in a phosphorylated state in both neonatal and adult cardiomyocytes (Beardslee et al., 2000; Laird et al., 1991). Phosphorylation occurs at several different sites on the molecule and regulates trafficking, assembly, turnover and

coupling (Lampe and Lau, 2000). Our previous studies suggested that mitotic stimulation may result in a PKC-mediated phosphorylation of Cx43 on S262 (Doble et al., 1996; Doble et al., 2000a). Whether Cx43 phosphorylation at any one site, including S262, can affect its ability to inhibit DNA synthesis is not known. In fact, the mechanism of cell cycle (DNA synthesis) inhibition by Cx43, and the role of intercellular communication is not well understood. It has been proposed that GJ-mediated intercellular communication is an integral part of the mechanism of inhibition, by allowing passage of growth affecting signals between cells (Goldberg et al., 2000; Loewenstein and Rose, 1992). There is, however, increasing evidence of channel-independent growth inhibition by Cx43 (Dang et al., 2003; Krutovskikh et al., 2000; Moorby and Patel, 2001; Moorby, 2000).

We present evidence that Cx43 can inhibit DNA synthesis also in primary cardiomyocytes. Furthermore, we have addressed several elements of the mechanism of Cx43-inhibition. These include the role of cell-cell contact and/or coupling, and the role of phosphorylation at a specific serine residue, S262.

Materials and Methods

Primary cell cultures

Neonatal rat cardiomyocytes were prepared from 1-day-old Sprague Dawley rat pups (obtained from the Central Animal Care Facility at the University of Manitoba) as previously described (Doble et al., 1996; Doble et al., 2000b). Our procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the local Animal Care Committee of the National Research Council of Canada.

For most experiments, myocytes were plated at 4×10^5 cells/35 mm collagen-coated dish, in 10% foetal bovine serum (FBS) in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 ng/ml FGF2 (conditions of maximal mitogenic stimulation). Under these conditions cells were about 60% confluent 1 day after plating. In some experiments, myocytes were seeded at low densities, at 5×10^4 cells/35 mm well. For scrape-loading, cardiomyocytes were plated at high density (9×10^6/35 mm dish) and maintained as described previously (Doble et al., 1996; Doble et al., 2000b).

Antibodies

Polyclonal anti-Cx43 antibodies, raised against residues 367-382, have been described previously (Doble et al., 2000b). Monoclonal anti-Cx43 antibodies (against residues 252-270) were purchased from Transduction Laboratories; their specificity for Cx43 was ascertained in previous studies (Doble et al., 1996). Rabbit polyclonal antibodies for phosphorylated (serine 262; P-262) Cx43 and anti-N-cadherin antibodies were purchased from Santa Cruz Biotechnology. Monoclonal anti-bromodeoxyuridine (BrdU), anti-rabbit immunoglobulin (biotinylated species-specific whole antibody), streptavidin-fluorescein and mouse immunoglobulin (whole antibody linked to Texas Red) were from Amersham Polysciences. Rabbit polyclonal anti-(rodent) Ki-67 antibodies were from Novocastra (UK).

Connexin43 mutagenesis

The plasmid pBSM13-Cx43 (rat origin; kindly provided by Dr E. Beyer) (Beyer et al., 1987) was used as the template for PCR-mediated site-directed mutagenesis. The primers: 5′-CGATCCTTACC-ACGCCACCACTGGCCCACTGAGCCCATCAAAAGACTGCGG-AgCTCCAAAATAC-3′ (S262A sense primer) and 5′-CGATCC-TTACCACGCCACCACTGGCCCACTGAGCCCATCAAAAGACT-GCGGAgaTCCAAAATAC-3′ (S262D sense primer) were used in combination with the primer 5′-CCATGCGATTTTGCTCTGCGC-TGTAG-3′ (antisense primer) to generate two 229 bp fragments, which were mutated in position 262 from either serine (S) to alanine (A) or from S to aspartate (D) in the connexin43 cDNA, respectively. The primers: 5′-GTTTTGCTCGCTAGCTTGCTTGTTGTAATTGC GGCACGcGGAATTGTTTCTG-3′ (S297A antisense primer) and 5′-CAGGCCGAGGCCTGCTGCTGGCGCGGCTGCTGGCTCTGCT-GGcAGGTCGTTGG-3′ (S364A antisense primer) were used in combination with the primer 5′-CGTTAAGGATCGCGTGAA-GGGAAGAAGC-3′ (sense primer) to generate either (i) a 222 bp fragment with S297 converted to A; or (ii) a 427 bp fragment with S364 converted to A; in the Cx43 cDNA, respectively. The PCR reactions were carried out as previously described (Jin et al., 1994). The PCR products were digested with *Xcm*I/*Nhe*I for the S262A, S262D and S297A mutants or with *Xcm*I/*Stu*I for the S364A mutant. The digested PCR products were then subcloned into the *Xcm*I/*Nhe*I or *Xcm*I/*Stu*I site of pBSM13 Cx43 plasmid to generate the pBSM13-Cx43/S262A, pBSM13-Cx43/S262D, pBSM13-Cx43/S297A and pBSM13-Cx43/S364A mutant constructs. Sequences were checked by the dideoxy method using the fmfol DNA Cycle Sequencing System Kit (Promega Corp., Madison, WI). The full length wild-type (wt) and mutated Cx43 fragments (1391 bp) were released by *Eco*RV/*Xba*I digestion and ligated into the *Eco*RV/*Xba*I site of the mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad,CA) for use in infection experiments.

Western blotting

Total cell protein was obtained using a lysis buffer composed of 1% SDS in 50 mM Tris-HCl pH 8.0. Subcellular fractionation to cytosolic, Triton-soluble and Triton-insoluble fractions were done as described by others (Musil and Goodenough, 1991). Protein content was determined using the BCA assay (Pierce). Samples were prepared in Laemmli sample buffer (Laemmli, 1970) and analyzed by SDS-PAGE (10% polyacrylamide gels) and western blotting as previously described (Doble et al., 2000b).

Adenoviral vectors

We have used replication-deficient adenovirus as a vehicle for highly efficient gene delivery (over 90%) in cardiomyocytes (Doble et al., 2000b; Kirshenbaum, 1997; Kirshenbaum et al., 1993). Recombinant adenoviral vectors carrying the 262A-, 262D- or wt-Cx43 genes under the control of the CMV promoter were constructed as described previously (Ping et al., 1999). Recombinant viruses were prepared as high-titer stocks through propagation in HEK 293 cells. Infection with Ad.CMV.β-gal was used in all experiments to control for non-connexin-related effects of viral infection.

Transient gene transfer

Adenovirally mediated gene transfer was achieved by adding the required viral construct (10 m.o.i.) to the myocyte cultures, one day after plating. For all experiments aimed at examining effects on DNA synthesis, myocytes were maintained in the presence of 10% FBS supplemented with 10 ng/ml FGF2 for up to 3 days. Non-myocyte contamination did not exceed 5% during this time. For 'scrape-loading', myocytes were infected with adenoviral vectors one day before the experiment.

Immunofluorescent labelling

Immunofluorescent labelling and nuclear staining with Hoechst 33342 was carried out as previously described (Doble et al., 1996; Doble et al., 2000b). Briefly, cells, grown on collagen-coated glass coverslips, were fixed with 4% paraformaldehyde in phosphate-buffered saline

(PBS) and permeabilized with 0.1% Triton X-100 in PBS. Our own rabbit polyclonal Cx43 antibody was used at a dilution of 1:5,000; the anti-BrdU monoclonal antibody was used undiluted. All other antibodies were used as per manufacturer's instructions. In all experiments, non-specific fluorescent staining was examined by incubating cells in the presence of equivalent concentrations of non-immune rabbit or mouse sera.

Determination of BrdU labelling index

Myocytes were allowed to express transiently introduced genes for up to 48 hours. They were incubated with 15 μg/ml BrdU (Sigma) for 6 hours. Subsequently, cells were fixed and processed for multiple fluorescence labelling. In most experiments, the identity of myocytes was confirmed by the characteristic striated staining of alpha-actinin or striated myosin in the cytosol, as we have shown previously (Pasumarthi et al., 1996). Staining of N-cadherin at intercellular contacts also served to identify myocytes. A minimum of eight random fields in each of at least three coverslips per group were assessed. Labelling index (LI) was determined by dividing the number of BrdU-positive myocyte nuclei by the total number of myocyte nuclei (Hoechst positive), per field. Labelling index for Ki-67 was obtained in a similar fashion.

Scrape loading

Confluent rat cardiomyocyte cultures were used and dye transfer assessed exactly as described previously (Doble et al., 1996; Doble et al., 2000b). Cells were scrape-loaded with 6-carboxyfluorescein and fixed 2 minutes later. Spread of fluorescence dye was measured at several points at the centre of the scrape line. Under these conditions, fluorescent dextran (10,000 Da) does not spread past the scrape line (Doble et al., 1996), indicating that the movement of carboxyfluorescein is achieved via gap junctions.

Statistics

Comparisons were made between cultures infected with vector (controls) and those infected with Cx43 (wt, S262A, or S262D) using the student-Newman-Keuls multiple comparisons post-hoc test, following analysis of variance (InStat, GraphPad Software, San Diego, CA).

Results

State of S262 phosphorylation in cardiomyocytes

Myocytes, maintained in low serum (0.5%) for 2 days in order to reduce baseline PKC activity, were stimulated with FGF2 or phorbol myristate acetate (PMA). Total cell lysates were then analyzed by western blotting, using either the rabbit polyclonal antibody that recognizes all Cx43 species irrespectively of phosphorylation (Fig. 1A), or a polyclonal antibody specific for those Cx43 species that are phosphorylated on S262 (Fig. 1B). Results are shown in Fig. 1. As expected, the first antibody detects Cx43 bands migrating with an apparent molecular mass of larger or equal to 45 kDa. This is typical of cardiomyocyte Cx43 (Doble et al., 1996; Doble et al., 2000a; Doble et al., 2001). Similar amounts of total Cx43 were present in all lanes (Fig. 1A), confirming that our gene transfer treatments did not alter Cx43 abundance. The anti-P-S262 antibody did not recognize the Cx43 bands before stimulation (Fig. 1B). Since the ~45 kDa Cx43 bands represent phosphorylated Cx43 (the unphosphorylated form migrates at 41 kDa) (Laird et al., 1991) our data indicate that sites other than S262 must be phosphorylated at this stage. FGF2 stimulation caused the

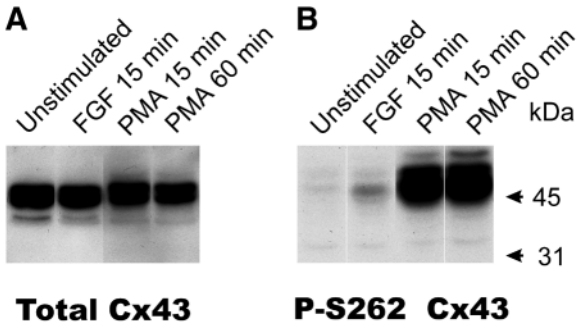

Fig. 1. Effect of FGF2 and PMA on Cx43 phosphorylation on S262. Western blots of total lysates from cardiomyocyte cultures (20 μg/lane) probed with antibodies recognizing (A) total, or (B) P-262-Cx43, as indicated. Cultures were stimulated with FGF2 (10 ng/ml) or PMA (100 nM) for 15-60 minutes, as indicated.

recognition by anti-P-S262 antibodies of band(s) at 46-47 kDa indicating additional, over 'baseline' phosphorylation of Cx43. PMA stimulation causes recognition of several 44-50 kDa bands and more prolonged incubation by this agent increased levels of the slowest migrating (presumably 'hyper'-phosphorylated) band (Fig. 1B). Phosphorylation at additional sites would account for the multiple bands recognized by anti-P-S262.

Expression and localization of wt-, 262A- and 262D-Cx43 in rat cardiomyocytes

Expression of the introduced Cx43 genes (wt-, 262A-, 262D-Cx43) following adenoviral gene delivery was evaluated by western blotting and immunolocalization. All cells treated with vectors expressing Cx43 and its mutants, as well as control myocytes produced immunoreactive Cx43 bands migrating mostly at 44-47 kDa. The amount of Cx43 present in 1 μg lysate from cultures treated with vectors expressing Cx43 or its mutants was comparable to endogenous Cx43 present in a 10-fold amount of lysate from vector-infected cells (Fig. 2A).

Fig. 2B-D shows Cx43 localization in control and overexpressing cultures. Cx43 was visualized by indirect immunofluorescence, using two different antibodies to Cx43. The polyclonal antibody ('green') detects Cx43 mainly at cell-cell contact areas, while the monoclonal antibody ('red') raised against a different region of the molecule, detects Cx43 at sites of cell-cell contact as well as synthesis and trafficking around the nucleus. Since both antibodies are specific for Cx43, lack of perinuclear staining by the polyclonal antibody is likely the result of epitope masking. Epitope masking of anti-Cx43 antibodies has been reported in other studies (Doble et al., 1996; Ochalski et al., 1995). Together the two antibodies provide a more comprehensive picture of Cx43 abundance compared to any one antibody used on its own. As expected, overall intensity of anti-Cx43 staining with both antibody preparations (resulting in yellow colour) was very strong at cell-cell contact areas in overexpressing cultures; intensity of anti-Cx43 staining was weak in vector-infected cultures (Fig. 2B). No differences in the pattern of subcellular Cx43 distribution between wt-, 262A- or 262D-Cx43 overexpressing cultures were evident. Identical results were obtained when wt and mutant connexins were transiently expressed in non

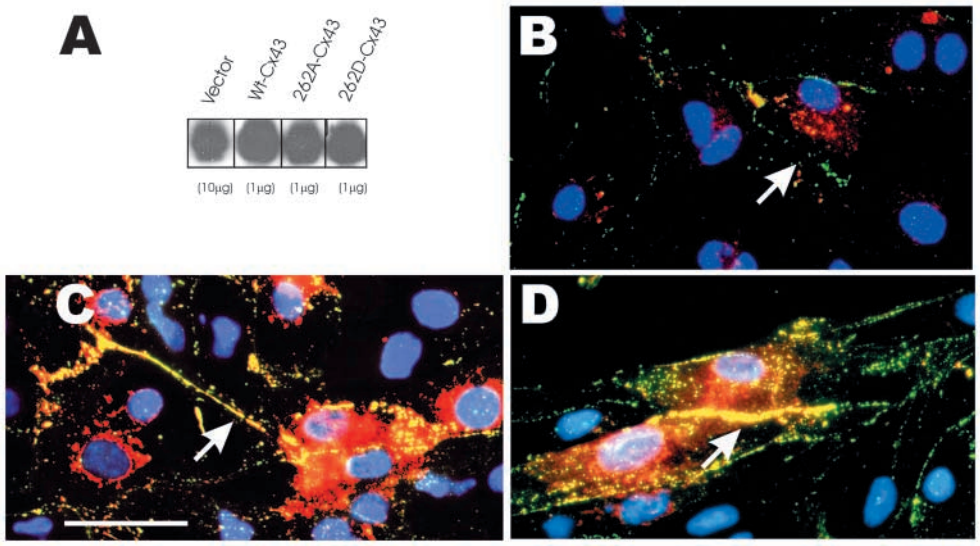

Fig. 2. Detection of overexpressed wt, 262A- and 262D-Cx43. (A) Western blot of lysates from vector-infected (10 μg/lane) or Cx43 (wt and mutant) overexpressing cultures (1 μg/lane; as indicated) probed with rabbit polyclonal anti-Cx43 antibodies. (B-D) Triple-fluorescence labelling, using rabbit polyclonal anti-Cx43 antibodies (green), mouse monoclonal anti-Cx43 antibodies (red) and Hoechst 33342 (blue). Myocyte cultures were infected with (B) vector, (C) 262A-Cx43 or (D) 262D-Cx43. Scale bar: 50 μm. Arrows point to areas of cell-cell contact.

cardiac cell lines expressing undetectable levels of endogenous Cx43, such as HEK293 cells (unpublished observations). We also compared the solubility properties of the overexpressed versus endogenous connexins. We have observed previously that unlike its properties in other cell types (Musil and Goodenough, 1991) Cx43 from non-ischemic cardiac myocytes shows very little solubility in 0.5-1% Triton-X-100, and this was true for both unphosphorylated and phosphorylated Cx43 (unpublished observations) (Doble et al., 1996). Cytosolic, Triton-X-100-soluble and Triton-X-100-insoluble fractions from control or overexpressing myocyte cultures were analyzed for Cx43 by western blotting. In all cases, Cx43 immunoreactivity was detectable only in the Triton-insoluble fraction, confirming that all overexpressed connexins retained similar aggregation properties as endogenous cardiac Cx43 (unpublished observations).

Effect of Cx43 and S262 on DNA synthesis in the presence or absence of cell-cell contact

To achieve cell-cell contact and at the same time avoid overcrowding, cardiomyocytes were plated at semi-confluent densities. Under these conditions virtually all myocytes were present in clusters, making contact with at least one more myocyte (Figs 3 and 4). Cells were maintained under maximal mitogen stimulation to facilitate detection and comparison of 'treatments' that would inhibit stimulation. Gene delivery by non-replicating adenoviral vectors were used to overexpress wt and mutant Cx43 species, achieving over 90% efficiency of gene transfer (verified in each experiment by anti-Cx43 immunostaining). Incorporation of BrdU was used to detect DNA synthesis in nuclei (Fig. 3). In parallel experiments nuclear labelling for Ki-67 was also used as a marker of cell proliferation (Fig. 4). Ki-67 detects cells in G1, S, G2 and early mitosis, but not in G0 (Brown and Gatter, 2002). These approaches gave similar results (Figs 3 and 4). Fig. 5A shows quantitative results on the effect of wt and mutant Cx43 overexpression on myocyte BrdU LI (fraction of BrdU-positive myocyte nuclei): expression of wt-Cx43 decreased the BrdU LI significantly, by 60% (P<0.001 compared to vector-infected

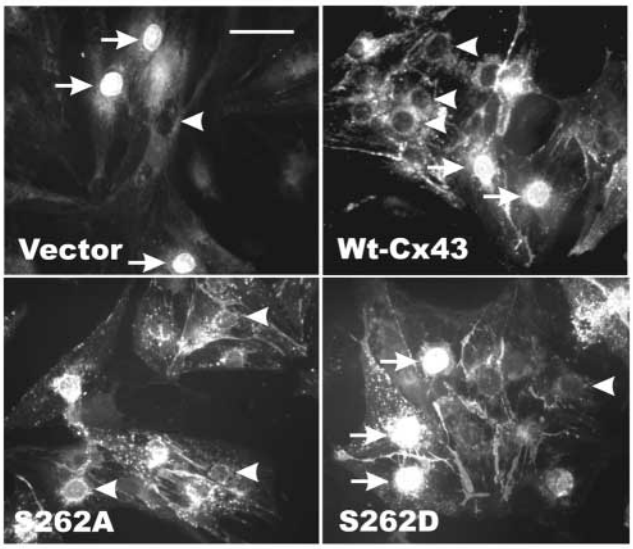

Fig. 3. Effect of wt or mutant Cx43 overexpression on cardiomyocyte anti-BrdU labelling. Myocyte cultures, infected with adenoviral vectors expressing wt-, 262A- and 262D-Cx43 (as indicated) were labelled simultaneously for BrdU (nucleus) and Cx43 (cell membrane), using monoclonal antibodies specific for each antigen. One colour is sufficient to detect both antigens, since they are found in different cellular locations. Arrows and arrowheads point to BrdU-positive and negative nuclei, respectively. Scale bar: 50 μm.

cells). Expression of 262A-Cx43 resulted in significantly more potent inhibition, by 84% (P<0.001 compared to vector-infected, and P<0.01 compared to the wt-Cx43 infected cells). Expression of 262D-Cx43 had no inhibitory effect at all (Fig. 5A) and was indistinguishable from vector-infected cells. The degree of Cx43 overexpression was similar between cultures treated with vectors expressing Cx43 or its mutants, and within each experimental series (Figs 2-4). The same pattern of response was obtained in three separate experiments, and when calcium-phosphate-mediated transient gene transfer was employed (Doble et al., 2000b).

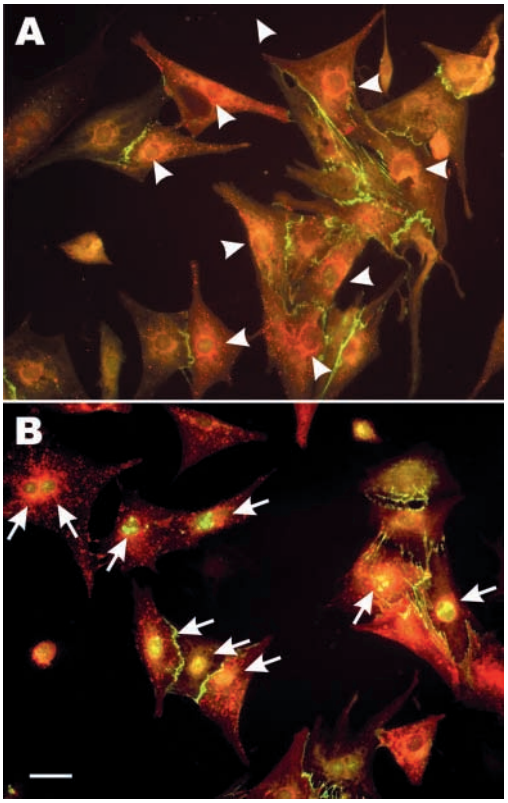

Fig. 4. Effect of 262A- and 262D-Cx43 overexpression on cardiomyocyte anti-Ki-67 labelling. Myocyte cultures infected with adenoviral vectors expressing (A) 262A-Cx43 or (B) 262D-Cx43 were labelled for N-cadherin (green; rabbit polyclonal antibodies to delineate cell-cell contact cardiomyocyte areas), Ki-67 (green; rabbit polyclonal to detect nuclei of proliferating cells; elicits speckled nuclear staining) and Cx43 (red; monoclonal antibodies). Arrowheads, non-proliferating myocytes; arrows, proliferating myocytes. Scale bar: 50 μm.

To examine whether Cx43 and its mutants had an effect on proliferation of non-interconnected cardiomyocytes (i.e. in the absence of GJ channels and cell-cell communication) we used sparsely seeded cultures. These cultures contained mostly isolated myocytes, although clusters of 2-4 myocytes were also occasionally present. The latter were excluded from our determinations. Results are summarized in Fig. 5B. Overexpression of wt, or either of the S262-Cx43 mutants caused a significant (P<0.01) drop in LI compared to vector-infected cultures. In this case however, there were no significant differences in LI between wt and either of the two mutated connexin-expressing cells. The same pattern of response was obtained in two separate experiments. Both BrdU and Ki-67 LI were obtained, giving qualitatively similar results.

Effect of S262 on dye transfer between cardiomyocytes

Cell-cell contact allows formation of GJ channels and cell-cell communication. The effect of S262 mutations on Cx43 inhibition of DNA synthesis may be mediated by changes in GJ permeability. A first step towards clarifying this would be to establish whether the S262 mutations alter GJ-mediated dye transfer between cells. Thus we used the scrape loading approach to compare the effects of overexpressing wt and mutant Cx43 on dye spread between cardiomyocytes. Results are summarized in Fig. 6. Dye spreading was not significantly different between vector, wt- and 262D-Cx43-infected cells. Dye spreading was significantly increased (P<0.05) in cultures overexpressing 262A-Cx43 compared to all other groups. Fig. 6 shows a representative image of fluorescent dye migration in myocyte cultures expressing 262A- or 262D-Cx43 (Fig. 6A and B, respectively). The same pattern of response was obtained in three different experiments.

Stimulation with the phorbol ester PMA (and the ensuing Cx43 phosphorylation on multiple sites) is well known to diminish dye coupling between cells (Kwak and Jongsma, 1996). To test whether S262 is solely responsible for mediating the effects of PMA, dye transfer was assessed after stimulating all myocyte cultures with this compound for 15 minutes. Under these conditions, dye spreading became minimal and similar in all groups. Fig. 6C summarizes the effect of PMA on dye spreading in cultures overexpressing 262A- or 262D-Cx43.

Discussion

We have found that Cx43 can inhibit DNA synthesis in cultured cardiomyocytes, and that inhibition does not depend on cell-cell contact or intercellular communication. We have also

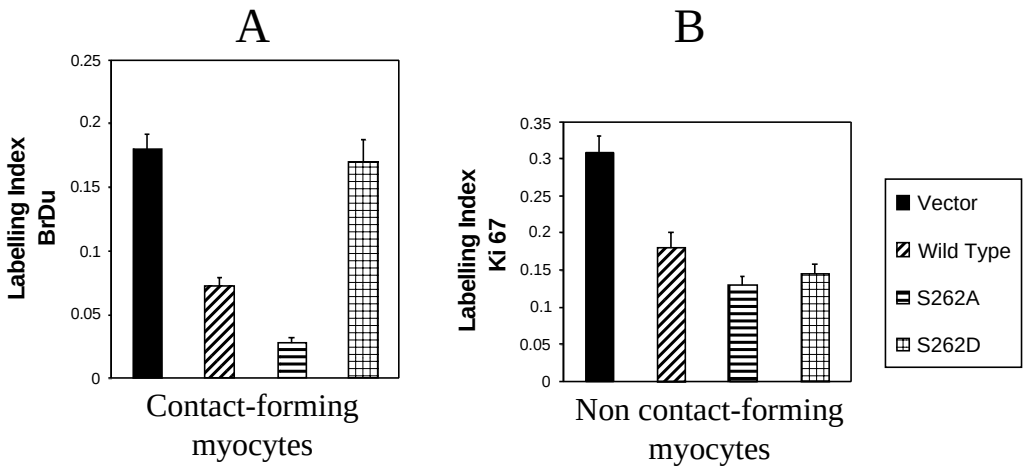

Fig. 5. Effect of wt-, 262A- and 262D-Cx43 on myocyte DNA synthesis. (A) BrdU labelling index (LI) in semi-confluent myocyte cultures infected with adenoviral vectors expressing wt-, 262A- and 262D-Cx43, as indicated (n=4-6). (B) Ki-67 LI in sparse cultures infected with adenoviral vectors expressing wt-, 262A- and 262D-Cx43, as indicated (n=4). Bars indicate s.e.m.

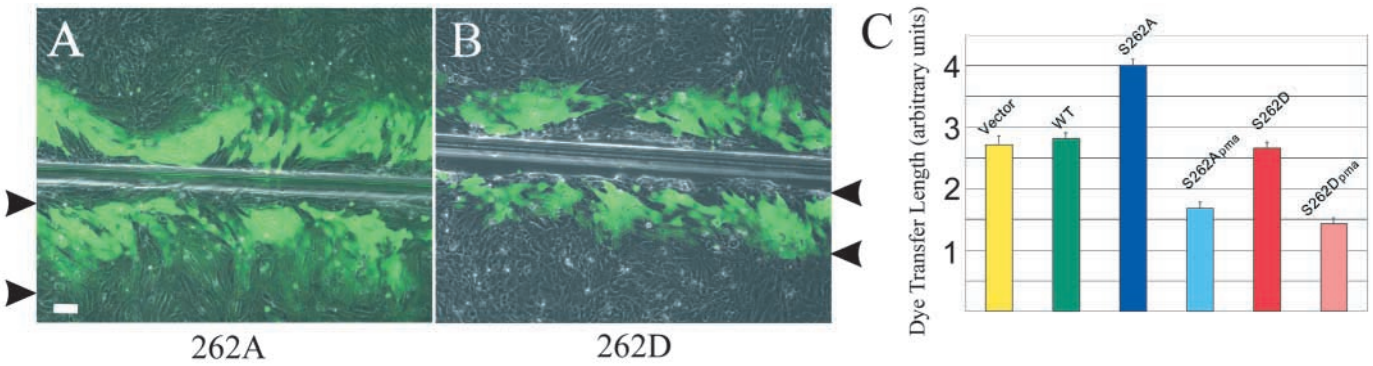

Fig. 6. Effect of Cx43 and S262 mutations on dye coupling. (A,B) Representative micrographs of dye migration (green), assessed by scrape-loading, in confluent cultures (seen by simultaneous phase contrast photography) expressing (A) 262A- or (B) 262D-Cx43. Arrowheads indicate length of dye spreading. In C dye spreading is plotted as a function of overexpressing wt-, 262A- (+/–PMA) and 262D-Cx43 (+/–PMA), compared to vector-infected cultures, as indicated (*n*=4). Bars indicate s.e.m.

found that cell-cell contact changes the mechanism of Cx43-mediated inhibition, rendering it dependent on the state of Cx43 phosphorylation on S262. Phosphorylation at S262, a PKC site that becomes phosphorylated in the cell environment in response to growth factor stimulation, cancels Cx43 inhibition only in contact-forming myocytes. Finally, S262 affected GJ-mediated dye transfer, suggesting that channel-permeable signals may be part of the mechanism by which S262 affects Cx43-mediated inhibition in contact-forming myocytes.

A role for Cx43 in cardiomyocyte DNA synthesis

The ability of cardiac myocytes to synthesise DNA and divide decreases after birth. This correlates with presence of multiple, well-established cell-cell contacts and Cx43-composed GJ at the intercalated discs between working cardiomyocytes. Cx43 is capable of inhibiting proliferation of neoplastic cells (Yamasaki et al., 1999), preventing the transition to the S-phase (Zhang et al., 2001). We show for the first time that primary neonatal cardiomyocytes are susceptible to Cx43-mediated inhibition of DNA synthesis, since overexpression of wt Cx43 reduced the fraction of myocytes incorporating BrdU or staining positive for Ki-67. Assuming that our data from cultured myocytes provide a good simulation of the situation in vivo, a channel protein, Cx43, in addition to ensuring electrical and metabolic coupling, may also be regarded as a potential suppressor of cardiomyocyte proliferation, at least in the embryonic and early post-natal stages.

Mechanism of growth inhibition by Cx43

An ongoing debate regarding the mechanism of growth inhibition by Cx43 is whether it requires the presence of active GJ channels and transmission of GJ-permeable signals. Several groups have suggested that inhibitory factors diffusing through the GJ channels mediate growth inhibition (Esinduy et al., 1995; Mehta et al., 1986; Mehta et al., 1991; Zhang et al., 2001). Others have reported that Cx43 can inhibit growth in a manner independent of channel permeability and/or subcellular localization (Krutovskikh et al., 2000; Moorby and Patel, 2001). In fact, the non-channel forming, C-terminal ‘tail’ of Cx43 retains growth inhibitory activity (Dang et al., 2003; Moorby and Patel, 2001). In our system, inhibition was detected irrespective of cell-cell contact (Fig. 5), supporting the notion that Cx43-mediated inhibition of cardiomyocyte DNA synthesis does not require formation of GJ channels. A possible mechanism may involve interactions of Cx43 with other proteins and/or protein complexes that can regulate the cell cycle. Proteins such as ZO-1, εPKC, Src or β-catenin, all of which have been implicated in regulation of cell proliferation, are known to interact with Cx43 (Ai et al., 2000; Doble et al., 2000b; Giepmans et al., 2001; Lin et al., 2001). It is also theoretically possible that Cx43 may affect gene expression directly, acting as a transcription factor, or indirectly by interacting with transcription affecting protein(s) or complexes. The C-terminal tail of Cx43 can localize to the nucleus (Dang et al., 2003) where it may have effects on gene expression.

The role of S262 phosphorylation and cell-cell contact on Cx43 inhibition of DNA synthesis

While Cx43 and FGF2 have opposing effects on cardiomyocyte DNA synthesis they are ‘linked’ by a regulatable event, Cx43 phosphorylation in response to FGF2 stimulation. We therefore asked the question whether the mitogen-induced and PKC-mediated phosphorylation of Cx43 (Doble et al., 1996; Doble et al., 2000b) were actually required for stimulation of DNA synthesis. To address this it was important to identify the particular serine(s) becoming phosphorylated on Cx43 in response to stimulation, and then create a model to examine functional consequences (inhibition of DNA synthesis, dye coupling) of selectively interfering with the phosphorylation status of that serine.

Our previous work pointed to S262 as a site that becomes phosphorylated in response to growth factor stimulation, in a PKC-dependent manner (Doble et al., 1996; Doble et al., 2000a). The recent availability of anti-P-S262-Cx43 antibodies enabled us to demonstrate directly that indeed FGF2, and PMA, stimulate phosphorylation of Cx43 on S262. The antibodies detected Cx43 only after PKC stimulation and the apparent size of immunoreactive bands (45-49 kDa) further corroborated their phosphorylated status. Since S262

phosphorylation was only detected after stimulation of an already phosphorylated protein, and was associated with only the slower migrating bands, it is possible that phosphorylation at other sites is necessary before S262 can become phosphorylated. PMA resulted in more extensively phosphorylated Cx43 (i.e. presenting slower electrophoretic mobility) compared to FGF2, reflecting, presumably, phosphorylation at sites in addition to S262. Other PKC sites that may become phosphorylated in cardiomyocytes include S364 (Britz-Cunningham et al., 1995), S372 and S368 (Saez et al., 1997).

We then created single mutations on Cx43 designed to simulate the charge effects incurred by lack of (S-to-A) or constitutive (S-to-D) phosphorylation on serine 262, and examined whether these mutants, overexpressed in proliferative myocyte cultures by adenoviral gene delivery, would affect cardiomyocyte DNA synthesis. Overexpression of wt-Cx43 provided an additional 'baseline' for comparison, in addition to non-overexpressing, vector-only-treated myocytes. Interestingly, we found that our results were dependent on whether cardiomyocytes were 'isolated' or in contact with each other: in the presence of cell-cell contact, prevention of phosphorylation (S262A) resulted in maximal inhibition of DNA synthesis (by 84%, compared to vector-infected cultures). Simulated constitutive phosphorylation at the same site (S262D) completely cancelled Cx43 inhibition. In the absence of cell contact, however, S262 did not affect Cx43-mediated inhibition of DNA synthesis. Overexpression per se was not responsible for the effects on DNA synthesis, since the same level of overexpression resulted in pronounced (S262A), intermediate (wt) and no (S262D) inhibition in contact-forming myocytes. A single amino acid therefore can cancel or potentiate growth inhibition by Cx43 depending on whether it is phosphorylated or not. We speculate that Cx43 phosphorylation on S262 may be a component of the mechanism by which mitogens promote DNA synthesis and proliferation in myocytes remaining in contact with each other, during embryonic and early post-natal stages. The effects of S262 mutations were not specific for cardiomyocytes. Transient expression of the wt and S262 mutants in cells with undetectable levels of endogenous Cx43 (HEK293) elicited identical effects on DNA synthesis (unpublished observations).

Cell-cell contact would allow GJ-mediated intercellular communication. Since S262 can affect gap junction dye permeability as presented here, one must consider the possibility that the mechanism by which S262 phosphorylation (or lack thereof) affects DNA synthesis includes as yet unknown GJ permeable signals. The absence of S262 regulation in the non-coupled myocytes offers support to this notion. This, however, remains to be demonstrated. Another possibility is that cell-cell contact promotes phenotypic changes that affect the mechanism by which Cx43 exerts inhibition. For example the protein(s) interacting with Cx43 may be different depending on cell contact, and S262 may only interfere with these interactions in contact-forming cells.

S262 affects GJ permeability to dyes

Overexpression of S262A-Cx43 enhanced dye transfer between myocytes, indicating that S262 affects GJ permeability. Increased coupling by S262A was not a result of overexpression per se since it was not observed in cultures overexpressing wt- or S262D-Cx43. The question arises as to whether our findings reflect permeability properties of channels composed mainly of the introduced S262A-Cx43 or an indirect effect of the introduced connexins on channels composed of endogenous Cx43. The first possibility appears more likely since all connexin constructs introduced by gene transfer retain intact connexin-forming domains, were expressed at an over 10-fold excess over the endogenous protein, and displayed a localization pattern identical to endogenous Cx43. In either case, however, the S262A mutation resulted in more permeable GJ between cardiomyocytes.

The S262 site was not sufficient to account for the effects of PMA on dye coupling. If it was, the S262A- or S262D-expressing cultures would not display decreased coupling in response to PMA (Fig. 6). Since PMA stimulates phosphorylation of additional sites on Cx43 (Lampe and Lau, 2000; Saez et al., 1997) (Fig. 1), these sites probably contribute to decreased channel permeability and mask the effects of the S262A mutation. One such site is serine 368 that accounts to a large degree for the PMA-induced effects on Cx43-mediated dye transfer in mouse fibroblasts (Lampe and Lau, 2000).

In conclusion

We have shown that overexpression of Cx43 inhibits DNA synthesis of neonatal cardiomyocytes in primary culture irrespectively of cell-cell contact and thus GJ-mediated communication. For the first time we provide evidence that regulation of inhibition by Cx43 differs depending on cell-cell contact and possibly GJ-mediated communication. In the absence of cell-cell contact inhibition is independent of S262, while in the presence of cell-cell contact it becomes dependent on the phosphorylation status of S262. What follows is the novel concept that cardiomyocytes need actually to be in contact with each other for mitogens to overcome Cx43-mediated inhibition of DNA synthesis by stimulating S262 phosphorylation. It remains to be determined whether our findings are applicable to cardiomyocytes in vivo.

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