CARDIAC GAP JUNCTIONS:
A TARGET OF GROWTH FACTOR SIGNALING

By

Bradley Wayne Doble

A thesis submitted to the Faculty of Graduate Studies
of the University of Manitoba in partial fulfillment of the
requirements for the degree of:

DOCTOR OF PHILOSOPHY

Department of Physiology
Faculty of Medicine

Cardiac Gap Junctions: A Target of Growth Factor Signaling

BY

Bradley Wayne Doble

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

Doctor of Philosophy

TABLE OF CONTENTS

ABSTRACT

Introduction: Gap junctions, specialized membrane structures containing numerous intercellular channels composed of connexins, permit interchange of molecules less than 1 kDa between coupled cells. In addition to ensuring electrical coupling, gap junctions regulate growth and differentiation. An inverse correlation exists between cell proliferation and gap junctional intercellular communication in many experimental systems. Fibroblast growth factor-2 (FGF-2) is a multifunctional mitogen stimulating cardiomyocyte proliferation and hypertrophy; its local levels increase upon injury. We sought to: (a) examine the effects of FGF-2 on cardiomyocyte gap junctions composed of connexin 43 (Cx43); (b) identify the kinase(s) responsible for mitogen induced Cx43 phosphorylation; (c) establish a cause and effect relationship between Cx43 phosphorylation and cardiomyocyte proliferation.

Methods: We used well coupled, primary neonatal rat cardiomyocytes as our model system. Cx43 protein and mRNA levels were determined by Western and Northern blotting, respectively. Labeling cells with [^{32}P], followed by Cx43 immunoprecipitation, gel electrophoresis and autoradiography was used to assess Cx43 phosphorylation. The identity of phosphorylated Cx43 residues was examined by phospho-amino acid analysis. Scrape-loading and microinjection of fluorescent dye were used to assess gap junction-mediated coupling. Genistein, an inhibitor of tyrosine phosphorylation, PD98059, an inhibitor of mitogen activated protein kinase (MAPK) kinase, and calphostin-C and chelerythrine, inhibitors of protein kinase C (PKC), were used to delineate FGF-2-initiated signals involved in regulating Cx43 phosphorylation and intercellular coupling. Dominant-negative PKCε and MAPK kinase mutants were used to assess the role of

PKCε and MAPK in the phosphorylation of Cx43 under control or phorbol 12-myristate, 13-acetate (PMA)-treatment conditions. Association between Cx43 and PKC isoforms was detected by co-immunoprecipitation and co-localization. Transient transfections with site-directed mutants converting consensus PKC-phosphorylation site serines (S) 262, 297 or 364 of rat Cx43 to alanines (A), were used to determine if phosphorylation of these residues affected the electrophoretic profile of Cx43 in Western blots. The S262A Cx43 as well as a mutant replacing S262 with aspartate (D), to simulate phosphorylation of S262, were used to assess the role of S262 phosphorylation on rat cardiomyocyte DNA synthesis (assessed by bromodeoxyuridine incorporation).

Results: FGF-2 stimulated Cx43 phosphorylation on serine with a concomitant decrease in dye-coupling; FGF-2 had no effect on Cx43 accumulation or distribution, but elicited masking of epitope(s) contained within residues 260-270 of Cx43. Pathways requiring activation of tyrosine phosphorylation and PKC, but not MAPK, mediated the FGF-2 effects on Cx43 phosphorylation and dye-coupling. An interaction between Cx43 and PKCε (but not PKCα), detected by co-immunoprecipitation, was strengthened by FGF-2 treatment. Also, more extensive co-localization of PKCε and Cx43, upon FGF-2 or PMA treatment, was detected by immunofluorescence. Overexpression of dominant-negative PKCε significantly reduced basal levels of Cx43 phosphorylation. Furthermore, only dominant-negative PKCε, not dominant-negative MAPK kinase, prevented hyperphosphorylation of Cx43 stimulated by PMA treatment. The S262A Cx43 mutant, but not the wild type Cx43 nor the S297A or S364A mutants, lacked the 'hyperphosphorylated', PMA-induced, 46 kDa Cx43 band, as detected in Western blots of cardiomyocytes or HeLa cells transfected with the corresponding plasmids. In addition,

expression of the S262A, but not wild-type or S262D Cx43, inhibited DNA synthesis of neonatal rat cardiomyocytes.

Conclusions: FGF-2 causes a reduction in dye coupling between neonatal rat cardiomyocytes through activation of its receptor tyrosine kinase and a PKC pathway resulting in serine phosphorylation of Cx43. PKCε is required for neonatal rat cardiomyocyte Cx43 phosphorylation and is directly responsible for PMA and FGF-2-induced phosphorylation of Cx43. S262 of Cx43 appears to be phosphorylated in the cellular milieu in response to PKC stimulation; its phosphorylation, triggered by mitogens, is likely required to cancel the growth-inhibitory effect exerted by Cx43. Targeting the phosphorylation state of S262 and/or other amino acids on Cx43 may provide a new approach to regulate the cardiomyocyte regenerative response.

ACKNOWLEDGEMENTS

Over the course of my graduate training, I have been fortunate to be assisted and guided by numerous people. I would like to thank the current and former members of the Kardami Laboratory for their assistance and camaraderie, especially Robert Fandrich, Ron Padua, Lei Liu, and Yijing Chen. I would also like to thank all of the principal investigators in the Institute of Cardiovascular Sciences for generously allowing me to use their laboratory resources including equipment, books and reagents as well as for sharing their knowledge and experience. Thanks also to the many graduate students, technicians, postdocs, research associates and support staff I have worked with at the Research Centre for their friendship and assistance. Thanks to the members of the Health Sciences campus who provided technical assistance, especially Yan Jin and Denis Bosc. I also thank the Heart and Stroke Foundation of Canada and the Manitoba Health Research Council for funding my studies. Thanks also to my graduate committee members, Dr. Judy Anderson, Dr. Peter Cattini, and Dr. Larry Hryshko for their guidance in my yearly progress and their evaluation of my thesis. I also reserve utmost thanks to Dr. Elissavet Kardami for her guidance, understanding and patience throughout my graduate training and preparation of my thesis. Furthermore, I have been supported by many friends and family members throughout my graduate training period and I would like to thank especially my immediate family: parents Ralph and Irene, sister Michelle, and brothers Jon and Chris; and my friends Ron, Kari and Adrea who have helped me to accomplish my goal of attaining a Ph.D.

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

6-CF 6-Carboxyfluorescein
A ..... Alanine
ANOVA ..... Analysis of Variance
Approx. ..... Approximately
BCA ..... Bicinchoninic Acid
bFGF ..... Basic Fibroblast Growth Factor
BrdU ..... Bromodeoxyuridine
BSA ..... Bovine Serum Albumin
cAMP ..... Cyclic Adenosine 5'-Monophosphate
cGMP ..... Cyclic Guanosine 5'-Monophosphate
CIB ..... Co-immunoprecipitation Buffer
CMF ..... Calcium- and Magnesium-Free
CMGM ..... Chicken Myocyte Growth Medium
Cx ..... Connexin
ECL ..... Enhanced Chemiluminescence
EDTA ..... Ethylenediaminetetraacetate
EGTA ..... Ethylene Glycol-Bis(2-aminoethylether)N,N,N',N'-Tetraacetic Acid
ERK ..... Extracellular Signal-Regulated Kinase
D ..... Aspartic Acid
DMEM ..... Dulbecco/Vogt modified Eagle's Minimal Essential Medium
DNA ..... Deoxyribonucleic Acid
FBS ..... Fetal Bovine Serum
FGF ..... Fibroblast Growth Factor
GAPDH ..... Glyceraldehyde-3-phosphate Dehydrogenase
GJ ..... Gap Junction
GJIC ..... Gap Junctional Intercellular Communication
HSM ..... High Serum Medium
IgG ..... Immunoglobulin
IP ..... Immunoprecipitation
IP_3 ..... Inositol-1,4,5-Trisphosphate

kDa ..... Kilodalton

LSM ..... Low Serum Medium

LY ..... Lucifer Yellow

MAPK ..... Mitogen Activated Protein Kinase

Mc ..... Monoclonal

MEK ..... MAPK or ERK Kinase

MEM ..... Minimal Essential Medium

μM ..... Micromoles per Litre

μm ..... Micrometer

NP-40 ..... Nonidet P-40

PAGE ..... Polyacrylamide Gel Electrophoresis

PBS ..... Phosphate Buffered Saline

Pc ..... Polyclonal

Pen / Strep ..... Penicillin / Streptomycin

PKA ..... cAMP-Dependent Protein Kinase

PKC ..... Protein Kinase C

PMA ..... Phorbol 12-myristate, 13-acetate

PMSF ..... Phenylmethylsulfonylfluoride

PPIC ..... Protease and Phosphatase Inhibitor Cocktail

RIPA ..... Radio-immunoprecipitation Assay

RPM ..... Rotations Per Minute

rrFGF-2 ..... Recombinant Rat FGF-2

S ..... Serine

SDS ..... Sodium Dodecyl Sulfate

SMP ..... Skim Milk Powder

TBST ..... Tris Buffered Saline with Tween

TPA 12-0-Tetradecanoylphorbol 13-Acetate

Note: PMA and TPA refer to the same chemical.

I. INTRODUCTION: REVIEW OF THE LITERATURE

A. GAP JUNCTIONS; DEFINITION AND FUNCTION

Gap junctions are aggregates of channels, composed of proteins called connexins, that serve as conduits linking the cytosols of adjacent cells (Beyer et al., 1990). Substances with masses less than 1 kDa, including ions, second messengers and metabolites can pass through these channels by passive diffusion, a process termed gap junctional intercellular communication (GJIC) (Loewenstein, 1981). The channels that make up gap junctions extend through a narrow extracellular gap that separates the plasma membranes of adjacent cells. This gap is a peculiar specialization of intercellular junctional membranes discovered by electron microscopists, and gave the gap junction its name (Revel and Karnovsky, 1967). Gap junctions play important roles in the development of multicellular organisms, the control of cellular growth, the conduction of action potentials in excitable tissues and the maintenance of intercellular homeostasis in differentiated tissues (reviewed in: Bruzzone et al., 1996; Goodenough et al., 1996).

B. GAP JUNCTION STRUCTURE

1. The Hierarchy of Gap Junction Structure; Connexins, Connexons and Gap Junctions

Individual connexin molecules are assembled into hexameric hemi-channels known as connexons. Connexons embedded in intracellular vesicles are targeted to the cell membrane where they become available for docking with connexons presented by an adjacent cell. Coupled connexons create a continuous cylindrical channel linking the cytoplasms of connected cells (Fig. 1). This model of gap junction structure was originally proposed by Makowski et al. in 1977, based on electron micrographic and x-ray diffraction data (Makowski et al., 1977) and is still valid.

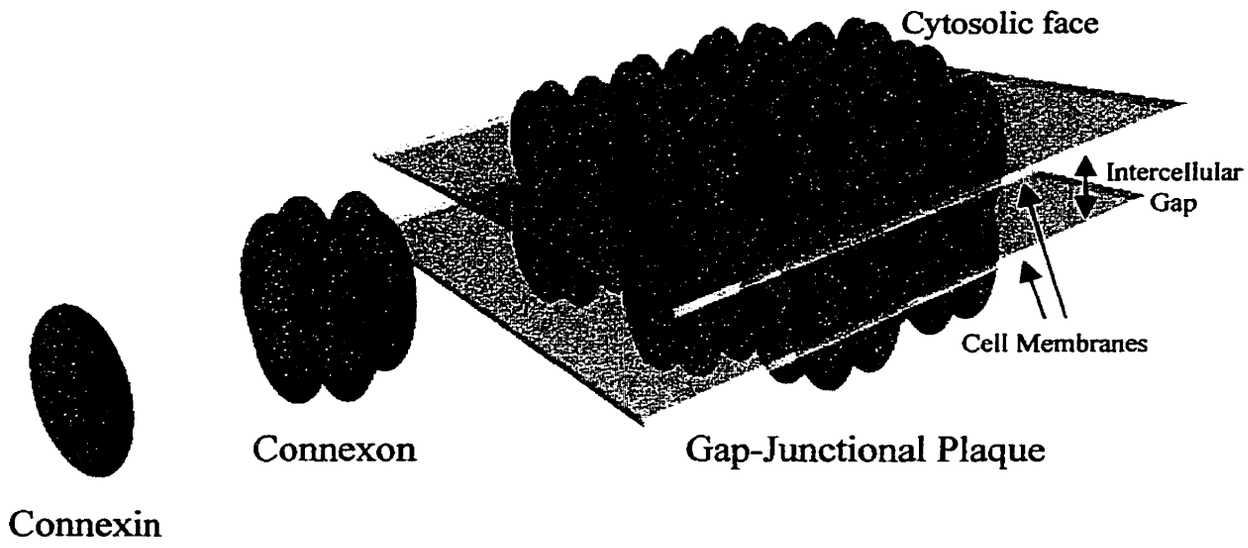


Figure 1. Components of the Gap Junction

2. Connexins

The connexins comprise a large family of structurally and functionally related proteins. The most commonly used nomenclature distinguishing between the different members of the connexin family specifies the species of origin and the predicted molecular mass of the protein, e.g. rat connexin 43 (Beyer et al., 1987). An alternate nomenclature in which connexins are classified as either α or β connexins, based on their similarity with respect to nucleotide and amino acid sequence and secondary structure, is used by some investigators (Kumar and Gilula, 1992). The currently known mammalian connexins, with both nomenclatures, and the tissues in which they are expressed are presented in Table 1. A recently cloned connexin, Cx36, is phylogenetically different from both the α and β connexins, and has prompted the addition of another class of connexins the γ connexins (Condorelli et al., 1998; O'Brien et al., 1998; Srinivas et al., 1999). General comments made about connexins in this text will refer only to the α and β connexins.

Table 1. The mammalian connexins and the tissues in which they are expressed.

Connexin Type	Alternate Name	Tissues with Detectable Expression of Connexin Isoform
Cx 26	β_2	liver, kidney, intestine, lung, spleen, stomach, testes, and brain (Zhang and Nicholson, 1989)
Cx 30.3	β_5	skin (Hennemann et al., 1992a)
Cx 31	β_3	placenta, Harderian gland (rodent lacrimal gland), skin, and eye (Hoh et al., 1991)
Cx 31.1	β_4	skin, testes (Haefliger et al., 1992; Hennemann et al., 1992a)
Cx 32	β_1	liver, kidney, intestine, lung, spleen, stomach, testes, and brain (Zhang and Nicholson, 1989)
Cx 33	α_7	testes (Haefliger et al., 1992)
Cx 36	γ_1	neurons of brain and retina (Condorelli et al., 1998)
Cx 37	α_4	highly expressed in lung, but also found in brain, kidney, skin, spleen, liver, intestine, and heart (Willecke et al., 1991)
Cx 40	α_5	lung, heart (conduction tissue and endothelial cells), uterus, ovary, and blood vessels (Bastide et al., 1993; Beyer et al., 1992; Hennemann et al., 1992b)
Cx 43	α_1	heart, ovary, uterus, kidney, brain, skin and lens epithelium (Beyer et al., 1987; Guo et al., 1992; Micevych and Abelson, 1991)
Cx 45	α_6	lung, brain, skin, heart, kidney, and intestine (Butterweck et al., 1994a; Butterweck et al., 1994b; Kanter et al., 1993b; Kanter et al., 1994)
Cx46	α_3	highly expressed in lens, but also found in myocardium and kidney (Paul et al., 1991)
Cx 50	α_8	lens (White et al., 1992)

a) Structure

(1) Primary and Secondary Structure of the Connexins

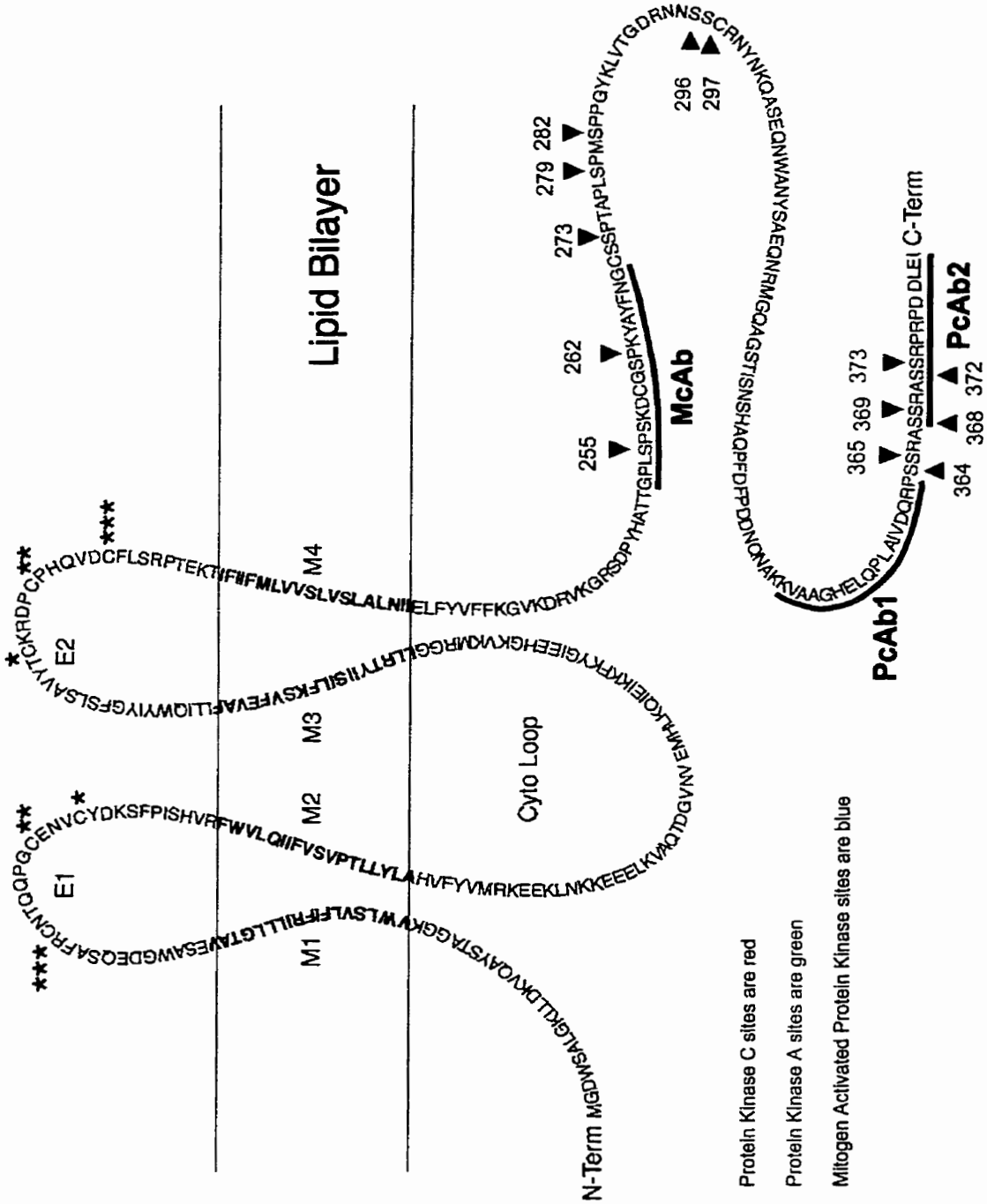
All connexin molecules have similar structures, with cytoplasmic amino and carboxy termini, four transmembrane domains (M1-M4), two extracellular loops (E1 and E2) and one intracellular loop (Fig. 2). The intracellular loop and the carboxy terminus of the connexins show considerable differences in amino acid sequence between different connexin isoforms, while the remaining regions have a high degree of similarity (Bennett et al., 1991).

(i) The Extracellular and Intracellular Loops

The extracellular loops of the connexins are involved in the docking of connexons, voltage gating properties and are in part responsible for determining the selectivity between connexon types in homo- and hetero-typic connexon interactions. Experiments examining the properties of chimeric connexins, composed of combinations of extracellular domains of Cx26 and Cx32 in the paired Xenopus oocyte system, revealed that E1 contributes to the voltage sensing mechanism of gap junctional channels (Rubin et al., 1992; White et al., 1995). The E2 domain, as assessed by similar experiments using chimeric connexins, plays a role in the specificity of interactions between different connexon types (Bruzzone et al., 1994; White et al., 1994; White et al., 1995). Incompatibility between Cx43 and Cx40 though seems to be determined by the intracellular loop and / or the cytoplasmic carboxy tail (Haubrich et al., 1996). Chimeras composed of Cx40 with both extracellular loops replaced by those of Cx43 were capable of forming functional channels with Cx40 hemichannels, while a further modification replacing the cytoplasmic loop of the chimera with that of Cx43 prevented channel

Figure 2. Topology of Connexin 43.

Cysteines labeled with the same number of asterisks form intramolecular disulfide bonds. Underlined stretches of sequence indicate residues used to raise the monoclonal antibody (McAb) and polyclonal antibodies (PcAb1 and PcAb2) used in this study. Some of the putative serine phosphorylation sites for well-studied kinases are indicated.



formation.

Each of the extracellular loops contains 3 cysteine residues that are conserved in all connexin family members except Cx31 (Hoh et al., 1991). Mutation of any one of the cysteine residues to a serine residue prevents the formation of functional channels (Dahl et al., 1992). Mapping of the disulfide bonds required for functional channel formation determined that each cysteine of E1 forms a disulfide bond with a partner cysteine in E2 (see Fig. 2 for location of partner cysteines) (Foote et al., 1998). The disulfide-coupled E1 and E2 domains are proposed to form antiparallel β-sheets that can interdigitate with similar β-sheets from partner connexins in a docking process between connexons to form a continuous cylindrical β-barrel structure sealing partner connexons together into a gap junctional channel. Electron crystallographic data confirms this model of interdigitation (Perkins et al., 1998). In light of this model, perhaps it is not incompatibility between E2 and the extracellular domains of partner connexons that prevents functional channels in experimental chimeras, but rather it is incompatibility between E1 and E2 domains of the chimeric connexin that prevents the necessary disulfide bonds required to form a structure compatible with connexon docking.

(ii) Transmembrane Domains

Electron crystallographic analyses of gap junctional channels formed from carboxy-truncated Cx43 indicate that the four transmembrane domains exist as α-helical rods (Unger et al., 1999a; Unger et al., 1999b; Yeager, 1998). The pore lining domain was initially thought to be the M3 domain based on the order of its conserved polar, basic, and acidic groups, which could be envisioned as forming an amphipathic helix with polar residues lining the pore of a channel (Bennett et al., 1991). More recent

studies on channel properties provide evidence indicating that it is actually M1 that lines the channel pore: i) chimeric connexins with different M1 domains took on the conductance and gating properties of the connexins from which the M1 domain originated (Hu and Dahl, 1999); ii) using another technique, cysteine scanning mutagenesis, once again M1 was implicated as being important for pore formation in Cx46 hemi-channels analyzed in Xenopus (Zhou et al., 1997); iii) site mutations in Cx32 M1 domain are associated with Charcot-Marie-Tooth disease and display reduced permeability or open channel probability of gap junctional channels (Oh et al., 1997). That both M1 and M3 are involved in creating the channel pore is consistent with the structural data from the electron crystallographic studies which indicate that one α-helix lines the pore along its entire length, while another helps make up the wider cytoplasmic entrance of the pore (Unger et al., 1999b).

(iii) The Carboxy Terminus

The carboxy terminus of the connexins is one of the least conserved regions between the different members of the connexin family. Not only are the amino acid sequences of the carboxy termini of the different connexins dissimilar, but there are large differences between the lengths of the carboxy termini between different connexin isoforms. For instance, while the carboxy terminus of Cx43 is 156 amino acids long, that of Cx26 is only 18 amino acids long (Bennett et al., 1991).

The carboxy terminus is not strictly required for the generation of functional gap junctional channels. This has been shown by generating truncation mutants that lack most of the carboxy terminal domain for each of the following connexins: Cx32 (Werner et al., 1991), Cx37 (Stergiopoulos et al., 1999), Cx40 (Stergiopoulos et al., 1999), Cx43

(Dunham et al., 1992), Cx45 (Koval et al., 1995) and Cx50 (Lin et al., 1998; Lin et al., 1997). The truncation mutants in all cases were still able to form functional channels, indicating that oligomerization, targeting, docking and pore formation were not affected.

It appears that the carboxy terminus is involved in the regulation of the properties of gap junctions, in many cases due to the phosphorylation of this domain. Indeed, the carboxy termini of many connexins contain a multitude of phosphorylatable residues. For instance, the carboxy terminus of Cx43 contains 21 serine, 7 tyrosine, and 5 threonine residues, all which are theoretically capable of being phosphorylated (see Fig. 2).

Several kinases targeting serine/threonine and tyrosine residues have been implicated in the phosphorylation of carboxy terminal residues of several different connexins.

(a) Significance of different length

The length of the carboxy terminus appears to be related to different functional properties of connexins showing large differences in carboxy terminal length. For instance, Cx32, with a carboxy-tail length of 76 amino acids, and Cx43, with a carboxy tail length of 156 amino acids, have significantly different gating properties in response to intracellular acidification, with Cx43 being more sensitive (closing at a higher pH). Truncation of the carboxy tail of Cx43, so that it was the same length as that of Cx32, resulted in channels that now had acidification mediated gating properties indistinguishable from those of Cx32 channels (Liu et al., 1993b).

The mechanism for the pH mediated closure of Cx43 channels has been studied extensively, and has given rise to a particle-receptor, or ball and chain model in which

essential segments of the carboxy terminus comprise a particle that is used to close the channel upon acidification (Ek-Vitorin et al., 1996; Morley et al., 1997; Morley et al., 1996). This model also appears to apply to growth factor (insulin and insulin-like growth factor) -mediated uncoupling of Cx43 channels expressed in Xenopus oocytes (Homma et al., 1998), and may be applicable to other phosphorylation modifications of the carboxy terminus mediated by other treatments. In fact, recent evidence indicates that even the pH sensitive regulation of gap junction conductance may be due to phosphorylation of the carboxy tail of Cx43 by an acid kinase (Yahuaca et al., 2000).

(2) Targeting and Oligomerization of Connexins

The synthesis of connexins and co-translational integration into the proper topography in membranes occurs in the endoplasmic reticulum (ER) as assessed by cell-free in vitro translation systems supplemented with ER microsomes (Falk and Gilula, 1998; Falk et al., 1994; Zhang et al., 1996). Although the expected co-translational insertion of connexins into the ER membrane via a signal recognition particle and an internal signal anchor appears to be the main route used to incorporate connexins in membranes, post-translation incorporation of some connexins into ER microsomes in the correct topography has been reported as well (Ahmad et al., 1999; Zhang et al., 1996).

The oligomerization of connexins into hexameric hemi-channels also occurs in the ER (Falk et al., 1997). This is not unexpected, as assembly in the ER has been shown for most known oligomeric membrane spanning proteins, such as ion specific channels that are structurally similar to the connexins (Green and Millar, 1995). Post-ER oligomerization has also been reported for both homomeric and heteromeric connexons (Ahmad et al., 1999; Musil and Goodenough, 1993). Cell-free translation reactions

containing mRNAs for both Cx26 and Cx32, when supplemented only with ER vesicles, formed homomeric connexons, but the addition of Golgi membranes resulted in heteromeric connexons as well (Ahmad et al., 1999). Thus, the Golgi may serve a critical role in the formation of heteromeric connexons. Connexons assembled in the ER pass through the Golgi apparatus and are targeted to the cell membrane according to the well-defined secretory pathway for membranous proteins. It is not clear whether connexons are targeted directly to gap junctional plaques or are first transported to nonjunctional membrane sites with subsequent recruitment to gap junctions through lateral movement in the lipid bilayer.

The role of phosphorylation in the regulation of connexin synthesis and trafficking is not clear. As we have seen, connexins without their carboxy tail, containing most known phosphorylation sites, can still form functional channels, so phosphorylation is not an absolute requirement for proper assembly and targeting to the cell membrane. Still, phosphorylated forms of connexins can be trapped before their incorporation into the cell membrane (Laird et al., 1995; Puranam et al., 1993) and upon their integration into Triton-insoluble gap junctional plaques at the interface of adjacent cell membranes (Musil et al., 1990; Musil and Goodenough, 1991). Thus, although not essential for the assembly of connexins into functional gap junctions, the existence of these early phosphorylation steps suggests a role for phosphorylation in the assembly or trafficking of full-length connexins into gap junctional plaques. Indeed, with wild-type Cx43, the ability to form communicating gap junctional plaques appears to require an early phosphorylation step in at least some conditions (Musil et al., 1990; Musil and Goodenough, 1991).

The point at which connexons dock to form continuous channels is also not clear. Two scenarios have been proposed by Bukauskas *et al.*: i) connexons from adjacent cells dock randomly, change their conformation, and form a nucleus for the generation of a gap junctional plaque, with recruitment of other channels; ii) other factors such as adhesion proteins may cause clustering of connexons of adjacent cells, bringing them into close enough contact to dock and form gap junctional plaques (Bukauskas et al., 2000). The second scenario is appealing since a role for adhesion molecules in the formation of gap junctions has been suggested by several studies (Hertig et al., 1996; Meyer et al., 1992; Musil et al., 1990; Prowse et al., 1997; Wang and Rose, 1997; Zuppinger et al., 2000). Adhesion of cells to extracellular matrix components through integrins has also been implicated in the regulation of gap junction formation and function (Lampe et al., 1998). The manner in which connexons dock to one another results in a very tight non-covalent bond between the connexon subunits. This bond, once formed, cannot be broken except with very harsh reagents such as detergents and urea (Ghoshroy et al., 1995; Laird and Revel, 1990).

An exciting new technique that has been recently applied to the study of connexin trafficking is the use of autofluorescent protein tags. By creating a chimeric protein fusing a fluorescent protein, such as green fluorescent protein (GFP), to the carboxy-tail of connexins, direct visualization, in real time, of connexin synthesis, assembly and trafficking in living cells has been possible (Bukauskas et al., 2000; Holm et al., 1999; Jordan et al., 1999; Windoffer et al., 2000). These studies revealed that gap junctions are incredibly plastic structures that can rapidly (within seconds) move laterally within the plane of the membrane. Portions of gap junctions can be seen to pinch off in vesicular

structures that are then shuttled to cytoplasmic locations where they are presumably either degraded or perhaps processed for recycling. These internalized vesicles containing gap junctions are thought to represent "annular gap junctions" that have been described in histological studies (Larsen et al., 1979; Mazet et al., 1985; Severs et al., 1989). The mechanisms by which connexons and gap junctions move laterally within the plasma membrane are not known but may require an interaction with actin microfilaments (Wang and Rose, 1995). The internalization process of annular gap junctions may also depend on microfilaments (Larsen et al., 1979; Murray et al., 1997).

The use of protein tags with different fluorescence excitation-emission spectra, cyan-fluorescent protein, green fluorescent protein and yellow fluorescent protein, each fused to a different connexin, have allowed for the direct demonstration of different connexins within a single gap junctional plaque (Falk, 2000). Connexins that have been shown to be compatible in the formation of heterotypic channels (Cx26 and Cx32) reveal direct overlap of fluorescent signals in gap junctional plaques, while incompatible connexins (Cx43 and Cx26) were found to localize to discrete domains within the plaques.

A study in which electrical coupling and gap junctional plaque formation (through the visualization of GFP-Cx43) were simultaneously assessed, suggests that a critical size of gap junctional plaque (several hundred channels) must be formed before electrical coupling can occur (Bukauskas et al., 2000). The data further suggest that very few channels within a communication-competent plaque are able to open at a given time, with approximately 2% active channels in the smallest functional plaques. The number of active channels increases with the size of the plaque, so that a plaque consisting of 2000

channels contains approximately 10-20% active channels.

Fluorescence of GFP-Cx43 at non-junctional plasma membrane sites has been reported (Bukauskas et al., 2000; Falk, 2000; Jordan et al., 1999) and probably reflects the presence of pre-assembled hemi-channels which have been described in other systems (presented in more detail below; page 33). The diffuse nature of non-junctional GFP-Cx43 fluorescence suggests that clustering of hemi-channels does not occur except at regions of intercellular contact (Bukauskas et al., 2000).

3. A Detailed Examination of Connexin 43

Connexin 43 is by far the most studied of the connexin family members. This is due to the following reasons: i) Cx43 was one of the first connexins to be cloned; ii) high quality monoclonal and polyclonal antibody reagents to Cx43 have been generated; iii) Cx43 is expressed in many cell lines that are convenient models for many researchers; iv) Cx43 is located in many different organs and tissues; v) Cx43 is the predominant connexin of the myocardium.

a) Regulation of GJIC Through Cx43 Channels

There are multiple control mechanisms that regulate intercellular communication through Cx43 gap junctions. Regulation of Cx43-containing gap junctions can occur due to modulation of the transcription, mRNA stability, translation, post-translational modification, assembly or degradation of Cx43. As well, factors that change the biochemical environment within which a gap junction resides, such as the pH or membrane composition, can alter the function of the gap junction dramatically. Changes in these parameters can lead to either increased or decreased GJIC through Cx43 channels which can occur rapidly, in time frames as short as milliseconds, or slowly over a period of several hours.

(1) Gene Silencing by Methylation

The HeLa cell line, derived from a human cervical carcinoma, is commonly used in cell and molecular biology studies due to its rapid growth, relative ease of maintenance and its amenability for gene transfer experiments (Jones et al., 1971). As HeLa cells, as a population, express little Cx43 and exhibit little to no GJIC (Eckert et al., 1993), they have been used in numerous experiments in which the effects on cell growth of transiently or stably expressed wild-type or mutant Cx43 were assessed (Elfgang et al., 1995; George et al., 1998; Haubrich et al., 1996; Mesnil et al., 1995; Zhu et al., 1997).

King et al. (2000) have proposed that the mechanism for the silencing of Cx43 expression in HeLa cells is due to the hypermethylation of the Cx43 promoter or a regulatory gene affecting this promoter, since treatment of Cx43-negative clones with 5-aza-2'-deoxycytidine, a nucleoside analogue incapable of being methylated, resulted in re-expression of endogenous Cx43 protein. Methylation of the Cx43 promoter has been reported previously in normal rat liver cells and was suggested to be a major negative regulator of Cx43 expression in this cell type (Piechocki et al., 1999). Hypermethylation of DNA may be a general mechanism for the loss of Cx43 expression in Cx43-negative tumor cells. The connexins are considered to form a novel class of tumor suppressors (see below, page 39). Thus, it is not suprising that hypermethylation of the Cx43 promoter occurs in some tumor cells, since hypermethylation has been implicated in the decreased expression of other tumor suppressor genes in various tumor cell types (Bender et al., 1998; Kane et al., 1997; Xing et al., 1999).

(2) Transcriptional Regulation of Cx43 mRNA Expression

Cx43 has exquisitely controlled tissue specific regulation of its mRNA

expression. This is best exemplified in the myometrium during labour, when the level of Cx43 mRNA and corresponding protein undergoes a dramatic increase in the hours just prior to parturition with no change in Cx43 expression seen in other organs containing Cx43 such as the heart (Risek et al., 1990). A component of this specific example of transcriptional regulation appears to be related to the fact that the Cx43 gene is responsive to the estrogen/progesterone ratio in myometrial cells (Grummer et al., 1999; Petrocelli and Lye, 1993).

(a) Transcription/Translation Regulatory Elements

Although still not fully characterized, analysis of the Cx43 promoter has revealed several transcription regulatory sequences that bind different transcription factors. One of the first identified transcription response elements in the Cx43 promoter was a binding site for the AP-1 transcription factor (Sullivan et al., 1993). Transcription activating protein 1 (AP-1), originally described as binding a 12-0-tetradecanoylphorbol 13-acetate (TPA*)-responsive element in the human metallothionein gene promoter (Lee et al., 1987), consists of c-fos and c-jun proteins that assemble to form a heterodimeric "leucine-zipper" structure that binds specific DNA sequences (Chiu et al., 1988; Curran and Franza, 1988; Landschulz et al., 1988). Elements responsive to AP-1 are found primarily in the promoters of genes that are involved in the regulation of cellular growth and proliferation (reviewed in: Karin et al., 1997). The promoter of Cx43 also contains an AP-2 regulatory site (Yu et al., 1994). The AP-2 transcription factor has been implicated in the control of cellular differentiation and the negative regulation of cell growth (Bar-Eli, 1999; Imagawa et al., 1987; Morriss-Kay, 1996; Zeng et al., 1997). A

* Another nomenclature (Phorbol 12-myristate, 13-acetate) yields the abbreviation PMA.

series of half-palindromic estrogen responsive elements are also found in the Cx43 promoter (Yu et al., 1994) as well as two consensus specificity protein 1 (Sp1) sites and an NF-κB consensus site (Echetebu et al., 1999). The Sp1 transcription factor is a general factor that is involved in the transcription of most genes (reviewed in: Suske, 1999). The transcription factor NF-κB regulates genes involved in immune responses, inflammation, liver development, programmed cell death, and cell proliferation (reviewed in: Chen and Ghosh, 1999b; Gilmore, 1999; Karin and Ben-Neriah, 2000; Pahl, 1999).

The upregulation of reporter genes driven by Cx43 promoter sequences in transfected cardiomyocytes co-cultured with cells overexpressing Wnt-1 suggests that the Cx43 promoter also contains a T cell factor / lymphoid enhancer binding factor (TCF / LEF) site that can be activated by β-catenin (Ai et al., 2000). Recently identified targets of TCF/LEF transcription factor regulation include the proto-oncogene c-myc, the matrilysin gene (which encodes a metalloproteinase) and the cyclin D1 gene (Waltzer and Bienz, 1999), all of which may play a role in cellular transformation. The presence of a TCF/LEF binding site in the promoter of Cx43 may again reflect the putative role of Cx43 in the regulation of cellular growth and proliferation.

Most protein synthesis in eukaryotes is initiated by the binding of the ribosome to a methylated cap structure at the 5' end of the mRNA (Banerjee, 1980). The mRNA for Cx43 has an unusually long (208 nucleotides) 5'-untranslated region (UTR), a feature that suggests it may be involved in the translational regulation of Cx43 protein production. Experiments in which the basal Cx43 promoter and the entire 5'-UTR was inserted between two luciferase cistrons (one for Renilla luciferase, the other for firefly luciferase), driven by the cytomegalovirus promoter in transiently transfected mammalian

cells, revealed that the 5'-UTR contains an internal ribosome entry site (IRES) (Schiavi et al., 1999). Compared to the IRES from encephalomyocarditis virus, used as a positive control, the Cx43 IRES activity was 18 times greater. Computer generated predictions of the secondary structure of the 5'-UTR of Cx43 mRNA reveal a Y-type structure at the end of a long stem loop, which is consistent with IRES elements found in other mRNAs (Le and Maizel, 1997; Schiavi et al., 1999).

The identification of IRES elements in the mRNAs of eukaryotic genes is a relatively new finding (early 1990s), as they were originally only thought to occur in picornaviral RNA (Oh and Sarnow, 1993). Although the reason for the presence of IRES elements in the mRNAs of only a small subset of genes is unclear, it has been suggested that they allow translation of proteins essential for survival throughout periods of cellular stress, when cap-dependent translation is inhibited (Schiavi et al., 1999). Consistent with this notion is the presence of IRES elements in the mRNAs of several genes known to be involved in responses to injury or stress such as immunoglobulin binding protein, VEGF and FGF-2 (Morris et al., 1997; Stein et al., 1998; Vagner et al., 1996). Several of the genes with IRES elements are also involved in cell proliferation such as oncogenes and growth factors. Misregulation of their IRES-mediated translation has been implicated in cellular transformation and the development of cancer (Chappell et al., 2000; Galy et al., 1999; Willis, 1999). The presence of a strong IRES element in the promoter of Cx43 thus suggests that Cx43 may play a role in situations of cellular stress and supports the idea that Cx43 plays a role in the control of cell growth.

(3) Post-translational Regulation of Cx43 Properties

(a) Phosphorylation / Dephosphorylation

Phosphorylation was the earliest identified type of post-translational modification of Cx43. Indeed, in some tissues, like the heart, phosphorylated forms of Cx43 predominate. Cx43 isolated from the heart and analyzed by Western blotting reveals several 40-47 kDa bands which can be reduced to a single 40 kDa band with phosphatase treatment (Laird et al., 1991; Laird and Revel, 1990). Phospho-amino acid analysis of Cx43 isolated from cardiac cells revealed that the major phosphorylated amino acid is serine, although some phospho-threonine was also detected (Laird et al., 1991; Lau et al., 1991).

(i) Serine versus Tyrosine Phosphorylation

The phosphorylation of Cx43 on tyrosine residues is generally associated with a decrease in GJIC, based on numerous studies using different models. Treatments that have been shown to cause tyrosine phosphorylation of Cx43 with an accompanying reduction in GJIC are: i) transforming cells with the viral oncogenes encoding the tyrosine kinases src (see below) and p130gag-fps (Kurata and Lau, 1994); ii) incubating normal cells with inhibitors of tyrosine phosphatases such as pervanadate (Mikalsen et al., 1997; Mikalsen and Kaalhus, 1996; Mikalsen and Kaalhus, 1997; Mikalsen and Kaalhus, 1998); iii) subjecting human umbilical vein endothelial cells to hypoxia-reoxygenation protocols (Zhang et al., 1999a; Zhang et al., 1999b).

Phosphorylation of Cx43 on serine residues appears to have variable effects, increasing GJIC in some and decreasing GJIC in others. As there are multiple serine phosphorylation sites in the carboxy-terminus of Cx43, several of which are within consensus sequences for different kinases (Kreegipuu et al., 1999), these disparate results with respect to serine phosphorylation probably arise due to different target serines being

phosphorylated by different kinases with different results.

In some cases, even the same kinase can cause different effects in different cell types. For example cyclicAMP-dependent protein kinase (PKA) is found to increase GJIC in most cell types (Stagg and Fletcher, 1990), but decreases it or has no effect in cardiomyocytes (Kwak and Jongsma, 1996), myometrial smooth muscle cells (Cole and Garfield, 1986), and Sertoli cells (Grassi et al., 1986). There are at least two explanations for this discrepancy: i) PKA is not directly involved in the phosphorylation of Cx43, but stimulates phosphorylation through the activation of another kinase which may have different isoforms in different tissues; ii) the basal level of phosphorylation of the Cx43 molecule, and the specific sites that are already phosphorylated may influence the effects of subsequent phosphorylation reactions.

(ii) Kinases implicated in Cx43 channel regulation

Numerous kinases have been proposed to regulate Cx43 with respect to GJIC, either due to direct phosphorylation of Cx43 or through indirect mechanisms affecting Cx43 synthesis, assembly or degradation. Some of the better studied kinases involved in Cx43 regulation are presented below:

(a) Src

One of the first kinases shown to phosphorylate Cx43 with a clear effect on GJIC was the viral oncogene pp60v-src, that encodes for a tyrosine kinase. Transformation of fibroblasts by infection with Rous sarcoma virus (RSV) was associated with a decrease in GJIC and phosphorylation of Cx43 on tyrosine residues (Crow et al., 1990; Filson et al., 1990). Conversion of tyrosine 265 of Cx43 to phenylalanine prevented src-mediated tyrosine phosphorylation and accompanying decreases in GJIC in RSV-infected Xenopus

oocytes (Swenson et al., 1990). That src was directly responsible for the tyrosine phosphorylation of Cx43 was established in studies comparing tryptic phosphopeptide profiles of Cx43 phosphorylated in vitro with Cx43 isolated from cells expressing active v-src and in co-immunoprecipitation studies using v-src transformed fibroblasts (Loo et al., 1995; Loo et al., 1999). The interaction between src and Cx43 appears to require the SH2 and SH3 domains of src and proline rich regions of Cx43 (Kanemitsu et al., 1997).

Most models of src mediated phosphorylation of Cx43 have been based on v-src transformed cells. Recently though, cellular c-src, the endogenous src found coupled to different signaling pathways in most cell types, has been implicated in the phosphorylation of Cx43 in two studies: i) A role for c-src in the reduction of GJIC observed in a variety of cell types treated with agonists of G-protein coupled receptors including lysophosphatidic acid, neuropeptides and thrombin, has been suggested (Postma et al., 1998); and, ii) Increased tyrosine phosphorylation of Cx43, paralleling a concomitant increase in levels of c-src activity, has been reported in the cardiomyopathic hamster during late phases of heart failure (Toyofuku et al., 1999). This indirect evidence linking c-src activation to Cx43 phosphorylation was supported by transfection studies in which constitutively active c-src was expressed in HEK293 and rat neonatal cardiomyocytes. When forced to express active c-src, both of these cell types revealed a decrease in GJIC and the HEK293 cells were shown to contain increased levels of tyrosine-phosphorylated Cx43 (experimental design did not allow for detection of tyrosine-phosphorylated Cx43 in the cardiomyocytes) (Toyofuku et al., 1999).

Interestingly, a detailed examination of the role of v-src in the regulation of Cx43 GJIC indicated that tyrosine phosphorylation of Cx43 was not actually required for the

acute reduction in GJIC that occurs upon src activation (Zhou et al., 1999). It appears that while tyrosine phosphorylation of Cx43 by src may affect channel trafficking and assembly, fast gating changes mediated by src may actually be due to serine phosphorylation through an intermediate kinase (speculated to be mitogen activated protein kinase (MAPK) or a related kinase) (Zhou et al., 1999).

(b) Protein Kinase A

As mentioned earlier (page 19), PKA is reported to exert cell-type specific effects on GJIC. In cardiac myocytes, a cell type that expresses predominantly one type of connexin (Cx43), conflicting results have been obtained. In some studies PKA enhanced GJIC between cardiomyocytes (Burt and Spray, 1988), while in others, PKA had no effect (Kwak and Jongsma, 1996). The reason for this discrepancy is unclear, but may be due to different culture conditions. The carboxy terminus of Cx43 contains four consensus sequence sites for PKA at serines 296, 365, 369 and 373 (Kreegipuu et al., 1999). It is not known whether PKA can directly phosphorylate Cx43 in vivo, but in some systems, treatments that increase PKA activity also increase Cx43 phosphorylation (Dowling-Warriner and Trosko, 2000; Ogawa et al., 2000; Sears et al., 1998) . A commonly reported phenomenon in models that show increased GJIC upon PKA activation is an increase in the clustering of Cx43 at the membrane of treated cells that occurs rapidly (Burghardt et al., 1995; Mehta et al., 1996; Paulson et al., 2000; Wang and Rose, 1995), or over a longer time period (Abudara et al., 1999; Banoub et al., 1996; Burghardt et al., 1996). This suggests that the mode of action of PKA in increasing GJIC is not through a direct gating mechanism, but is rather through the increased expression of Cx43 and/or the recruitment of more channels to the membrane.

(c) Mitogen Activated Protein Kinase

Mitogen activated protein kinase (MAPK) is an essential serine / threonine kinase implicated in signal transduction pathways resulting in increased proliferation of various cell types (Ferrell, 1996). Several lines of experimentation have indicated that MAPK is capable of phosphorylating Cx43 *in vitro* and *in vivo* (Hii et al., 1994; Kanemitsu and Lau, 1993; Vikhamar et al., 1998; Warn-Cramer et al., 1998; Warn-Cramer et al., 1996). The first study to implicate MAPK as being involved in the phosphorylation of Cx43 used T51B rat liver epithelial cells stimulated with EGF (Kanemitsu and Lau, 1993). EGF caused decreased GJIC and increased serine phosphorylation of Cx43 even when prolonged pre-treatment with PMA was used to down-regulate PMA-sensitive protein kinase C (PKC) isoforms. Two-dimensional analysis of phosphorylated tryptic and chymotryptic Cx43 peptides derived from EGF-treated cells or from Cx43 that was phosphorylated with purified MAPK in vitro, revealed that the in vitro phosphopeptides overlapped with a subset of the phosphopeptides derived from the treated cells.

The minimal recognition sequence for MAPK is a serine residue followed by a proline (Clark-Lewis et al., 1991). The carboxy terminus of Cx43 contains four such sites at serines 255, 273, 279, and 282. Three of these fall into the more restricted consensus sequence for MAPK which is Pro-$X_{1,2}$-Ser/Thr-Pro, where X can be any basic or neutral amino acid and the number of X's is either one or two (Clark-Lewis et al., 1991; Gonzalez et al., 1991). In vitro incubation of a glutathione-S-transferase (GST) fusion protein linked to the carboxy terminus of Cx43 (residues 236-282) with purified MAPK, resulted in phosphorylation of all three consensus MAPK target sites (Warn-Cramer et al., 1996). Alternate sites of MAPK phosphorylation at serines 272 and 273

were identified when the in vitro MAPK assay utilized a mutated version of the GST-Cx43 carboxy-terminus fusion in which all three MAPK consensus site serines were mutated to alanine. These sites (S272 and S273) were only phosphorylated in the absense of the consensus MAPK targets.

A direct role for MAPK in the regulation of Cx43 channel permeability in a cell free liposomal system has been reported (Kim et al., 1999). In this study, liposomes containing purified Cx43 connexons from rat brain were assessed for their permeability to sucrose and/or lucifer yellow with the Cx43 in its native, dephosphorylated (with alkaline phosphatase), or MAP kinase-phosphorylated state. Dephosphorylated Cx43-containing liposomes were the most permeable, while MAPK treatment dramatically reduced the fraction of permeable liposomes (79% reduction). The MAPK-mediated reduction in liposome permeability was reversed with alkaline phosphatase treatment.

As a follow-up to earlier studies implicating MAPK involvement in EGF-mediated GJIC reduction, Warn-Cramer et al. have conducted experiments using a MAPK kinase (MEK1) inhibitor and site-directed Cx43 mutants to assess the importance of MAPK in the EGF-mediated regulation of Cx43 GJIC in vivo (Warn-Cramer et al., 1998). A cell line derived from Cx43 knockout mice (Martyn et al., 1997) was used to create clones expressing either wild-type ($Cx43^{WT}$) or mutant Cx43 in which all three consensus MAPK sites had their serines mutated to alanine residues ($Cx43^{255,279,282}$). The $Cx43^{WT}$ cells responded to EGF with significantly reduced dye-coupling and junctional conductance, while the $Cx43^{255,279,282}$ cells were unresponsive to EGF with respect to these parameters. The EGF-mediated decrease in dye-coupling in the $Cx43^{WT}$ cells was also prevented by pre-treatment with the MEK1 inhibitor PD98059. In an attempt to

further define which of the three residues are necessary for the EGF effects, another Cx43 mutant, S255D, was stably transfected into HeLa cells. The S255D-Cx43 HeLa cells did not differ from HeLa cells expressing wild-type Cx43 with respect to GJIC assessed by dye coupling, and the S255D-Cx43 was still phosphorylated upon EGF treatment with a concomitant decrease in GJIC. Thus, the authors suggest that serine 255 of Cx43 is not required for EGF effects on Cx43 GJIC while phosphorylation of ser 279 and/or ser 282 are still implicated (Warn-Cramer et al., 1998).

The in vitro and in vivo data taken together strongly support a role for MAPK in the reduction of Cx43-mediated GJIC upon EGF treatment, but the direct involvement of MAPK in the phosphorylation of Cx43 is not certain. Several lines of evidence indicate that MAPK activation does not necessarily correlate with a decrease in GJIC (Hossain et al., 1999a; Hossain et al., 1999b). In a model in which PDGF was used to stimulate T51B rat liver epithelial cells expressing wild-type or mutant PDGF receptors incapable of activating defined downstream effectors, it was concluded that although MAPK is not required for the direct phosphorylation of Cx43, it is still a necessary component, in combination with PKC and other unknown factors, for the reduction in GJIC seen upon PDGF treatment (Hossain et al., 1999b). In the same model, stimulation of MAPK with H_2O_2 or sorbitol was not sufficient for Cx43 phosphorylation (assessed by Cx43 band mobilities in Western blots) or a reduction in GJIC (Hossain et al., 1999a).

(d) Protein Kinase C

Studies utilizing phorbol esters revealed that, as well as having the ability to transform cells, they were able to decrease GJIC (Madhukar et al., 1983; Mosser and Bols, 1982; Newbold and Amos, 1981; Rivedal et al., 1985; Ruch et al., 1987; Shiba et

al., 1989; Walder and Lutzelschwab, 1984; Yamasaki et al., 1985; Yamasaki et al., 1983). Since phorbol esters activate PKC, it has long been hypothesized that phosphorylation of Cx43 by PKC underlies the decreased GJIC in phorbol ester treated cells (Asamoto et al., 1991; Aylsworth et al., 1989; Berthoud et al., 1992; Brissette et al., 1991; Budunova et al., 1994; Budunova et al., 1993; Davidson et al., 1985; Husoy et al., 1993; Munster and Weingart, 1993; Reynhout et al., 1992). Using an internet based search tool to scan through the rat Cx43 sequence for the PKC consensus site X-Ser/Thr-X-Arg/Lys (where X can be any amino acid), 7 potential sites for PKC phosphorylation are found, threonines 186 and 204 and serines 262, 297, 364, 368, and 372 (Kreegipuu et al., 1999). Only very recently, a specific target site for PKC was determined to reside on the carboxy terminus of Cx43 at residue 368 (Lampe et al., 2000).

The method used to determine that ser 368 was a target for PKC in vivo included comparison of two-dimensional tryptic phosphopeptide maps of Cx43 derived from [^{32}P]-labeled, PMA-treated cells, versus maps of immunopurified Cx43 phosphorylated in vitro with a purified PKC preparation from rat brain and maps of single peptides that were also phosphorylated in vitro. One radioactive spot was common to Cx43 phosphopeptide maps from PMA-treated cells, in vitro phosphorylated Cx43 and a peptide spanning residues 360-382 of Cx43. Sequencing of the spots by Edman degradation indicated that serine 368, not 372 was phosphorylated in vitro and in vivo. Site-directed mutagenesis converting ser 368 to alanine prevented PMA-induced reduction of dye-coupling in one clone of T51B rat epithelial cells expressing the S368A mutant, but not another. Furthermore, a 50 pS conductance state event hypothesized to be caused by PKC phosphorylation of Cx43 at ser 368 was still present, although at a lower frequency in the

cells expressing the S368A mutant. Thus, although ser 368 appears to be phosphorylated upon PKC activation the authors speculate that other kinases may also phosphorylate Cx43 at other sites to give rise to the 50 pS events (Lampe et al., 2000). It should be noted that other phosphorylated spots on the tryptic phosphopeptide maps from cells and Cx43 phosphorylated in vitro showed overlap, but were ignored, and that the PKC preparation used to phosphorylate Cx43 in vitro only contained α, β and γ isoforms of PKC. Thus, other sites for PKC phosphorylation of Cx43 have not been ruled out by this study.

In cardiac myocytes activation of PKC with PMA has been shown to increase Cx43 phosphorylation with a concomitant decrease in intercellular coupling as measured by intercellular dye transfer, but an increase in gap junctional conductance as measured electrophysiologically (Kwak and Jongsma, 1996; Kwak et al., 1995). This apparent contradiction was explained by Kwak et al. as resulting in a shift in the single channel subconductance state to a lower value, but with an overall increase in the frequency of the open state of the channels. Thus, while the dye was prevented from moving between coupled cardiomyocytes due to a restricted channel pore opening, ion flow was increased due to a higher open probability of the channel. These discrepancies between dye permeability of gap junctional channels and junctional conductance values have been reported in other systems and are elaborated on further below (page 37).

The findings of Kwak et al. (Kwak and Jongsma, 1996; Kwak et al., 1995) are supported by another study using neonatal rat cardiomyocytes in which the broad-specificity kinase inhibitor staurosporine decreased gap junctional conductance, but PMA treatment reversed the effect (Saez et al., 1997). No analysis of dye coupling was carried

out in this study. A role for Cx43 phosphorylation was speculated to be important for the PMA-mediated increase in conductance, but direct phosphorylation of Cx43 by PKC was deemed unlikely based on analysis of tryptic phosphopeptides derived from a C-terminal Cx43 peptide phosphorylated by purified PKC in vitro or Cx43 phosphorylated in vivo in PMA-stimulated [^{32}P]-labeled cells (Saez et al., 1997). The purified PKC preparation from rat brain used in the in vitro phosphorylation reaction (Saez et al., 1997) only contains two isoforms; one 78 kDa, the other 80 kDa (Woodgett and Hunter, 1987), while rat cardiomyocytes express at least 6 isoforms (Disatnik et al., 1994). Thus, direct phosphorylation of Cx43 by PKC isoforms other than the two used in the in vitro kinase assay could be responsible for the PMA-mediated effects on gap junctional conductance.

A study by Bastide et al. contradicts the results of Kwak et al. with respect to decreased dye-coupling caused by PMA treatment of neonatal rat cardiomyocytes (Bastide et al., 1994). Using fluorescence recovery after photobleaching to quantitate changes in gap junction dye permeability, Bastide et al. reported that 10^{-7} M PMA had no effect on dye-coupling between pairs of neonatal rat cardiomyocytes (Bastide et al., 1994). Bastide et al. cultured their myocytes under very high serum conditions (10% FBS + 10% HS), while Kwak et al. maintained their myocytes in 5% FBS (Kwak et al., 1995). Perhaps under the very high serum conditions used by Bastide et al., PKC was maximally stimulated, rendering PMA treatment ineffective in mediating any response.

(b) Turnover of Cx43

Initial studies of Cx43 degradation were done in cultured cardiomyocytes, where it was determined by pulse-chase labeling with [^{35}S]-methionine that the half-life of Cx43 was only 1-2 hours (Laird et al., 1991). This is very rare as most membrane proteins

have half-lives that exceed 24 hours (Hare and Taylor, 1991). As Cx43 moves from the nucleus through the Golgi and to the cell membrane it undergoes phosphorylation steps (Laird et al., 1995; Puranam et al., 1993). A phosphorylated form of Cx43 that can be trapped in the Golgi by brefeldin-A treatment is relatively resistant to degradation (Laird et al., 1995). Thus, it appears that connexin degradation occurs when it is further phosphorylated and targeted to the cell membrane.

There are two proteolytic pathways that have been implicated in the degradation of Cx43. These are the lysosomal pathway and the proteasomal pathway. The lysosomal pathway requires endocytosis of membrane proteins and subsequent degradation of internalized material by the lysosome. Evidence for gap junction structures in lysosomes obtained through electron microscropy has been available for many years (Ginzberg and Gilula, 1979; Larsen et al., 1979; Severs et al., 1989). The proteasomal degradation pathway utilizes a multi-protein degradation complex that most commonly degrades cytosolic and nuclear proteins with short half-lives (reviewed in: Ciechanover, 1994). Proteins that are degraded by the proteasome are usually tagged with phosphorylation and/or polyubiquitin modifications to target them to the proteasome.

It was suggested that the short half-life of Cx43 in cultured cardiomyocytes was merely an artifact of the cell culture in which disaggregated myocytes are actively re-establishing GJIC (Beardslee et al., 1998). To test this, an ex-vivo model of a Langendorff perfused adult rat heart was used to establish the half-life of Cx43 in the multicellular ex-vivo rat heart. This system revealed that Cx43 still had a very short half-life (1.3 hours), even in the intact heart, comparable to that seen in cell culture models (Beardslee et al., 1998). The degradation of Cx43 in the adult rat heart was shown to

occur by both the ubiquitin-proteasomal pathway and the lysosomal pathway. Interestingly, blockade of the proteasomal pathway resulted in accumulation of Cx43 that was non-phosphorylated, while blockade of lysosomal degradation resulted in accumulation of phosphorylated Cx43. When assessed by confocal immunofluorescent microscopy, accumulated Cx43 resulting from blockade of either the lysosomal or proteasomal pathways was found at cell membrane sites. The accumulation of dephosphorylated Cx43 at the membrane indicates that either it underwent dephosphorylation in situ, or that phosphorylation of Cx43 is not required for channel assembly and insertion into the membranes of cardiomyocytes (Beardslee et al., 1998).

Since Cx43 has such a short half-life it can be appreciated that its degradation may play a functional role in the regulation of GJIC. In scenarios such as myocardial ischemia, where degradative pathways may not balance with synthetic pathways, dire consequences may ensue. A recent study of acute ischemia in the canine heart revealed that after a one hour period of ischemia induced by ligation of the left anterior descending coronary artery there was a heterogeneous decrease in the amount of Cx43 in the ventricular myocardium (Huang et al., 1999). Longer periods of ischemia led to more dramatic loss of Cx43 immunostaining as assessed by confocal immunofluorescence visualization. Reduced levels of immunodetectable Cx43 are found in human ventricles subjected to reversible ischemia induced by exercise (Guerrero et al., 1997) or in ischemic "hibernating" myocardium, viable tissue with impaired contractile function that can be returned to a healthy state with surgical intervention (coronary bypass surgery) (Kaprielian et al., 1998). Animal models with decreased Cx43 and studies on ischemic and infarcted hearts strongly imply that decreased Cx43 content predisposes the heart to

arrhythmias, and it has been suggested that pharmacological interventions that could alter Cx43 degradation might be beneficial in the prevention of lethal cardiac arrhythmias (Severs, 2000).

Supporting the possibility for chemical regulation of connexins by altering their degradation, a study by Musil et al. indicates that blockade of the proteasomal degradation pathway, but not the lysosomal pathway, or blockade of protein synthesis can be used to not only alter gap junction quantity, but also function (Musil et al., 2000). Inhibitors of proteasomal degradation and of protein synthesis caused a striking increase in the size and number of immunodetectable gap junctions with a concomitant increase in GJIC assessed by dye-coupling analysis. Although initially counter-intuitive, the authors suggest that the reason protein synthesis blockade results in an accumulation of gap junctions and increased GJIC is due to the inhibition of the synthesis of an accessory protein that is necessary for the degradation of Cx43 channels. Thus, even under conditions of reduced Cx43 production, the half-life of Cx43 increases so that an overall accumulation of Cx43 at the membrane is achieved.

4. Heterotypic and Heteromeric Channels

Theoretically, an individual connexon could be composed of different connexin molecules (a heteromeric connexon) and connexons composed of different connexins can be envisioned as being able to dock to form heterotypic channels. With only two different connexins an incredible number of combinations are possible: 14 different heteromeric connexons would allow for 196 different channel configurations. It appears that this additional level of gap junction complexity does not only exist in theory, but also in experimental systems and *in vivo*.

The first evidence indicating that connexons composed of different connexins

could form functional channels was in experiments utilizing paired Xenopus oocytes (Swenson et al., 1989; Werner et al., 1989). These experiments revealed that endogenous Xenopus connexin (Cx38) could form functional heterotypic channels with exogenously expressed Cx43 and that exogenously expressed Cx43 and Cx32 could also form functional hybrid channels. Similar experiments with exogenous Cx32 and Cx26 showed that these two connexins are also capable of forming heterotypic channels in the Xenopus system, although these channels had properties different from the expected properties of the individual constituent connexons in homotypic configurations (Barrio et al., 1991). Specifically, while homotypic channels composed of either Cx26 or Cx32 responded symmetrically to transjunctional voltages of either sign, heterotypic channels responded in an asymmetric manner with reduction in junctional conductance with relative positivity on the Cx26 side of the channel, but no change with transjunctional voltage of opposite polarity. Not all connexins are able to pair to form functional channels in the Xenopus system, as oocytes expressing Cx50 are incapable of forming functional channels with oocytes expressing Cx43 (White et al., 1994).

Although the Xenopus model proved very useful in determining compatibility of different connexons in the formation of heterotypic channels, a mammalian model in which connexins could undergo post-translational processing and reside in a more functionally relevant intracellular milieu was required. To this end, transfected HeLa cells have been one of the most utilized models, serving as a mammalian analogue of the Xenopus system.

The main reason for using HeLa cells is that they are generally accepted as being deficient in GJIC with little to no detectable connexin expression (Eckert et al., 1993),

although a recent report indicates that HeLa cells are actually a heterogeneous population of connexin-expressing and connexin-deficient cells (King et al., 2000). Nonetheless, HeLa cells stably transfected with different connexin isoforms served as one of the first models to illustrate that heterotypic channels can indeed form between mammalian cells and that there is a discrimination between different connexon types in functional channel formation (Elfgang et al., 1995). The unusual properties noted for Cx26/Cx32 heterotypic channels described in Xenopus oocytes were also seen in paired HeLa cells expressing one of each connexin subtype (Bukauskas et al., 1995).

The presence of heterotypic and heteromeric gap junctional channels is now thought to be common, with heterotypic channels occurring between cells that express different connexins, and heteromeric connexons found in cells that express more than one connexin. Examples of heterotypic channels have been found in the following tissues: i) epithelial cells of the ciliary body of the eye (Wolosin et al., 1997); ii) mammalian glial cells (Zahs, 1998); iii) rat vascular smooth muscle cells (Li and Simard, 1999); iv) cochlear supporting cells of the ear (Zhao and Santos-Sacchi, 2000). Heteromeric connexons have been found in these tissues: i) rat vascular smooth muscle cells (He et al., 1999; Li and Simard, 1999); ii) multiple tissues of the eye (Jiang and Goodenough, 1996; Vaney and Weiler, 2000); iii) guinea pig liver (Diez et al., 1999). Interestingly, heteromeric channels do not necessarily attain graded degrees of function, with an averaging of properties of each of the component connexins; rather, it appears that single connexins can be dominant with respect to the function of the channel. For instance, wild-type Cx32 channels and mutant Cx32 channels that are more responsive to intracellular acidification take on the properties of the wild-type Cx32 when assembled in

heteromeric channels (Wang and Peracchia, 1998).

Since many tissues and individual cells express more than one type of connexin, an incredible number of channel configurations appear to be possible; this implies the presence of exquisite control of intercellular communication.

5. Non-Junctional Hemi-channels:

Typically, gap junctional channels are conceptualized as requiring two hemi-channels docked together, but there is increasing evidence to support the notion that some connexins can form functional hemi-channels in cell-surface membranes without docking with another connexon. Studies utilizing *Xenopus* oocytes have revealed that endogenous Cx38 (Ebihara, 1996), and exogenously expressed Cx46 or Cx56 can form functional, voltage-gated hemi-channels in non-junctional membranes (Ebihara et al., 1995; Ebihara and Steiner, 1993; Paul et al., 1991; Trexler et al., 1996). Measurement of hemi-channel properties using traditional patch-clamp techniques in the *Xenopus* oocyte model indicate that the functional properties of hemi-channels composed of Cx46 or Cx56 are very similar whether they are in apposition with other hemi-channels or not, and that whole channel conductance and permeability properties of Cx46 channels can be explained by adding the properties of two hemi-channels in series (Ebihara et al., 1995; Verselis et al., 2000). Thus, for some connexins, hemi-channel analysis through traditional patch-clamp techniques can provide similar information about single channel properties as is obtained from the much more technically demanding dual-cell patch-clamp techniques. These results present a possible alternate function for connexins: the gated transport of small molecules across non-junctional cellular membranes.

Voltage gated non-junctional hemi-channels have also been described in mammalian HeLa cells expressing connexins 30, 46 or 50 (Valiunas and Weingart,

2000). Although closed under physiological conditions, these hemi-channels opened when the resting membrane potential and/or the extracellular calcium concentration was reduced. A possible physiologically relevant function for non-junctional hemi-channels composed of Cx43 has been suggested by a recent study utilizing a variety of mammalian cell types. Cell types that express endogenous Cx43, but not those lacking or deficient in expressed connexins, increased in volume when external calcium was reduced within a physiologically relevant range (Quist et al., 2000). Thus, non-junctional hemi-channels may play a role in the regulation of cell volume.

In the heart, hemi-gap junctional plaques have been observed through the use of atomic force microscopy, although it was unclear whether they were endogenous to the preparation examined or were a result of the gap junction isolation procedure (Lal et al., 1995). More recent evidence appears to support the idea of nonjunctional hemi-channels in rat myocytes which were shown to open under conditions of low calcium or metabolic inhibition such as ischemia (John et al., 1999). These findings provide a novel mechanism for the calcium paradox in which cardiomyocytes subjected to low calcium and then returned to physiological calcium show severely compromised viability. Gap junctions have been suggested to play a role in the calcium paradox before, but the previous study did not consider the presence of non-junctional hemi-channels (Diederichs, 1995). The opening of gap junctional hemi-channels through metabolic inhibition has also been proposed to be part of the mechanism for the genesis of ischemia-induced arrhythmias due to altered ionic fluxes (Kondo et al., 2000).

C. GAP JUNCTION FUNCTION

Gap junctions play important roles in both excitable and non-excitable tissues. In excitable tissues, a major role of gap junctions is to serve as low resistance pathways for

the electrotonic propagation of action potentials between cells. Additionally, in most cell types, excitable or not, gap junctions serve to allow for what is known as metabolic coupling, the interchange of small metabolites and second messengers between cells (Gilula et al., 1972).

1. Ionic Coupling in Excitable Tissues

Ionic coupling through gap junctions occurs only in those cells that are electrically excitable such as smooth muscle cells, cardiac myocytes, and neurons. In smooth muscle and cardiac myocytes, gap junctions allow the coordination of contractions (recently reviewed in: Brink et al., 2000; Severs, 2000). In neurons, gap junctions may serve as a novel way of integrating electric signals, permitting non-synaptic transmission of information between coupled cells (recently reviewed in: Reuss and Unsicker, 1998; Rozental et al., 2000).

2. Metabolic Coupling

The metabolic coupling of cells through gap junctions has been implicated in several important processes related to the development of multicellular organisms and the control of cellular growth and proliferation.

a) Role of Gap Junctions in Development / Differentiation

Communication through gap junctions appears to be necessary in key developmental processes. The importance of connexins in differentiation and development has been underscored by gene knock-out and overexpression studies of specific connexins, including knockout models of Cx43, Cx46, Cx37, Cx40 and Cx26. In all of these experiments, serious developmental defects, some causing embryonic lethality, were observed (White and Paul, 1999).

b) Selectivity of Gap Junctions

Originally, gap junctions were thought to function simply as non-specific aqueous pores bridging cells, so the diversity of the connexin family was hard to explain (Beyer et al., 1990). Now we know that different connexins have different properties and that the specific expression of different types of connexins relates to their function, i.e. ionic and metabolic coupling between cells. Since connexins have different permeabilities to different molecules, specialized cells may require specific connexin family members in order to maintain the proper flow of metabolites and other small molecules between them.

Selectivity for different small permeants, of varying charge and shape, between gap junctional channels composed of different connexin isoforms is apparent, but is much less than the selectivity of ion-specific channels (Veenstra, 1996). Unlike what would be expected of a nonspecific aqueous pore, the differences in selectivity and permeability noted between channels composed of different connexins expressed in Xenopus ooocytes occur independently of the channel conductance (Veenstra et al., 1995). Thus, the channel pore diameter does not define channel conductance; rather, electrostatic interactions between permeants and the channel pore are thought to play a role in conductance, permeability and selectivity. These findings are not only applicable to connexins expressed in Xenopus oocytes, but have been reproduced in transfected HeLa cells where it was determined that the size of permeants alone was not sufficient to predict their permeability through different types of gap junction channels (Cao et al., 1998; Elfgang et al., 1995).

Is gap junction selectivity physiologically relevant? In order to answer this

question, studies have been conducted to determine if actual cellular metabolites showed any differences in their permeability through gap junctions composed of different connexins. In a liposomal system containing reconstituted connexin channels either homomeric for Cx32 or heteromeric for both Cx32 and Cx26, clear connexin-dependent differences in the permeability of radio-labeled cyclic nucleotides and uncharged maltose polymers were reported (Bevans et al., 1998). The permeability of HeLa cells transfected with connexins 26, 32, or 43 to the second messenger inositol 1,4,5-trisphosphate (IP_3) has also been shown to vary depending on the expressed connexin isoform, with Cx32 channels being more permeable than either Cx26 or Cx43 channels (Niessen et al., 2000). Hepatocytes from Cx32 knock-out mice (retaining only Cx26) reinforce the HeLa cell data since these cells, compared to wild-type hepatocytes, are less able to transmit calcium waves between coupled cells upon IP_3 injection, and require 25 times more IP_3 to induce propagation of calcium waves between doublet cells (Niessen and Willecke, 2000).

A new method has been developed to study the transfer of endogenous cellular metabolites between coupled cells that screens naturally occuring permeants in an unbiased manner. This technique involves labeling a donor population of cells with a fluorescent dye and loading these cells with glucose that has all 6 of its carbons radiolabeled. Donor cells are mixed with an excess of unlabeled acceptor cells for a predetermined time to allow establishment of gap junctions that are chemically blocked with a reversible inhibitor (18α-carbenoxolone; ACO). The ACO is washed out of the system to allow GJIC for a short time period. Then the cells are separated using a fluorescence activated cell sorter and the radiolabeled metabolic contents of donor and

acceptor cells are assessed by HPLC and TLC. These studies have shown that Cx32 and Cx43 channels in transfected C6 cells, transfer endogenous metabolites such as ATP and ADP at significantly different rates (Cx43 transfers nucleotides 10 times faster than Cx32 channels) (Goldberg et al., 1999; Nicholson et al., 2000). Different metabolites had different permeabilities through the Cx32 and Cx43 channels that could not be predicted from the size and charge of the metabolites.

The experiments described above have shown that gap junctions composed of different connexins can discriminate between physiologically relevant molecules and imply that different connexins have different functions. Clear evidence for distinct functional roles for different connexins can be found in a "knock-in" mouse model in which the gene for Cx43 was replaced with either the gene for Cx32 (Cx43KICx32) or Cx40 (Cx43KICx40) (Plum et al., 2000). If the connexins are functionally redundant, and only serve as aqueous channels, replacement of one connexin type with another should have no functional consequences. The "knock-in" experiments indicate that this clearly is not the case. Both the Cx43KICx32 and Cx43KICx40 mice were viable, but demonstrated different phenotypes. The Cx43KICx32 mice had heart defects similar to, but less severe than, those of Cx43 knock-outs, while the Cx43KICx40 mice had no obvious cardiac phenotypic anomalies, although they showed a susceptibility to spontaneous arrhythmias. The Cx43KICx32 mice also had defective mammary glands and were unable to lactate, while the Cx43KICx40 mice had normal mammary function. Suprisingly, both the Cx43KICx32 and Cx43KICx40 males were sterile (Plum et al., 2000). Thus, connexin isoforms serve highly specific roles in the development and function of specialized tissues and cannot be used interchangeably.

c) Role of Gap Junctional Communication in Growth Control

It has long been postulated that intercellular communication through gap junctions is inversely correlated to the growth potential or tumorigenicity of cells. This idea originated from the discovery that many tumor cells and tumorigenic cell lines display decreased connexin expression and/or decreased intercellular communication (Loewenstein and Kanno, 1966). Conversely, overexpression of connexins in cancer cell lines has been found to reduce the growth and oncogenicity of these cells to varying degrees. Thus, a tumor suppressor role for the connexins has been proposed (Levine, 1993).

There are numerous examples in the literature in which the forced expression of connexins in a tumorigenic cell line has resulted in the reduction of the growth or tumorigenicity of the cells. Table 2 outlines cell lines tested, connexins transfected, and the effect on the growth or tumorigenicity of the cells. In the case of HeLa cells, growth and tumorigenicity could be decreased by transfection with Cx26, but not Cx40 or Cx43 (Mesnil et al., 1995), while in the case of C6 glioma cells Cx43 transfection could normalize growth but Cx32 transfection could not (Bond et al., 1994; Zhu et al., 1991). This again points to the specificity of connexin function and how connexins of different types cannot be assumed to impart the same result on the growth parameters of transfected cells.

(1) GJIC-independent Connexin effects on Growth

When attributing a tumor- suppressor role to the connexins, it has been assumed that the mechanism by which connexins are able to alter cellular growth is through increasing GJIC, and in many instances this appears to be the case. This may not be the

TABLE 2. Effect of transfected connexins on growth and tumorigenicity of several cell lines.

Cell Line	Cx(s)	Effect on Growth / Tumorigenicity
SkHep1 (human hepatoma cells)	Cx32	• Unchanged growth in culture • Suppression of tumorigenicity (Eghbali et al., 1991)
10T 1/2 (mouse fibroblasts)	Cx43	• Decreased growth rate in culture (same as normal mouse fibroblasts) • Decreased saturation density in cultures (Mehta et al., 1991)
MCA-10 (mouse sarcoma cells)	Cx43	• Decreased growth rate in culture • Suppression of tumorigenicity in nude mice (Rose et al., 1993)
TRMP (canine kidney epithelial cells)	Cx43	• Decreased growth rate in culture • Decreased tumorigenicity (Chen et al., 1995)
HeLa (human cervix cells)	Cx26, Cx40 & Cx43	• Only Cx26 reduced tumorigenicity and growth rate in culture (Mesnil et al., 1995)
C6 (rat glioma cells)	Cx43	• Decreased growth rate in culture • Decreased glioma formation *in vivo* • Co-culture of Cx43-transfected cells with wild-type C6 cells, reduced growth rate of wild-type cells (Naus et al., 1992; Zhu et al., 1991; Zhu et al., 1992)
C6	Cx32	• Unchanged growth in culture • Decreased growth *in vivo* (Bond et al., 1994)
MDA-MB-435 (human mammary epithelial cells)	Cx26 & Cx43	• Decreased growth rate in culture • Decreased tumorigenicity • Restored capacity for differentiation (Hirschi et al., 1996)
LNCaP (human prostate cancer cells)	Cx32 & Cx43	• Decreased growth rate in culture • Decreased tumorigenicity • Restored capacity for differentiation (Mehta et al., 1999)
Jeg-3 (Human trophoblast cells; choriocarcinoma)	Cx26, Cx40 &Cx43	• Decreased growth rate in culture • Cx26 transfectants displayed highest degree of restored differentiation (Hellmann et al., 1999)

only mechanism by which connexins can have an inhibitory effect on cellular growth though, as there are two studies that suggest otherwise: i) the tumorigenicity of a rat bladder carcinoma cell line (BC31) that is GJIC proficient, can be suppressed by forced expression of wild-type or communication-incompetent Cx43 (Krutovskikh et al., 2000); ii) the Cx43 mutant A253V inhibited the growth inhibitory function of wild-type Cx43 in transfected C6 glioma cells, even though it was capable of GJIC as assessed by dye transfer assays with the fluorescent dye Lucifer Yellow. These studies suggest that connexin molecules may regulate growth by a mechanism other than regulating GJIC, perhaps by interacting with signaling molecules of signal transduction pathways involved in growth processes.

In support of this notion, it has recently been shown that Cx43 can be upregulated by the Wnt signal transduction pathway in cardiomyocytes, and that it can bind the signaling molecule β-catenin (Ai et al., 2000). The Wnt pathway utilizes the multifunctional protein, β-catenin, originally described as a structural component of the adherens junction, as a transcriptional transactivator of certain target genes, of which Cx43 appears to be one (reviewed in: Behrens, 2000). Beta-catenin can only function as a transcriptional transactivator when it is allowed to accumulate in the cytosol (instead of being degraded by the proteasome) and then translocate to the nucleus (Behrens, 2000). Since Cx43 is able to bind β-catenin, it may sequester it from the cytoplasm, preventing transcription of Cx43 itself and other responsive genes, several of which are known to be involved in growth stimulation such as c-myc, cyclin D1, and c- jun (Behrens, 2000). Indeed, forced expression of Cx43 in cardiomyocytes reduced the expression of a reporter gene driven by β–catenin transactivation (Ai et al., 2000).

3. Gap Junction Defects in Human Disease

The importance of connexin diversity and the specificity of the function of individual connexin isoforms has been highlighted by the discovery of connexin gene mutations as being the cause of several human diseases (Krutovskikh and Yamasaki, 2000), including the following:

a) X-linked Charcot-Marie-Tooth Disease

The first disease to be directly linked to mutations in a connexin gene was X-linked Charcot-Marie-Tooth Disease (CMTX), which is a hereditary neuropathy with demyelination (Bergoffen et al., 1993). The affected connexin in this disease is Cx32. Over 160 different Cx32 gene mutations, linked to the genesis of CMTX, and affecting trafficking or channel properties, have been identified (reviewed in: Abrams et al., 2000).

b) Deafness

The first indication that a connexin was involved in some forms of deafness was when mutations in Cx26 were linked to hereditary non-syndromic deafness, implicating an important role for Cx26 in the function of the cochlea (Kelsell et al., 1997). Subsequently, mutations in two other beta connexins, Cx30 and Cx31, have also been associated with deafness, and the range of types of deafness associated with connexin defects has expanded to include recessive and dominant, and non-syndromic (with a single phenotype) and syndromic (with multiple phenotypes) types (Rabionet et al., 2000).

c) Cataracts

The prismatic fibers of the lens of the eye are interconnected by gap junctions which are the only means of maintaining correct ion and metabolite concentrations in these avascular cells (Goodenough, 1992). It is thus not suprising that defects in the

connexins involved in lens gap junction formation (Cx50 and Cx46) lead to the formation of cataracts, caused by improper homeostasis of the lens (Gong et al., 1997; Mackay et al., 1999; Pal et al., 2000; Shiels et al., 1998; Steele et al., 1998; White et al., 1998).

D. GAP JUNCTIONS IN THE HEART

1. Localization

In the mammalian myocardium, individual myocytes are coupled to their neighbors through numerous gap junctions. A typical canine myocyte has approximately 100 gap junctions on its surface and is coupled to an average of approximately 10 other cells (Hoyt et al., 1989; Luke and Saffitz, 1991). Gap junctions are found concentrated in intercalated disks, specialized structures involved in both the mechanical and electrical coupling of cardiomyocytes. Although intercalated disks and associated gap junctions occur predominantly at the ends of myocytes, they can be found anywhere along the length of a myocyte (Luke et al., 1989).

2. Function

A major function of gap junctions in the heart is to allow for unimpeded action potential propagation between myocytes. This allows the cells of the heart to be stimulated synchronously, an absolute requirement for the heart to work as a pump (Giros and Jongsma, 1996). As with all cells coupled with gap junctions though, GJIC also allows for homeostasis of small signaling molecules and metabolites between coupled cells.

3. Major Connexins in the Mammalian Myocardium

The myocytes of the mammalian heart express at least three different Cx isoforms, each with different patterns of expression throughout the development of the heart.

a) Connexin 40

(1) Localization

In rodents, Cx40 is found primarily in the vascular endothelium and in the specialized cells that make up the conduction tissue of the heart (Bastide et al., 1993; Beyer et al., 1992; Bruzzone et al., 1993; Hennemann et al., 1992b). Specifically, Cx40 of rat myocytes is found in the His bundle, the bundle branches and Purkinje fibers, where it is thought to mediate fast conduction; it is not found in myocytes of the working ventricular myocardium (Davis et al., 1994; Gros et al., 1994). Connexin 40 is not able to form heterotypic channels with Cx43 in paired Xenopus oocytes (Bruzzone et al., 1993), but recently, heterotypic channels have been suggested to occur between HeLa cells expressing these connexins (Valiunas et al., 2000).

(2) Function

Evidence as to the function of Cx40 has come from analysis of mutations associated with human heart disease and from the generation of a Cx40-null mouse. A gene defect causing dysfunctional atrioventricular conduction, reduced contractility and progressive cardiomyopathy was found to map to a small region of human chromosome 1, where Cx40 resides (Kass et al., 1994). Mice lacking Cx40 have defects in atrioventricular conduction and are predisposed to arrhythmias (Kirchhoff et al., 1998; Simon et al., 1998). Thus, Cx40 is an important component of the conduction tissue of the heart necessary for fast propagation of action potentials from nodal tissue to the ventricular myocardium. A detailed follow-up study on the morphology of hearts from Cx40-null mice indicated that approximately 50% of these mice died during embryogenesis during septation of the heart, 16% died shortly after birth and the remaining viable mice had anatomic defects in the atrio-ventricular junction and/or

interventricular septum (Kirchhoff et al., 2000), indicating a role for Cx40 in cardiac development.

b) Connexin 45

(1) Localization

Studies looking at Cx45 in the heart sustained a major setback when it was discovered that antibodies that were assumed to be specific for Cx45, actually cross-reacted with Cx43 (Coppen et al., 1998). Several studies that used antibodies directed against residues 285-298 of canine Cx45, shown by Coppen et al. (Coppen et al., 1998) to contain a region of homology to Cx43 resulting in cross-reactivity of these antibodies, have to be re-interpreted (Chen et al., 1994; Darrow et al., 1995; Davis et al., 1994; Davis et al., 1995; Kanter et al., 1993a; Kanter et al., 1993b; Kanter et al., 1992). An affinity-purified antiserum raised against residues 354-367 of human Cx45, with no cross-reactivity with Cx43, revealed that the distribution of Cx45 in mouse and rat hearts was localized to very discrete regions largely overlapping with Cx40-expressing cells of the conduction system (Coppen et al., 1998). Further studies indicate that Cx45 is actually the connexin isoform most continuously associated with the conduction tissue of the rodent heart, as it is located in peripheral regions of the conduction network that are Cx40 negative, where merging of Cx45 and Cx43 staining occurs (Coppen et al., 1999a; Coppen et al., 1999b). These results explain why Cx40 null mice did not show a more drastic alteration in phenotype, as Cx45 can most likely compensate for most of Cx40's function.

(2) Function

The importance of Cx45 in the development of the rodent heart has recently been revealed in a mouse model in which Cx45 was replaced by a nls-lacZ gene by the Cre-

loxP system. Mice deficient in Cx45 died from heart failure at embryonic day 10 due to conduction block and an endocardial cushion defect (Kumai et al., 2000). In the heart, the first synthesized connexin molecule appears to be Cx45 according to RNA and protein expression analysis in mice (Alcolea et al., 1999). It appears that this connexin isoform is required for the coordination of the first heart beats of the embryonic mouse, as no other connexin isoforms are detectable at this stage of mouse development. During development, Cx45 is down-regulated and it becomes undetectable by immuno-fluorescence after birth of the mouse, except in some regions of the interventricular septum and a few small areas of the free ventricular wall (Alcolea et al., 1999).

c) Connexin 43

(1) Localization

Connexin 43 is the predominant connexin of the myocardium and is found between the working myocytes of the atria and ventricles (Beyer et al., 1987; Davis et al., 1994; Laird and Revel, 1990; van Kempen et al., 1991). It is not found in the His bundle or proximal regions of the bundle branches of the rat heart, but is found in more distal regions where it colocalizes with Cx45 at the periphery of conduction tissue (Coppen et al., 1999a; Coppen et al., 1999b; Gros et al., 1994).

(2) Function

As the connexin which directly links the cardiomyocytes of the heart, Cx43 functions to synchronize the contractions of the myocardium. Different transgenic mice have been created to assess Cx43 function and are described below:

(a) Cx43-null mice

Mice lacking Cx43 are able to survive to term, but die shortly after birth due to defects in the development of the pulmonary outflow tract of the heart (Reaume et al.,

1995). Similar defects occur using a dominant negative approach (Sullivan et al., 1998). Thus, although Cx43 is expressed in many tissues, there must be functional redundancy that permits the proper development of the majority of mouse organs in the absence of Cx43.

(b) Overexpression of Cx43

Mice overexpressing Cx43 under the regulation of the constitutively active cytomegaloviral (CMV) promoter, exhibit both heart and neural tube defects (Ewart et al., 1997). Defects of the heart conotruncus were explained as occurring due to disruption of developmental processes involving neural crest cells, as subpopulations of these cells are the target of CMV-directed transcription. Follow up studies have also indicated a role for Cx43 in the regulation of neural crest cell migration and survival during development (Bannerman et al., 2000; Huang et al., 1998a; Huang et al., 1998b; Lo et al., 1997; Lo et al., 1999; Sullivan et al., 1998; Waldo et al., 1999). These experiments imply that Cx43 is required for proper neural crest cell function during development and that the level of Cx43 expressed in neural crest cells must be precisely regulated for proper development to ensue.

(c) Cx43 +/- heterozygous model

Deletion of one allele of Cx43 led to a reduction in the propagation of action potentials through the ventricular myocardium with a decrease in ventricular epicardial conduction of 44% compared to Cx43 +/+ controls in adult mice (Guerrero et al., 1997). Even more dramatic results were obtained when the hearts from Cx43 +/- mice were subjected to ischemia in an isolated perfused heart setup. Compared to ischemic Cx43 +/+ hearts, ischemic Cx43 +/- hearts developed more arrhythmias and developed them more quickly (Lerner et al., 2000). These results suggest that the level of Cx43 expressed

in mouse hearts is a critical parameter of cardiac action potential conduction, with more Cx43 giving rise to more stable impulse propagation and less Cx43 predisposing hearts to arrhythmias due to ischemic insult, possibly due to disruption of limiting numbers of functional Cx43 gap junctions.

E. FIBROBLAST GROWTH FACTORS

1. The Fibroblast Growth Factor Family

The fibroblast growth factors (FGFs) comprise a family of structurally related heparin binding polypeptides (reviewed in: Galzie et al., 1997). A recent scan of the Genbank database reveals that this family contains at least 23 different members. Most signaling initiated by extracellular FGFs occurs through their binding to high affinity transmembrane FGF receptors (FGFR) of the tyrosine kinase type (RTK) (Jaye et al., 1992), with subsequent activation of intracellular signal transduction pathways. The FGF activation of RTKs is modulated by interactions with heparan sulfate proteoglycans (HSP) which can be found in the extracellular matrix, embedded in the cell membrane or strongly associated with the exterior surface of cells (McKeehan et al., 1998). The HSPs have been suggested to stabilize FGF-FGFR interactions to promote signaling and to serve as extracellular storage "sinks" for FGF family members. The FGFs play roles in cell growth, survival, differentiation and migration for numerous cell types in many different tissues (Galzie et al., 1997).

2. FGF-2

One of the prototypical FGFs that has been extensively studied and characterized is FGF-2 (a.k.a. basic fibroblast growth factor; bFGF) (Bikfalvi et al., 1997; Nugent and Iozzo, 2000; Okada-Ban et al., 2000). Using heparin affinity chromatography, FGF-2 was initially purified from bovine pituitary extracts as a 13.3 kDa polypeptide (a

proteolytic degradation product of an 18 kDa form) that was a strong mitogen for 3T3 fibroblasts (Gospodarowicz, 1975). In the human, FGF-2 is now known to exist as at least five different isoforms, which arise due to alternative translation from four CUG start codons upstream of the conventional AUG start codon (Okada-Ban et al., 2000). Thus, in humans, there are 18, 22, 22.5, 24 and 34 kDa isoforms of FGF-2. The isoforms larger than 18 kDa all possess a nuclear localization signal, which targets them to the nucleus where they are involved in intracrine signaling (Delrieu, 2000). The high molecular weight forms of FGF-2 have also been shown to be exported from the cell and can act as ligands for FGFR-mediated regulation of cell proliferation and migration (Piotrowicz et al., 1999).

Most secreted proteins contain a signal peptide sequence that targets them through the ER and Golgi apparatus and out of the cell (Rothman and Orci, 1992). The 18 kDa isoform of FGF-2 does not contain such a signal sequence but can be exported from the cell through an alternative energy-dependent system that is ER/Golgi-independent (Florkiewicz et al., 1995). Sublethal and lethal injury of cellular membranes, and mechanical strain caused by stretching have also been implicated in the release of FGF-2 from cells (Cheng et al., 1997; Cheng et al., 1996; Ku and D'Amore, 1995; McNeil et al., 1989), including a reported beat-to-beat release of FGF-2 in cardiomyocytes (Clarke et al., 1995; Kaye et al., 1996a).

a) Receptor Mediated FGF-2 Signal Transduction Pathway

The signal transduction pathway for FGF-2 is initiated by binding of FGF-2 to a FGFR. There are four different FGF tyrosine kinase receptors that are structurally related (Partanen et al., 1993). The FGFRs consist of the following domains listed in order from

the extracellular amino terminus: i) two or three tandem extracellular immunoglobulin (Ig)-like domains (determined by alternative splicing affecting the third Ig domain); ii) an acidic box, unique to FGFRs between the first two Ig domains; iii) a single transmembrane domain; iv) a cytoplasmic juxtamembrane domain; and v) a cytoplasmic tyrosine kinase domain split by a kinase insert (Johnson and Williams, 1993). With the aid of heparan sulfate proteoglycans that are thought to either facilitate or stabilize ligand-receptor interactions (Ornitz, 2000), FGF-bound receptors dimerize and undergo a process of autophosphorylation in which the cytosolic tyrosine kinase domains of both receptors phosphorylate each other (Jaye et al., 1992).

Activated FGFRs are able to recruit and phosphorylate a set of signaling molecules that mediate the cellular responses to FGF-2 stimulation. For FGFR1, at least the following signaling molecules can be activated upon receptor dimerization and autophosphorylation: i) phospholipase C-γ (PLC-γ); ii) Src ; iii) FGF receptor substrate 2 (FRS2); iv) Grb2; v) Phosphatidylinositol-3'-kinase; vi) SH2 domain-containing phosphotyrosine phosphatase 2 (SHP-2); vii) focal adhesion kinase; viii) Nck; ix) Shb; and x) Shc (reviewed in: Klint and Claesson-Welsh, 1999). Modulation of the availability of these mediators of FGF-2 signaling and their downstream targets determines the end biological effect of FGF-2 stimulation.

With respect to cell proliferation, FRS2, Shc, PLC-γ and Src are all potential contributors to FGFR1-mediated mitogenesis (Klint and Claesson-Welsh, 1999). The molecules Shc and FRS2 serve as adaptor proteins, which upon tyrosine phosphorylation by an FGFR bind another adaptor protein, Grb2, that is complexed to the nucleotide exchange factor Sos (Kouhara et al., 1997; Rozakis-Adcock et al., 1992). Sos catalyzes

GDP/GTP exchange on the small G-protein Ras which causes Ras to be activated (Pawson, 1995). Activated Ras then binds to and activates the serine/threonine kinase Raf that initiates a cascade of phosphorylation events resulting in MAP kinase activation (Lewis et al., 1998). Receptor-mediated tyrosine phosphorylation of PLC-γ results in the hydrolysis of phosphatidylinositol 4, 5-bisphosphate to inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). The resultant IP_3 causes calcium release from the ER/sarcoplasmic reticulum while DAG serves to activate members of the PKC family (Bell et al., 1986; Berridge, 1993).

3. FGF-2 in the heart

FGF-2 is found in the heart and is expressed by several cell types including both neonatal and adult cardiomyocytes (Casscells et al., 1990; Kardami and Fandrich, 1989; Kardami et al., 1990; Speir et al., 1989). FGF-2 can be detected by immunofluorescence in nuclear, cytoplasmic and extracellular locations of cardiomyocytes (Kardami et al., 1991; Kardami et al., 1990). There is a developmental shift from a high molecular weight (21.5-22 kDa) isoform of FGF-2 in embryonic and neonatal rat hearts to a low molecular weight isoform (18 kDa) in adult hearts (Liu et al., 1993a). Cardiomyocytes have been identified as expressing both high and low affinity FGF receptors. The high affinity FGF receptor expressed in neonatal and adult heart myocytes is FGFR1 and different splice variants have been identified, which are developmentally regulated (Jin et al., 1994; Kardami et al., 1995; Liu et al., 1995; Speir et al., 1992). Cultured embryonic, neonatal and even adult cardiomyocytes can respond to FGF-2 with increased DNA synthesis (Pasumarthi et al., 1994; Pasumarthi et al., 1996; Speir et al., 1992). Both MAP kinase and PKC signaling are activated by FGF-2 treatment of cardiomyocytes (Bogoyevitch et al., 1994; Disatnik et al., 1995; Padua et al., 1998).

Numerous functions have been ascribed to FGF-2 in the heart including: i) cardioprotection from ischemia-reperfusion injury (Cuevas et al., 1997; Cuevas et al., 2000; Cuevas et al., 1999; Padua et al., 1998; Padua et al., 1995); ii) angiogenesis (reviewed in: Battegay, 1995; Goncalves, 1998; Slavin, 1995); iii) hyperplasia / hypertrophy (Armstrong et al., 2000; Hudlicka and Brown, 1996; Kaye et al., 1996b; Parker, 1995; Parker et al., 1991; Schneider et al., 1992; Schultz et al., 1999); and iv) reduced death of myocytes after myocardial infarction (Hasegawa et al., 1999; Horrigan et al., 1999; Miyataka et al., 1998; Sasame et al., 1999; Yanagisawa-Miwa et al., 1992).

F. PROTEIN KINASE C

1. Protein Kinase C Family

Currently, there are at least ten different isoforms identified in the protein kinase C (PKC) family of serine/threonine protein kinases (for a recent review see Liu and Heckman, 1998). The PKCs transduce signals mediated by phospholipid hydrolysis that occurs subsequent to activation of G-protein- or tyrosine kinase- coupled receptors or activation of non-receptor tyrosine kinases. There are three main classes of PKCs based on differences in structure and co-factor requirements. The conventional or calcium-dependent PKCs (cPKC), α, βI, βII, and γ are regulated by diacylglycerol (DAG), Ca^{2+} and phosphatidylserine (PS). The novel PKCs (nPKC), δ, ε, θ and η are regulated by DAG, PS and unsaturated fatty acids but not Ca^{2+}, as they lack a Ca^{2+} binding domain. The atypical PKCs (aPKC), ζ, λ and ι are activated by PS, unsaturated fatty acids and phosphoinositides, but are unaffected by Ca^{2+} or DAG. The λ and ι isoforms are species variants of the same isoenzyme.

The PKCs are composed of a single polypeptide with an amino-terminal regulatory region and a carboxy terminal catalytic region. The regulatory region of all

three classes of PKCs contains a stretch of amino acids known as the "pseudosubstrate" which is bound by the substrate acceptor portion of the catalytic region, maintaining the enzyme in an inactive state (Newton, 1995; Newton, 1997).

a) The PKC Signal Transduction Pathway

It is widely accepted that when inactive, PKCs reside in various locations throughout the cytosol and can be isolated from cells without the use of detergents. The generation of DAG by phospholipase C or indirectly through phospholipase D, recruits conventional or novel PKCs to membranes where specific binding between the enzyme and both DAG and PS causes a conformational change that releases the pseudosubstrate from the catalytic domain of the PKC (Newton, 1995; Newton, 1997). Released from its autoinhibition, the PKC enzyme is free to phosphorylate its true target substrates. The binding between PKC and membrane components upon activation is so strong that it becomes necessary to use detergents to isolate the active form from cells. The difference in solubility between non-active and active forms of PKC, so called translocation of PKC to membranes, has been used extensively by investigators to follow PKC isoform activation profiles (Kraft and Anderson, 1983; Kraft et al., 1982; Wolf et al., 1985). Although translocation of PKC isoforms to membrane compartments is generally thought to correspond to their activation, other factors such as the phosphorylation state of PKC can present exceptions to this rule (Lee et al., 2000).

How different PKC isoforms are able to preferentially act on specific substrates is thought to be mediated through the positioning of the isoforms near their targets via PKC binding proteins. Several types of PKC binding proteins have been identified including the receptors for activated C kinase (RACKS), the perinuclear binding protein PICK1,

and the A-kinase anchoring proteins (AKAPS) which have been found to act as scaffold proteins for several different enzymes including PKC (Colledge and Scott, 1999; Mochly-Rosen, 1995; Mochly-Rosen and Gordon, 1998; Staudinger et al., 1997; Staudinger et al., 1995).

2. PKCs in the heart

The rabbit heart expresses all of the PKC isoforms although at different levels and with different responses to stimuli (Ping et al., 1997): i) The cPKCs are more abundant than the nPKCs, with PKCα being the most abundant cPKC and PKCε being the most abundant nPKC; ii) In response to a preconditioning protocol, only PKCε and PKCη were translocated from the cytosolic to the particulate fraction. Neonatal rat heart myocytes contain at least the following PKC isoforms: α, βI, βII, δ, ε, and ζ (Disatnik et al., 1994).

3. PKC epsilon

The epsilon isoform of PKC is a major isoform found in the adult mammalian heart (Bogoyevitch et al., 1993). Analysis of protein levels of PKCε in developing rat hearts indicates that although expressed at the highest levels in neonatal heart preparations it is still found in relatively high levels in the adult heart (Clerk et al., 1995; Rybin and Steinberg, 1994). Further assessment of PKCε levels on a per myocyte basis indicated that, although total heart PKCε levels are reduced in adult compared to neonatal hearts (<25%), the levels of PKCε per myocyte are actually higher in adult myocytes (10X), although activated levels (in particulate fractions) are less (Clerk et al., 1995). Cultured neonatal rat cardiomyocytes express PKCε that is highly responsive to phorbol-12-myristate-13-acetate (PMA) treatment as assessed by translocation from cytosolic to

particulate fractions (Clerk et al., 1995; Puceat et al., 1994).

The relatively high levels of PKCε in the heart have prompted many investigators to decipher its physiological function in this organ. Numerous studies now implicate PKCε as a mediator of cardioprotection derived from ischemic preconditioning for the following reasons: i) ischemic preconditioning can be blocked by treatment with inhibitors of PKC, thus implicating these enzymes in the process (Armstrong et al., 1996); ii) PKCε is activated during the initial ischemic rounds of the ischemia-reperfusion protocol (Kawamura et al., 1998; Ping et al., 1997; Yoshida et al., 1997); iii) specific activation of PKCε and PKCδ can mimic ischemic preconditioning in an assay utilizing isolated rabbit myocytes (Armstrong and Ganote, 1994); iv) specific blockade of PKCε using an inhibitory peptide can block the cardioprotective effects of hypoxic preconditioning in a cell culture model (Gray et al., 1997); v) specific activation of PKCε using an engineered peptide enhances cardioprotection against prolonged ischemia (Dorn et al., 1999).

The mechanism by which PKCε acts to protect the heart from ischemic damage is unclear, but may involve the regulation of mitochondrial K(ATP) channels. Treatment of hearts with the drug diazoxide, a mitoK(ATP) channel opener, protects against ischemic heart injury, and appears to require PKC activity since the protection is blocked by PKC inhibitors (Wang and Ashraf, 1999; Wang et al., 1999). Specifically, PKC isoforms delta, epsilon and alpha were activated by diazoxide, although the localization of activated isoforms differed, with PKCα translocating to the sarcolemma, PKCδ translocating to the intercalated disk and mitochondria and PKCε translocating only to the intercalated disk. Translocation of activated PKCε to the intercalated disk region of

cardiomyocytes has also been documented by other investigators and PKCε-specific RACKs are found to reside in cross striations and intercalated disks of cardiomyocytes (Disatnik et al., 1994; Mochly-Rosen et al., 2000). PKCε is not only implicated in cardioprotection induced by ischemic preconditioning, but has also been linked to cardioprotection conferred by growth factors and other agents such as ethanol (Chen et al., 1999a; Miyamae et al., 1998; Padua et al., 1998).

Another function for PKCε in the heart is suggested to be the mediation of the development of compensatory hypertrophy. Transgenic overexpression of constitutively active PKCε is found to cause mild concentric hypertrophy that does not alter normal cardiac function (Takeishi et al., 2000). Increased activation of PKCε in a model of cardiac hypertrophy, due to the overexpressed G-protein Galpha(q), enhanced contractile function of the hypertrophic heart, while inhibition of PKCε activation led to decompensated lethal dilated hypertrophy (Wu et al., 2000). A constitutive role for PKCε in the regulation of myocardial growth in physiological hearts is also suggested by experiments from the same laboratory (Mochly-Rosen et al., 2000). Considering that in other cell systems PKCε is a potent activator of cell replication and is even considered an oncogene for some cell types (Cacace et al., 1993), perhaps it is not suprising that PKCε should have a growth regulatory role for cardiomyocytes.

G. RATIONALE AND HYPOTHESES

From the preceding review of the literature it is clear that gap junctions play a role in the regulation of cellular growth in many experimental systems. We have seen that a general inverse correlation between GJIC and cellular growth exists in many models. Connexins, perhaps independently of their role in gap junctional channels, have been

suggested to function as tumor suppressors. Several growth factors have been shown to affect gap junction and connexin properties. These changes include increased phosphorylation of connexins on tyrosine or serine residues, often with concomitant changes in the function of the gap junctions containing the modified connexins.

The fibroblast growth factors, specifically FGF-2, have been shown to play a role in the regulation of hypertrophic and hyperplastic growth of the rat heart. The signaling pathway for FGF-2 includes activation of both the MAPK and PKC pathways. Both of these enzymes have been implicated in the phosphorylation of Cx43, the primary connexin of the mammalium myocardium. Several consensus phosphorylation sites for both PKC and MAPK are found on the carboxy terminus of Cx43, including the MAPK phosphorylation site S262.

Based on these key findings, the following general hypothesis and specific hypotheses became the foundation of this thesis:

GENERAL HYPOTHESIS: FGF-2, an endogenous cardiac growth factor, will affect GJ properties. A corollary to this hypothesis is that modulation of GJ function may be a component of the mechanism mediating the growth stimulatory effects of FGF-2.

SPECIFIC HYPOTHESES: i) FGF-2 signaling will decrease dye coupling between neonatal rat cardiomyocytes; ii) The MAPK and/or PKC pathways are involved in FGF-2 -mediated effects on cardiac gap junctions; iii) Growth-factor stimulated phosphorylation of specific serine(s) on Cx43 is required to cancel or decrease growth inhibition by Cx43.

II. MATERIALS AND METHODS

A. MATERIALS

1. General Chemicals and Supplies:

Chemicals were purchased from Sigma unless otherwise noted. Laboratory supplies were purchased either from VWR-Canlab Canada or Fisher Scientific Canada unless otherwise noted. The MEK1 inhibitor PD98059 was purchased from New England Biolabs. The PKC inhibitors, calphostin-C and chelerythrine, were purchased from Research Biochemicals International. The tyrosine kinase inhibitor, genistein was purchased from Calbiochem. Recombinant human FGF-2 was obtained from Upstate Biotechnology. Recombinant rat FGF-2 (rrFGF-2) was prepared as previously described (Padua et al., 1998).

2. Primary Antibodies:

Two rabbit polyclonal (Pc) antisera recognizing Cx43 were used. The first antiserum, raised against residues 346-363 of rat Cx43, was used in the experiments presented in Chapter One of the Results section and has been previously characterized (Berthoud et al., 1992; Yamamoto et al., 1990). The second antiserum was produced by Quality Controlled Biochemicals (QCB) by immunization of rabbits with an antigenic peptide (residues 368-382 of rat Cx43) conjugated to keyhole limpet hemocyanin. Five production bleeds were obtained from QCB, each producing antisera with a higher specificity and titre than the previous one. The antiserum derived from the fifth bleed was capable of detecting Cx43 bands in 2 μg of total protein lysate from neonatal rat cardiomyocytes when used at a dilution of 1:50 000 in Western blots. This bleed was used for all experiments requiring polyclonal anti-Cx43 antiserum in Chapters Two and Three of the Results section. A monoclonal (Mc) antibody raised against residues 252-

270 of rat Cx43 was purchased from Biodesign International (No. K22755, clone CON 11-2, lot #791) and was used for experiments in Chapter One of the Results section. This product was discontinued by Biodesign International, but was re-introduced into the market by Transduction Laboratories. The monoclonal anti-Cx43 antibody from Transduction Laboratories was used in Chapter Two of the Results section.

Polyclonal rabbit antibodies against PKCε and PKCα were purchased from Santa Cruz Biotechnology. A monoclonal antibody for PKCε was obtained from Transduction Laboratories. A rabbit polyclonal antibody recognizing dually phosphorylated MAP kinase (anti-ACTIVE MAPK) was purchased from Promega. A mouse monoclonal antibody recognizing BrdU was purchased from Amersham/Pharmacia Biotechnology. A monoclonal antibody against sarcomeric myosin (clone MF-20) was obtained from the Developmental Studies Hybridoma Bank at the University of Iowa.

3. Peptides:

Synthetic peptides consisting of residues 252-270 (GPLSPSKDCGSPKYAYFNG) or 261-270 (GSPKYAYFNG) of rat Cx43 were obtained from ImmunoDynamics Inc. A peptide composed of residues 252-260 (GPLSPSKDC) was purchased from Genemed Biotechnologies Inc. In experiments designed to use the peptides as competitive inhibitors of antibody-antigen binding, the peptides were used at a concentration of 10 μg / mL.

B. METHODS

1. Neonatal Rat Cardiomyocyte Culture Preparation:

Cell culture media, horse and chicken serum, trypsin and antibiotics were purchased from Life Technologies (formerly Gibco/BRL). Fetal bovine serum (FBS) was obtained from Hyclone. Cultureware was purchased from Corning. To collagen-

coat 35 mm dishes or 35 mm dishes containing autoclaved coverslips, 1 mL of rat tail collagen I (Upstate Biotechnology), dissolved in water to a final concentration of 0.25 mg/mL, was applied to each dish and was allowed to air dry overnight under ultraviolet illumination in a laminar flow hood.

Myocyte cultures were obtained from neonatal rat hearts that were disaggregated into single cells by limited tryptic proteolysis, according to previously published methods (Pasumarthi et al., 1996). Briefly, the ventricles from 36 Sprague-Dawley rat pups (1-2 days old) were minced in Ca^{2+}-free F-10 medium and then incubated in a water-jacketed spinner flask, maintained at 37 °C, for ten consecutive ten minute digestion periods in a 0.1% trypsin / F10 solution. Liberated cells were decanted off the remaining undigested tissue after each incubation period. After the final incubation, the remaining intact tissue was passed several times in and out of a 10 mL pipette to break it down into single cells.

To isolate the myocytes from the resultant heterogeneous mixture of cardiac cell types, the cells were applied to a discontinuous Percoll gradient which was capable of separating myocytes from non-myocytes based on their densities (Shubeita et al., 1992). Myocytes purified in this manner contained less than 5% contaminating cells and were plated out on collagen-coated tissue culture dishes at a density of 6-8 X 10^5 cells / 35 mm culture dish in F-10 medium containing 10% fetal bovine serum (FBS), 10% horse serum, and 1X penicillin / streptomycin (pen/strep). Cells were left in this medium overnight, after which they were rinsed three times with calcium- and magnesium-free phosphate buffered saline (CMF-PBS) solution to remove dead cells and debris and placed into new culture medium. Cells were maintained in a humidified incubator (37 °C, 5% CO_2).

Depending on the experiment in which the myocytes were used, different cell culture media were used to replace the initial plating medium. Low serum medium (LSM) consisting of DMEM/F12 medium containing 0.5% FBS, 20 nmol/L selenium, 10 μg / mL insulin, 10 μg / mL transferrin, 2 mg / mL bovine serum albumin, 20 μg / mL ascorbic acid and 1X pen/strep, was used for most experiments. Cells were maintained in this medium for 6-7 days, replacing the medium every 2 days. High serum medium (HSM) consisting of 10% FBS in DMEM and 1X pen/strep was used for myocytes that were to be transfected using the calcium phosphate precipitation method.

2. Embryonic Chicken Cardiomyocyte Culture:

All cell culture reagents were purchased from Life Technologies unless otherwise noted. Embryonic chicken cardiomyocytes were prepared as previously described (Pasumarthi et al., 1994). Briefly, hearts were dissected from 5 day old chicken embryos and ventricles were disaggregated into individual cells with an enzymatic solution consisting of 0.25 mg/mL trypsin, 0.025 mg/mL collagenase I (Sigma), 5.5 μg / mL pancreatin, and 0.4% chicken serum prepared in PBS. Ventricles from 12 dozen embryos were incubated in 10 mL of the enzyme mix at 37 °C for 8 minutes. The enzyme reaction was stopped by adding an equal volume of FBS. The ventricles were then washed three times with DMEM and were resuspended in 10 mL of DMEM containing 30 Units of bovine pancreatic deoxyribonuclease I (Sigma). The ventricles were incubated in this DNAse solution for 5 minutes at 37 °C. The ventricles were then passed in and out of a Pasteur pipette repeatedly until no aggregates of cells were visible with the naked eye. The resulting cell suspension was filtered through a nylon sieve material (Nytex), centrifuged in a benchtop swinging bucket centrifuge at 170 x g for 5 minutes and the

resulting pellet was resuspended in chicken myocyte growth medium (CMGM) consisting of 2% FBS / DMEM plus pen/strep. Cells were plated at a density of 7-8 X 10^5 so that they would be 70-80% confluent one day after plating and were maintained in CMGM.

3. FGF-2 , Phorbol Ester and Inhibitor Treatments

All treatments with FGF-2 used the growth factor at a final concentration of 10 ng / mL. The phorbol ester, phorbol 12-myristate, 13-acetate (PMA), was used at a final concentration of 100 nM. The tyrosine kinase inhibitor genistein was used at a final concentration of 20 μM in a pretreatment for 30 minutes prior to FGF-2 addition. The MEK1 inhibitor, PD98059, was used at a concentration of 50 μM in a pre-treatment period of 1 hour prior to FGF-2 addition. The PKC inhibitors chelerythrine and calphostin-C were both used at a final concentration of 1 μM and were applied to cells 15 minutes prior to FGF-2.

4. RNA Isolation and Northern Blotting:

RNA was isolated from control or FGF-2-treated myocytes using the acid phenol-guanidine isothiocyanate method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Total RNA (10 μg/lane) was run on a 1% agarose gel containing 0.22 M formaldehyde, transferred to a nylon membrane (Nytran®-Plus, Schleicher and Schuell) and probed with a 1.4 kb EcoRI cDNA fragment specific for Cx43 (Beyer et al., 1987). Scanning densitometry of autoradiograms from blots reprobed with a 700 bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe (GenBank/EMBL: M17851) were used to normalize loading. Probes were labeled with [^{32}P]-dCTP (DuPont) by the random oligonucleotide primer procedure (LifeTechnologies kit).

5. Preparation of Total Protein Extracts

Total protein was extracted from cardiomyocytes by lysing them in a buffer containing the strong ionic detergent sodium dodecyl sulfate (SDS) to solubilize all cytosolic and membrane-spanning proteins. Cells were rinsed with ice-cold PBS three times and then lysed with SDS lysis buffer (1% SDS, 50 mM Tris pH 8.0). Both the wash buffer and the lysis buffer were supplemented with a phosphatase and protease inhibitor cocktail (PPIC) consisting of the following: 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, and 2 μg/mL each of leupeptin, pepstatin, aprotinin and E-64. Lysates were scraped off the culture plates, transferred to microcentrifuge tubes and then boiled for 5 minutes. To shear DNA that can interfere with SDS-polyacrylamide gel electrophoresis (SDS-PAGE), the lysates were sonicated with three 5 second pulses with a probe sonicator set at the maximum output that did not cause excessive frothing of the lysate. Lysates were then clarified with a ten minute centrifugation at 14 000 rpm in a benchtop microfuge at room temperature. The supernatants, containing all SDS-soluble cellular proteins, were then prepared for SDS-PAGE by mixing them with a concentrated SDS-PAGE sample buffer (1X SSB contains: 5% 2-mercaptoethanol, 62.5 mM Tris pH 6.8, 10% glycerol, 1% SDS, 0.01% bromophenol blue). An aliquot of the supernatant was also used to determine its protein concentration using the bicinchoninic acid assay (BCA) purchased from Pierce. The standard curve was generated with known amounts of BSA.

6. Immunoprecipitation of Tyrosine Phosphorylated Proteins

Total protein lysates prepared as above were diluted 1:10 with the following immunoprecipitation buffer (IP): 1% Triton-X-100, 0.5% Nonidet P-40 (NP-40), 10 mM Tris pH 7.4, 150 mM NaCl, plus PPIC. Aliquots containing 150 μg of total protein were

incubated with 25 μL of a slurry containing monoclonal anti-phosphotyrosine IgG conjugated to agarose beads (clone 4G10, Upstate Biotechnology Inc.) for one hour at 4 °C with continuous mixing in a rotary mixer. The immune complexes were then washed five times with IP buffer by centrifuging the samples briefly in a benchtop microfuge to pellet the agarose beads, removing the supernatant and resuspending the beads in IP buffer. Tyrosine phosphorylated proteins were eluted from the beads by boiling the beads in double-strength SSB and run on 10% SDS-polyacrylamide gels.

7. Western Blotting:

Proteins in SDS-polyacrylamide gels were transferred to Immobilon-P membrane (Millipore) by electroblotting. Membranes were blocked in 5% skim milk powder / PBS for 1 hour at room temperature to prevent non-specific binding of antibody to protein-free regions of the membrane. The various antibodies were diluted as follows, in 1% skim milk powder (SMP) / Tris buffered saline containing 0.1% Tween-20 (TBST): i) Pc Cx43, 1 : 20 000; ii) Pc PKCε and PKCα, 1 : 2000; iii) Anti-ACTIVE MAPK, 1 : 20 000. Blots were incubated with primary antibodies for 1 hour at room temperature and were then washed three times with 1% SMP / TBST. The secondary antibody, goat-anti-rabbit IgG conjugated to horseradish peroxidase (BioRad), diluted 1: 10 000 in 1% SMP / TBST was then incubated on the blots for another hour at room temperature. Blots were then washed at least five times with TBST and were processed for enhanced chemiluminescence (ECL) using the Pierce SuperSignal ECL kit and autoradiography.

8. Metabolic Labeling and Immunoprecipitation of Cx43

Metabolic labeling with aqueous [^{32}P]-orthophosphate (NEN) or [^{35}S]- cysteine /methionine (EasyTag™ EXPRE^{35}S^{35}S protein labeling mix from NEN) was carried out in phosphate-deficient or cysteine/methionine-deficient media (Sigma), respectively.

Both [^{32}P] and [^{35}S] labels were used at 100 μCi / mL unless otherwise noted. Incubation periods were 1.5 hours for the experiment on Cx43 protein synthesis in Chapter One of the Results section, 3 hours for all immunoprecipitation experiments requiring [^{32}P]-label and 4 hours for the protein labeling experiment in Chapter Three of the Results section. All treatments with growth factors or other agents were coordinated so that they ended at the end of the labeling period. Following the labeling period, cells were washed three times with ice-cold PBS containing PPIC. The cells were then either flash-frozen with liquid nitrogen and stored at –70°C until convenient to lyse them or were lysed immediately. Cells were lysed in 1% SDS, 50 mM Tris pH 8.0 (with PPIC), boiled 5 minutes, and diluted 1:10 with RIPA correction buffer resulting in a final lysate in RIPA buffer with the following composition: 1% Nonidet P-40, 0.1% SDS, 0.25% sodium deoxycholate, 150 mM NaCl, 10 mM Tris pH 8.0 plus PPIC. Lysates were passed through 24 gauge needles to shear DNA and centrifuged at 14 000 rpm in a benchtop microfuge. Protein content of the supernatants was determined using the BCA assay.

Aliquots containing 100 μg of total protein were incubated with 2 μL of Pc anti-Cx43 antibody at 4 °C with continuous mixing in a rotary mixer. To collect the antibody-antigen complexes, 100 μL of a 1:3 dilution of protein A–Sepharose beads (Pharmacia) in RIPA buffer were added, and the whole suspension was incubated for another hour at 4°C. Immunoprecipitated proteins, immobilized on the protein-A beads, were pelleted by brief centrifugation in a benchtop microfuge and washed four times with RIPA buffer through consecutive resuspensions and centrifugations in fresh buffer. Proteins were eluted from the beads by boiling in double-strength SSB and were run on 10% polyacrylamide gels.

Gels were stained with Coomassie Blue, dried and processed for autoradiography using a cassette with appropriate intensifying screen. In the case of the [^{35}S]-labeled proteins, gels were processed with the fluorographic agent Amplify (Amersham) according to the manufacturer's instructions, prior to drying the gel and processing for fluorography. Typical exposure times for both [^{35}S]- and [^{32}P]-labeled proteins were 24 - 48 hours.

9. Scrape-loading:

Scrape loading was carried out as described by el-Fouly (el-Fouly et al., 1987) with minor modifications. Confluent monolayers of control or treated myocytes were washed 3 times with warm (37 °C) calcium- and magnesium- free (CMF) PBS and were then covered with a pre-heated (37 °C) solution of 0.05% 6-carboxyfluorescein (6-CF) and 0.05% tetramethylrhodamine dextran dissolved in CMF-PBS. For each dish of cells, four parallel scrape lines were made through the monolayer with a sharp scalpel. Two minutes after scraping, the dye was removed, the plate was washed with PBS and the cells were fixed with ice-cold 1% paraformaldehyde in PBS (pH 7.4). Dye migration from primary loaded cells along the scrape line to adjacent cells beyond the scrape line was then assessed using epifluorescence microscopy.

10. Microinjection and Dye Transfer:

Myocyte microinjections were done essentially as previously described (Shubeita et al., 1992; Traub et al., 1994), with the help of a Narishige micromanipulator (Nikon), a Medical Systems (Greenvale NY) picoinjector, and a Nikon Diaphot microscope equipped with epifluorescence and phase-contrast capabilities. Myocytes were kept at 37 °C with a stage heater (Medical Systems) and the pH maintained at 7.4 by adding 15 mM HEPES to the medium. A 0.05% solution of 6-CF dissolved in 100 mM KCl, 5 mM KH_2PO_4, pH 7.2, was back-loaded into a glass micropipette pulled by a Sutter

Instrument Co. (Novato, CA) pipette puller set at heat=740, pull=90, voltage=120, time=250, pressure=600. Cells were injected for 30 msec with a pressure of 20 psi and then viewed with epifluorescence and low-light phase-contrast for 30 seconds. Cells that had filled with dye during this period were scored and placed, by comparing fluorescence and phase-contrast views, in three categories (Traub et al., 1994): (i) first order, primary cells, i.e. cells directly connected to the injected; (ii) second order, cells located immediately next to the primary cells; and (iii) third order, cells located next to the second-order group and distal from the first order group.

11. Immunofluorescence:

Myocytes were grown on collagen-coated glass coverslips in 35 mm dishes. Coverslips were rinsed three times with ice-cold CMF-PBS, fixed with 1% fresh paraformaldehyde/CMF-PBS for 15 minutes at 4 °C, rinsed three times with CMF-PBS, permeabilized with 0.1% Triton X-100 in CMF-PBS for 15 minutes at 4 °C and then rinsed thoroughly with CMF-PBS. Primary antibodies were diluted in 1% BSA / PBS and were used at the following dilutions : i) Pc rabbit anti-Cx43, 1: 2000; ii) Mc mouse anti-Cx43, 1:50; iii) Mc mouse anti-BrdU, undiluted; iv) Mc mouse anti-sarcomeric myosin (MF20), 1:100; v) Pc anti-PKCε, 1:200; vi) Mc anti-PKCε, 1: 100. After incubating with the primary antibody for either one hour at room temperature or overnight at 4 °C, coverslips were rinsed 3X with CMF-PBS, and then incubated with secondary antibodies (purified anti-mouse immunoglobulin (IgG) conjugated to Texas Red or purified anti-rabbit IgG conjugated to biotin (Amersham) for the monoclonal and polyclonal antibodies, respectively) diluted 1:25 in 1% BSA/PBS, for 1 hour at room temperature. Subsequently, monoclonal IgG-treated coverslips were rinsed and mounted, while rabbit IgG-treated coverslips required a further 1 hour incubation at room

temperature with streptavidin-fluorescein (Amersham Corp.) diluted 1:25 in 1% BSA/PBS. Coverslips were rinsed 5X with CMF-PBS, mounted on slides with ProLong antifade medium (Molecular Probes), and viewed with a Nikon Diaphot epifluorescence microscope, as described previously (Kardami and Fandrich, 1989).

In peptide blocking experiments, the primary antibody was preincubated for one hour at room temperature in 1% BSA / PBS containing synthetic peptides (0.01 mg/mL) before addition to coverslips.

12. Phosphoaminoacid analysis:

This procedure was performed as described previously (Apel et al., 1991; Boyle et al., 1991). Briefly, immunoprecipitated [^{32}P]-labelled Cx43 (from 150 μg total protein) was recovered from a gel slice after SDS-PAGE and autoradiography and was then hydrolysed with constant boiling HCl for 1 hour at 110 °C. The hydrolyzed samples were lyophilized and mixed with a solution of all unlabelled phosphoserine, phosphotyrosine, and phosphothreonine dissolved in pH 1.9 buffer (2.5% (vol/vol) formic acid (88% purity grade), 7.8% (vol/vol) glacial acetic acid). Samples were electrophoresed on cellulose thin-layer chromatography plates (Merck) with the pH 1.9 buffer in the first dimension for 45 minutes at 1000V, and in pH 3.5 buffer [0.5% (vol/vol) pyridine, 5% (vol/vol) glacial acetic acid] in the second dimension for 15 minutes at 1000V. Standards were visualized by ninhydrin staining and the plates were placed in a phosphorimager (Molecular Dynamics) cassette for 24 hours. The image from the phosphorimager was exported as a TIFF file which was filtered (3X3 median filter; to reduce background), cropped and labelled using the program NIH Image.

13. Determination of Cx43 protein synthesis:

Myocytes were labelled for 2 hours in methionine-depleted DMEM supplemented

with 100 μCi/mL EXPRE^{35}S^{35}S protein labelling mix (NEN) as previously described (Darrow et al., 1995). Treatment with 10 ng/mL FGF-2 was during the last 30 minutes of the labeling period. Total protein extracts were prepared as described above, and protein content was determined with the BCA assay. Samples containing 100 μg of total protein were immunoprecipitated with 2 μL of rabbit anti-Cx43 serum, followed by collection of antibody-antigen complexes with Protein-A-Sepharose. Precipitates were washed four times with RIPA buffer and then analyzed by SDS-PAGE, followed by gel staining with Coomassie blue, destaining, incubation in Amplify fluorographic reagent (Amersham) for 30 minutes, gel drying, and autoradiography for 48 hours. The density of bands was determined by scanning densitometry.

14. Cx43/PKCε Co-immunoprecipitation:

Myocytes maintained in 0.5% FBS / DMEM / F12 medium for 6 days were treated with 10 ng/mL rrFGF-2 or vehicle for 5 minutes at 37 °C. After rinsing with ice-cold PBS, cells were lysed in 500 μL ice-cold co-immunoprecipitation buffer (CIB): 1% NP-40, 10% glycerol, 50 mM Hepes pH 7.5, 100 mM NaCl with PPIC; and incubated for 30 minutes on ice. Cleared lysates (100 μg protein in 500 μL CIB), obtained after centrifugation at 14 000 rpm for 15 min, were used in immunoprecipitation with 2 μL of rabbit anti-Cx43 serum as described above. Immunoprecipitates were washed three times with CIB, eluted into 40 μL Laemmli sample buffer by boiling for 5 minutes, run on 10% polyacrylamide gels, transferred to PVDF membranes (Roche) by electroblotting and probed with anti-PKCε or anti-PKCα antibodies (1:2000 dilution for both). Blots were processed for enhanced chemiluminescence as described above.

15. Transfection of Rat Cardiomyocytes with Truncated PKCε (1-401):

We used a cDNA plasmid containing the regulatory domain of murine PKCε (1-401) in the pSVK3 vector. This plasmid construction was a gift from Dr. Geoffrey Cooper of the Dana-Farber Cancer Institute in Boston, MA and is described in full detail by Cai et al (Cai et al., 1997). Cardiomyocytes that had been plated out 1 day earlier were switched to fresh high serum medium (HSM), consisting of 10% FBS in DMEM, 3 hours prior to transfection. A modified calcium phosphate transfection method in which precipitates were only allowed to form for 1 minute was used (Jordan et al., 1996). Six micrograms of DNA were used to form a precipitate in a final volume of 350 μL that was added drop-wise to myocytes plated on a 35 mm dish containing 2 mL of HSM.

16. Transfection and Detection of a β-galactosidase Reporter Construct

The lacZ (β-galactosidase) gene from the reporter construct pCH110 (Amersham) was removed by digestion with HindIII and BamHI and inserted into the HindIII and BamHI sites of pcDNA3.1(+) (Invitrogen), putting it under the control of a CMV promoter. Six micrograms of this construct were transfected into myocytes on a 35 mm dish as described above. Expression of β-galactosidase activity was determined by staining cells, 48 hours after transfection, using the β-galactosidase staining kit from Roche, according to the manufacturer's instructions. The resulting blue-stained β-galactosidase-positive cells were observed by light microscopy and photographed.

17. Infection with Adenovirally-driven Dominant Negative PKCε and Dominant Negative Map Kinase Kinase-1 (MKK1):

A dominant negative mutant of PKCε (DN) was obtained through site- directed mutagenesis of the rabbit PKCε cDNA as described recently in detail by Ping et al (Ping et al., 1999). Similarly, a dominant negative mutant of MKK1 was also obtained through

site- directed mutagenesis by the same laboratory. We obtained the recombinant adenoviruses expressing the PKCε (DN) or MKK1 (DN) as gifts from the laboratory of Dr. PeiPei Ping of the University of Louisville and Jewish Hospital Heart and Lung Institute, Louisville, KY. Replication deficient adenoviruses were generated by cloning the corresponding cDNA into the E1 region of human adenoviral type 5 genomic DNA, and propagated in H293 cells transformed with E1 genes, as described (Ping et al., 1999). Neonatal cardiomyocytes maintained in culture for 6 days in LSM were infected with adenovirus expressing PKCε (DN) or MKK1 (DN) at a multiplicity of infection of 50. Infection with non-exogenous protein-expressing virus was used in control cultures.

18. Connexin43 mutagenesis:

The plasmid pBSM13-Cx43 (kindly provided by Dr. E. Beyer) was used as the template for PCR site-directed mutagenesis (Beyer et al., 1987). All mutant Cx43 plasmid constructions used in this study were prepared by Ms. Yan Jin from the laboratory of Dr. Peter Cattini, of the Dept. of Physiology at the University of Manitoba. The primers 5′-CGATCCTTACCACGCCACCACTGGCCCACTGAGCCCATCAAA AGACTGCGGagCTCCAAAATAC-3′ (S262A sense strand primer) and 5′CGATCCT TACCACGCCACCACTGGCCCACTGAGCCCATCAAAAGACTGCGGAgaTCCAA AATAC-3′ (S262D sense strand primer) were used in combination with the primer 5′CCATGCGAT TTTGCTCTGC GCTG TAG-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′-GTTTTGCTCGCTAGCTTGC TTGTTGTAATTGCGGCACGcGGAATT GTTTCTG-3′ (S297A antisense strand primer) and 5′CAGGCCGAGGCCTGCTGCTGGCGCGGC

TGCTGGCTCTGCTGGcAGGTCGTT GG-3′ (S364A antisense strand primer) were used in combination with the primer 5′-CGTTAAGGATCGCGTGAAGGGAAGAAG C-3′ (sense primer) to generate either (i) a 222 bp fragment with S297 converted to A, or (ii) a 427 bp fragment with S364 coverted to A; in the connexin43 cDNA, respectively. The PCR reactions were carried out as previously described (Jin et al., 1994). The PCR products were digested with XcmI/NheI for the S262A, S262D and S297A mutants or with XcmI/StuI for the S364A mutant. The digested PCR products were then subcloned into the XcmI/NheI or XcmI/StuI site of pBSM13 connexin43 vector to generate the pBSM13-Cx43/S262A, pBSM13-Cx43/S262D, pBSM13-Cx43/S297A and pBSM13-Cx43/S364A mutant constructs. The full length wild type and mutated connexin43 fragments (1391 bp) were released by EcoRV/XbaI and ligated into the EcoRV/XbaI site of the mammalian expression vector pcDNA3.1(+) (Invitrogen, Carlsbad, CA) for use in transfection experiments.

The sequences of each mutant were confirmed by the dideoxy method (*f*-mol sequencing Kit from Promega Corp., Madison, WI, USA). Mismatched nucleotides in the primers are indicated by lower case letters.

19. Densitometric Analysis of Cx43 Phosphorylation Isoforms in Western Blots of Transfected Cardiomyocytes:

The volume analysis tools of the software program, Molecular Analyst, that accompanies the BioRad scanning densitometer system, were used to determine the band intensities of Cx43 in Western blots. Intensities of individual bands P2 and P3 or pooled bands NP and P1 were determined. The relative contribution of each band (or pooled bands) to the total amount of Cx43 detected by Western blotting was determined by

dividing the individual band intensity values by the sum of all band values in a given lane and multiplying the quotient by 100%.

20. Immunoprecipitations from Metabolically Labeled Chicken Myocytes:

Transfected chicken myocytes were metabolically labeled with 250 μCi/mL [^{32}P]-orthophosphate for three hours and Cx43 immunoprecipitates were prepared as described above.

21. Transfection of Chicken and Rat Cardiomyocytes with Cx43 Constructs:

All transfections were done on cells that were 70-80% confluent that had been plated out the previous day in the maintenance medium appropriate for the cell type. Three hours prior to the transfections, the myocytes were rinsed with DMEM to remove dead cells and were placed in fresh growth medium (2% FBS/DMEM/1X penicillin/streptomycin for chicken cardiomyocytes; 10% FBS/DMEM/1X penicillin/streptomycin for rat cardiomyocytes). Cells were transfected with 6 μg / 35 mm plate of the different Cx43 constructs described above using a modification of the standard calcium phosphate transfection method (Jordan et al., 1996).

22. BrdU Labeling and Determination of BrdU Labeling Index in Transfected Rat Cardiomyocytes

Rat cardiomyocytes on collagen-coated coverslips were allowed to express transiently transfected genes for 48 hours, during which time they were maintained in 10% FBS/DMEM. The cells were then incubated with 15 mg / mL bromodeoxyuridine (Sigma) for a 6 hour labeling period at 37 °C. Subsequently, the cells were fixed and stained for BrdU as previously described (Pasumarthi et al., 1996). The cells were also immunostained for Cx43 and myosin as described above. Sixteen random fields from each of five coverslips were assessed. Nuclei were visualized by Hoechst 33342 staining

under UV fluorescence, BrdU incorporation was visualized by Texas Red fluorescence localized in nuclei, and myocytes were visualized by Texas Red fluorescence of MF-20. The labeling index was determined by dividing the number of BrdU positive myocyte nuclei by the total number of myocyte nuclei and multiplying the quotient by 100.

23. Statistical Analyses

Analysis of variance (ANOVA), followed by Fisher's protected least significant difference post hoc test, was used to evaluate the significance of the effect of FGF-2 on Cx43 mRNA levels and protein levels at various time points up to 24 hours after FGF-2 administration. No significant changes were seen ($P > 0.05$). ANOVA was also used to evaluate the statistical significance of changes in Cx43 phosphorylation (immunoprecipitated Cx43 from [^{32}P]-labeled cardiomyocytes) at several time points of incubation with FGF-2 ($P < 0.01$). An unpaired t-test was used to determine statistical significance of differences between cardiomyocyte [^{35}S]-Cx43 synthesis in the absence or presence of FGF-2 and between the number of cardiomyocytes loaded with 6-CF in the absence and presence of FGF-2. In the latter case, the t-test was done at three "distances" from the originally injected cell ($P < 0.01$, $P < 0.001$). Results are presented as the mean +/- the standard error of at least three separate experiments. These calculations were obtained by a Macintosh computer running StatView 4.0 (Abacus Concepts).

To determine the statistical significance of the difference in the relative amounts of PKC co-immunoprecipitated with Cx43, an unpaired Student's t-test was used. The same test was used to compare the relative degree of Cx43 phosphorylation in vector versus dominant negative PKCε –treated myocytes. Labeling indices for each group of Cx43-transfected cardiomyocytes (vector, Cx43 wild type, Cx43 S262A, and Cx43 S262D) were assessed by analysis of variance followed by the Student-Newman-Keuls

multiple comparisons post-hoc test using the Macintosh statistical program InStat (GraphPad Software, SanDiego, CA).

III. RESULTS

A. EFFECT OF FIBROBLAST GROWTH FACTOR-2 ON CARDIOMYOCYTE CX43

INTRODUCTION:

As reviewed in the Introduction, the regulation of gap junction function occurs at many levels including: i) connexin transcription / translation; ii) connexin trafficking; iii) connexin stability / degradation; and iv) connexin gating by voltage, pH, high intracellular Ca^{2+}, or post-translational modification such as phosphorylation (Bruzzone et al., 1996; Goodenough et al., 1996). In several different experimental systems, growth factors have been shown to regulate gap junctions through different mechanisms. Table 3 presents some of these growth factors, the systems in which they were tested, their reported effects on Cx43 gap junctions and suggested mechanisms by which they exert their effects.

These precedents linking gap junction function and growth factors in other systems prompted us to determine if a similar relationship between FGF-2, an endogenous growth factor of the heart, and Cx43, the major connexin of cardiac myocytes, might exist. Any effect of FGF-2 on gap junctions in the heart could have implications related to the function of cardiac gap junctions as mediators of intercellular action potential propagation (Gros and Jongsma, 1996). In addition, since gap junctions are associated with a growth regulatory function in many cell types (Yamasaki and Naus, 1996), FGF-2 regulation of cardiac gap junctions could also have relevance with respect to cardiac myocyte hyperplasia and / or hypertrophy.

Table 3: Effects of various growth factors on GJIC in several cell systems.

Growth Factor	Experimental System	Effect on Gap Junction Function and Mode of Action
EGF	Human Kidney Epithelial Cells	Increased GJIC possibly due to increased synthesis or transport of Cx43 to plaques (Vikhamar et al., 1998)
EGF	Rat Liver Epithelial Cells	Decreased GJIC due to Cx43 phosphorylation (Kanemitsu and Lau, 1993; Lau et al., 1992)
PDGF	Rat Liver Epithelial Cells	Decreased GJIC due, in part, to Cx43 phosphorylation (Hossain et al., 1998; Hossain et al., 1999a; Hossain et al., 1999b)
PDGF	C3H/10T1/2 Mouse Fibroblasts	Decreased GJIC due to Cx43 phosphorylation (Pelletier and Boynton, 1994)
IGF	Xenopus Oocytes	Decreased GJIC due to gating via ball and chain mechanism (Homma et al., 1998)
FGF-2	Mouse Osteoblastic Cell Line	Decreased GJIC due to decreased Cx43 mRNA and protein (Shiokawa-Sawada et al., 1997)
FGF-2	Rat cortical and Striatal Astroglial Cells	Decreased GJIC due to decreased Cx43 mRNA and protein (Reuss et al., 1998)
FGF-2	Bovine Microvascular Endothelial Cells	Increased GJIC due to increased Cx43 mRNA and protein (Pepper and Meda, 1992)
FGF-2	Rat Cardiac Fibroblasts and Human Skin Fibroblasts	Increased GJIC due to increased Cx43 mRNA and protein (Abdullah et al., 1999; Doble and Kardami, 1995)

Technical Considerations:

We maintained our cardiomyocyte cultures in low serum conditions for at least six days in order for the myocytes to develop a synchronously contracting confluent monolayer that has numerous gap junctions and is amenable to dye transfer studies (Oyamada et al., 1994). Cardiomyocytes maintained for this length of time contract more synchronously, have higher numbers of gap junctions and are better dye-coupled than cardiomyocytes in short term (2-4 day) culture (Oyamada et al., 1994). Cardiomyocytes were treated with 10 ng/mL FGF-2 in all experiments since this concentration is sufficient to fully activate signaling mediated by the high affinity FGF receptor in these cells as assessed by DNA synthesis studies (Kardami, 1990) and analysis of the generation of tyrosine-phosphorylated proteins (Padua et al., 1996).

1. Effect of FGF-2 on Metabolic Coupling

We used two techniques to determine the degree of intercellular communication between cultured cardiomyocytes: scrape-loading and microinjection of the fluorescent dye 6-carboxyfluorescein (6-CF).

a) Scrape Loading

Confluent monolayers of neonatal rat cardiomyocytes, maintained in low serum conditions for at least six days, were treated with 10 ng/mL FGF-2 for thirty minutes, with or without a thirty minute pre-treatment with the tyrosine kinase inhibitor, genistein. Cells bathed in a 6-CF solution in CMF-PBS were then scraped with a scalpel, incubated for two minutes, rinsed with PBS, fixed and immediately examined and photographed through an inverted microscope capable of detecting epifluorescence. Under these

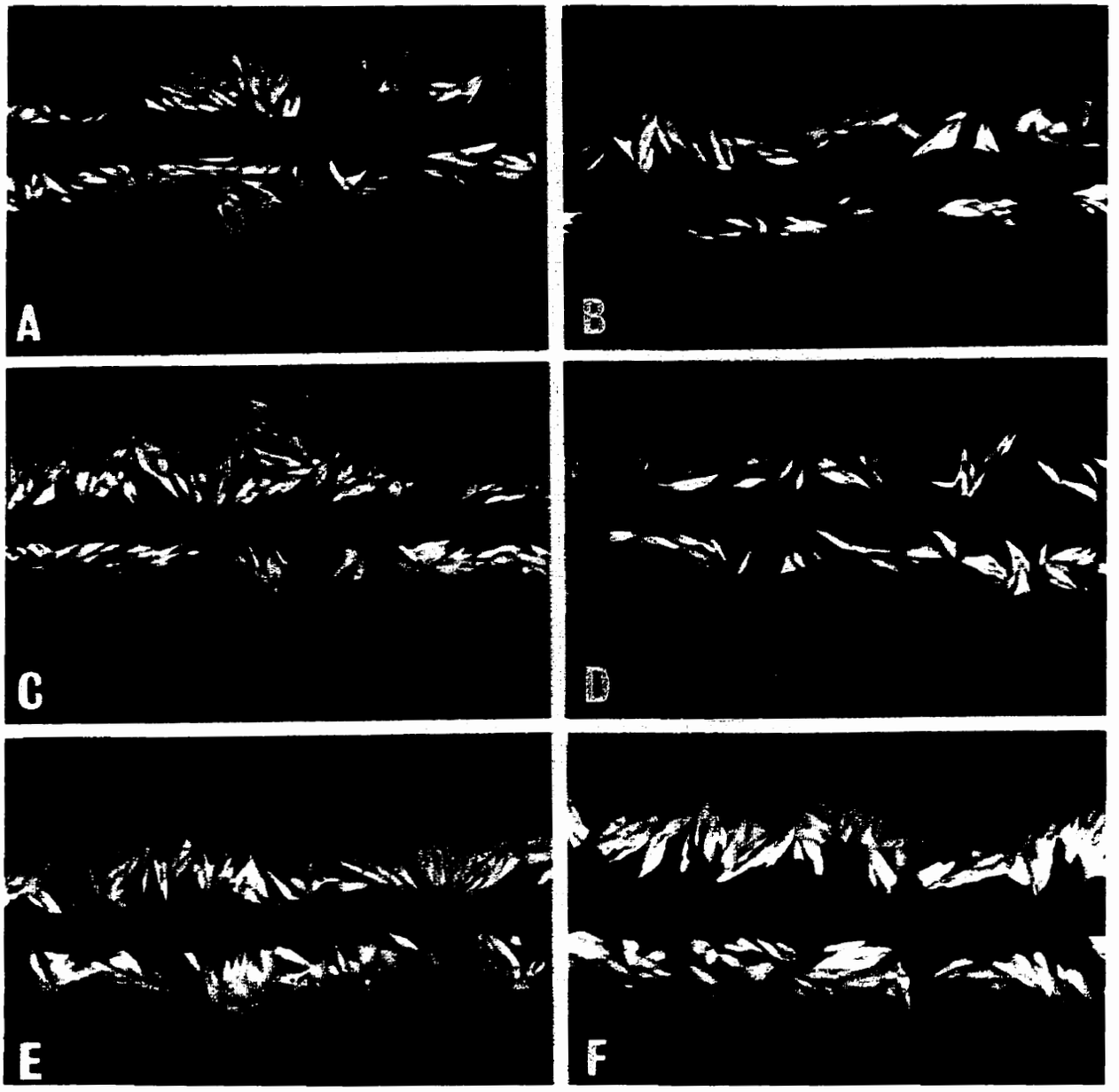
conditions, cells directly along the scrape line are able to take up 6-CF and transfer it to other more distal cells through gap junctions.

Untreated myocytes, Figure 3A and 3C, reveal extensive dye-coupling, as fluorescent dye can be seen extending through several orders of cells distal to the primary loaded cells along the scrape line. Similar results were seen with another fluorescent dye of similar size, Lucifer Yellow (LY). The effect of FGF-2 treatment on dye-coupling is seen in panels 3B and 3D. The extent of dye migration from the scrape line is clearly reduced in these cells compared to control cells (compare 3A and 3C to 3B and 3D). Note that the dye along the scrape line in FGF-2 treated cells appears to be more intense than that along the scrape line of control cells, indicating a reduction in the ability of the FGF-2 to diffuse from the primary cells to other coupled cells. These results with FGF-2 were seen consistently in repeated scrape-loading experiments (at least three repetitions).

The FGF-2-mediated reduction in dye-coupling could be blocked by a 30 minute pre-treatment with the tyrosine kinase inhibitor genistein (20 μM) (Figure 3E), indicating that activation of tyrosine kinase signaling, presumably through a FGF receptor tyrosine kinase, was required. Genistein pretreatment alone did not cause any change in intermyocyte dye coupling compared to that obtained under control conditions (compare Figure 3A and Figure 3F).

Figure 3. Effect of FGF-2 on intercellular communication between cardiomyocytes as assessed by scrape loading.

Myocytes were loaded for 2 minutes with a 0.05% solution of 6-CF. A and C, control myocytes, untreated with FGF-2. B and D, Myocytes treated with 10 ng/ml FGF-2 for 30 minutes before dye loading. E, Myocytes treated with 20 μmol/L genestein 30 minutes before loading. F, Myocytes treated with FGF-2 and genestein.



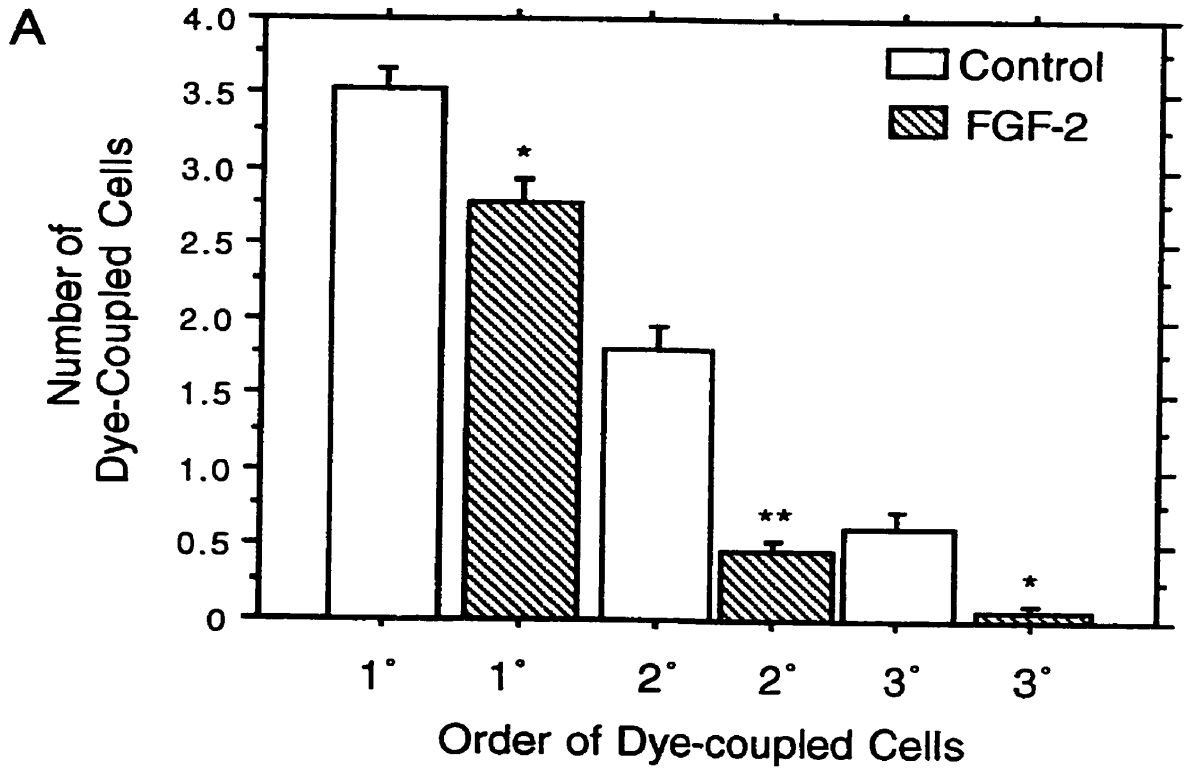
b) Microinjection

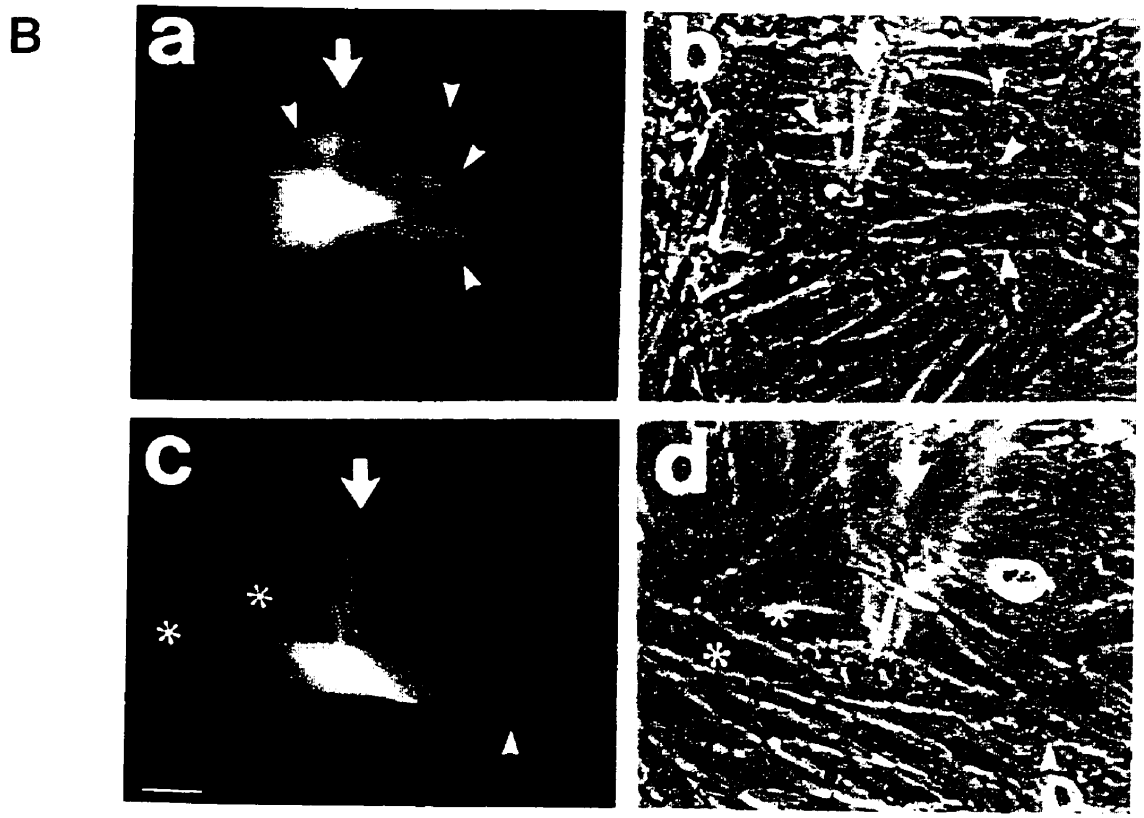
Intermyocyte dye coupling was also examined by microinjection of 6-CF and LY according to published procedures (Shubeita et al., 1992; Traub et al., 1994). Microinjection of either dye into cardiomyocytes resulted in migration of the dye from the injected cell to surrounding cells coupled to the injected cell with gap junctions. We scored the number of cells that took up the dye that were immediately adjacent to the injected cell (first order) as well as those more distal to the injected cell (second and third order). Myocytes were maintained under physiological culture conditions throughout the injection period.

Quantitative results from the microinjection experiments are presented in Figure 4A. In the presence of FGF-2, the dye did not diffuse as readily to coupled cells. Significantly reduced numbers of dye-coupled cells were seen at all distances from the primary injected cell, with a more marked decrease in the number of cells receiving dye in the second and third order cells. In total, dye was transferred to 6.0 ± 0.3 neighbor cells in control cultures compared with 3.3 ± 0.2 cells in FGF-2 treated cultures, indicating a significant ($p < 0.0001$, unpaired t-test) reduction in dye coupling induced by FGF-2. Similar results were seen with the dye LY. Figure 4B shows phase contrast and fluorescent microscopic photographs of typical injections with LY in the absence (Fig. 4B panels a and b) or presence of FGF-2 (Fig. 4B panels c and d).

Figure 4. Effect of FGF-2 on intermyocyte dye coupling as assessed by microinjection of fluorescent dye.

A, Control or FGF-2-treated (30 minutes) cardiomyocytes were injected with 6-CF and then viewed with epifluorescence and low-light phase-contrast optics for 30 seconds. Cells that had filled with dye during this period were scored and placed, by comparing fluorescence and phase-contrast views, into three categories: first order (1°), primary cells (i.e. cells directly connected to the injected cell); second order (2°), cells located immediately next to the primary loaded cells; and third order (3°), cells located next to the 2° group and distal from the 1° group. FGF-2 decreased the number of dye-loaded cells in all groups. Each group was assessed individually for statistical significance using an unpaired t-test (* $P < 0.001$, ** $P < 0.0001$, respectively). Sixty cells were injected under control conditions and 55 were injected under FGF-treatment conditions. Vertical bars denote SEM. B, Cardiomyocytes were microinjected with LY and then photographed within 30 seconds of dye injection. Panels Ba and Bb show identical fields from non-treated myocyte cultures viewed under epifluorescence and phase-contrast optics, respectively. Panels Bc and Bd show identical fields from FGF-2-treated myocyte cultures viewed under epifluorescence or phase-contrast optics, respectively. Arrows indicate the direction of the injection. Arrowheads denote myocytes adjacent to the LY-injected cell that receive the dye. Asterisks indicate myocytes adjacent to the injected cell that do not take up the dye. Bar = 30 μm.


A
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
Number of Dye-Coupled Cells
Control
FGF-2
*
**
*
1°
1°
2°
2°
3°
3°
Order of Dye-coupled Cells


B
a
b
c
d

2. Effect of FGF-2 on Cx43 Expression

To determine if FGF-2 affected Cx43 synthesis or accumulation / degradation, we used Western and Northern blotting techniques to assess steady-state Cx43 protein and mRNA levels, respectively. Levels of Cx43 mRNA were detected using a Cx43-specific radio-labeled probe that hybridized strongly to a 3.0 kb Cx43 transcript in blots containing electrophoretically separated total RNA (10 μg). A probe specific for a "housekeeping" gene, GAPDH, was used to normalize Cx43 levels between samples. Quantitative densitometric analysis of normalized Cx43 mRNA levels examined at times 0.5 to 24 hours post FGF-2 treatment is presented in figure 5A1 and a representative northern blot in which both GAPDH and Cx43 probes were used simultaneously, is presented in figure 5A2. No changes in Cx43 mRNA levels were seen upon FGF-2 treatment (n=3).

Relative Cx43 protein levels in extracts containing 10 μg of total protein from untreated or FGF-2 treated cardiomyocytes were analyzed by Western blotting with the polyclonal rabbit antibody raised against residues 346-363 of rat Cx43. Bands on autoradiograms resulting from chemiluminescent detection of Cx43 were quantified by scanning densitometry. Equal loading of protein samples was assessed by post-staining the ECL-processed blots with the protein-specific dye, amido black. Major protein bands detected in this manner showed less than 5% variation between lanes. Quantitative evaluation of Cx43 protein levels in cells treated for 0.5-24 hours with FGF-2 is presented in Figure 5B1. Representative blots indicating the Cx43 band pattern and relative levels are shown in Figure 5B2. No statistically significant changes in Cx43 protein levels were detected with FGF-2 treatment (n=3).

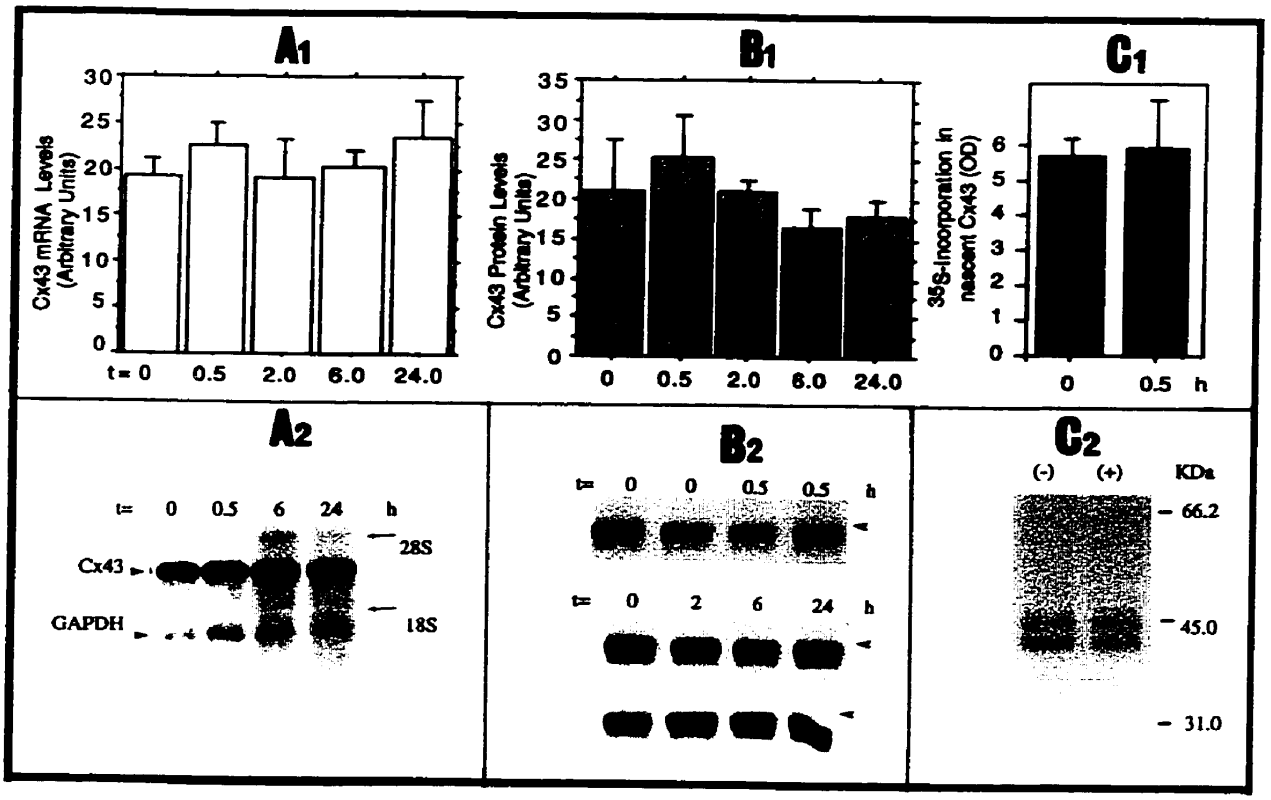
Levels of newly synthesized Cx43 were assessed by metabolic labeling with [^{35}S]-methionine/cysteine, immunoprecipitation of Cx43 with the polyclonal Cx43 antibody, SDS-PAGE and fluorography. Quantitative results indicating levels of Cx43 synthesized over a 2 hour labeling period ± FGF-2 treatment during the last 30 minutes of labeling are presented in Figure 5C1. A representative autoradiogram obtained by fluorography of a gel containing [^{35}S]-Cx43 is presented in Figure 5C2. No changes in Cx43 synthesis were apparent (n=3). Note that the newly synthesized Cx43 migrated with a mobility corresponding to relative molecular masses of 41 kDa and 43-45 kDa. This is expected as it has been documented that Cx43 is initially synthesized as a 41 kDa polypeptide that becomes phosphorylated during its transit through the ER and on its insertion into the plasma membrane (Laird et al., 1995; Musil et al., 1990; Musil and Goodenough, 1991; Musil and Goodenough, 1993).

3. Effect of FGF-2 on Cx43 Localization and Antibody Recognition

The change in the permeability of cardiomyocyte gap junctions could possibly be due to changes in the localization of Cx43, so we examined the localization of Cx43 in control and FGF-2 treated cardiomyocytes using immunofluorescent visualization with two anti-Cx43 antibodies. The polyclonal rabbit antiserum raised against residues 346-363 of rat Cx43 revealed strong punctate staining of gap junctions located primarily at the cell membrane interface between adjacent myocytes, in both control and FGF-2-treated cultures (Figures 6a and 6b, respectively). Some staining was also apparent in peri-

Figure 5. Effect of FGF-2 on cardiomyocyte Cx43 expression.

Cells were treated with 10 ng/mL FGF-2 for varying lengths of time, as indicated. Panels A1 and A2 illustrate the effect of FGF-2 on Cx43 mRNA accumulation. A1 shows cumulative data from three separate experiments. A2 is a representative Northern blot; arrows indicate migration of 28S and 18S RNA, and bands corresponding to Cx43 and GAPDH mRNAs are indicated. Panels B1 and B2 illustrate the effect of FGF-2 on Cx43 protein accumulation. B1 shows cumulative data from three separate experiments. B2 shows representative photographs of Western blots from two separate experiments, one (upper photograph) examining the effect of Cx43 protein at t=0 and 0.5 hours and the other (lower composite photograph) at times 0-24 hours. Arrowheads indicate where the 45 kDa protein ovalbumin migrates in these gels. Panels C1 and C2 illustrate the effect of FGF-2 on Cx43 protein synthesis with or without addition of FGF-2 during the last 30 minutes of a 2 hour labeling period with [^{35}S]-methionine. C1 shows cumulative data from three experiments. C2 is a representative autoradiogram showing SDS-PAGE analysis of immunoprecipitated [^{35}S]-Cx43 before (-) and after (+) FGF-2 treatments. [^{35}S]-Cx43 migrated as two major bands at 41 and 43 kD. Data in panels A1 and B1 were analyzed by ANOVA while those in C1 were analyzed by an unpaired t-test. Vertical bars in all graphs indicate SEM.


A1
Cx43 mRNA Levels (Arbitrary Units)
30
25
20
15
10
5
0
t= 0
0.5
2.0
6.0
24.0
B1
Cx43 Protein Levels (Arbitrary Units)
35
30
25
20
15
10
5
0
0
0.5
2.0
6.0
24.0
C1
35S-incorporation in nascent Cx43 (OD)
6
5
4
3
2
1
0
0
0.5
h
A2
t= 0 0.5 6 24 h
Cx43
GAPDH
28S
18S
B2
t= 0 0 0.5 0.5 h
t= 0 2 6 24 h
C2
(-)
(+)
KDa
66.2
45.0
31.0

nuclear locations. Staining with this antiserum was effectively competed by incubating the antibody with its immunizing peptide, indicating the specificity of this antibody (Berthoud et al., 1992; Yamamoto et al., 1990).

The monoclonal anti-Cx43 antibody also revealed a punctate staining pattern between adjacent cardiomyocytes in control cultures (Figure 6c). This staining pattern was very similar to that observed with the polyclonal antiserum (Figure 6b). Some perinuclear staining was also apparent with the monoclonal anti-Cx43 antibody. After 30 minutes of FGF-2 treatment, a dramatic decrease in immunofluorescent intensity at sites of intercellular contact was observed (compare Figure 6d with 6c). Since staining with the polyclonal antibody remained unchanged, loss of monoclonal antibody recognition indicated that FGF-2 caused masking of Cx43 epitopes.

4. The Epitope Recognized by the Monoclonal Anti-Cx43 Antibody Resides Between Residues 261-270

To characterize the epitope recognized by the monoclonal anti-Cx43 antibody, synthetic peptides consisting of the whole immunizing peptide (residues 252 to 270 of rat Cx43), fragments of this peptide consisting of residues 252-260 or 261-270, as well as an unrelated peptide containing residues 346-363 were used. Immunofluorescent signal due to staining with the monoclonal antibody under control conditions (Figure 7a) was competed by preabsorption with the synthetic peptides spanning residues 252-270 and 261-270 (Figure 7b and 7c, respectively). Immunofluorescent staining was not blocked by preincubation of the monoclonal antibody with peptides containing residues 252-260 nor 346-363 (Figure 7d and 7e, respectively). Thus, the epitope(s) recognized by the monoclonal anti-Cx43 antibody likely reside(s) between residues 261-270 of rat

Figure 6. Effect of FGF-2 on Cx43 localization in rat cardiac myocytes. Immunofluorescent staining of control myocytes (a and c) and FGF-2-treated myocytes (b and d), using a rabbit polyclonal anti-Cx43 antiserum (a and b) or a mouse monoclonal anti-Cx43 preparation (c and d). Curved arrows indicate gap junctional staining between cells. Although staining with the rabbit antibody remains unchanged, staining with the monoclonal anti-Cx43 antibody is reduced in FGF-2-treated myocytes. Bar is 50 μm.

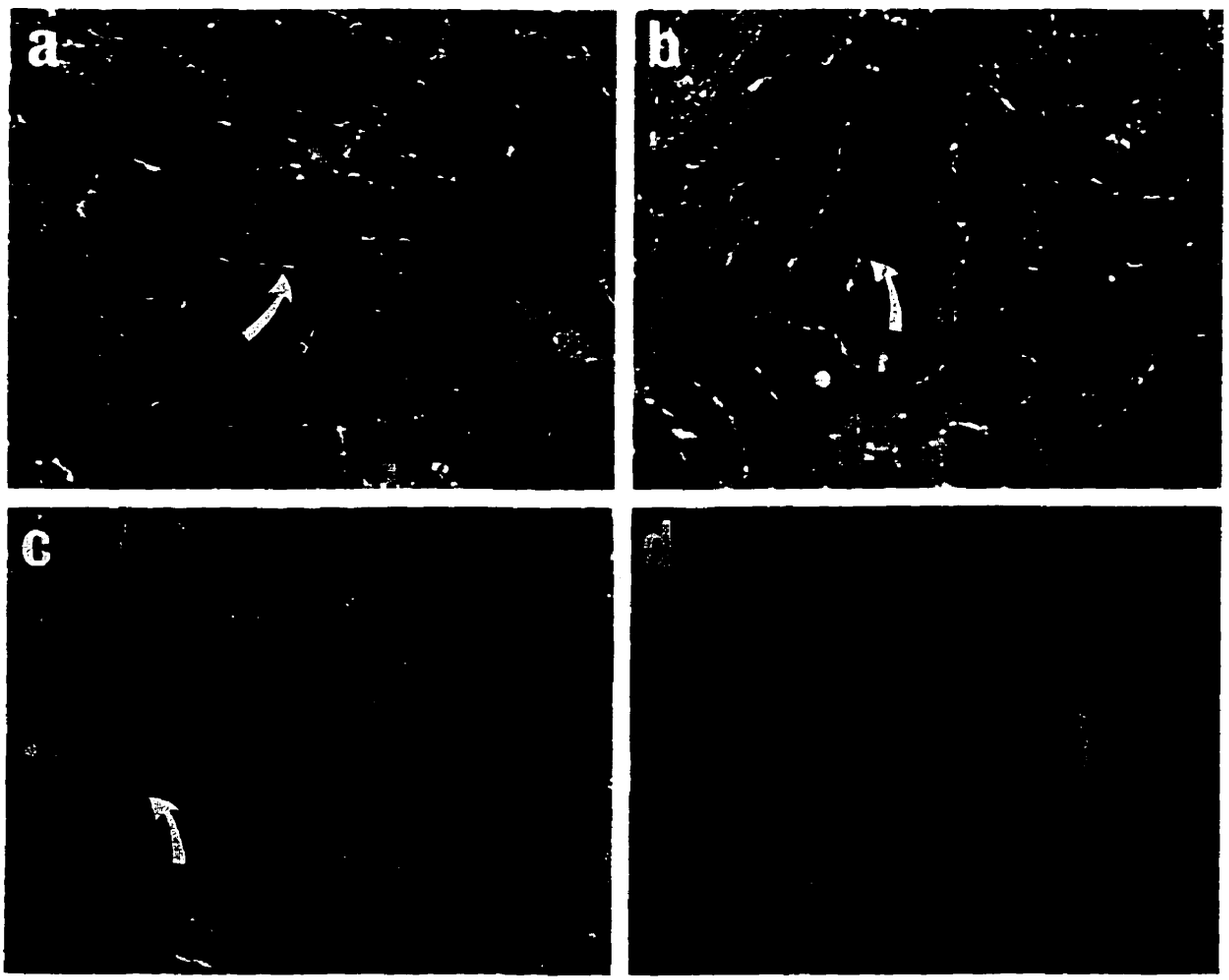
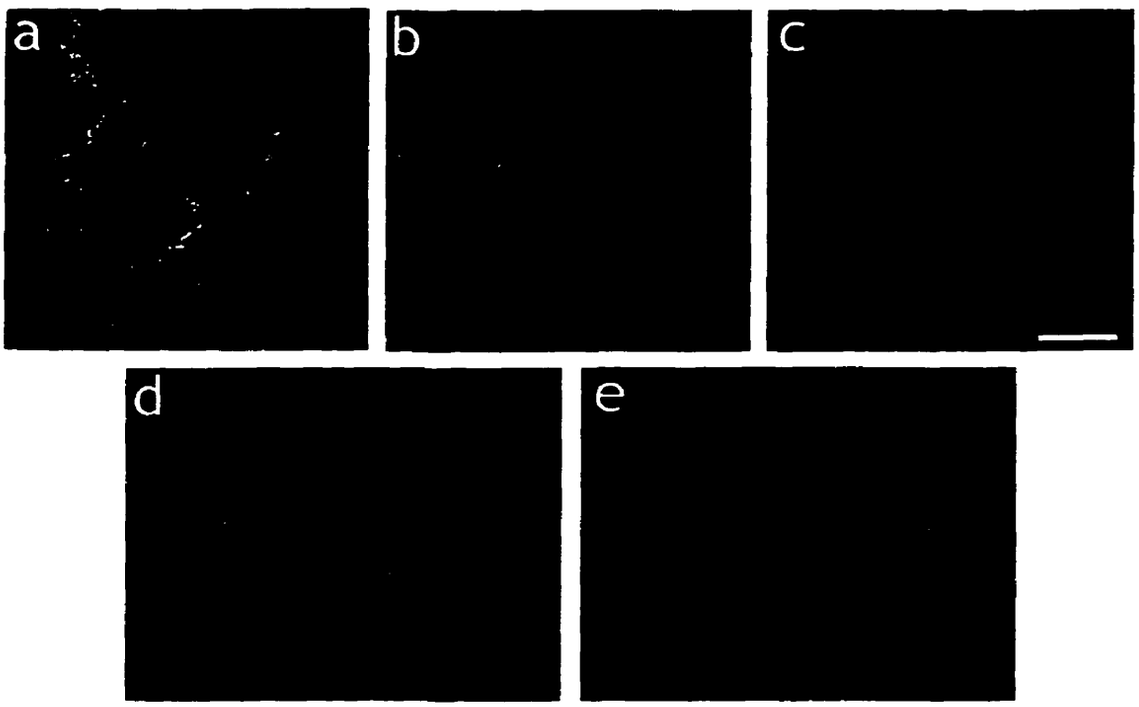


Figure 7. Blocking of the monoclonal anti-Cx43 antibody with synthetic peptides. Immunofluorescent staining of cardiac myocytes incubated with the monoclonal anti-Cx43 antibody in the absence (a) or presence (b-e) of synthetic peptides (0.01 mg / mL) spanning different regions of the carboxy terminus of rat Cx43. The peptides used are as follows: panel b, residues 252-270; panel c, residues 261-270; panel d, residues 252-260; panel e, residues 346-363. Staining was completely abolished by peptides 261-270 and 252-270 but was not affected by peptides 252-260 nor 346-363. Bar = 50 μm.



Cx43.

5. Effect of FGF-2 on Cardiomyocyte Cx43 Phosphorylation

As there were no changes in Cx43 localization or protein levels with FGF-2 treatment, yet there were still changes in GJ function, we tested if Cx43 phosphorylation, a post-translational modification that is known to affect gap junction channel properties (Bruzzone et al., 1996; Goodenough et al., 1996), was altered. We used metabolic labeling with [^{32}P]-orthophosphoric acid and immunoprecipitation of Cx43, followed by SDS-PAGE and autoradiography to test the effects of FGF-2 on Cx43 phosphorylation. Cardiomyocytes were labeled for a period of three hours at 37 °C with FGF-2 added for various lengths of time (2-30 minutes) with the end of the FGF-2 treatment synchronized with the end of the labeling period. A typical autoradiogram from one experiment is shown in Fig. 8A. Only a single diffuse band centered at 45 kDa is detected in each lane, indicating the specificity of the immunoprecipitation reaction for phosphorylated Cx43. Combined results from three separate experiments (Fig. 8B) reveal that at 30 minutes after FGF-2 addition the amount of phosphorylated Cx43 more than doubles upon FGF-2 treatment ($p < 0.01$). Therefore, FGF-2 triggers phosphorylation of Cx43. This phosphorylation could occur on serine, threonine or tyrosine residues.

6. Role of Tyrosine Phosphorylation in Mediating the Effects of FGF-2 on Cardiomyocyte Gap Junctions

The biological effects of FGF-2 are mediated by binding to tyrosine kinase receptors (FGFR) at the plasma membrane. Cardiac myocytes express FGFR at all developmental stages (Kardami et al., 1995; Liu et al., 1995). Ligand binding activates

the tyrosine kinase activity of FGFR, initiating a cascade of tyrosine phosphorylation events affecting numerous cellular proteins (Kardami et al., 1995; Padua et al., 1996).

Since the effects of FGF-2 on dye-coupling were blocked by the tyrosine kinase inhibitor genistein, we examined whether Cx43 was phosphorylated on tyrosine as a result of FGF-2 treatment. Myocyte extracts were immunoprecipitated with anti-phosphotyrosine antibodies covalently coupled to agarose beads. Immunoprecipitated proteins, containing phosphorylated tyrosine residues, were then analyzed by probing Western blots with the polyclonal anti-Cx43 antiserum. A typical result of such an experiment is shown in Fig. 9A. Immunoprecipitates from control cultures contained no bands detected by the anti-Cx43 antiserum (Fig. 9A lane 2), while Cx43 immunoreactive bands were clearly seen after FGF-2 treatment (Fig. 9A lane 3). Genistein pretreatment for 30 minutes (20 μM) prevented the immunoprecipitation of Cx43 immunoreactive bands from lysates obtained from both control and FGF-2 treated myocytes (Fig. 9A, lanes 4 and 5, respectively). The specificity of the anti-phosphotyrosine antibody was confirmed by performing immunoprecipitations in the presence of excess phosphotyrosine, phosphoserine or phosphothreonine (Figure 9B). Only immuno-precipitation reactions containing excess phosphotyrosine were incapable of precipitating Cx43 immunoreactive bands, indicating that only free phosphotyrosine could competitively block the antigen binding sites of the monoclonal antibody. There are two possible interpretations of these results. The first is that FGF-2 treatment induces a direct tyrosine phosphorylation of Cx43. The second is that FGF-2 induces tyrosine phosphorylation of another protein or protein complex that binds tightly to Cx43 and co-precipitates with Cx43 under the immunoprecipitation conditions used. To discriminate

Figure 8. Effect of FGF-2 on cardiomyocyte Cx43 phosphorylation.

Cardiac myocytes were labeled with [^{32}P]-orthophosphate, treated with FGF-2, lysed, immunoprecipitated with the polyclonal rabbit antiserum and analyzed by autoradiography. Results from a representative experiment are shown in panel A. Lanes 1, 2, 3, and 4 show [^{32}P]-Cx43 from 100 μg protein obtained from cardiomyocytes treated with FGF-2 for 0, 2, 10, and 30 minutes, respectively. Migration of molecular mass markers is indicated in kilodaltons. In panel B, quantitative results from three separate experiments compare phosphorylated Cx43 levels in FGF-2 treated myocytes with those of untreated cells (set arbitrarily at 100%), shown as a function of time of incubation. Asterisk denotes statistical significance ($P<0.01$, ANOVA with Fisher's protected least significant difference post hoc test). Vertical bars are SEM.

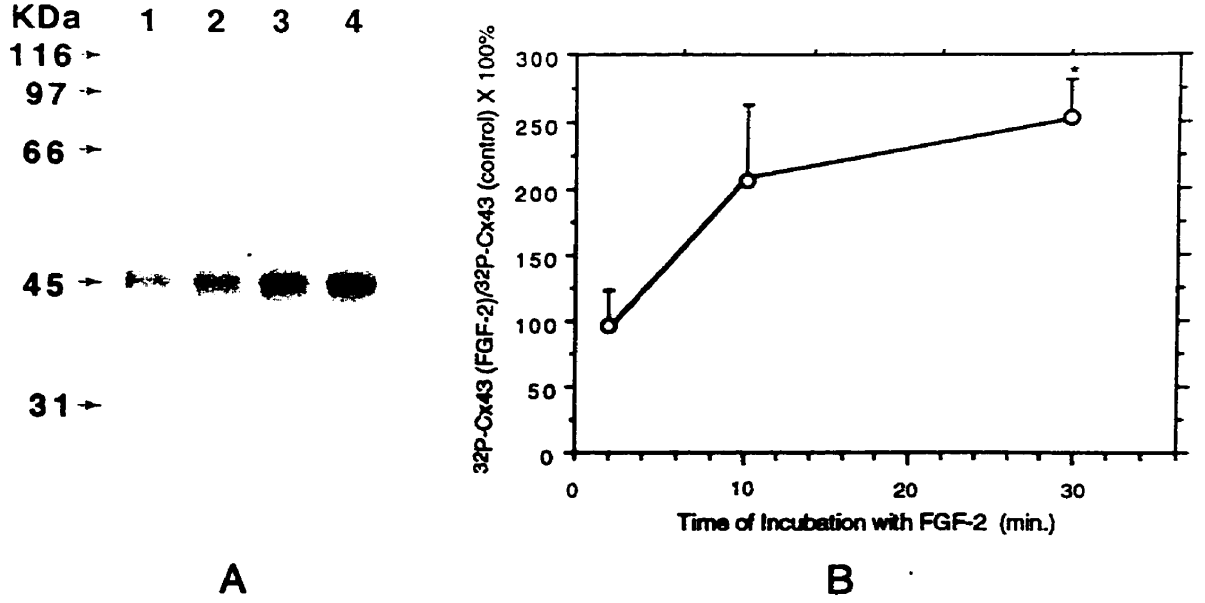
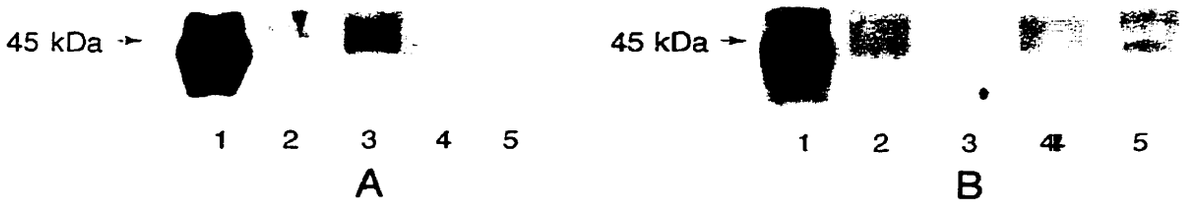


Figure 9. Immunoprecipitation of Cx43 with anti-phosphotyrosine antibodies after FGF-2 treatment.

Cardiac myocytes were treated with FGF-2, lysed and immunoprecipitated with anti-phosphotyrosine-agarose antibodies as described in the Materials and Methods section. Immunoprecipitated proteins were analyzed by Western blotting with the polyclonal anti-Cx43 antiserum. A rat heart gap junction-enriched membrane preparation was also run as a positive control for Cx43 immunostaining. In panel A, the lanes contain the following: 1, heart GJ membranes (2 μg); 2-5, immunoprecipitated proteins from 150 μg lysate from cardiac myocytes. The cardiac myocytes were treated in the following ways.: lane 2, untreated; lane 3, FGF-2 treated; lane 4, 20 μM genistein treatment; lane 5, 20 μM genistein treatment followed by FGF-2 treatment. Treatment with FGF-2 resulted in immunoprecipitation of Cx43 by anti-phosphotyrosine; this effect was blocked by genistein. In panel B, lane 1 contains heart GJ membranes, while the other lanes contain immunoprecipitated proteins from 150 μg of lysate from cardiac myocytes treated with FGF-2. Immunoprecipitations were performed in the presence of no phospho-amino acid addition (lane 2), or in the presence of 200 μM of the following amino acids: phospho-tyrosine (lane 3); phospho-threonine (lane 4); phospho-serine (lane 5). Only phospho-tyrosine blocked Cx43 from being immunoprecipitated by anti-phosphotyrosine antibody.



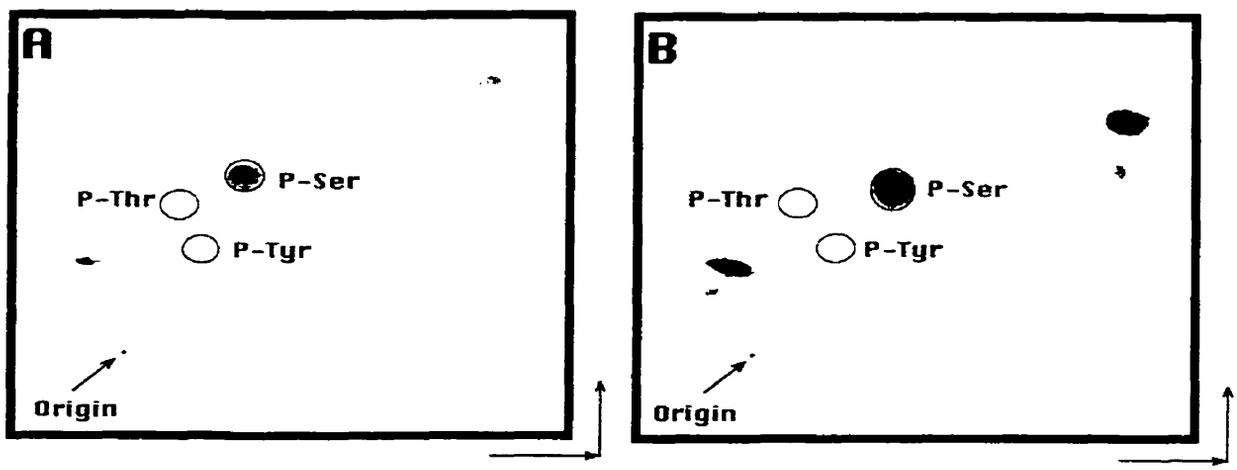
between the two possibilities, we proceeded to directly analyze the phospho-amino acid composition of Cx43.

7. Phospho-Amino Acid Analysis of Cx43

To assess which type of amino acid residues were phosphorylated in [^{32}P]-Cx43 under control and FGF-2 treatment conditions, we directly determined the phospho-amino acid profile of immunoprecipitated [^{32}P]-Cx43. Phospho-amino acids derived from [^{32}P]-Cx43 by acid hydrolysis were separated using electrophoretic techniques and autoradiograms of separated phospho-amino acid spots on thin layer (TLC) plates were compared with stained phospho-amino acid standards that were separated on the same TLC plate. As shown in Figure 10, only phospho-serine was identified in both control and FGF-2 treated samples. Thus, it would appear that FGF-2 treatment increases serine phosphorylation of Cx43 and promotes interaction of Cx43 with tyrosine phosphorylated protein(s).

Figure 10. Phospho-amino acid analysis of [^{32}P]-Cx43.

A, Cx43 from control cells. B, Cx43 from FGF-2 treated cells. The directions of migration for the first dimension (pH 1.9) and second dimension (pH 3.5) on thin-layer chromatography plates are indicated by arrows (horizontal and vertical, respectively). Circles outline the position of ninhydrin-stained unlabeled phospho-amino acid standards, that were separated with the unknown samples.



B. PKCε IS REQUIRED FOR CX43 PHOSPHORYLATION IN NEONATAL RAT CARDIOMYOCYTES

INTRODUCTION:

We have shown that FGF-2 treatment results in Cx43 phosphorylation on serine residues and decreases GJIC in neonatal rat cardiomyocytes. Two serine kinases that have been implicated in the phosphorylation of Cx43 with a resultant decrease in GJIC are MAPK and PKC (Budunova et al., 1994; Kanemitsu and Lau, 1993; Koo et al., 1997; Kwak and Jongsma, 1996; Kwak et al., 1995; Warn-Cramer et al., 1998; Warn-Cramer et al., 1996). Both of these kinases have been proposed to directly phosphorylate Cx43 on carboxy terminal serine residues in epithelial cell lines (Lampe et al., 2000; Warn-Cramer et al., 1998; Warn-Cramer et al., 1996).

Both PKC and MAPK have been shown to be activated by FGF-2 treatment in cardiomyocytes by us and others (Bogoyevitch et al., 1994; Disatnik et al., 1995; Padua et al., 1998). Thus, we hypothesized that the FGF-2-mediated effects on Cx43 gap junctions in neonatal rat cardiomyocytes might involve the activation of these kinases. To identify whether the activation of PKC and/or MAPK was necessary for the FGF-2-mediated effects on cardiomyocyte gap junctions, we used strategies to inhibit these enzymes including the use of specific inhibitors and forced expression of dominant negative enzymes.

1. Effect of the MAPK Inhibitor PD98059 on FGF-2-Mediated Changes in Cx43 Phosphorylation and GJIC

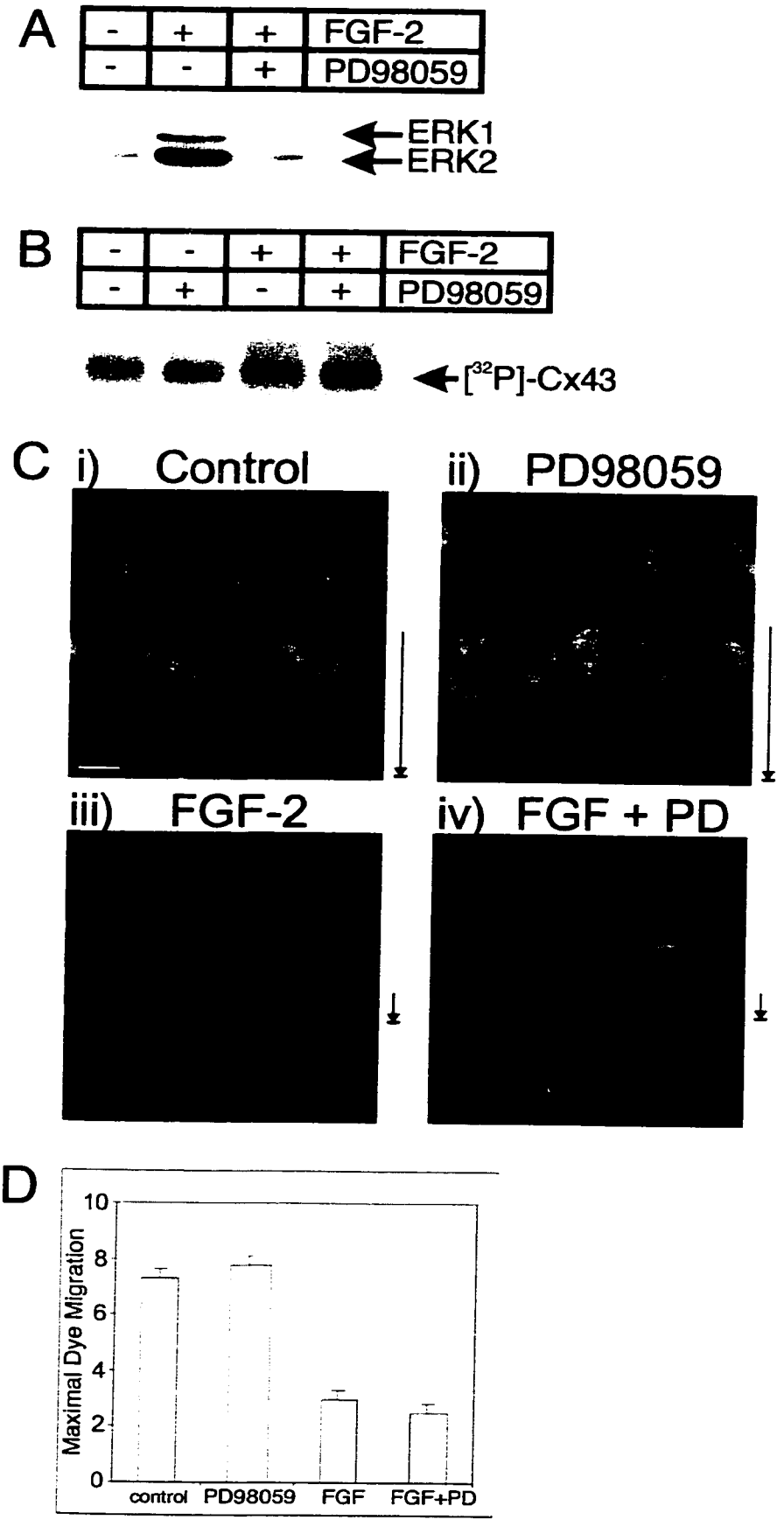
To determine the activation status of MAPK in cardiomyocytes, we used an antibody preparation that specifically recognizes only the dually phosphorylated

(activated) form of MAPK in Western blots of cell lysates. As shown in Fig. 11A, untreated control cardiomyocytes had barely detectable levels of activated MAPK. Treatment of cardiac myocytes with FGF-2 stimulated the activation of both the 42- and 44-kDa isoforms of MAPK (also known as extracellular signal regulated kinase; ERK) that was readily detected by the dual phospho-specific antibody (Fig. 11A, lane 2). Pretreatment with the MAPK / extracellular signal-regulated kinase kinase-1 (MEK1) inhibitor, PD98059 (50 μM for 1 hour) completely blocked the FGF-2 activation of MAPK (Fig. 11A, lane 3).

Having established the effectiveness of FGF-2 in stimulating MAPK in neonatal rat cardiomyocytes, and the ability of PD98059 to completely block MAPK activation, we proceeded to determine whether MAPK was necessary for the FGF-2 induced increase in Cx43 phosphorylation. As can be seen in Fig. 11B, even with complete blockade of MAPK activation, FGF-2 treatment for 15 minutes was still able to induce an increase in Cx43 phosphorylation.

We then examined whether MAPK was necessary for the FGF-2-mediated decrease in GJIC as assessed by scrape-loading of the fluorescent dye 6-CF. Under control conditions, dye movement from the primary-loaded cells along the scrape line through adjacent cells was clearly evident (Fig. 11C-i). As we saw in the previous chapter, FGF-2 induced a clear decrease in dye-coupling between myocytes (Fig. 11C-iii). PD98059 pretreatment did not affect control nor FGF-2 reduced levels of GJIC as assessed by scrape loading (Fig. 11C-ii and 11C-iv), indicating that MAPK activation was not required for FGF-2-mediated effects on dye coupling. Combined semiquantitative data on dye migration from 3 experiments are shown in Fig. 11D.

Figure 11. Effect of MAPK inhibition on FGF-2-mediated Cx43 phosphorylation. A. Western blot of myocyte lysates from cultures treated with the MEK1 inhibitor PD98059 and/or FGF-2, as indicated, and probed with anti-ACTIVE™ MAPK antibodies. B. Autoradiographic detection of [^{32}P]-Cx43 immunoprecipitated from cardiac myocytes treated with PD98059 and/or FGF-2, as indicated. C. Images of fluorescent dye (6-CF) transfer (introduced by scrape loading) between myocytes treated with PD98059 and/or FGF-2, as indicated. Length of arrows indicates the distance of dye migration from the primary-loaded cells at the scrape line. Size bar = 50 μm. D. Combined data from 3 experiments shown in panel C, comparing maximal dye migration distance under different conditions, as indicated. Data are mean ± SEM.


A
- + + FGF-2
- - + PD98059
ERK1
ERK2
B
- - + + FGF-2
- + - + PD98059
[32P]-Cx43
C
i) Control
ii) PD98059
iii) FGF-2
iv) FGF + PD
D
Maximal Dye Migration
10
8
6
4
2
0
control
PD98059
FGF
FGF+PD

2. Effect of the PKC Inhibitors Calphostin C and Chelerythrine on FGF-2-Mediated Changes in Cx43 Phosphorylation and GJIC

Cardiac myocytes were pre-incubated with the PKC inhibitors calphostin C and chelerythrine (1 μM) before FGF-2 for 15 minutes. Pretreatment with chelerythrine had no effect on basal Cx43 phosphorylation, but completely blocked any increase in Cx43 phosphorylation induced by 15 minutes of FGF-2 treatment (Fig. 12A). Calphostin C pretreatment not only blocked the FGF-2-mediated increase in Cx43 phosphorylation, but also reduced basal levels of Cx43 phosphorylation (Fig. 12B). Chelerythrine pretreatment had no effect on the activation of MAPK by FGF-2 as assessed by Western blotting with the ACTIVE™-MAPK antibody preparation (Fig. 12C).

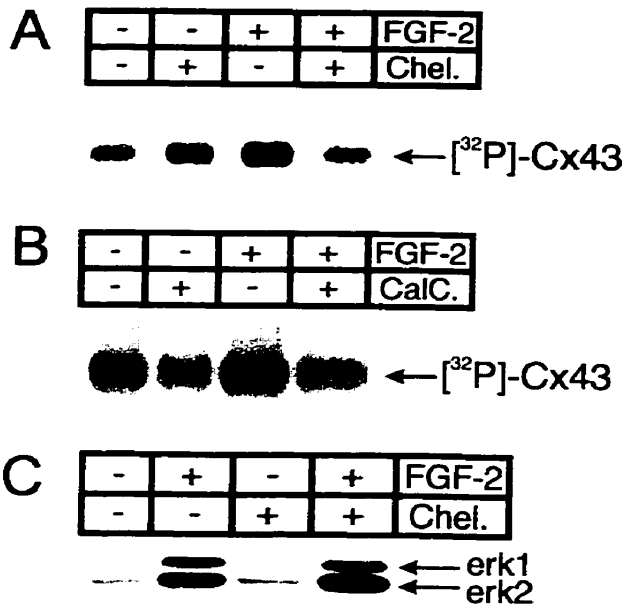
To assess the role of PKC in the FGF-2-mediated reduction in dye-coupling, pretreatment with chelerythrine was used in scrape loading assays. Chelerythrine did not affect basal levels of dye coupling (Fig. 12D-ii), but prevented the decrease in GJIC initiated by FGF-2 treatment (Fig. 12D-iv). Combined semiquantitative data of dye migration from 3 experiments are shown in Figure 12E. Taken together, these data implicate PKC in the transduction of FGF-2 effects on Cx43 phosphorylation and GJIC. Experiments in our laboratory had indicated that of all the PKCs present in cardiomyocytes, PKCα and PKCε, were localized to cell-cell contact sites. Thus, we investigated the involvement of PKCε and PKCα in FGF-2 induced effects on gap junctions.

3. Effect of FGF-2 Treatment on PKCε Localization.

In unstimulated cardiomyocytes, staining with a polyclonal antibody to PKCε produced an interrupted pattern of localization at regions of intercellular contact (Fig.

Figure 12. Effect of PKC inhibition on FGF-2-mediated Cx43 phosphorylation.

A. Autoradiographic detection of [^{32}P]-Cx43 immunoprecipitated from cardiac myocytes treated with chelerythrine and/or FGF-2, as indicated. B. Autoradiographic detection of [^{32}P]-Cx43 immunoprecipitated from cardiac myocytes treated with calphostin-C and/or FGF-2, as indicated. C. Western blot of myocyte lysates from cultures treated with chelerythrine and/or FGF-2, as indicated, and probed with anti-ACTIVE™ MAPK antibodies . D. Images of fluorescent dye (6-CF) transfer between myocytes treated with chelerythrine and/or FGF-2, as indicated. Length of arrows indicates the distance of dye migration from the primary-loaded cells at the scrape line. Size bar=50 μm. E. Combined data from 3 experiments shown in panel D, comparing maximal dye migration distance under different conditions, as indicated. Data are mean ± SEM.


A
- - + + FGF-2
- + - + Chel.
←[32P]-Cx43
B
- - + + FGF-2
- + - + CalC.
←[32P]-Cx43
C
- + - + FGF-2
- - + + Chel.
←erk1
←erk2
D
i) Control
ii) Chel.
iii) FGF-2
iv) FGF-2 + Chel.
E
Maximal Dye Migration
10
8
6
4
2
0
control
chel.
FGF
FGF+ chel.

13A). This pattern was very similar to that seen with immunofluorescent visualization of cadherin, an intercellular attachment protein found at cardiomyocyte intercalated disks in adherens junctions and desmosomes (Kaufmann et al., 1999). In contrast, cardiomyocytes stimulated with FGF-2 revealed a more continuous staining for PKCε along the cell membranes of adjacent cells (Fig. 13D).

Staining for Cx43 with a monoclonal antibody under control conditions produced punctate staining characteristic of gap junctions (Fig. 13B). Simultaneous visualization of both Cx43 and PKCε immunofluorescence indicated regions of colocalization between Cx43 and PKCε (yellow) as well as regions of exclusive localization of PKCε (green) and Cx43 (red). A representative image showing dual immunofluorescent staining for Cx43 and PKCε under control conditions is presented in Fig. 13C. With FGF-2 stimulation, more extensive colocalization between Cx43 and PKCε was observed (Fig. 13F). No differences in PKCα localization were observed as a result of FGF-2 treatment (not shown).

4. Effect of PMA Treatment on PKCε Localization

Treatment of cardiomyocytes with 100 nM PMA for 15 minutes produced identical changes in PKCε localization as those induced by FGF-2 treatment (Fig. 14). Under control conditions, anti-PKCε immunostaining was discontinuous along cell-cell contacts (Fig. 14A). PMA-treatment resulted in a more continuous, yet irregular, staining for PKCε along cell-cell contact sites (Fig. 14B). The incidence of exclusive Cx43 staining, with no overlapping PKCε staining, appeared higher in control (Fig. 14C) versus PMA-treated (Fig. 14D) cardiomyocytes.

Figure 13. Localization of PKCε and Cx43 to sites of intercellular contact.

A,B and D,E: Double immunofluorescence labeling of cardiac myocytes for PKCε and Cx43 (green and red, respectively). C, F: Simultaneous staining for both fluorochromes. A-C: Myocytes under control, unstimulated conditions. D-F: Myocytes stimulated with FGF-2. Areas of co-localization appear yellow. Arrows in B and C point to regions where Cx43 and εPKC do not overlap. In panels E and F, arrows indicate regions of overlap between PKCε and Cx43 staining. Bar=50 μm.

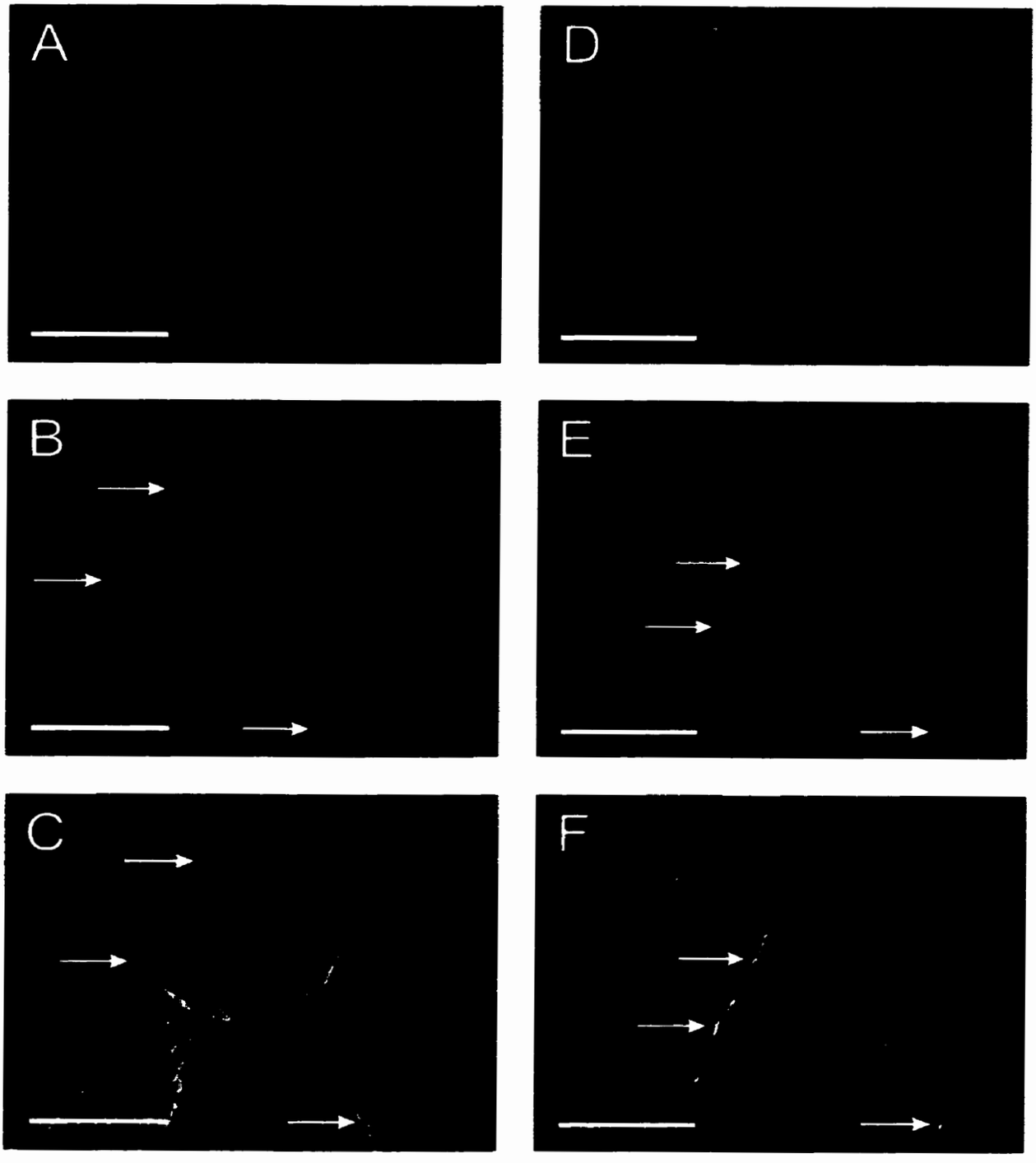
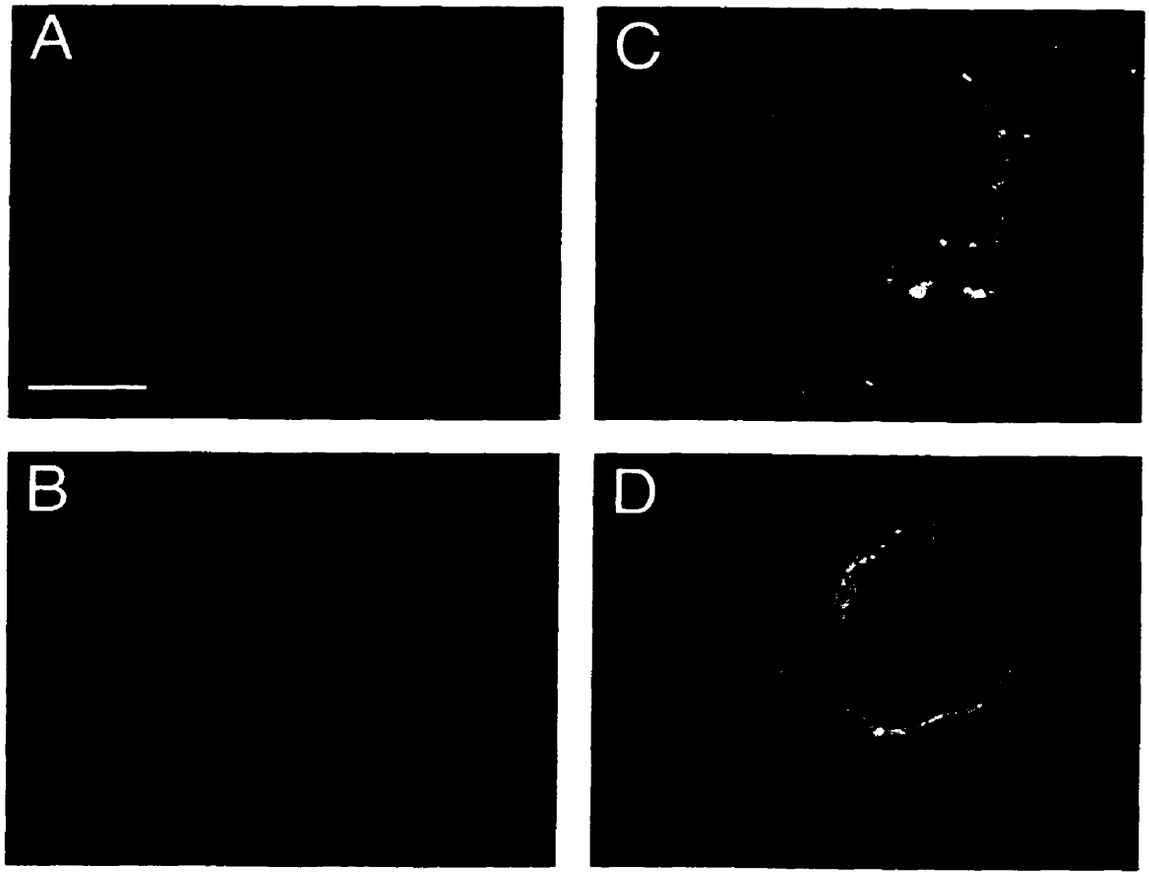

A
D
B
E
C
F

Figure 14. Immunolocalization of PKCε and Cx43 in control and PMA treated cardiomyocytes.

Cells were stained for εPKC (green) and Cx43 (red). A, C, unstimulated myocytes. B,D, PMA-stimulated myocytes. Bar=20 μm.



5. Effect of FGF-2 Treatment on Co-immunoprecipitation of Cx43 with PKCε and PKCα

To determine whether there might be a direct interaction between Cx43 and PKCε, we used anti-Cx43-specific antibodies to immunoprecipitate Cx43 and interacting protein(s) from control and FGF-2 stimulated cell lysates. Typical results are shown in Fig. 15. Western blot analysis of immunoprecipitated proteins revealed the presence of an anti-PKCε immunoreactive band migrating at 90 kDa (Fig. 15A). As a rabbit antibody was used to detect PKCε, and rabbit IgGs were also used in the immunoprecipitation, a broad band at 55 kDa corresponding to the rabbit IgGs was detected by the anti-rabbit horse-radish peroxidase conjugate used for enhanced chemiluminescent visualization of blotted proteins.

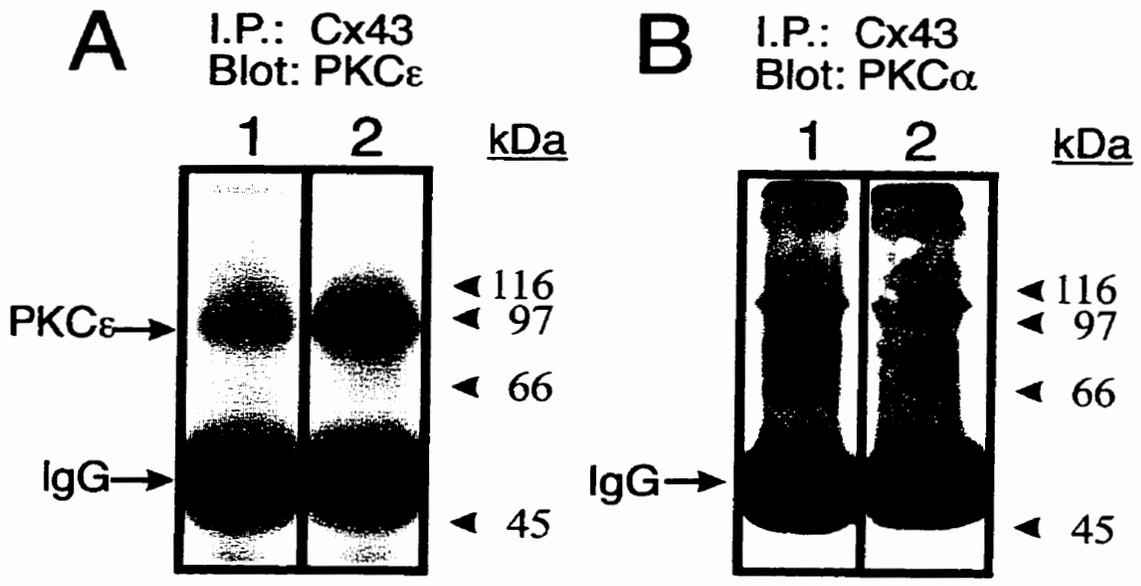
In contrast, when an anti-PKCα antibody was used to probe the same samples, no band corresponding to the expected molecular mass of PKCα (approx. 82 kDa) was visualized, even after an extended exposure time. Under conditions of long exposure, some bands were detected, presumably non-specifically, and the intensity of these bands did not change with FGF-2 treatment (Fig. 15B). Levels of the 90 kDa PKCε immunoreactive band were significantly elevated in immunoprecipitates from FGF-2 treated samples compared to control samples (Fig. 15C, n=4).

6. Effect of Expression of the Truncation Mutant PKCε(1-401) on Cx43 Phosphorylation

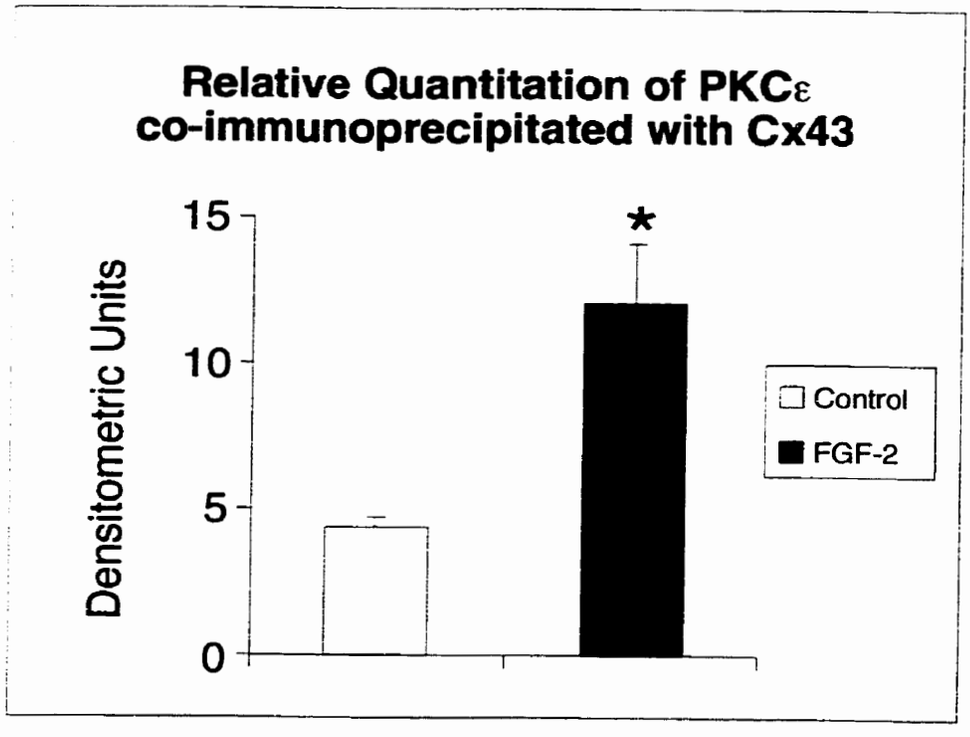
Cardiac myocytes were transiently transfected with a truncated form of murine PKCε, $PKC\varepsilon_{(1\text{-}401)}$, which has been shown previously to act as a dominant negative inhibitor of PKCε (Cai et al., 1997). Using a modified calcium phosphate transfection protocol (Jordan et al., 1996), transfection efficiencies of nearly 20% were achieved.

Figure 15. Co-precipitation of PKCε with anti-Cx43 antibodies.

(A): Western blot analysis of protein immunoprecipitated with polyclonal anti-Cx43 antibodies and probed for PKCε. Lane 1, protein from unstimulated myocytes, Lane 2, protein from FGF-2-stimulated myocytes. Migration of immunoglobulin (IgG), PKCε and molecular mass markers is indicated. (B): Western blot analysis of protein immunoprecipitated with polyclonal anti-Cx43 antibodies and probed for PKCα. Lane 1, protein from unstimulated myocytes, Lane 2, protein from FGF-2-stimulated myocytes. Migration of immunoglobulin (IgG), and molecular mass markers is indicated. (C). Densitometric quantitation of co-precipitated PKCε, as shown in panel A. Asterisk indicates statistically significant differences between FGF-2-treated and control samples ($n=4$, $P<0.05$, Student's t-test). Data are mean ± SEM.


A
I.P.: Cx43
Blot: PKCε
1
2
kDa
PKCε
IgG
116
97
66
45
B
I.P.: Cx43
Blot: PKCα
1
2
kDa
IgG
116
97
66
45

C


Relative Quantitation of PKCε
co-immunoprecipitated with Cx43
Densitometric Units
15
10
5
0
*
Control
FGF-2

A representative picture of neonatal rat cardiomyocytes transfected using this technique with a β-galactosidase reporter gene allowed to express for 48 hours and then stained for β-galactosidase activity (blue) is presented in Fig. 16. Expression of the truncated PKCε isoform in transfected cells was assessed by Western blotting with an antibody raised against the amino-terminus of PKCε that detected a band at approximately 50 kDa, corresponding to the truncated protein (Fig. 17A). Native PKCε levels (90 kDa band) were unchanged in cells transfected with vector or $PKC\varepsilon_{(1\text{-}401)}$. Levels of Cx43 phosphorylation, determined by immunoprecipitation of [^{32}P]-Cx43 and corrected for variations in total Cx43 present in the samples, were significantly decreased in cultures expressing the PKCε(1-401) compared with vector-transfected controls (Fig. 17B and 17C).

7. Effect of Infection with the Dominant Negative PKCε-expressing Adenovirus Ad. PKCε(DN) on Cx43 Phosphorylation

Infection of cardiomyocytes with an adenovirus expressing a dominant-negative form of PKCε , Ad. PKCε(DN), was performed as previously described (Ping et al., 1999). The dominant negative PKCε encoded by Ad.PKCε(DN) is still capable of membrane translocation, but cannot phosphorylate its target based on a mutation to its kinase domain (Ping et al., 1999). Very high levels of expression of the dominant negative PKCε isoform were detected when myocytes were infected at a multiplicity of infection of 50 infectious viral particles per cell. Using Western blotting and ECL conditions that do not detect endogenous PKCε, very high levels of the dominant negative PKCε were detected (Fig. 18A). Longer ECL exposure times resulted in detection of the endogenous isoform (as in 17A) in the lane containing lysates from

Figure 16. Transfection of neonatal rat cardiomyocytes with a β-galactosidase reporter construct.

Cells were transfected, using a modified calcium phosphate technique, with 6 μg of a plasmid construct encoding β-galactosidase under the control of a CMVpromoter and were allowed to express the transfected protein for 48 hours. Cells were then fixed and stained for β-galactosidase activity as indicated in the Methods section. A. Field of cardiomyocytes photographed with a 20X objective lens; bar = 50 μm. B. Field of cardiomyocytes photographed with a 4X objective lens; bar = 100 μm.

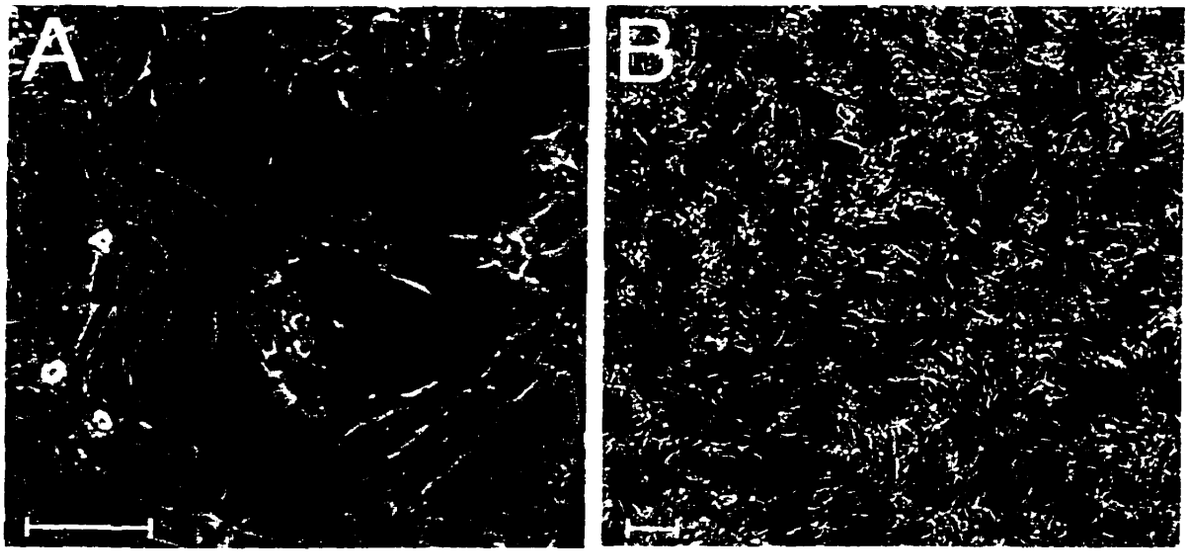


Figure 17. Effect of PKC$\varepsilon_{(1\text{-}401)}$ on Cx43 phosphorylation.

A. Detection of PKC$\varepsilon_{(1\text{-}401)}$ expression by Western blotting. Myocytes were transfected by the calcium phosphate method with vector or the cDNA coding for PKC$\varepsilon_{(1\text{-}401)}$, as indicated. Migration of the endogenous PKCε (at 90 kDa) and the truncated PKC$\varepsilon_{(1\text{-}401)}$ is indicated. [^{32}P]-labeled Cx43 was quantified by immunoprecipitation from labeled myocyte lysates and analysis by SDS-PAGE, followed by autoradiography and densitometry. Levels of [^{32}P]-Cx43 were corrected for the Cx43 content from each lysate, determined by parallel Western blotting and densitometry. Cx43 phosphorylation in control cultures was arbitrarily defined as 100%, and values from the treated cultures were expressed relative to control values. Cultures were transfected with cDNA for PKC$\varepsilon_{(1\text{-}401)}$ or vector, as indicated (n=4, * $p < 0.05$, unpaired t-test). C. Representative autoradiograph of [^{32}P]-Cx43 immunoprecipitated from myocytes transfected with vector or PKC$\varepsilon_{(1\text{-}401)}$ cDNA. Migration of molecular mass markers (A and C) is shown in kDa.

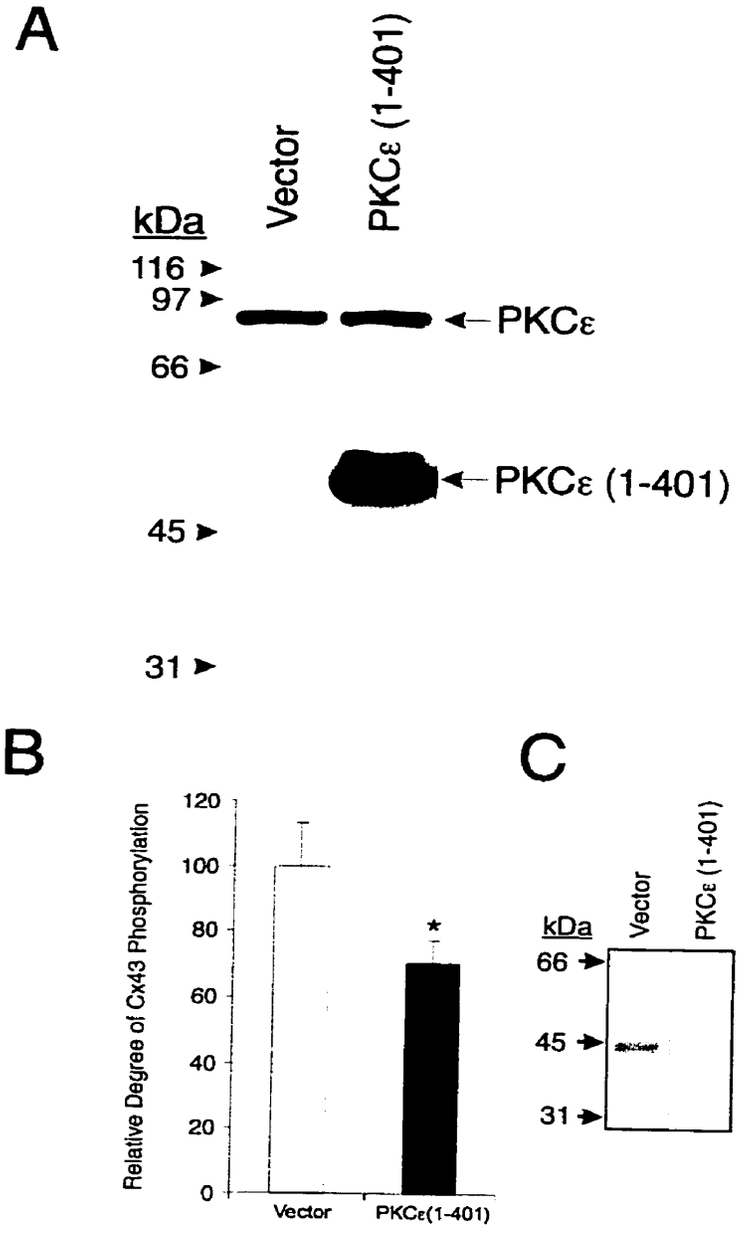
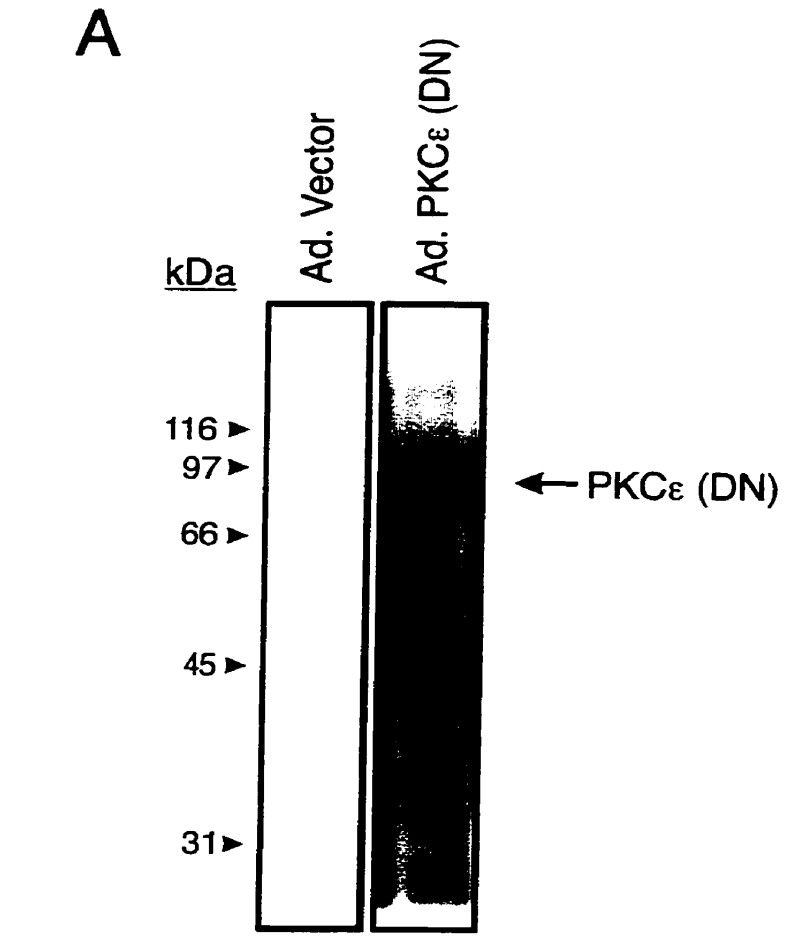
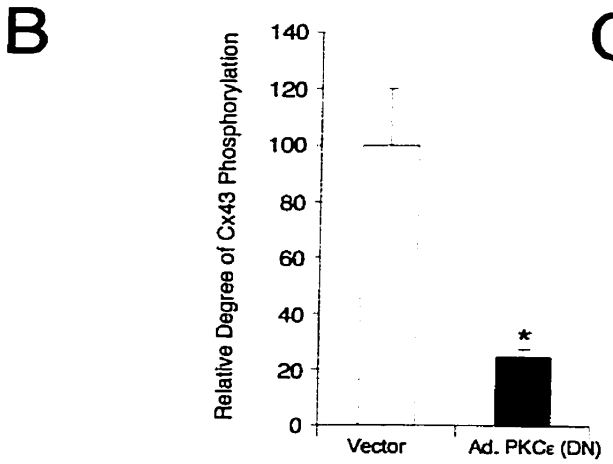
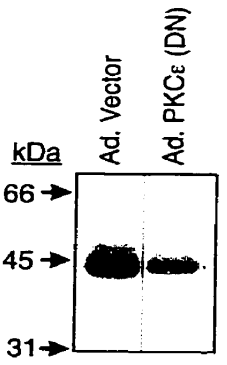

A
Vector
PKCε (1-401)
kDa
116
97
66
45
31
PKCε
PKCε (1-401)
B
Relative Degree of Cx43 Phosphorylation
120
100
80
60
40
20
0
*
Vector
PKCε(1-401)
C
kDa
Vector
PKCε (1-401)
66
45
31

Figure 18. Effect of Ad.PKCε(DN) on Cx43 phosphorylation.

A. Detection of Ad.PKCε(DN) by Western blotting. Myocytes were infected with adenovirus alone (vector) or with Ad.PKCε(DN), as indicated. B. Procedures as in Fig. 17B. Cultures were infected with adenovirus alone or Ad.PKCε(DN) (n=3, * $p < 0.05$, unpaired t-test). C. Representative autoradiograph of [^{32}P]-Cx43 immunoprecipitated from myocytes transfected with vector alone or Ad.PKCε(DN). Migration of molecular mass markers (A and C) is shown in kDa.


A
Ad. Vector
Ad. PKCε (DN)
kDa
116
97
66
45
31
PKCε (DN)


B
Relative Degree of Cx43 Phosphorylation
140
120
100
80
60
40
20
0
*
Vector
Ad. PKCε (DN)


C
Ad. Vector
Ad. PKCε (DN)
kDa
66
45
31

vector-infected cells, but the lane corresponding to Ad.PKCε(DN) was then completely black. Levels of [^{32}P]-Cx43, normalized to total Cx43 levels, decreased sharply in cultures expressing Ad.PKCε(DN) compared with controls treated with a vector control virus (Fig. 18B and 18C).

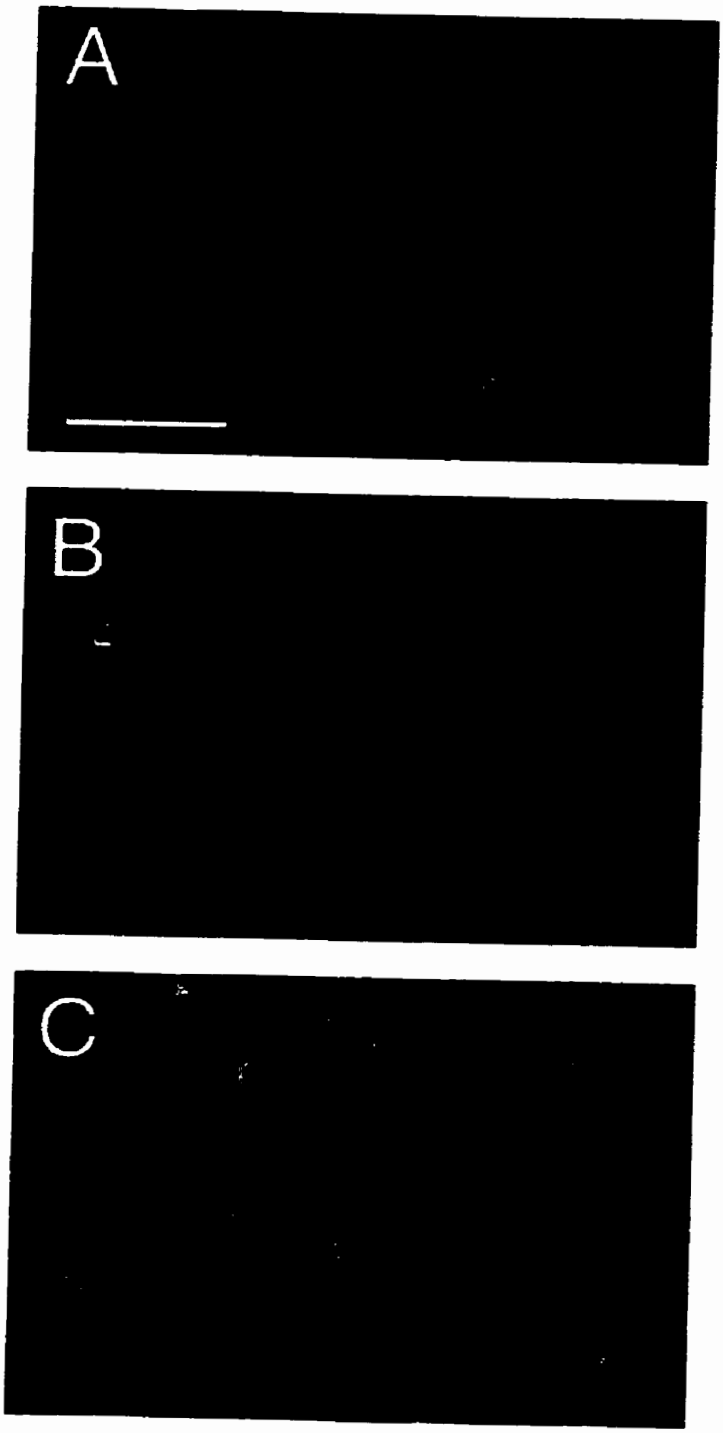
The adenoviral expression system allowed for very high transfection efficiency with greater than 95% of cardiac myocytes over-expressing PKCε. This was determined by staining cardiomyocytes with a monoclonal anti-PKCε antibody preparation. In control cells (infected with vector virus), this antibody reacts weakly with endogenous PKCε. (Fig. 19A). In Ad.PKCε(DN) infected cells there is very strong staining of cytosolic and intercellular contact sites (Fig. 19B and 19C). Figure 19B was photographed under the same exposure conditions as 19A, while 19C was photographed under reduced exposure conditions to allow better visualization of PKCε staining.

8. Effect of Dominant Negative PKCε and MKK1 on PMA-stimulated Phosphorylation of Cx43

We used the Ad.PKCε(DN) adenovirus and another adenovirus encoding a dominant negative isoform of MKK1 (MEK1), to determine the effects of inhibiting these signaling molecules on Cx43 phosphorylation due to PMA stimulation of neonatal rat cardiomyocytes. Cells were labeled with [^{35}S]-methionine/cysteine for a period of four hours, treated with 100 nM PMA during the last 15 minutes of labeling and then processed for immunoprecipitation of Cx43 and fluorography. PMA treatment caused a hyperphosphorylation of Cx43 that resulted in a shift in migration of Cx43 from a series of bands ranging in molecular mass from 41 to > 45 kDa, to a broad band of Cx43 (possibly a doublet or multiplet), migrating above 45 kDa, with very little of the lower molecular weight isoforms of Cx43 remaining (compare Fig. 20A control, vs PMA).

Figure 19. Expression of Ad.PKCε (DN) in cardiac myocytes.

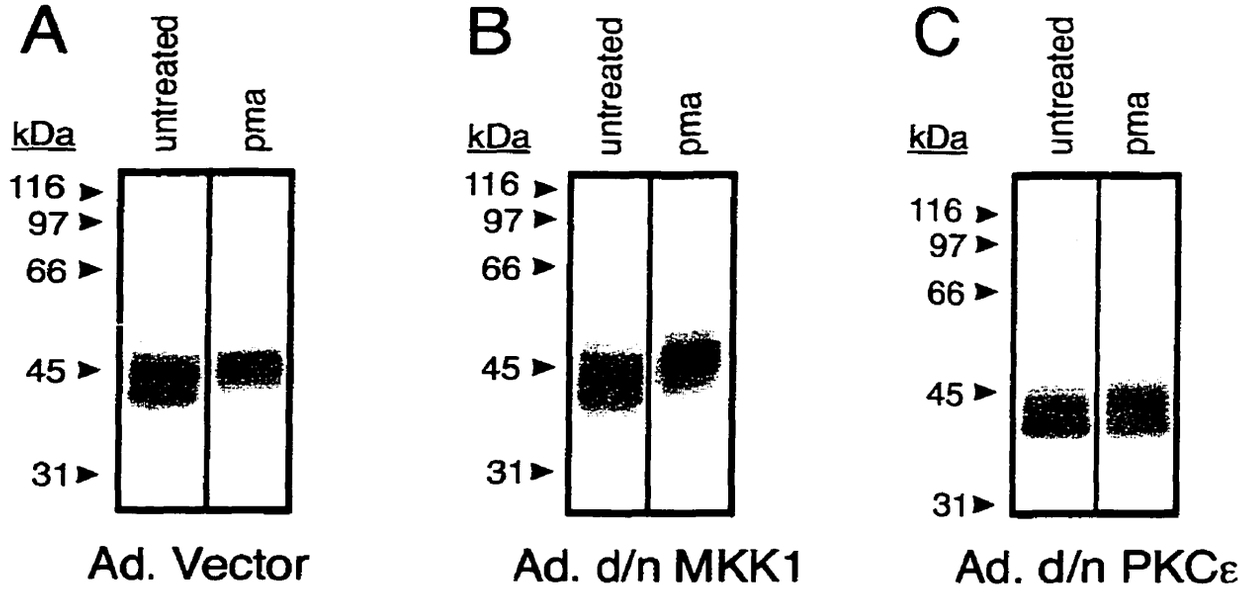
Immunofluorescence staining for PKCε (red) using the monoclonal antibody preparation. Nuclear staining (blue) with HOE 33342 is also shown. (A). Myocytes infected with adenovirus alone show background levels of anti-PKCε staining. (B). Myocytes infected with Ad.PKCε(DN) and photographed under identical conditions of film exposure as in (A) show strong expression of PKCε(DN). (C). Same field as in B, photographed under decreased conditions of film exposure. Bar=50 μm.


A
B
C

Cells that were expressing a dominant negative isoform of MKK1, that should block signaling through MAPK, still responded to PMA with a hyperphosphorylated pattern of Cx43 mobility (Fig. 20B). Cells that expressed dominant negative PKCε, on the other hand, did not respond to PMA with an obvious hyperphosphorylation of Cx43 (Fig. 20C). These results indicate that Cx43 hyperphosphorylation induced by PMA treatment in neonatal rat cardiomyocytes requires the epsilon isoform of PKC, and does not require MAPK activation.

Figure 20. Effect of dominant negative MKK1 and PKCε on PMA stimulation of Cx43 phosphorylation.

Lysates from cells metabolically labeled with [^{35}S]-methionine/cysteine that were either treated or untreated with 100 nM PMA during the last 15 minutes of labeling, were adjusted to contain identical amounts of total Cx43. Cx43 was immunoprecipitated from these samples, run on SDS-PAGE gels, and processed for fluorography. A. Cells treated with vector virus at m.o.i. 50, either untreated or treated with PMA as indicated. B. Cells treated with adenovirus encoding a dominant negative isoform of MKK1 (m.o.i. 50), either untreated or treated with PMA as indicated. C. Cells treated with adenovirus encoding a dominant negative isoform of PKCε (m.o.i. 50), either untreated or treated with PMA as indicated.



C. SERINE 262 OF CX43 AFFECTS CARDIOMYOCYTE DNA SYNTHESIS, AND IS REQUIRED TO OBTAIN THE PHOSPHORYLATED SPECIES OF LOWEST ELECTROPHORETIC MOBILITY

INTRODUCTION:

In the previous chapters, we found that FGF-2, a known mitogen for cardiomyocytes, causes PKC-dependent phosphorylation of Cx43 and decreased GJIC in neonatal rat cardiomyocytes. Basal as well as PMA-induced Cx43 phosphorylation could be reduced by inactivating the specific PKC isoform, PKCε, with strategies utilizing dominant negative PKCε mutants. Increased co-immunoprecipitation and colocalization of PKCε and Cx43 upon FGF-2 treatment suggested that PKCε may act directly on Cx43 to phosphorylate it on serine (S) residues. Serine is a polar amino acid with the following defining side chain: $HO\text{-}CH_2\text{-}$. There are five consensus phosphorylation sites for PKC in the carboxy terminus of Cx43 targeting the following residues: i) S262; ii) S297; iii) S364; iv) S368 and; v) S372 (Kreegipuu et al., 1999).

Indirect evidence from our laboratory suggests that the FGF-2 induced phosphorylation of Cx43 and reduced GJIC may be related to the mitogenic effect of FGF-2 on neonatal rat cardiomyocytes. For instance, TGFβ, a factor that cancels the mitogenic effects of FGF-2 on cardiomyocytes, prevented FGF-2 induced phosphorylation of Cx43 (Kardami and Doble, 1998). This notion is in keeping with several studies that have shown a positive correlation between reduced GJIC and increased cellular growth (reviewed in: Yamasaki and Naus, 1996). Our initial studies (Results: Chapter 1) on FGF-2-induced masking of Cx43 epitope(s) suggested that affected amino acids were located between residues 261-270 of rat Cx43. Since S262, a

potential PKC target, resides in this span of amino acids, we decided to study the function of this residue, and for comparison, two other potential PKC targets, S297 and S364, through the use of site-directed mutagenesis. We hypothesized that if phosphorylation of Cx43 on S262 was contributing to the FGF-2 effects on cardiomyocyte proliferation and / or growth, mutating this residue might affect cardiomyocyte growth properties in cells overexpressing the mutant connexin.

In addition, since phosphorylation of Cx43 affects its electrophoretic mobility, we hypothesized that mutating S262 to an unphosphorylatable amino acid residue such as alanine (A), that has a defining side chain consisting of a single methyl (CH_3-) group, might prevent the shift to lower mobility species seen in gels of wild type Cx43. Thus, this chapter addresses the role of S262: i) in the regulation of the electrophoretic mobility of Cx43 (indirect evidence of the ability to become phosphorylated in the physiological environment of the cell as well as the degree of phosphorylation); and ii) in the regulation of cardiomyocyte DNA synthesis.

1. Expression of Mutant Cx43 in Embryonic Chicken Cardiomyocytes by Transient Gene Transfer:

Site-specific mutants targeting three serines of the carboxy terminus of Cx43 were constructed using mutagenic PCR primers. The mutations were confirmed by DNA sequencing as described in Materials and Methods. Serine residues were mutated to alanine residues to render these positions in the Cx43 molecule unphosphorylatable. We chose to transfect embryonic chicken cardiomyocytes with our Cx43 constructions for the following reasons: i) these cells are efficiently transfected using the calcium phosphate technique; ii) they express very little immunodetectable Cx43; iii) they provide the

intracellular environment of a cardiomyocyte for the expression of our connexin mutants. Using a modified calcium phosphate transfection method, relatively high (approx. 20%) and consistent levels of transfection were obtained as assessed by Western blotting and visualization, through indirect immunofluorescence microscopy, of cells over-expressing Cx43.

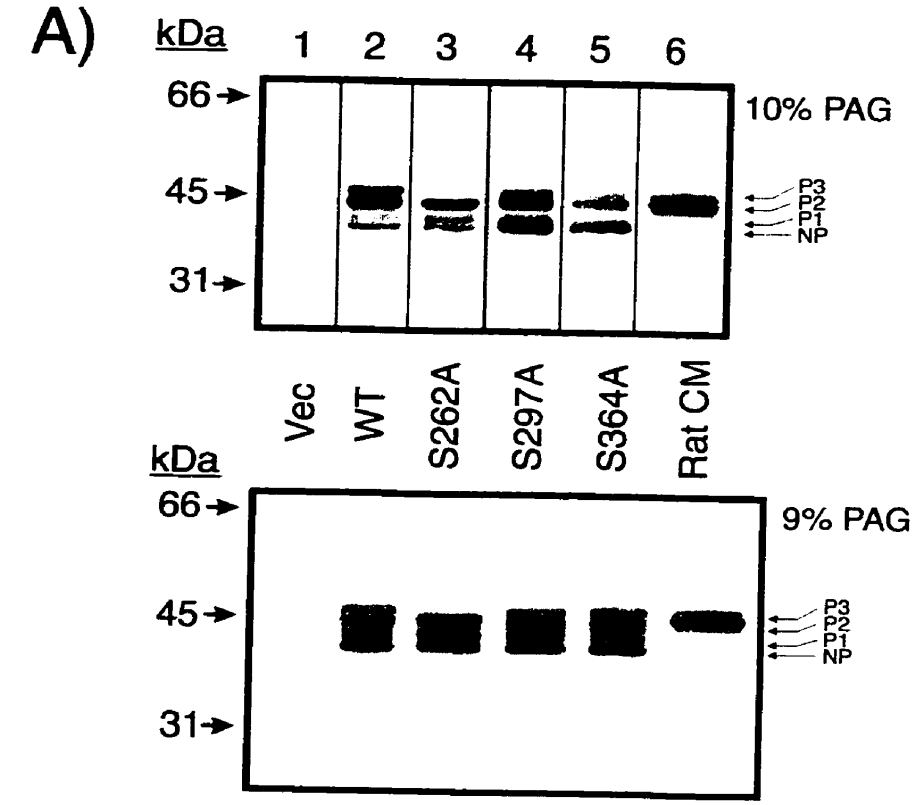
Transfection of chicken cardiomyocytes with wild-type and mutant rat Cx43 plasmids resulted in levels of Cx43 protein that were easily detectable by Western blotting with a polyclonal antibody to Cx43. Two representative Western blots, each from an independent transfection experiment, are shown in Fig. 21A. Protein levels were adjusted so that all lanes contained equal amounts of protein (20 μg). Optimal exposure times for the detection of transfected Cx43 by enhanced chemiluminescence revealed no bands in the lane corresponding to vector-transfected cardiomyocytes (Figure 21A, lane 1). This indicates that any endogenous Cx43 present in the chicken cardiomyocytes is undetectable when compared to transfected Cx43. Thus, Western blots for Cx43 from Cx43 transfected chicken cardiomyocytes display only the transfected Cx43 isoforms. A lane containing 1 μg of total protein lysate from neonatal rat cardiomyocytes was run as a positive control for Cx43 immunoreactivity (Fig. 21A, lane 6).

Expression of wild type Cx43 produced four Cx43 immunoreactive bands, labeled, in order of decreasing mobility and increased phosphorylation, as: NP (for non-phosphorylated), P1, P2 and P3 (Fig. 21A, lane 2). Four bands were also produced when mutant connexins S297A and S364A were expressed (Fig. 21A, lanes 4 and 5, respectively), and they seemed to display similar electrophoretic mobility as those in cells expressing the wild type Cx43. Resolution of the first blot shown in Fig. 21A (10%

polyacrylamide gel; PAG), combined with higher relative levels of the faster migrating forms did not allow complete separation between NP and P1 bands. Additional analysis

Figure 21. Effect of S-to-A mutations on the electrophoretic mobility of Cx43 expressed in chicken cardiomyocytes.

A. Immunoblots from two independent transfection experiments, using 10% and 9% polyacrylamide gels (PAG), respectively, probed with polyclonal anti-Cx43 antibodies. Lanes 1-5 were loaded with 20 μg lysate protein from chicken cardiomyocyte cultures transfected with vector or the different Cx43 plasmids, as indicated. Lane 6 was loaded with 1 μg of rat cardiomyocyte lysate. Arrows point to the different Cx43 species (NP, P1, P2, P3). Electrophoretic motility of molecular mass markers is indicated in kDa. B. Immunoblot obtained by stripping the 9% PAG blot in A and reprobing it with monoclonal anti-Cx43 antibodies specific for the NP form of Cx43. C. Autoradiogram of [^{32}P]-Cx43 immunoprecipitated from chicken cardiomyocyte cultures transfected with wild type or mutant Cx43 and labeled with [^{32}P]-orthophosphate, as indicated.


A)
kDa
1 2 3 4 5 6
66
45
31
10% PAG
P3
P2
P1
NP
Vec
WT
S262A
S297A
S364A
Rat CM
kDa
66
45
31
9% PAG
P3
P2
P1
NP
B)
kDa
45
31
NP
C)
WT
S262A
S297A
S364A
kDa
66
45
31

(9% PAG), shown in the second immunoblot, provided improved separation between the NP and P1 bands. In all cases, expression of the S262A Cx43 mutant produced three bands, corresponding in electrophoretic motility to the NP, P1 and P2 bands, while no band corresponding to the slowest migrating (≈ 46 kDa) P3 band was present. In total, this experiment was repeated three times, with similar results: a P3 band was always absent from the S262A transfectants, while four bands were expressed, to variable degrees, by the other transfectants.

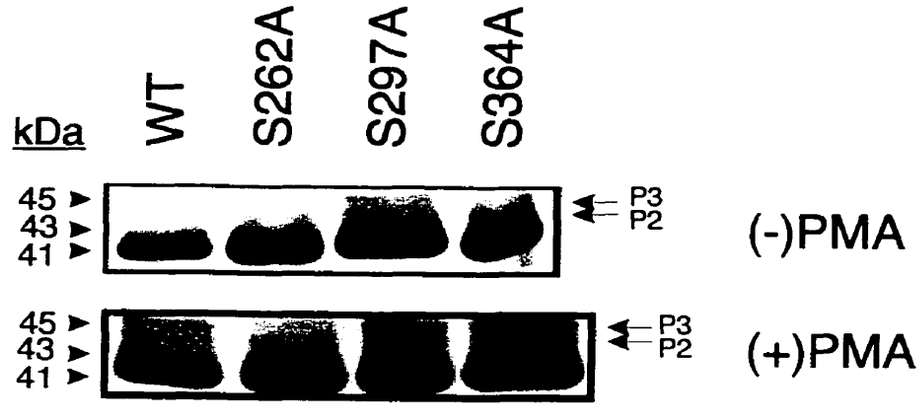
The 9% PAG blot shown in Fig.21A was reprobed with a monoclonal antibody preparation specific for the 41 kDa non-phosphorylated Cx43 in cardiomyocytes (Nagy et al., 1997). As shown in Fig. 21B, only the faster migrating 41 kD Cx43 band was detected in all transfectants. Finally, Cx43 was immunoprecipitated from chicken cardiomyocyte transfected cultures labeled with [^{32}P]-orthophosphate. [^{32}P]-Cx43 migrated as a broad band in the range of 43-46 kDa (Fig. 21B), as expected for the P1, P2, P3 species. This is similar to the pattern obtained from [^{32}P]-incorporation in endogenous Cx43 in rat cardiomyocytes (see Chapter 1). Although the resolution of the method did not allow separation of individual Cx43 species, [^{32}P]-Cx43 from the S262A transfectant had a slightly faster motility compared to all the others, consistent with the absence of the slowest migrating P3 band.

2. Transfection of Cx43 Site-Directed Mutants into HeLa cells:

To assess whether mutating S262 would have similar effects on Cx43 mobility in a mammalian system, the same plasmids that were transfected into the chicken cardiomyocytes were transfected into HeLa cells. The cells were maintained in low serum to reduce phosphorylation that might occur due to growth stimulatory pathways

Figure 22. Effect of S-to-A mutations on the electrophoretic mobility of Cx43 expressed in Hela cells.

Western blots of lysates from HeLa cells transfected with wild type or mutant Cx43s, as indicated, before or after 15 minute incubation with 100 nM PMA (±PMA, as indicated). The intensely labeled band at around 41-42 kD is presumed to represent both the NP and P1 species. Slower migrating Cx43 species are indicated as P2 and P3.



and some were treated with the phorbol ester PMA. HeLa cells, like the chicken cardiomyocytes, express no immunodetectable Cx43, so only transfected Cx43 was detected in Western blots. Without PMA stimulation (Figure 22, as indicated), the transfected Cx43 species exist predominantly in high mobility forms corresponding to low levels of endogenous phosphorylation. With the addition of PMA for 15 minutes (Fig. 22, as indicated), there is the clear appearance of two lower mobility forms of Cx43, P2 and P3. As in the transfected chicken myocytes, only WT, S297A and S364A-transfected cells presented both P2 and P3 Cx43 isoforms; the S262A transfectants clearly had no band corresponding to P3.

3. Effect on DNA synthesis in Neonatal Rat Cardiomyocytes Transiently Transfected With S262A Cx43

The S262A mutant and a new mutant that replaces S262 with a negatively charged aspartate residue (D), mimicking serine phosphorylation at this site, were transfected, along with WT Cx43 and a vector control, into neonatal rat cardiomyocytes. We chose to transfect these cells as opposed to the HeLa cells or chicken cardiomyocytes as we are most interested in the effects of the Cx43 mutants in a mammalian cardiomyocyte system. We used the incorporation of the thymidine analogue, bromodeoxyuridine (BrdU), to assess the degree of DNA synthesis in these transfected cells.

An average transfection efficiency of 7.2 +/- 2.4% was obtained for all transfectants. Transfection efficiency was assessed by counting myocytes clearly overexpressing Cx43 (identified by intense cytoplasmic and cell membrane immunofluorescent staining) and determining the ratio of these overexpressing cells to

the total number of myocytes in a microscopic field. This method underestimates the actual transfection efficiency as only strong overexpressors are scored positive for transfection. Transfection with the reporter gene β-galactosidase, assayed by histochemical visualization of β-galactosidase activity, revealed that the transfection method used here actually results in cardiomyocyte transfection efficiencies of approximately 20% (see Fig. 16 of the previous chapter). The introduction of plasmids expressing wild type, S262A or S262D Cx43 resulted in all cases in a 2-fold increase in total immunoreactive Cx43 assessed by Western blotting (Fig. 23); this was consistent between plates. Labeling indices determined by normalizing the number of myocyte nuclei actively synthesizing DNA (BrdU positive), to the total number of myocyte nuclei in a given field, are plotted in Fig. 24. The WT and S262D-transfected cells had labeling indices not significantly different from the vector control, but the S262A mutant had a labeling index approximately 50% of the vector control. The decrease in labeling index was statistically significant ($p < 0.05$, ANOVA with Student-Newman-Keuls multiple comparisons post-hoc test). Also, the labeling index of S262D-transfected cultures was significantly higher than the index of the S262A-transfected cultures ($p < 0.05$, unpaired Student's t-test).

4. Localization of Overexpressed Cx43 in Chicken and Rat Cardiomyocytes:

Wild-type and all serine mutants of Cx43 expressed in chicken cardiomyocytes were localized to sites of intercellular contact between expressing cells and diffusely in the cytoplasm (Figure 25). A similar localization was seen in similarly transfected rat cardiomyocytes (Figure 26). The presence of transfected Cx43 at the cell membrane

Figure 23. Levels of Cx43 protein in neonatal rat cardiomyocytes transfected with plasmids encoding WT and mutant Cx43.

Total protein lysates (10 μg) isolated from transfected neonatal rat cardiomyocytes were analyzed by SDS-PAGE (10% PAG) and Western blotting with the rabbit polyclonal anti-Cx43 antiserum. Lanes containing lysates from vector- as well as wild type- (WT), S262A-, and S262D- transfected myocytes are as indicated. The migration of molecular mass standards is indicated in kilodaltons.

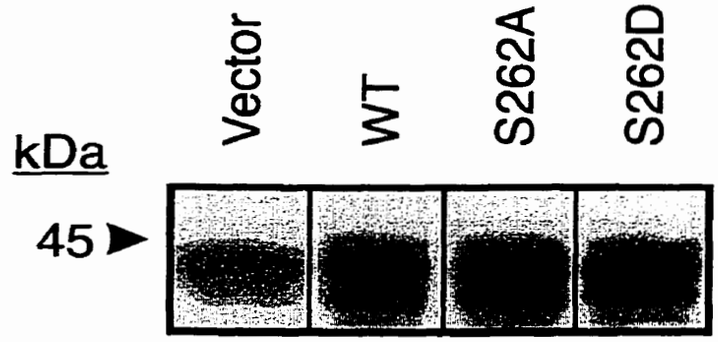
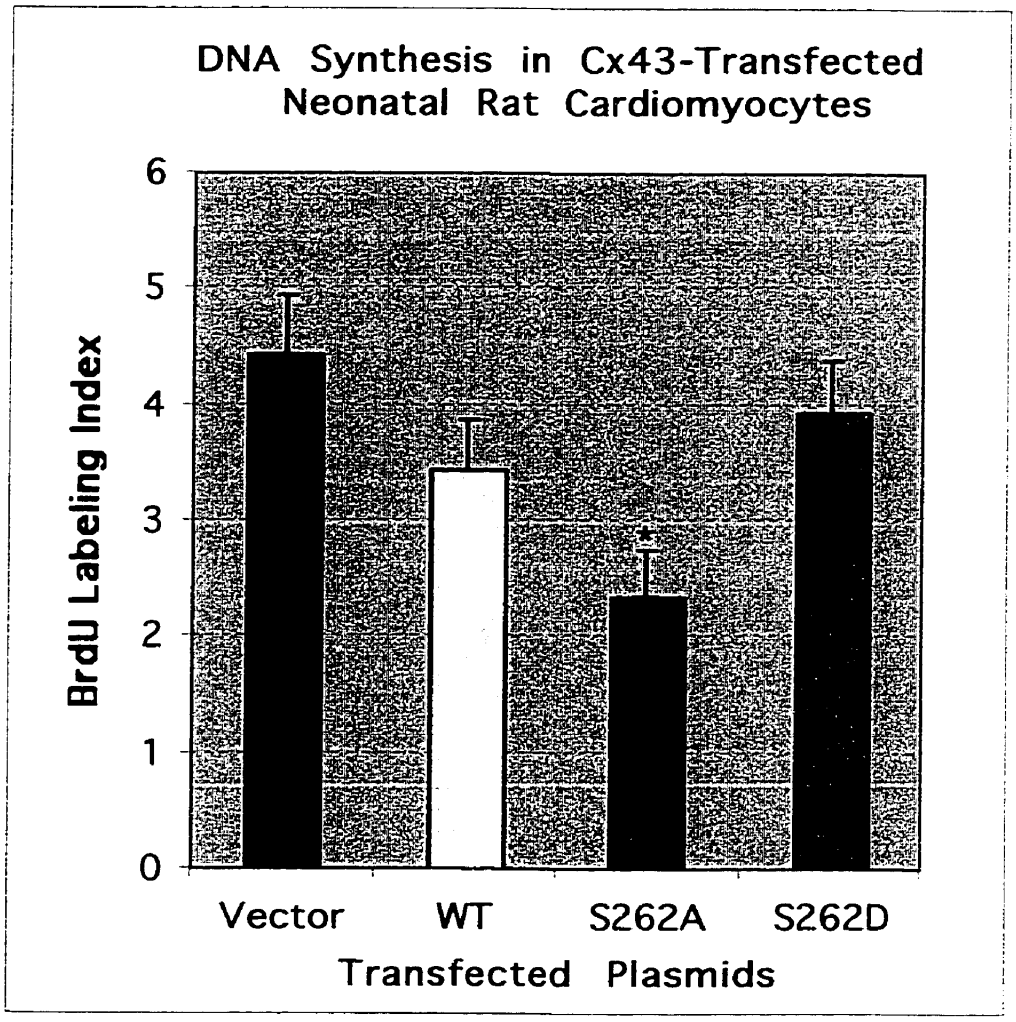


Figure 24. Effect of mutations on S262 of Cx43 on DNA synthesis of neonatal rat cardiomyocytes.

Neonatal myocytes, maintained in 10% FBS, were transfected with vector, wild type, S262A and S262D Cx43 cDNAs, and processed for immunofluorescence labeling 48 hours later, after labeling with BrDU for 6 h. Labeling Index, ratio of BrDU positive myocyte nuclei to total number of myocyte nuclei, is indicated in the vertical axis (n=5). Error bars are SEM. *denotes $p < 0.05$ between vector-transfected and S262A Cx43 expressing cultures (ANOVA with Student-Newman-Keuls multiple comparisons post-hoc test).


DNA Synthesis in Cx43-Transfected Neonatal Rat Cardiomyocytes
BrdU Labeling Index
6
5
4
3
2
1
0
*
Vector
WT
S262A
S262D
Transfected Plasmids

indicates that the mutants are still properly targeted, and are most likely able to form intercellular gap junctional structures. In both rat and chicken cells, strong perinuclear staining was also observed. This staining most probably indicates Cx43 in endoplasmic reticular and Golgi sites as it is being synthesized and processed for membrane insertion.

Figure 25. Localization of introduced Cx43 and Cx43 mutants in chicken cardiomyocytes.

Double-immunofluorescence labeling for myosin (A, B, C, D) or Cx43 (E, F, G, H), respectively, of chicken cardiomyocyte cultures transfected with wild type and S-to-A mutants of Cx43, as indicated. Arrows point to membrane sites of intercellular contact. Bar = 20 μm.

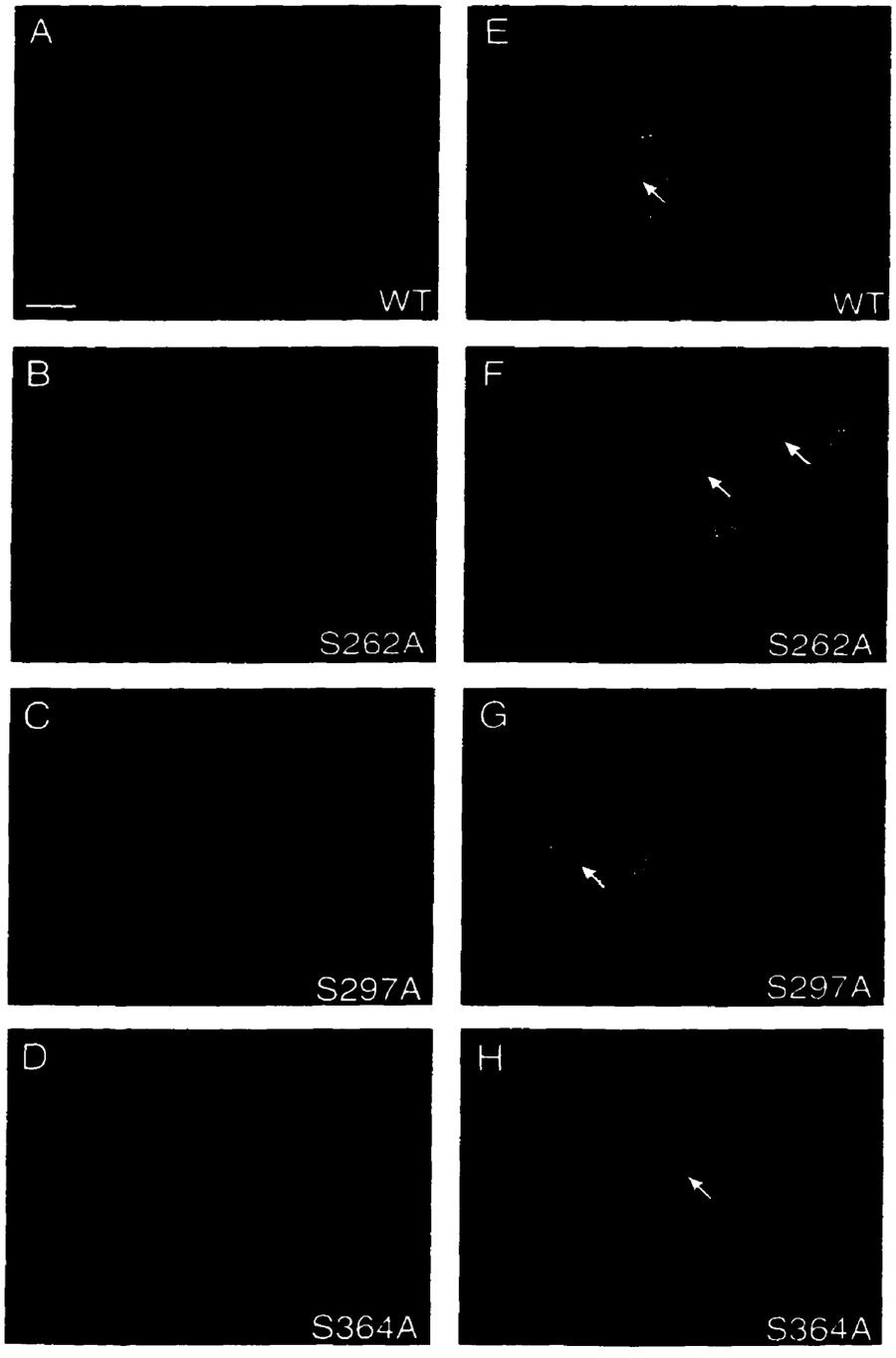
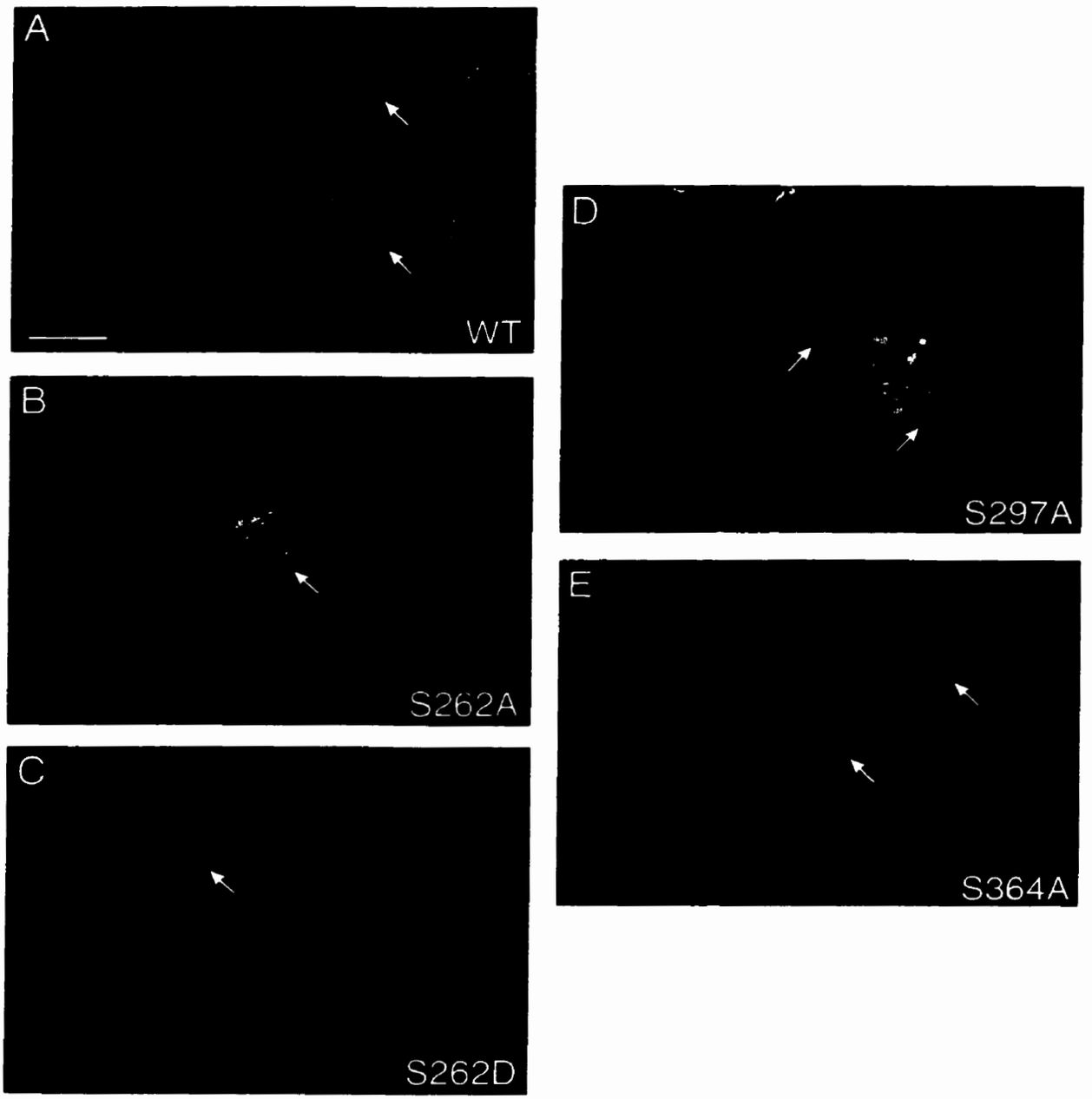


Figure 26. Localization of introduced Cx43 and Cx43 mutants in rat cardiomyocytes.

Immunofluorescence staining of rat cardiomyocyte cultures expressing introduced wild type or mutant Cx43, as indicated. Arrows point to membrane sites of intercellular contact. Bar=20 μm.



IV. DISCUSSION

A. FIBROBLAST GROWTH FACTOR-2 DECREASES METABOLIC COUPLING AND STIMULATES PHOSPHORYLATION AS WELL AS MASKING OF CONNEXIN43 EPITOPES IN CARDIAC MYOCYTES

FGF-2 is known to be released from myocytes during contraction and especially after injury, and is expected to exert both acute and long-term effects during the injury-repair response. We have examined the acute changes in intercellular communication and Cx43 in response to FGF-2. We initially used the scrape-loading technique to assess, in a relatively quick and easy manner, whether FGF-2 treatment had any effect on GJIC in cardiomyocytes. This technique is widely used (Blomstrand et al., 1999; Ciray et al., 2000; Kang et al., 2000), although it has been suggested that it be used only as an initial screening tool to be followed with a more quantitative technique (McKarns and Doolittle, 1992). In a recent *Methods* paper, Trosko et al. proposed that quantitative data can be obtained from scrape-loaded cells with appropriate digitization and analysis of collected scrape-loading images (Trosko et al., 2000).

Our scrape-loading data clearly implicated FGF-2 as inducing a reduction in the dye-coupling between cardiomyocytes. These data were fully supported by the more quantitative technique of fluorescent dye microinjection. In these experiments FGF-2 significantly reduced transfer of injected dye from the injected cell to adjacent neighboring cells. Microinjection also had the advantage of allowing dye-coupling measurements to be determined with the cells bathed in their maintenance cell culture medium, containing physiological levels of extracellular ions. In contrast, scrape-loading is done under reduced calcium conditions to prevent calcium-mediated closure of GJs

upon wounding (and loading) the cells with the bathing solution containing 6-CF.

It has become evident that Cx43 is the predominant connexin in rat ventricular myocytes, therefore, the FGF-2-mediated effect on dye-coupling in our study most likely reflects a change in the permeability of gap junctional channels composed of Cx43. Although earlier studies had suggested that Cx45 is also expressed by ventricular cardiomyocytes, this view is no longer held (Coppen et al., 1998). Instead, Cx45 is expressed primarily in cardiac conduction tissue (Coppen et al., 1998).

The reduction in dye-coupling caused by FGF-2 treatment is in contrast to our findings in rat heart fibroblasts (Doble and Kardami, 1995) and the findings of others in different cell types (Abdullah et al., 1999; Pepper and Meda, 1992). On the other hand, studies in mouse osteoblasts and rat cortical and striatal astroglial cells support a role for FGF-2 in the reduction of GJIC (Shiokawa-Sawada et al., 1997). A possible explanation for these differential effects is that different cells express different receptor subtypes that signal through alternative pathways. In the case of FGF, not only can different high affinity receptors lead to alternative signaling, but different types of low affinity heparan sulfate proteoglycans can also modulate the effects of FGF stimulation (McKeehan et al., 1998; Quarto and Amalric, 1994). Another possibility is that even when the same receptors are expressed by different cell types, the result of stimulation by a single growth factor may differ due to the existence of different intracellular signaling components. For instance, both PC12 cells and Swiss 3T3 fibroblasts express only FGFR1, but PC12 cells respond to FGF-2 by differentiating, while 3T3 cells respond by proliferating (Maher, 1999). The difference in FGF-2 effects in these cells is thought to be due to different levels of the signaling molecule p38 MAPK in the two cell types

(Maher, 1999). Thus, results obtained from growth factor stimulation of one cell type do not necessarily apply to other cell types.

Decreases in GJIC may arise due to several mechanisms: i) a reduction in connexin synthesis (Reuss et al., 1998; Shiokawa-Sawada et al., 1997); ii) an increase in connexin degradation (Musil et al., 2000); iii) a reduction in gap junction plaque size due to altered connexin trafficking or aggregation (Bukauskas et al., 2000); iv) changes in gap junction properties that result in reduced channel permeability (Kwak and Jongsma, 1996; Nicholson et al., 2000); and v) interaction with another protein(s) that leads to blockage of the gap junction pore (Peracchia et al., 2000). Gap junctions have been shown to be incredibly plastic structures that can move laterally in the plane of cell membranes and that can be pinched off in vesicles for trafficking to recycling or degradation compartments in time courses measured in minutes (Bukauskas et al., 2000; Falk, 2000; Holm et al., 1999; Jordan et al., 1999; Martin et al., 2000; Windoffer et al., 2000). In cultured neonatal rat cardiomyocytes, as well as in intact rat hearts, Cx43 has been shown to have an unusually fast turnover rate (approx. 1.5 hours), much higher than that of most membrane proteins (Beardslee et al., 1998; Hare and Taylor, 1991; Laird et al., 1991). Thus, an imbalance between degradation and synthesis, favoring degradation, could lead to very rapid decreases in Cx43 content at cell membranes that would be apparent by immunofluorescent detection of Cx43. In addition, changes in plaque size from large plaques to smaller plaques would also be obvious through immunofluorescent detection of Cx43. A critical plaque size appears to be necessary for a communicating gap junction (Bukauskas et al., 2000), so a marked decrease in plaque size could be responsible for reduced dye coupling. We used two different Cx43-specific antibodies,

one monoclonal, one polyclonal, in immunofluorescence studies to determine if FGF-2 was causing any gross changes in Cx43 abundance and / or distribution.

The two antibodies used were raised against different regions of the carboxy terminus of Cx43. Immunostaining with the rabbit antibody revealed no change in Cx43 distribution or quantity after FGF-2 treatment. This was also confirmed by Western blotting. On the other hand, a dramatic decrease in Cx43 staining by the monoclonal antibody was evident after FGF-2 treatment, indicating that the epitope(s) recognized by this antibody were altered / masked. Such an alteration in recognition by an antibody could be due to: i) a conformational change in the antigen caused by changes in the local environment (such as pH or Ca^{2+} levels); ii) a direct modification of a reactive epitope due to a post-translational modification such as phosphorylation; iii) interaction of the antigen with another molecule(s) that blocks antibody access to reactive epitopes; or any combination of the above. Our data do not exclude any of these scenarios as mechanisms for the observed masking of epitopes recognized by the monoclonal antibody. Local intracellular increases in Ca^{2+} levels have been shown to occur in rat cardiomyocytes upon FGF-2 treatment (Merle et al., 1995; Merle et al., 1997). This change in Ca^{2+} could possibly contribute to changes in Cx43 epitope conformation and also to the observed decrease in GJIC. Also, direct modification of Cx43 by phosphorylation and a possible interaction with an unidentified protein did occur in our experimental system, as discussed.

As described in the Introduction, Cx43 phosphorylation regulates its function at gap junctions (see pages 17-26). Both tyrosine and serine phosphorylation of Cx43 can cause rapid decreases in GJIC (Crow et al., 1990; Filson et al., 1990; Kanemitsu and Lau,

1993; Kwak and Jongsma, 1996). Thus, we examined whether part of the mechanism for the FGF-2 induced reduction in GJIC, and perhaps for the epitope masking phenomenon, might be due to increased Cx43 phosphorylation. Cardiac phosphorylated Cx43 migrates at 43-47 kDa (Laird and Revel, 1990; Lau et al., 1991). FGF-2 consistently stimulated [^{32}P]-labeling of Cx43, migrating at approx. 46 kDa. Autoradiographs of [^{32}P]-labeled Cx43 resulted in broad, rather diffuse bands, so we cannot rule out that subtle shifts in mobility may have occurred that are undetectable by this technique.

Phospho-amino acid analysis of immunoprecipitated [^{32}P]-Cx43 determined that Cx43 was phosphorylated on serine residues in both control and stimulated conditions. Therefore, the additional phosphorylation induced by FGF-2 concerned serine residues. This finding was in apparent contrast to our data showing immunoprecipitation of Cx43 with anti-phosphotyrosine antibodies after FGF-2 stimulation. We cannot completely rule out the possibility that our technique for detecting phospho-amino acids was not sensitive enough to detect low levels of tyrosine-phosphorylated amino acids. However, in view of the significant increase in serine phosphorylation caused by FGF-2, we propose that the anti-phosphotyrosine antibody may have immunoprecipitated another tyrosine-phosphorylated protein, or complex of proteins, associated with Cx43. Kinases such as PKC and MAPK have been proposed to phosphorylate Cx43 on serine residues (Kwak and Jongsma, 1996; Lampe et al., 2000; Warn-Cramer et al., 1998; Warn-Cramer et al., 1996) and are also capable of being tyrosine phosphorylated (Cobb et al., 1991; Konishi et al., 1997). Proteins such as these, which can be activated by FGF-2 in rat cardiomyocytes (Bogoyevitch et al., 1994; Disatnik et al., 1995; Padua et al., 1998), may interact with Cx43 to yield the anti-phosphotyrosine immunoprecipitation data we have

obtained.

To summarize: i) FGF-2 induces Cx43 phosphorylation on serine residues accompanied by a reduction in GJIC and a masking of Cx43 epitopes recognized by a monoclonal antibody; ii) an interaction between Cx43 and an unknown tyrosine-phosphorylated protein is strongly suggested by immunoprecipitation studies. The reduction in GJIC caused by FGF-2 treatment of neonatal rat cardiomyocytes may have relevance with respect to the growth potential of cardiomyocytes in hyperplasia or hypertrophy and / or may have implications in cardiomyocyte conduction of action potentials. Thus, it is important to determine the signal transduction pathway intermediates that are involved in the FGF-2-mediated phosphorylation of Cx43 and decreased GJIC.

B. PKCε IS REQUIRED FOR PHOSPHORYLATION OF CX43 IN NEONATAL RAT CARDIOMYOCYTES

Having established that FGF-2 treatment of neonatal rat cardiomyocytes leads to Cx43 phosphorylation on serine with a concomitant decrease in GJIC, we proceeded to identify which category of kinase(s) might be involved. Two kinases known to be coupled to FGF signal transduction in cardiomyocytes, PKC and MAPK (Bogoyevitch et al., 1994; Disatnik et al., 1995; Padua et al., 1998), have also been linked to the direct phosphorylation of Cx43 in other experimental systems (Lampe et al., 2000; Warn-Cramer et al., 1998; Warn-Cramer et al., 1996). Therefore, we asked whether MAPK and / or PKC mediate the effects of FGF-2 on gap junctions in cardiomyocytes.

The MEK1 inhibitor PD98059, which completely blocked the FGF-2-induced activation of MAPK, failed to prevent the FGF-2-induced increase in Cx43

phosphorylation and reduction in GJIC. Furthermore, MAPK remained active under conditions (chelerythrine treatment) that completely block the FGF-2 effects on Cx43 phosphorylation and dye coupling. Overall, our findings are consistent with a lack of involvement of the MAPK pathway on the FGF-2- induced effects on Cx43 in cardiac myocytes. This is in contrast to studies that examined the EGF stimulation of T51B rat liver epithelial cells and cells derived from Cx43 knockout mice (Kanemitsu and Lau, 1993; Warn-Cramer et al., 1998). These studies suggested that EGF stimulates MAPK to phosphorylate Cx43 directly on serine residues with a resultant reduction in GJIC. Similar studies using PDGF stimulation of T51B cells suggested an indirect role for MAPK in PDGF-mediated reduction of GJIC, in which MAPK does not phosphorylate Cx43 directly, but is still required for PDGF effects on gap junctions in combination with other signaling molecules such as PKC (Hossain et al., 1999b). Activation of MAPK alone did not appear to be sufficient for Cx43 phosphorylation or decreased GJIC in T51B cells, since treatment of these cells with sorbitol or H_2O_2, which clearly activated MAPK, had no effect on Cx43 phosphorylation or GJIC (Hossain et al., 1999a). As outlined earlier (page 77), different cell types can respond differently to growth factors with respect to gap junction properties. Thus, although FGF-2, EGF, sorbitol, H_2O_2 and PDGF all activate MAPK, perhaps it is not surprising that the result of MAPK activation upon stimulation with these different factors appears to be different with respect to Cx43 phosphorylation and GJIC. It appears that the integration of different signals initiated by each factor is cell-type specific.

We then investigated the involvement of PKC activation in mediating the effects of FGF-2 on cardiac GJ. Both PKC inhibitors, chelerythrine and calphostin C, used at

doses that have been shown to inhibit PKC activity in rat hearts and isolated myocytes (Kashiwagi et al., 1998; Yoshida et al., 1997), prevented the FGF-2-induced increase in Cx43 phosphorylation, indicating that PKC activation is required. Furthermore, chelerythrine blocked the effect of FGF-2 on dye migration, indicating that PKC activation was required for the FGF-2 decrease in cardiomyocyte dye-coupling, and strengthening the link between Cx43 phosphorylation and the regulation of gap junction permeability. Our data are in agreement with several previous reports that have shown that PKC activation by phorbol esters decreases coupling and increases Cx43 phosphorylation (Berthoud et al., 1993; Kenne et al., 1994; Kwak et al., 1995; Matesic et al., 1994; Shiokawa-Sawada et al., 1997).

Cx43 is an integral plasma membrane protein. Having implicated PKC activation in the mediation of FGF-2 effects on Cx43, we investigated the possibility that PKC, which translocates to membrane and sarcolemmal (Padua et al., 1998) sites upon activation, might be directly involved in the phosphorylation of Cx43. The carboxy terminus of Cx43 contains five consensus sites for PKC phosphorylation. Thus, direct phosphorylation of Cx43 by PKC is theoretically possible. Neonatal rat cardiomyocytes express at least 6 different PKC isoforms at levels detectable by Western blotting (Disatnik et al., 1994). Colocalization of specific PKC isoforms with their putative substrates in a cell is proposed to enhance the specificity and speed of kinase action, and there is increasing evidence suggesting that PKC binds to specific anchoring proteins, such as the RACKS (receptors for activated C kinase) located at various subcellular sites (Mochly-Rosen and Gordon, 1998).

The calcium-independent PKC isoform, PKCε, has been reported to localize to

intercalated disk-like sites upon stimulation with PMA and the drug diazoxide, a mitoK(ATP) channel opener (Disatnik et al., 1994; Wang and Ashraf, 1999; Wang et al., 1999). PKCε is stimulated by FGF-1 (Disatnik et al., 1995) and FGF-2 (Padua et al., 1998) in rat cardiomyocytes. Activation of PKCε by FGF-2 was detected as an increased association of PKCε with the cardiac myocyte membrane fraction in neonatal and adult cardiomyocytes (Doble et al., 1998; Padua et al., 1998). Thus, based on the above findings, we thought PKCε was a good candidate for a direct interaction with Cx43. We used the same polyclonal antibody used by Disatnik et al. to visualize PKCε (Disatnik et al., 1994).

In our cultures of neonatal rat cardiomyocytes, we detected PKCε at membrane sites of intercellular contact irrespectively of stimulation with FGF-2 or PMA, in contrast to the report of Disatnik et al. in which little membrane staining was seen before PMA treatment, while intensified intercalated disk staining was apparent after PMA treatment (Disatnik et al., 1994). This difference in staining pattern detected with the same antibody could be due to different fixation or permeabilization protocols used to prepare the samples for immunofluorescent staining, or could be due to different culture conditions (e.g. substrate, media, serum, etc.). We did notice a change from an interrupted, intercalated disk-like pattern of PKCε staining in control cells to a more continuous staining along cell-cell contacts after PMA or FGF-2 treatment. Our findings suggest that there is always a detectable quantity of PKCε localized to the plasma membrane under all conditions.

Our immunofluorescence findings have been confirmed by Western blotting analysis of cardiac membrane fractions (enriched in sarcolemmal membranes) from non-

stimulated adult hearts and neonatal myocytes (Doble et al., 1998; Padua et al., 1998): a 90 kD band, recognized by three different anti-PKCε antibody preparations was present in these fractions. Furthermore, PKCε co-precipitated with Cx43 irrespectively of stimulation, a finding that further reinforces the validity of our immunofluorescence studies.

The continuous pattern of PKCε localization at intercellular contact sites upon stimulation, in conjunction with the increased association of PKCε with membranes detected by Western blotting (Doble et al., 1998), would suggest that additional PKCε is translocated to previously unoccupied plasma membrane sites. It is also possible that some re-distribution of membrane-associated PKCε may occur upon stimulation. FGF-2 and PMA induced the same qualitative changes in the pattern of distribution of membrane PKCε, in agreement with the notion that FGF-2 stimulates this PKC isoform in cardiac myocytes.

The co-localization and co-immunoprecipitation studies point to an interaction between PKCε and Cx43 at cell-cell contact sites, an interaction that becomes more extensive in stimulated cells. Interaction between a serine-threonine kinase and a potential substrate under activation conditions which are known to result in increased substrate phosphorylation, offers strong support to the notion that PKCε can phosphorylate Cx43 directly upon activation.

To further examine whether there was a cause and effect relationship between PKCε activation and Cx43 phosphorylation in the intact myocyte, we used expression of a dominant negative truncated PKCε, PKCε(1-401) (Cai et al., 1997). In cultures expressing the PKCε(1-401) a statistically significant reduction of Cx43 phosphorylation

was achieved, despite the relatively low transfection efficiency (20%) of cardiomyocytes under these conditions. One problem, however, with this approach is that the truncated PKCε, lacking the catalytic C-terminal may not target to the appropriate cellular sites (Acs et al., 1997).

To circumvent transfection efficiency problems, and any doubts as to the effectiveness of truncated PKCε as a dominant negative reagent, we also used an adenovirus expressing a site-directed dominant-negative mutant of PKCε, PKCε(DN), that differs from wild type PKCε by only one amino acid that renders its kinase domain inactive but does not affect its ability to be properly targeted to membranes (Ping et al., 1999). Adenoviral infection ensured high levels of PKCε(DN) expression in virtually all treated myocytes allowing for a very effective dominant negative strategy (Ping et al., 1999). Under these conditions we were able to show a dramatic decrease in Cx43 phosphorylation in cultures expressing PKCε(DN) but not the vector, demonstrating that active PKCε is required for Cx43 phosphorylation in neonatal rat cardiomyocytes.

Another PKC isoform, PKCα, was also a candidate for direct interaction with Cx43 because it is found localized to the plasma membrane of neonatal cardiomyocytes (Disatnik et al., 1994), and is activated by FGF-2. Under the same immunoprecipitation conditions that revealed an association between PKCε and Cx43, PKCα did not co-precipitate with Cx43. This suggested that a selective interaction exists between Cx43 and PKCε. In addition, expression of a truncated form of PKCα, shown to act in a dominant negative fashion (Doble et al., 1998), had no effect on Cx43 phosphorylation of myocytes (data not shown).

PKCε has been implicated in a number of processes in the heart such as

contractility, cardioprotection, preconditioning and hypertrophy (see page 55). It has been hypothesized that the mechanism by which PKCs protect hearts from ischemic damage is through the regulation of mitochondrial K(ATP) channels (Wang and Ashraf, 1999; Wang et al., 1999). The mitoK(ATP) channel opener, diazoxide, activates three PKC isoforms, alpha, delta and epsilon, but only delta is actually seen to translocate to the mitochodria (Wang and Ashraf, 1999; Wang et al., 1999). PKCε translocates only to the intercalated disk, the site of gap junction localization (Wang and Ashraf, 1999; Wang et al., 1999). Perhaps, gap junctions, targeted by PKCε, also contribute towards the overall cardioprotective effect induced by PKCε stimulated by cardioprotective agents. In support of this notion are studies that have shown that anesthetics such as halothane, that cause decreased gap junctional permeability, are also cardioprotective (Cope et al., 1997; He and Burt, 2000).

The mechanisms regulating cardiomyocyte growth and hypertrophy appear to be manifold, involving numerous signaling pathways that respond to stimuli such as mechanical stretch, cellular stress or injury, and local or circulating extracellular factors (Hefti et al., 1997; Omens, 1998; Yamazaki et al., 1998). One factor that has been implicated in the upregulation of cardiomyocyte DNA synthesis and hypertrophy is FGF-2 (Bogoyevitch et al., 1994; Hefti et al., 1997; Kardami, 1990; Parker et al., 1991; Pasumarthi et al., 1994; Pasumarthi et al., 1996; Scheinowitz et al., 1998; Schneider et al., 1992; Schultz et al., 1999; Speir et al., 1992). Perhaps the most direct evidence linking FGF-2 to cardiac hypertrophy is the finding that FGF-2 knockout mice show significantly less hypertrophy due to pressure overload compared to normal mice (Schultz et al., 1999). FGF-2 is found in the heart at all developmental stages, as is one

of its high affinity receptors, FGFR1 (Casscells et al., 1990; Jin et al., 1994; Kardami and Fandrich, 1989; Kardami et al., 1995; Kardami et al., 1990; Liu et al., 1995; Speir et al., 1992; Speir et al., 1989). Although many targets of FGF-2 signal transduction have been identified, there are still gaps in our knowledge as to how FGF-2 mediates its effects.

Interestingly, PKCε has also been strongly implicated in the development of compensatory hypertrophy and in the regulation of normal myocardial growth in physiological hearts (Mochly-Rosen et al., 2000; Takeishi et al., 2000). Overexpression of PKCε in a transgenic mouse leads to mild concentric cardiac hypertrophy with no detriment to cardiovascular function (Takeishi et al., 2000). In cells that are capable of hyperplasia, PKCε has been shown to act as an oncogene (Cacace et al., 1993). For instance, when PKCε was overexpressed in rat-6 embryo fibroblasts, it increased the rate of cell division, reduced contact inhibition and promoted tumorigenesis of these cells when injected in nude mice (Cacace et al., 1993).

In numerous non-cardiac systems, a strong inverse relationship between cellular growth and GJIC has been illustrated in mice (Yamasaki et al., 1999; Yamasaki and Naus, 1996). Indeed, even in cardiomyocytes, there are several studies that have shown decreased Cx43 protein, often with altered localization, in hypertrophic hearts (Hall et al., 2000; Peters et al., 1993). Although most of these reports assumed that the decrease in Cx43 was a result of the hypertrophy, not the other way around, some recent evidence suggests that alterations in Cx43 expression are an early event in the genesis of cardiac hypertrophy and myocardial dysfunction (Hall et al., 2000). Interestingly, in developing human hearts, the distribution of Cx43 assessed by immunofluorescent-confocal microscopy gradually changes from a scattered distribution of Cx43 immunoreactivity

over the entire surface of cardiomyocytes in neonatal hearts to the restricted intercalated disk-associated localization of Cx43 in the adult heart (Peters et al., 1994). This change in distribution correlates with the gradual decrease in hypertrophic growth of the heart that occurs during postnatal human development.

Although these studies link gap junction density and localization with cardiac hypertrophy, more subtle changes in gap junction function that do not cause overt changes in localization may also play a role in hypertrophy. In light of recent findings that indicate that small gap junction plaques are much less permeable than large plaques (Bukauskas et al., 2000), the scattered distribution of Cx43 in neonatal human hearts may indicate that their myocytes are not as well coupled as those from adult hearts that contain large gap junctional plaques localized to intercalated disks. It may be that reduced metabolic coupling between myocytes in neonatal human hearts allows them to grow, and that this growth is arrested by the development of better intercellular coupling. Other mechanisms which reduce intercellular coupling, such as connexin phosphorylation, may also be capable of regulating the hypertrophic response.

Having identified Cx43 as a target for PKCε action, and with the knowledge that FGF-2, PKCε and Cx43 have all been implicated in the generation of cardiac hypertrophy, perhaps all three molecules function in concert to regulate cardiac growth. Regulation of connexin levels or functional properties may not only be a possible modulator of cardiac hypertrophic growth, but may also play a role in the control of cardiomyocyte hyperplasia.

C. PHOSPHORYLATION OF CX43 ON SERINE 262 AFFECTS NEONATAL RAT CARDIOMYOCYTE DNA SYNTHESIS AND THE

ELECTROPHORETIC MOBILITY OF CX43

We have shown that FGF-2, a potent mitogen for neonatal rat cardiomyocytes, stimulates serine phosphorylation of Cx43 and reduces GJIC in these cells. In other cellular systems, treatments with mitogens such as EGF or PDGF also cause serine (S) phosphorylation of Cx43 as well as reduced GJIC and a concomitant increase in cellular proliferation (Hossain et al., 1998; Hossain et al., 1999b; Kanemitsu and Lau, 1993; Lau et al., 1992). Having identified Cx43 as a target for PKCε in neonatal rat cardiomyocytes, we were interested in the precise site of phosphorylation, since changes in the phosphorylation state of this residue might be sufficient to regulate Cx43 function with respect to cellular proliferation / DNA synthesis. There are five possible PKC phosphorylation sites in the carboxy terminus of Cx43 (Kreegipuu et al., 1999), one of which is serine 262 (S262). We were most interested in this residue because it resides within the epitope recognized by the monoclonal antibody; this epitope appears to be masked upon FGF-2 treatment. We sought to determine if mutating this single serine residue or two other putative PKC phosphorylation sites, S297 or S364, to the unphosphorylatable amino acid alanine (A), would affect the phosphorylation pattern and function of Cx43 relating to cardiomyocyte DNA synthesis.

Our previous studies on Cx43 phosphorylation and gap junction permeability were conducted using neonatal rat cardiomyocytes, but this model was not appropriate for examining expression and electrophoretic migration of transiently expressed site-specific connexin mutants since the high endogenous Cx43 levels would make it impossible to distinguish between endogenous and mutant Cx43 by Western blotting. To circumvent this problem, we used primary cell cultures derived from embryonic chicken

ventricles that express very little immunodetectable endogenous Cx43. We did not use Cx43 "tagged" with a fused peptide such as GFP, since we did not want to alter the cytoplasmic termini of Cx43 in any way that might affect the conformation, phosphorylation or regulation of Cx43.

Western blots of lysates from embryonic chicken cells transfected with S262A, S297A and S364A Cx43 mutants indicated that the slowest migrating Cx43 isoform (P3) seen in wild-type transfectants was missing only in the S262A transfectants. It is well established that phosphorylation affects the electrophoretic migration of Cx43. Multiple sites become phosphorylated, resulting in progressively slower migration of the molecule (43-47 kDa) (Kadle et al., 1991; Laird et al., 1991; Moreno et al., 1994). Mitogenic stimulation is reported to induce additional phosphorylation of Cx43, indicated by bands migrating at 46-47 kDa (Lau et al., 1992; Xie et al., 1997). Thus, it would appear that the presence of S262 is required to produce the slowest migrating band, presumably by phosphorylation, a finding that also implies that S262 becomes phosphorylated in the physiological environment of the cell. The effect on electrophoretic motility was not simply due to substitution of serine with alanine since it was not observed in the other two mutants, both of which continued to display the slowest migrating bands.

We sought to confirm these results in a mammalian system in which endogenous Cx43 expression is very low. We chose the HeLa cell line for transient transfections of the Cx43 site-directed mutants since these cells have been used extensively by several groups to perform structure-function analysis of connexins (Eckert et al., 1993; Elfgang et al., 1995; Mesnil et al., 1995). In this system, and under low serum conditions, relative levels of the high molecular mass phosphorylated species of the transfected Cx43 mutants

were minimal. Upon a fifteen-minute treatment with PMA though, a similar pattern as seen in the chicken myocytes maintained in high serum was obtained, i.e. WT, S297A, and S364A Cx43 transfectants gave rise to a slow migrating Cx43 band of approx. 46 kDa (P3), while the S262A transfectants did not. These results point not only to a role for S262 in the phosphorylation of Cx43, but also imply that its phosphorylation is dependent on PKC activation. Taken together, the experiments from the chicken myocytes and the HeLa cells strongly implicate S262 of rat Cx43 as being a target for PKC under stimulating conditions such as high serum in the chicken cells or presence of PMA in the HeLa cells. PMA treatment of HeLa cells also stimulated increases of a ~44 kDa Cx43 species, indicating the presence of additional PKC-dependent phosphorylation sites; a likely candidate for direct phosphorylation by PKC is S368 (Lampe et al., 2000).

Additional molecular modifications may also affect Cx43 motility, and there have been reports of co-migration of more than one phosphorylated Cx43 species (Cruciani and Mikalsen, 1999). It is therefore possible that each band (P1, P2, P3) in our analyses may contain more than one Cx43 species, and/or that the bands designated as P1-3 from one transfectant are not identical in composition to the comigrating bands from another transfectant. Two-dimensional electrophoresis would help resolve this issue. However, Cx43 is a very basic protein and, to the best of our knowledge, there have been no reports of successful separation of the various Cx43 species by this method.

It is not clear whether phosphorylation at a single Cx43 site is sufficient to cause a detectable shift to a slower migrating band in one dimensional electrophoresis, or whether phosphorylation of two or more sites are needed per motility shift. It is however generally accepted that slower migration signifies more extensive phosphorylation within

the individual molecule. The complete absence of a band migrating at the P3 position in the S262A transfectants therefore indicated that phosphorylation at this site is required to achieve the more extensively phosphorylated species of Cx43 within the cellular environment.

S262 was not required to produce the less phosphorylated P1 or the intermediate and overall more abundant P2 Cx43 species. It is suggested that newly made Cx43 is first undergoing phosphorylation at amino acids other than S262, leading to the P1 and P2 species. The bulk of neonatal rat cardiomyocyte Cx43 migrates predominantly as the P2 species, while growth factor or PMA stimulation results in the appearance of a slower migrating, ~46 kDa Cx43 (see Fig. 8).

With the knowledge that S262 of Cx43 appeared to be a target for phosphorylation, we sought to assess the functional consequences of this post-translational modification on a growth factor end-point such as stimulation of DNA synthesis. We used the S262A mutation and another site-directed mutation S262D, in which S262 was replaced with the negatively charged aspartate residue (D), to simulate phosphorylation of this residue. The strategy of mutating serine to aspartate has been used successfully by other investigators to simulate phosphorylation of serine residues of several proteins, including Cx43 (Beck et al., 1998; Warn-Cramer et al., 1998). Expression of the S262A mutant significantly decreased the fraction of cells synthesizing DNA (BrdU labeling index), while expression of the S262D Cx43 mutant had no effect on cardiomyocyte DNA synthesis. Thus, it appears that phosphorylation of S262, a consensus site for PKC phosphorylation, may play a role in the regulation of DNA synthesis in cultured neonatal cardiomyocytes.

The possibility that cell proliferation and intercellular communication were inversely related was first suggested by Loewenstein in the 1960s (Loewenstein, 1968; Loewenstein and Kanno, 1966). In order to test the correlation between tumor progression / formation GJIC, the tumor promoter PMA has been used extensively (see page 24). PMA was shown to be a very potent tumor promoter that also caused a marked reduction in GJIC. Numerous follow-up experiments on the effects of PMA on Cx43 phosphorylation and gap junction function have revealed that PMA causes an increase in the amount of Cx43 phosphorylation with a concomitant reduction in GJIC, presumably through PKC activation (Madhukar et al., 1983; Mosser and Bols, 1982; Newbold and Amos, 1981; Rivedal et al., 1985; Ruch et al., 1987; Shiba et al., 1989; Walder and Lutzelschwab, 1984; Yamasaki et al., 1985; Yamasaki et al., 1983) (see page 24).

Only recently has direct phosphorylation of Cx43 by PKC been examined. Our own studies strongly implicate the epsilon isoform of PKC as a direct mediator of Cx43 phosphorylation in cultured rat cardiomyocytes, and a study by Lampe et al. (Lampe et al., 2000) identifies S368 of rat Cx43 as a probable site of direct PKC phosphorylation. The results of Lampe et al. convincingly support a role for S368 as a substrate for PKC, but do not rule out the phosphorylation of other Cx43 sites by PKC, since S368A mutants still show Cx43 band shifts to slower mobilities upon PMA treatment. Also, single channel events thought to occur due to PKC phosphorylation of S368 are still found in the S368A mutant. As the identification of S368A as a direct PKC target in this study was accomplished by comparing two-dimensional tryptic phosphopeptide maps of Cx43 from PMA stimulated cells and from peptides phosphorylated *in vitro* by a mixture of PKC isoforms α, β, and γ, sites phosphorylated by other PKC isoforms may have been

missed.

Studies that followed up on Loewenstein's original theory proposing decreased GJIC as a key to tumorigenesis have found that, in most tumor cells, GJIC is reduced, and conversely, when connexins and GJIC are re-established in communication deficient cells, these cells lose their tumorigenic properties (Yamasaki et al., 1999; Yamasaki and Naus, 1996). The general theory is that cells require growth inhibitory signals from their neighbors in order to keep their own growth in check. The identification of the signals that are communicated through gap junctions to maintain growth control has not yet been achieved, but cyclic nucleotides, IP_3, DAG, Ca^{2+}, Na^+ and K^+ have all been suggested as possible mediators of growth effects (Bevans et al., 1998; Cao et al., 1998; Kam et al., 1998; Nicholson et al., 2000; Niessen et al., 2000; Yamasaki et al., 1999; Yamasaki and Naus, 1996).

The mechanism by which gap junctions composed of Cx43 are gated appears to be strongly reliant on the carboxy terminus of this molecule. Delmar et al. have proposed a particle-receptor model of Cx43 gating in which the carboxy terminus of Cx43 acts as a particle that, upon a conformational change induced by intracellular acidification or growth factor activation, leads to the closure of gap junctional channels (Ek-Vitorin et al., 1996; Morley et al., 1997; Morley et al., 1996). Regions of the carboxy terminus that are thought to be important for the regulation of intercellular communication in the particle-receptor model are residues 261-300 and 374-382 for pH-mediated closure, and amino acids 261-280 for growth factor-mediated closure.

Recent experiments have also implicated the carboxy terminus of Cx43 in the regulation of the growth parameters of HeLa and 363 A31 fibroblast cells. Stable

transfectants of 363 A31 fibroblasts, expressing a truncation mutant of Cx43 consisting of amino acids 1-256 of rat Cx43, were shown to have a decreased growth rate and a lowered mitogenic response to platelet derived growth factor compared to that of untransfected or vector transfected cells (Moorby, 2000; Moorby and Gherardi, 1999). These fibroblasts, expressing truncated Cx43, had decreased BrdU incorporation following growth factor stimulation, with an apparent arrest of growth in the G2 or M phases of the cell cycle and also revealed reduced cell motility (Moorby, 2000; Moorby and Gherardi, 1999). Similar experiments in HeLa cells have also revealed a vital role for the carboxy terminus in the regulation of Cx43 properties related to growth. HeLa cells transfected with full-length Cx43 showed no major differences in tumorigenic or growth properties, while those transfected with a truncation mutant suppressed anchorage-independent cell growth and delayed the appearance of tumors in nude mice (Omori and Yamasaki, 1999).

Thus, it appears that the carboxy terminus is very important for the regulation of Cx43 gating and regulation of its properties related to growth. The full length Cx43 molecule has several phosphorylation sites in its carboxy terminus that are probably phosphorylated by different kinases and are involved in regulating the permeability of Cx43 channels. Loss of this regulation, through the truncation of the carboxy tail, leads to a Cx43 channel that is constitutively open, resulting in decreased growth due to intercellular diffusion of the yet unknown negative growth regulatory factors.

In the present study, mutation of S262 to an unphosphorylatable residue was able to decrease the DNA synthesis of whole populations of cardiomyocytes, even though the apparent transfection efficiency was only 7.2 +/- 2.4%. As the transfection efficiency

was determined by counting the number of myocytes clearly overexpressing immunodetectable Cx43, this value underestimates the actual transfection efficiency which is probably closer to the 20% level we obtained by assaying myocytes, transfected with a reporter plasmid expressing β-galactosidase, for enzyme activity (see Fig. 16).

Since PMA-mediated phosphorylation of Cx43, presumably through PKC, leads to a decrease in intercellular communication (Kwak and Jongsma, 1996; Kwak et al., 1995), it is likely that the phosphorylation of S262, a putative PKC substrate, contributes to decreased GJ permeability (perhaps in addition to effects of S368 phosphorylation), although this remains to be tested. Our cardiomyocytes, maintained in 10% FBS, would have had a strong activation of many growth-related signaling pathways, including the PKC pathway. Thus, cardiomyocytes transfected with vector only displayed maximal DNA synthesis, accompanied by extensive Cx43 phosphorylation. Prevention of the phosphorylation of S262 will theoretically release some of the inhibition of intercellular communication between myocytes, thus restricting growth of the cells through the communication of growth inhibitory factors. Since the inhibition of growth is thought to be due to the increased diffusion of negative growth factors, a single cell expressing the mutant (open) channels may exert its effect on all cells it is in contact with. Thus, the S262A channels may exert a dominant effect, causing the opening of hemi-channels with which they dock.

Considering the exciting new data obtained through the use of Cx43-GFP fusion proteins (Holm et al., 1999; Jordan et al., 1999; Martin et al., 2000; Windoffer et al., 2000), we now know that gap junctions are extremely motile structures that move laterally within the membrane, and pinch-off and fuse with the membrane via vesicular

trafficking. It is possible that connexins from one cell can be endocytosed by a partner cell and then re-targeted to a membranous plaque, allowing for the flip-flop of different connexin types between adjacent cells. This would also allow for another way for mutant channel properties to be manifested in cells not originally expressing the mutant.

In cells overexpressing wild type Cx43, there was a trend towards decreased DNA synthesis. This is not surprising, as decreased cell proliferation has been reported in other cell types that have been transfected with Cx43 (Chen et al., 1995; Mehta et al., 1991; Mehta et al., 1999; Naus et al., 1992; Rose et al., 1993; Zhu et al., 1991). Perhaps expression of excess Cx43 overwhelms the mechanisms which regulate its function in gap junction channels. Alternatively, Cx43 may function to suppress cell growth via mechanisms other than regulation of GJIC (discussed below).

Since lack of phosphorylation of S262 caused decreased DNA synthesis, one could expect that its constitutive phosphorylation might potentiate DNA synthesis. Under the conditions of our experiments the S262D mutation did not enhance DNA synthesis above control levels. This might be because the cells were maintained in 10% FBS and were theoretically maximally stimulated. Future experiments should examine the effect of the S262D mutation under non-stimulated conditions. One can also speculate that phosphorylation at S262 may be permissive for the manifestation of the effects of mitogens, rather than contributing directly to stimulation of DNA synthesis. Still, there was a statistically significant difference between the labeling indices of cells transfected with S262A and S262D, indicating that simulated phosphorylation or prevention of phosphorylation on this residue gives rise to different effects on DNA synthesis.

The exact mechanism by which the S262A mutation results in inhibition of cardiomyocyte DNA synthesis remains to be established. It is assumed that it prevents gap junction uncoupling caused by mitogenic stimulation; this needs to be addressed by electrophysiological and dye transfer studies. On the other hand, Yamasaki et al. have provided some evidence for connexin growth regulation independent of intercellular communication (Krutovskikh et al., 2000; Yamasaki et al., 1999). Thus, we cannot exclude the possibility that the mutant S262A had additional or alternate effects, independent of or concurrent with effects on coupling. These might include interaction with intracellular signaling molecules and / or molecular complexes.

Several groups have demonstrated that expression of Cx43 results in formation of functional gap junction channels in various cell types (Elfgang et al., 1995; Koval et al., 1995). It is therefore likely that this is the case in our experimental systems as well. Formation of intercellular channels is a function of the amino (N)-terminal half of the molecule (Fishman et al., 1991). The N-terminal half of Cx43 remained identical in all our Cx43 transfectants, therefore, no differences in localization were anticipated. Our immunolocalization studies confirmed that wild type as well as mutant Cx43 localized in association with plasma membranes at sites of cell-to-cell contact. Some accumulation was also observed in the cytosol; this was presumably a result of overexpression and saturation of plasma membrane sites. Certainly wild type and all mutant Cx43s were phosphorylated to a substantial degree and all presented the slower migrating bands (predominance of P2) presumed to be present at functional, plasma membrane gap junction channels (Musil and Goodenough, 1991).

Cardiomyocytes derived from embryonic or neonatal rat hearts are still capable of

DNA synthesis and mitosis (Ueno et al., 1988), but it is generally accepted that shortly after birth they lose these abilities and become terminally differentiated (Long et al., 1990; Ueno et al., 1988). Thus, the heart is left with no mechanism to replace irreversibly damaged cardiomyocytes that result after a myocardial infarction, but can compensate only by increasing the size of remaining myocytes (hypertrophy). The critical factors involved in the permanent withdrawal of cardiac myocytes from the cell cycle are still unclear. Thus, it is important to identify all mechanisms that are involved in the regulation of cardiomyocyte proliferation, hypertrophy and differentiation.

Recent experiments in our laboratory (Banerji, Doble, Ping, Agustin and Kardami, manuscript *in preparation*), have demonstrated that PKCε is essential for the manifestation of growth-factor stimulation of cardiomyocyte DNA synthesis. Use of PKCε(DN), conditions that prevent growth factor or PMA induced phosphorylation of Cx43 (as in Chapter 2), also completely blocked cardiomyocyte DNA synthesis. Therefore, a close relationship exists between PKC activation, Cx43 phosphorylation and stimulation of DNA synthesis. Our data indicate that phosphorylation of Cx43 on S262 is an important element of the mechanism by which growth factors stimulate cardiomyocyte proliferation.

The cardiomyocytes of the heart are coupled through numerous gap junctions that are vital for coordinated contraction of the heart; proper coupling is also important in preventing arrhythmias. The necessity for electrical coupling through gap junctions may be part of the mechanism that precludes proliferation of differentiated cardiomyocytes, in view of the inverse relationship between GJIC and cellular proliferation. Thus, manipulations targeting the phosphorylation status of specific sites on Cx43 may provide

an important component of strategies to induce cardiac regeneration.

D. CONCLUDING REMARKS

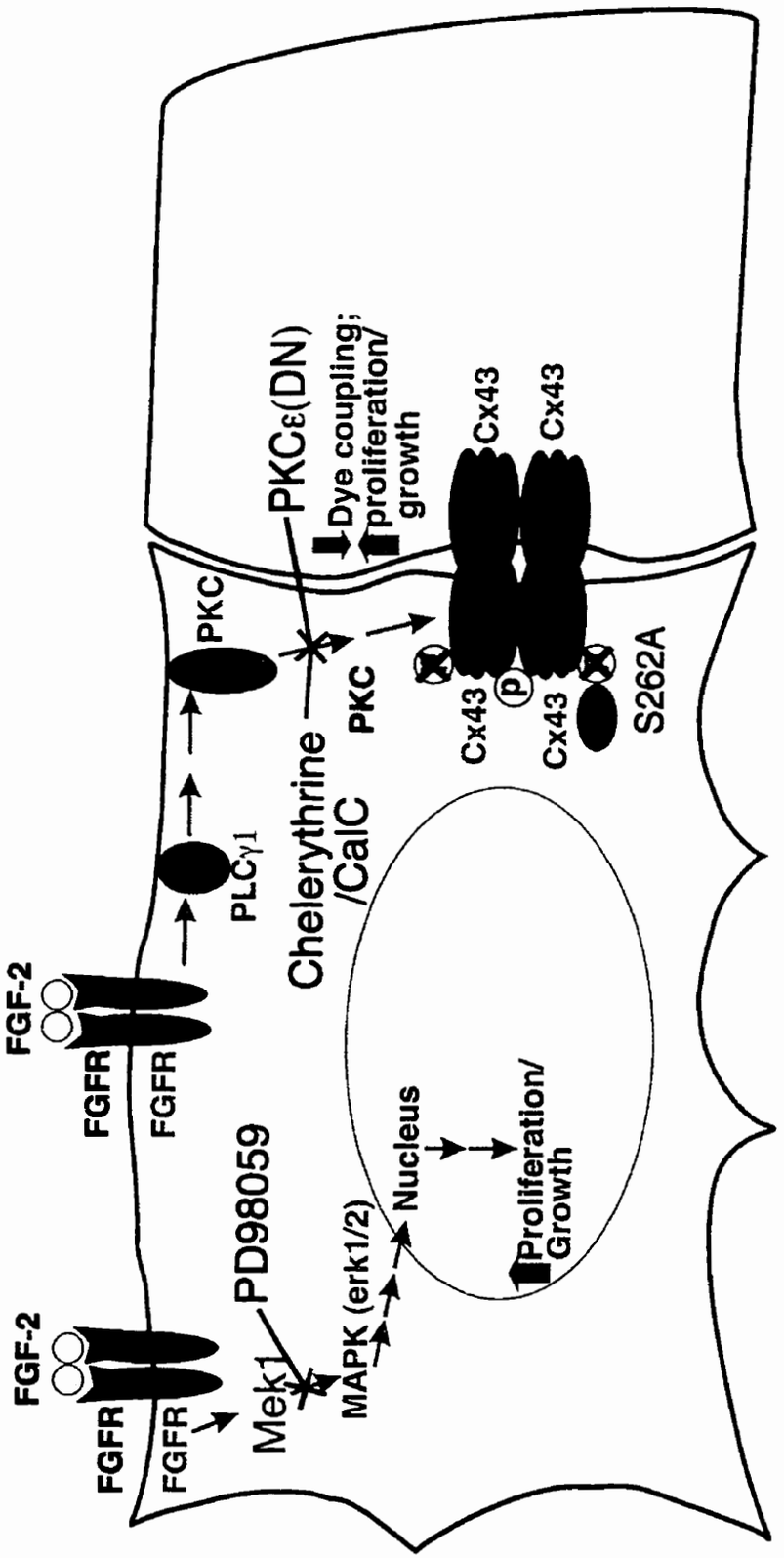
Taken together, the data presented in this thesis provide new information on the regulation of cardiomyocyte gap junction function as it relates to cardiomyocyte growth. FGF-2 has been shown to induce Cx43 phosphorylation and decrease GJIC through a PKC-mediated pathway. We have identified Cx43 as a substrate for the specific PKC isoform PKCε, and have provided evidence indicating a role for phosphorylation of Cx43 on S262 in the regulation of cardiomyocyte DNA synthesis. A schematic overview of these findings is presented in Fig. 27.

Our studies have been limited somewhat by currently available methodology and the nature of our experimental model. These limitations, and suggestions of how they can be overcome with future experiments and new techniques, are discussed below.

Limitations of Our Study and Future Directions of Study:

Our approach to studying cardiovascular gap junctions has been to use a neonatal rat cardiomyocyte cell culture model. While these myocytes are capable of DNA synthesis, it is generally accepted that adult cardiomyocytes are not. Thus, our studies on the effects of Cx43 phosphorylation on cardiomyocyte DNA synthesis are not directly applicable to adult cardiomyocytes. Future experiments should be carried out to determine if altering Cx43 phosphorylation, using for instance, the S262D mutant, in adult cardiomyocytes, has any effect on the DNA synthesis of these "terminally differentiated" cells. Studies on adult cardiomyocytes would require a different transfection system than the calcium phosphate technique used for the neonatal cells since these cannot be transfected with this technique. An adenoviral expression system, such as the one used to introduce dominant-negative PKCε in our neonatal

Figure 27. Schematic diagram of the regulation of neonatal rat cardiomyocyte gap junctions by FGF-2 signal transduction and phosphorylation of Cx43.



cardiomyocytes should be effective in introducing mutant connexins into adult cardiomyocytes (Kirshenbaum et al., 1993).

It would also be very interesting to examine the effects of Cx43 phosphorylation mutants in a transgenic mouse system, in which the effect of heart-targeted Cx43 phosphorylation mutants on parameters such as cell number and size could be determined. A transgenic model in which Cx43 phosphorylation mutants (e.g. S262D) were targeted to the myocardium, but were also under control of an inducible promoter, might be especially informative and could even be required if the phosphorylation mutant had severe adverse consequences during fetal development. The tetracycline inducible systems (Gossen and Bujard, 1992; Gossen et al., 1995) marketed by Clontech would be ideal for these experiments. In the "Tet-On" model, a transactivator activated by tetracycline would be put under the control of a cardiac specific promoter, while the mutant Cx43 gene would be under the control of the tetracycline responsive transactivator. Addition of tetracycline would activate the transactivator and initiate transcription of the Cx43 mutant.

Another limitation of our study was that we assessed coupling by dye transfer alone. As dye-coupling and ionic (electric) coupling do not always parallel each other (Kwak et al., 1995), it would be interesting to apply electrophysiological techniques used to measure gap junctional conductance to all of our experiments in which dye coupling was assessed. Analysis of both dye and electrical coupling would also be of interest with respect to the Cx43 phosphorylation mutants we have generated.

Our assessment of Cx43 phosphorylation used immunoprecipitation of [^{32}P]-Cx43 or analysis of band shifts in Western blots. These methods, although widely used to

examine Cx43 phosphorylation, provide information only on changes in phosphorylation levels, but not on which specific residues are being phosphorylated. Thus, we currently do not know through direct assessment which residue(s) of Cx43 are phosphorylated upon FGF-2 treatment. It will be vital to determine the actual sites that are phosphorylated on Cx43 under various conditions to help clarify data that imply a change in Cx43 function upon phosphorylation; that is, the exact stoichiometry of phosphorylation of Cx43 in numerous experimental models needs to be determined.

Towards that end, refinements in mass spectrometry instrumentation and sample preparation achieved within the last decade have led to the development of new applications for this technique that are becoming increasingly popular with biochemists and molecular biologists. While traditional mass spectroscopy techniques allowed only for the analysis of relatively small, stable molecules, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) mass spectrometry allow for the analysis of both small and large (several hundreds of kilodaltons) biomolecules (Kaufmann, 1995; Siuzdak, 1994; Stults, 1995). These new mass spectrometry techniques have become the method of choice for identifying unknown proteins isolated by techniques such as 2-dimensional electrophoresis, and are gaining popularity in determining post-translational modifications of proteins and even non-covalent interactions between proteins (Winston and Fitzgerald, 1997; Yates, 1998).

With respect to studying Cx43, MALDI and ESI could prove invaluable in helping to determine phosphorylation sites under control and treatment conditions in different cell systems. This technique has the power to determine phosphorylated residues in proteins derived from Coomassie-blue stained gels (Lennon and Walsh, 1999;

Matsumoto et al., 1998; Zhang et al., 1998). Thus, the exact stoichiometry and location of phosphorylated residues necessary to yield the multi-band electrophoretic profile of Cx43 from rat hearts could be resolved by analyzing each band individually.

Determination of phosphorylation sites may give some indication as to the kinases that are involved in Cx43 phosphorylation, but direct proof of kinase interaction with Cx43 must be determined for all kinases implicated in Cx43 phosphorylation. The current strategies used to find kinases involved in Cx43 phosphorylation have been based on screening likely candidates for Cx43 phosphorylation. An unbiased method, such as the yeast two hybrid system (ras-rescue), may help in identifying interacting kinases or other interacting molecules without bias. The ras-rescue system (Aronheim, 1997; Aronheim et al., 1997; Broder et al., 1998) uses a special strain of yeast that contains a temperature-sensitive (ts) mutation in the cdc25 gene. This strain of yeast can only survive at 37 °C if it is complemented with membrane-associated hSos gene product. The ts yeast are cotransformed with a "library" of membrane anchored "target" proteins and a "bait" protein (e.g. Cx43 carboxy terminus) fused to hSOS. A positive interaction between the "bait" and "target" will provide membrane-associated hSOS for the ts yeast, allowing for ras-signaling and survival of the yeast at 37 °C. Yeast in which no interaction occurs will die at the restrictive temperature. This yeast two-hybrid system is commercially available from Stratagene as the CytoTrap™ system. This system would be ideal for identifying interactions between Cx43 and unknown proteins since interactions occur in the cytoplasm near the plasma membrane, and both "bait" and "target" polypeptides can be post-translationally modified.

The Near Future of Connexin Research:

Connexin mutations have been identified as the cause of several human diseases including deafness (Kelsell et al., 1997; Rabionet et al., 2000), cataracts (Gong et al., 1997; Mackay et al., 1999; Pal et al., 2000; Shiels et al., 1998; Steele et al., 1998; White et al., 1998) and neuro-degeneration (Abrams et al., 2000). As with other genetic diseases in which the cause is known, gene therapy aimed at restoring defective connexins in the above diseases is sure to be explored in the near future.

Although still somewhat controversial, a direct role for connexin alterations in the development of cardiac arrhythmias is gaining increasing support from knock-out mice (Cx40) (Kirchhoff et al., 1998; Simon et al., 1998) and other transgenic models (Cx43 +/-) (Lerner et al., 2000). More research is needed to determine what sort of connexin modifications may be responsible for the genesis of arrhythmias in humans. Questions that remain to be addressed are: i) Are post-translational modifications of connexin, such as phosphorylation / dephosphorylation, sufficient to predispose a heart to arrhythmias, or are alterations in Cx43 protein levels or localization required? As current strategies for reducing arrhythmias in humans do not target connexins, there exists a strong potential for the development of new anti-arrhythmic therapeutic strategies once the nature of the role connexins play in arrhythmogenesis is discerned.

Another "hot" area of recent connexin research is connexin trafficking and degradation (see pages 27-30). The connexins have a very short half-life (1.5 hours for Cx43) (Laird et al., 1991), which may have severe consequences if the connexin synthetic machinery is disrupted. As Cx43 has been shown to be degraded by both the proteasomal and lysosomal systems (Beardslee et al., 1998), it will be important to determine what cues Cx43 to be degraded by either pathway. Also, the mechanism by which connexins

are targeted to the plasma membrane and recruited into gap junctional plaques requires further study.

The last decade of gap junction research has led from the evolution of gap junctions being considered as passive aqueous channels with limited regulation, to being recognized as dynamic, regulatable, motile structures that may serve as important targets for therapeutics aimed at human disease. The manifold layers of regulation controlling gap junction function, from their synthesis, degradation, phosphorylation, and targeting to their modulation by environmental factors such as pH and Ca^{2+}, as well as the possibility for numerous combinations of heteromeric or heterotypic channels, are only beginning to be understood. Many fruitful years of gap junction research are still to come.

V. REFERENCES

1. Abdullah, K.M., G. Luthra, J.J. Bilski, S.A. Abdullah, L.P. Reynolds, D.A. Redmer, and A.T. Grazul-Bilska. 1999. Cell-to-cell communication and expression of gap junctional proteins in human diabetic and nondiabetic skin fibroblasts: effects of basic fibroblast growth factor. *Endocrine*. 10:35-41.

2. Abrams, C.K., S. Oh, Y. Ri, and T.A. Bargiello. 2000. Mutations in connexin 32: the molecular and biophysical bases for the X- linked form of Charcot-Marie-Tooth disease. *Brain Res Brain Res Rev*. 32:203-14.

3. Abudara, V., G. Garces, and J.C. Saez. 1999. Cells of the carotid body express connexin43 which is up-regulated by cAMP. *Brain Res*. 849:25-33.

4. Acs, P., K. Bogi, P.S. Lorenzo, A.M. Marquez, T. Biro, Z. Szallasi, and P.M. Blumberg. 1997. The catalytic domain of protein kinase C chimeras modulates the affinity and targeting of phorbol ester-induced translocation. *J Biol Chem*. 272:22148-53.

5. Ahmad, S., J.A. Diez, C.H. George, and W.H. Evans. 1999. Synthesis and assembly of connexins in vitro into homomeric and heteromeric functional gap junction hemichannels. *Biochem J*. 339:247-53.

6. Ai, Z., A. Fischer, D.C. Spray, A.M. Brown, and G.I. Fishman. 2000. Wnt-1 regulation of connexin43 in cardiac myocytes. *J Clin Invest*. 105:161-71.

7. Alcolea, S., M. Theveniau-Ruissy, T. Jarry-Guichard, I. Marics, E. Tzouanacou, J.P. Chauvin, J.P. Briand, A.F. Moorman, W.H. Lamers, and D.B. Gros. 1999. Downregulation of connexin 45 gene products during mouse heart development. *Circ Res*. 84:1365-79.

8. Apel, E.D., D.W. Litchfield, R.H. Clark, E.G. Krebs, and D.R. Storm. 1991. Phosphorylation of neuromodulin (GAP-43) by casein kinase II. Identification of phosphorylation sites and regulation by calmodulin. *J Biol Chem*. 266:10544-51.

9. Armstrong, M.T., D.Y. Lee, and P.B. Armstrong. 2000. Regulation of proliferation of the fetal myocardium. *Dev Dyn*. 219:226-36.

10. Armstrong, S., and C.E. Ganote. 1994. Preconditioning of isolated rabbit cardiomyocytes: effects of glycolytic blockade, phorbol esters, and ischaemia. *Cardiovasc Res*. 28:1700-6.

11. Armstrong, S.C., D.B. Hoover, M.H. Delacey, and C.E. Ganote. 1996. Translocation of PKC, protein phosphatase inhibition and preconditioning of rabbit cardiomyocytes. *J Mol Cell Cardiol*. 28:1479-92.

12. Aronheim, A. 1997. Improved efficiency sos recruitment system: expression of the

mammalian GAP reduces isolation of Ras GTPase false positives. *Nucleic Acids Res.* 25:3373-4.

13. Aronheim, A., E. Zandi, H. Hennemann, S.J. Elledge, and M. Karin. 1997. Isolation of an AP-1 repressor by a novel method for detecting protein- protein interactions. *Mol Cell Biol.* 17:3094-102.

14. Asamoto, M., M. Oyamada, A. El Aoumari, D. Gros, and H. Yamasaki. 1991. Molecular mechanisms of TPA-mediated inhibition of gap-junctional intercellular communication: evidence for action on the assembly or function but not the expression of connexin 43 in rat liver epithelial cells. *Mol Carcinog.* 4:322-7.

15. Aylsworth, C.F., J.E. Trosko, C.C. Chang, K. Benjamin, and E. Lockwood. 1989. Synergistic inhibition of metabolic cooperation by oleic acid or 12-0-tetradecanoylphorbol-13-acetate and dichlorodiphenyltrichlorethane (DDT) in Chinese hamster V79 cells: implication of a role for protein kinase C in the regulation of gap junctional intercellular communication. *Cell Biol Toxicol.* 5:27-37.

16. Banerjee, A.K. 1980. 5'-terminal cap structure in eucaryotic messenger ribonucleic acids. *Microbiol Rev.* 44:175-205.

17. Bannerman, P., W. Nichols, S. Puhalla, T. Oliver, M. Berman, and D. Pleasure. 2000. Early migratory rat neural crest cells express functional gap junctions: Evidence that neural crest cell survival requires gap junction function. *J Neurosci Res.* 61:605-15.

18. Banoub, R.W., M. Fernstrom, A.M. Malkinson, and R.J. Ruch. 1996. Enhancement of gap junctional intercellular communication by dibutyryl cyclic AMP in lung epithelial cells. *Anticancer Res.* 16:3715-9.

19. Bar-Eli, M. 1999. Role of AP-2 in tumor growth and metastasis of human melanoma. *Cancer Metastasis Rev.* 18:377-85.

20. Barrio, L.C., T. Suchyna, T. Bargiello, L.X. Xu, R.S. Roginski, M.V. Bennett, and B.J. Nicholson. 1991. Gap junctions formed by connexins 26 and 32 alone and in combination are differently affected by applied voltage [published erratum appears in Proc Natl Acad Sci U S A 1992 May 1;89(9):4220]. *Proc Natl Acad Sci U S A.* 88:8410-4.

21. Bastide, B., J.C. Herve, and J. Deleze. 1994. The uncoupling effect of diacylglycerol on gap junctional communication of mammalian heart cells is independent of protein kinase C. *Exp Cell Res.* 214:519-27.

22. Bastide, B., L. Neyses, D. Ganten, M. Paul, K. Willecke, and O. Traub. 1993. Gap junction protein connexin40 is preferentially expressed in vascular endothelium and conductive bundles of rat myocardium and is increased under hypertensive conditions. *Circ Res.* 73:1138-49.

23. Battegay, E.J. 1995. Angiogenesis: mechanistic insights, neovascular diseases, and therapeutic prospects. *J Mol Med.* 73:333-46.

24. Beardslee, M.A., J.G. Laing, E.C. Beyer, and J.E. Saffitz. 1998. Rapid turnover of connexin43 in the adult rat heart [see comments]. *Circ Res.* 83:629-35.

25. Beck, E.J., R.G. Sorensen, S.J. Slater, and M. Covarrubias. 1998. Interactions between multiple phosphorylation sites in the inactivation particle of a K+ channel. Insights into the molecular mechanism of protein kinase C action. *J Gen Physiol.* 112:71-84.

26. Behrens, J. 2000. Control of beta-catenin signaling in tumor development. *Ann N Y Acad Sci.* 910:21-33; discussion 33-5.

27. Bell, R.M., Y.A. Hannun, and C.R. Loomis. 1986. Mechanism of regulation of protein kinase C by lipid second messengers. *Symp Fundam Cancer Res.* 39:145-56.

28. Bender, C.M., M.M. Pao, and P.A. Jones. 1998. Inhibition of DNA methylation by 5-aza-2'-deoxycytidine suppresses the growth of human tumor cell lines. *Cancer Res.* 58:95-101.

29. Bennett, M.V., L.C. Barrio, T.A. Bargiello, D.C. Spray, E. Hertzberg, and J.C. Saez. 1991. Gap junctions: new tools, new answers, new questions. *Neuron.* 6:305-20.

30. Bergoffen, J., S.S. Scherer, S. Wang, M.O. Scott, L.J. Bone, D.L. Paul, K. Chen, M.W. Lensch, P.F. Chance, and K.H. Fischbeck. 1993. Connexin mutations in X-linked Charcot-Marie-Tooth disease. *Science.* 262:2039-42.

31. Berridge, M.J. 1993. Inositol trisphosphate and calcium signalling. *Nature.* 361:315-25.

32. Berthoud, V.M., M.L. Ledbetter, E.L. Hertzberg, and J.C. Saez. 1992. Connexin43 in MDCK cells: regulation by a tumor-promoting phorbol ester and Ca2+. *Eur J Cell Biol.* 57:40-50.

33. Berthoud, V.M., M.B. Rook, O. Traub, E.L. Hertzberg, and J.C. Saez. 1993. On the mechanisms of cell uncoupling induced by a tumor promoter phorbol ester in clone 9 cells, a rat liver epithelial cell line. *Eur J Cell Biol.* 62:384-96.

34. Bevans, C.G., M. Kordel, S.K. Rhee, and A.L. Harris. 1998. Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules. *J Biol Chem.* 273:2808-16.

35. Beyer, E.C., D.L. Paul, and D.A. Goodenough. 1987. Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J Cell Biol.* 105:2621-9.

36. Beyer, E.C., D.L. Paul, and D.A. Goodenough. 1990. Connexin family of gap junction proteins. *J Membr Biol.* 116:187-94.

37. Beyer, E.C., K.E. Reed, E.M. Westphale, H.L. Kanter, and D.M. Larson. 1992. Molecular cloning and expression of rat connexin40, a gap junction protein expressed in vascular smooth muscle. *J Membr Biol.* 127:69-76.

38. Bikfalvi, A., S. Klein, G. Pintucci, and D.B. Rifkin. 1997. Biological roles of fibroblast growth factor-2. *Endocr Rev.* 18:26-45.

39. Blomstrand, F., N.D. Aberg, P.S. Eriksson, E. Hansson, and L. Ronnback. 1999. Extent of intercellular calcium wave propagation is related to gap junction permeability and level of connexin-43 expression in astrocytes in primary cultures from four brain regions. *Neuroscience.* 92:255-65.

40. Bogoyevitch, M.A., P.E. Glennon, M.B. Andersson, A. Clerk, A. Lazou, C.J. Marshall, P.J. Parker, and P.H. Sugden. 1994. Endothelin-1 and fibroblast growth factors stimulate the mitogen- activated protein kinase signaling cascade in cardiac myocytes. The potential role of the cascade in the integration of two signaling pathways leading to myocyte hypertrophy. *J Biol Chem.* 269:1110-9.

41. Bogoyevitch, M.A., P.J. Parker, and P.H. Sugden. 1993. Characterization of protein kinase C isotype expression in adult rat heart. Protein kinase C-epsilon is a major isotype present, and it is activated by phorbol esters, epinephrine, and endothelin. *Circ Res.* 72:757-67.

42. Bond, S.L., J.F. Bechberger, N.K. Khoo, and C.C. Naus. 1994. Transfection of C6 glioma cells with connexin32: the effects of expression of a nonendogenous gap junction protein. *Cell Growth Differ.* 5:179-86.

43. Boyle, W.J., P. van der Geer, and T. Hunter. 1991. Phosphopeptide mapping and phosphoamino acid analysis by two- dimensional separation on thin-layer cellulose plates. *Methods Enzymol.* 201:110-49.

44. Brink, P.R., J. Ricotta, and G.J. Christ. 2000. Biophysical characteristics of gap junctions in vascular wall cells: implications for vascular biology and disease. *Braz J Med Biol Res.* 33:415-22.

45. Brissette, J.L., N.M. Kumar, N.B. Gilula, and G.P. Dotto. 1991. The tumor promoter 12-O-tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. *Mol Cell Biol.* 11:5364-71.

46. Broder, Y.C., S. Katz, and A. Aronheim. 1998. The ras recruitment system, a novel approach to the study of protein- protein interactions. *Curr Biol.* 8:1121-4.

47. Bruzzone, R., J.A. Haefliger, R.L. Gimlich, and D.L. Paul. 1993. Connexin40, a component of gap junctions in vascular endothelium, is restricted in its ability to interact with other connexins. *Mol Biol Cell.* 4:7-20.

48. Bruzzone, R., T.W. White, and D.L. Paul. 1994. Expression of chimeric connexins reveals new properties of the formation and gating behavior of gap junction channels. *J Cell Sci.* 107:955-67.

49. Bruzzone, R., T.W. White, and D.L. Paul. 1996. Connections with connexins: the molecular basis of direct intercellular signaling. *Eur J Biochem.* 238:1-27.

50. Budunova, I.V., L.A. Mittelman, and J. Miloszewska. 1994. Role of protein kinase C in the regulation of gap junctional communication. *Teratog Carcinog Mutagen.* 14:259-70.

51. Budunova, I.V., G.M. Williams, and D.C. Spray. 1993. Effect of tumor promoting stimuli on gap junction permeability and connexin43 expression in ARL18 rat liver cell line. *Arch Toxicol.* 67:565-72.

52. Bukauskas, F.F., C. Elfgang, K. Willecke, and R. Weingart. 1995. Heterotypic gap junction channels (connexin26-connexin32) violate the paradigm of unitary conductance. *Pflugers Arch.* 429:870-2.

53. Bukauskas, F.F., K. Jordan, A. Bukauskiene, M.V. Bennett, P.D. Lampe, D.W. Laird, and V.K. Verselis. 2000. Clustering of connexin 43-enhanced green fluorescent protein gap junction channels and functional coupling in living cells. *Proc Natl Acad Sci U S A.* 97:2556-61.

54. Burghardt, R.C., R. Barhoumi, T.C. Sewall, and J.A. Bowen. 1995. Cyclic AMP induces rapid increases in gap junction permeability and changes in the cellular distribution of connexin43. *J Membr Biol.* 148:243-53.

55. Burghardt, R.C., R. Barhoumi, M. Stickney, M. Monga, C.Y. Ku, and B.M. Sanborn. 1996. Correlation between connexin43 expression, cell-cell communication, and oxytocin-induced Ca2+ responses in an immortalized human myometrial cell line. *Biol Reprod.* 55:433-8.

56. Burt, J.M., and D.C. Spray. 1988. Inotropic agents modulate gap junctional conductance between cardiac myocytes. *Am J Physiol.* 254:H1206-10.

57. Butterweck, A., C. Elfgang, K. Willecke, and O. Traub. 1994a. Differential expression of the gap junction proteins connexin45, -43, - 40, -31, and -26 in mouse skin. *Eur J Cell Biol.* 65:152-63.

58. Butterweck, A., U. Gergs, C. Elfgang, K. Willecke, and O. Traub. 1994b. Immunochemical characterization of the gap junction protein connexin45 in mouse

kidney and transfected human HeLa cells. *J Membr Biol.* 141:247-56.

59. Cacace, A.M., S.N. Guadagno, R.S. Krauss, D. Fabbro, and I.B. Weinstein. 1993. The epsilon isoform of protein kinase C is an oncogene when overexpressed in rat fibroblasts. *Oncogene.* 8:2095-104.

60. Cai, H., U. Smola, V. Wixler, I. Eisenmann-Tappe, M.T. Diaz-Meco, J. Moscat, U. Rapp, and G.M. Cooper. 1997. Role of diacylglycerol-regulated protein kinase C isotypes in growth factor activation of the Raf-1 protein kinase. *Mol Cell Biol.* 17:732-41.

61. Cao, F., R. Eckert, C. Elfgang, J.M. Nitsche, S.A. Snyder, D.F. Hülser, K. Willecke, and B.J. Nicholson. 1998. A quantitative analysis of connexin-specific permeability differences of gap junctions expressed in HeLa transfectants and Xenopus oocytes. *J Cell Sci.* 111:31-43.

62. Casscells, W., E. Speir, J. Sasse, M. Klagsbrun, P. Allen, M. Lee, B. Calvo, M. Chiba, L. Haggroth, J. Folkman, and et al. 1990. Isolation, characterization, and localization of heparin-binding growth factors in the heart. *J Clin Invest.* 85:433-41.

63. Chappell, S.A., J.P. LeQuesne, F.E. Paulin, M.L. deSchoolmeester, M. Stoneley, R.L. Soutar, S.H. Ralston, M.H. Helfrich, and A.E. Willis. 2000. A mutation in the c-myc-IRES leads to enhanced internal ribosome entry in multiple myeloma: A novel mechanism of oncogene de-regulation. *Oncogene.* 19:4437-40.

64. Chen, C.H., M.O. Gray, and D. Mochly-Rosen. 1999a. Cardioprotection from ischemia by a brief exposure to physiological levels of ethanol: role of epsilon protein kinase C. *Proc Natl Acad Sci U S A.* 96:12784-9.

65. Chen, F.E., and G. Ghosh. 1999b. Regulation of DNA binding by Rel/NF-kappaB transcription factors: structural views. *Oncogene.* 18:6845-52.

66. Chen, S.C., L.M. Davis, E.M. Westphale, E.C. Beyer, and J.E. Saffitz. 1994. Expression of multiple gap junction proteins in human fetal and infant hearts. *Pediatr Res.* 36:561-6.

67. Chen, S.C., D.B. Pelletier, P. Ao, and A.L. Boynton. 1995. Connexin43 reverses the phenotype of transformed cells and alters their expression of cyclin/cyclin-dependent kinases. *Cell Growth Differ.* 6:681-90.

68. Cheng, G.C., W.H. Briggs, D.S. Gerson, P. Libby, A.J. Grodzinsky, M.L. Gray, and R.T. Lee. 1997. Mechanical strain tightly controls fibroblast growth factor-2 release from cultured human vascular smooth muscle cells. *Circ Res.* 80:28-36.

69. Cheng, G.C., P. Libby, A.J. Grodzinsky, and R.T. Lee. 1996. Induction of DNA synthesis by a single transient mechanical stimulus of human vascular smooth muscle cells. Role of fibroblast growth factor-2. *Circulation.* 93:99-105.

70. Chiu, R., W.J. Boyle, J. Meek, T. Smeal, T. Hunter, and M. Karin. 1988. The c-Fos protein interacts with c-Jun/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell.* 54:541-52.

71. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. *Anal Biochem.* 162:156-9.

72. Ciechanover, A. 1994. The ubiquitin-proteasome proteolytic pathway. *Cell.* 79:13-21.

73. Ciray, H.N., X. Fu, M. Olovsson, G. Ahlsen, C. Shuman, B. Lindblom, and U. Ulmsten. 2000. Presence and localization of connexins 43 and 26 in cell cultures derived from myometrial tissues from nonpregnant and pregnant women and from leiomyomas. *Am J Obstet Gynecol.* 182:926-30.

74. Clark-Lewis, I., J.S. Sanghera, and S.L. Pelech. 1991. Definition of a consensus sequence for peptide substrate recognition by p44mpk, the meiosis-activated myelin basic protein kinase. *J Biol Chem.* 266:15180-4.

75. Clarke, M.S., R.W. Caldwell, H. Chiao, K. Miyake, and P.L. McNeil. 1995. Contraction-induced cell wounding and release of fibroblast growth factor in heart. *Circ Res.* 76:927-34.

76. Clerk, A., M.A. Bogoyevitch, S.J. Fuller, A. Lazou, P.J. Parker, and P.H. Sugden. 1995. Expression of protein kinase C isoforms during cardiac ventricular development. *Am J Physiol.* 269:H1087-97.

77. Cobb, M.H., D.J. Robbins, and T.G. Boulton. 1991. ERKs, extracellular signal-regulated MAP-2 kinases. *Curr Opin Cell Biol.* 3:1025-32.

78. Cole, W.C., and R.E. Garfield. 1986. Evidence for physiological regulation of myometrial gap junction permeability. *Am J Physiol.* 251:C411-20.

79. Colledge, M., and J.D. Scott. 1999. AKAPs: from structure to function. *Trends Cell Biol.* 9:216-21.

80. Condorelli, D.F., R. Parenti, F. Spinella, A. Trovato Salinaro, N. Belluardo, V. Cardile, and F. Cicirata. 1998. Cloning of a new gap junction gene (Cx36) highly expressed in mammalian brain neurons. *Eur J Neurosci.* 10:1202-8.

81. Cope, D.K., W.K. Impastato, M.V. Cohen, and J.M. Downey. 1997. Volatile anesthetics protect the ischemic rabbit myocardium from infarction. *Anesthesiology.* 86:699-709.

82. Coppen, S.R., E. Dupont, S. Rothery, and N.J. Severs. 1998. Connexin45 expression

is preferentially associated with the ventricular conduction system in mouse and rat heart. *Circ Res*. 82:232-43.

83. Coppen, S.R., I. Kodama, M.R. Boyett, H. Dobrzynski, Y. Takagishi, H. Honjo, H.I. Yeh, and N.J. Severs. 1999a. Connexin45, a major connexin of the rabbit sinoatrial node, is co- expressed with connexin43 in a restricted zone at the nodal-crista terminalis border. *J Histochem Cytochem*. 47:907-18.

84. Coppen, S.R., N.J. Severs, and R.G. Gourdie. 1999b. Connexin45 (alpha 6) expression delineates an extended conduction system in the embryonic and mature rodent heart. *Dev Genet*. 24:82-90.

85. Crow, D.S., E.C. Beyer, D.L. Paul, S.S. Kobe, and A.F. Lau. 1990. Phosphorylation of connexin43 gap junction protein in uninfected and Rous sarcoma virus-transformed mammalian fibroblasts. *Mol Cell Biol*. 10:1754-63.

86. Cruciani, V., and S.O. Mikalsen. 1999. Stimulated phosphorylation of intracellular connexin43. *Exp Cell Res*. 251:285-98.

87. Cuevas, P., F. Carceller, R.M. Lozano, A. Crespo, M. Zazo, and G. Gimenez-Gallego. 1997. Protection of rat myocardium by mitogenic and non-mitogenic fibroblast growth factor during post-ischemic reperfusion. *Growth Factors*. 15:29-40.

88. Cuevas, P., F. Carceller, V. Martinez-Coso, E. Asin-Cardiel, and G. Gimenez-Gallego. 2000. Fibroblast growth factor cardioprotection against ischemia-reperfusion injury may involve K+ ATP channels. *Eur J Med Res*. 5:145-9.

89. Cuevas, P., F. Carceller, V. Martinez-Coso, B. Cuevas, A. Fernandez-Ayerdi, D. Reimers, E. Asin-Cardiel, and G. Gimenez-Gallego. 1999. Cardioprotection from ischemia by fibroblast growth factor: role of inducible nitric oxide synthase. *Eur J Med Res*. 4:517-24.

90. Curran, T., and B.R. Franza, Jr. 1988. Fos and Jun: the AP-1 connection. *Cell*. 55:395-7.

91. Dahl, G., R. Werner, E. Levine, and C. Rabadan-Diehl. 1992. Mutational analysis of gap junction formation. *Biophys J*. 62:172-80; discussion 180-2.

92. Darrow, B.J., J.G. Laing, P.D. Lampe, J.E. Saffitz, and E.C. Beyer. 1995. Expression of multiple connexins in cultured neonatal rat ventricular myocytes. *Circ Res*. 76:381-7.

93. Davidson, J.S., I.M. Baumgarten, and E.H. Harley. 1985. Studies on the mechanism of phorbol ester-induced inhibition of intercellular junctional communication. *Carcinogenesis*. 6:1353-8.

94. Davis, L.M., H.L. Kanter, E.C. Beyer, and J.E. Saffitz. 1994. Distinct gap junction

protein phenotypes in cardiac tissues with disparate conduction properties. *J Am Coll Cardiol.* 24:1124-32.

95. Davis, L.M., M.E. Rodefeld, K. Green, E.C. Beyer, and J.E. Saffitz. 1995. Gap junction protein phenotypes of the human heart and conduction system [see comments] [published erratum appears in J Cardiovasc Electrophysiol 1996 Apr;7(4):383-5]. *J Cardiovasc Electrophysiol.* 6:813-22.

96. Delrieu, I. 2000. The high molecular weight isoforms of basic fibroblast growth factor (FGF-2): an insight into an intracrine mechanism. *FEBS Lett.* 468:6-10.

97. Diederichs, F. 1995. Protection of isolated rat heart against the Ca2+ paradox. Are gap junction channels involved? *J Mol Cell Cardiol.* 27:1301-9.

98. Diez, J.A., S. Ahmad, and W.H. Evans. 1999. Assembly of heteromeric connexons in guinea-pig liver en route to the Golgi apparatus, plasma membrane and gap junctions. *Eur J Biochem.* 262:142-8.

99. Disatnik, M.H., G. Buraggi, and D. Mochly-Rosen. 1994. Localization of protein kinase C isozymes in cardiac myocytes. *Exp Cell Res.* 210:287-97.

100. Disatnik, M.H., S.N. Jones, and D. Mochly-Rosen. 1995. Stimulus-dependent subcellular localization of activated protein kinase C; a study with acidic fibroblast growth factor and transforming growth factor-beta 1 in cardiac myocytes. *J Mol Cell Cardiol.* 27:2473-81.

101. Doble, B.W., and E. Kardami. 1995. Basic fibroblast growth factor stimulates connexin-43 expression and intercellular communication of cardiac fibroblasts. *Mol Cell Biochem.* 143:81-7.

102. Doble, B.W., P.-L. Merle, and E. Kardami. 1998. Involvement of protein kinase C-ε in mediating growth factor induced phosphorylation of connexin43 at gap junctions of the intact cardiomyocyte. *Circulation.* 98:I-677 (abstract).

103. Dorn, G.W., 2nd, M.C. Souroujon, T. Liron, C.H. Chen, M.O. Gray, H.Z. Zhou, M. Csukai, G. Wu, J.N. Lorenz, and D. Mochly-Rosen. 1999. Sustained in vivo cardiac protection by a rationally designed peptide that causes epsilon protein kinase C translocation. *Proc Natl Acad Sci U S A.* 96:12798-803.

104. Dowling-Warriner, C.V., and J.E. Trosko. 2000. Induction of gap junctional intercellular communication, connexin43 expression, and subsequent differentiation in human fetal neuronal cells by stimulation of the cyclic AMP pathway. *Neuroscience.* 95:859-68.

105. Dunham, B., S. Liu, S. Taffet, E. Trabka-Janik, M. Delmar, R. Petryshyn, S. Zheng, R. Perzova, and M.L. Vallano. 1992. Immunolocalization and expression of functional

and nonfunctional cell- to-cell channels from wild-type and mutant rat heart connexin43 cDNA. *Circ Res.* 70:1233-43.

106. Ebihara, L. 1996. Xenopus connexin38 forms hemi-gap-junctional channels in the nonjunctional plasma membrane of Xenopus oocytes. *Biophys J.* 71:742-8.

107. Ebihara, L., V.M. Berthoud, and E.C. Beyer. 1995. Distinct behavior of connexin56 and connexin46 gap junctional channels can be predicted from the behavior of their hemi-gap-junctional channels. *Biophys J.* 68:1796-803.

108. Ebihara, L., and E. Steiner. 1993. Properties of a nonjunctional current expressed from a rat connexin46 cDNA in Xenopus oocytes. *J Gen Physiol.* 102:59-74.

109. Echetebu, C.O., M. Ali, M.G. Izban, L. MacKay, and R.E. Garfield. 1999. Localization of regulatory protein binding sites in the proximal region of human myometrial connexin 43 gene. *Mol Hum Reprod.* 5:757-66.

110. Eckert, R., A. Dunina-Barkovskaya, and D.F. Hulser. 1993. Biophysical characterization of gap-junction channels in HeLa cells. *Pflugers Arch.* 424:335-42.

111. Eghbali, B., J.A. Kessler, L.M. Reid, C. Roy, and D.C. Spray. 1991. Involvement of gap junctions in tumorigenesis: transfection of tumor cells with connexin 32 cDNA retards growth in vivo. *Proc Natl Acad Sci U S A.* 88:10701-5.

112. Ek-Vitorin, J.F., G. Calero, G.E. Morley, W. Coombs, S.M. Taffet, and M. Delmar. 1996. pH regulation of connexin43: molecular analysis of the gating particle. *Biophys J.* 71:1273-84.

113. El-Fouly, M.H., J.E. Trosko, and C.C. Chang. 1987. Scrape-loading and dye transfer. A rapid and simple technique to study gap junctional intercellular communication. *Exp Cell Res.* 168:422-30.

114. Elfgang, C., R. Eckert, H. Lichtenberg-Frate, A. Butterweck, O. Traub, R.A. Klein, D.F. Hulser, and K. Willecke. 1995. Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J Cell Biol.* 129:805-17.

115. Ewart, J.L., M.F. Cohen, R.A. Meyer, G.Y. Huang, A. Wessels, R.G. Gourdie, A.J. Chin, S.M. Park, B.O. Lazatin, S. Villabon, and C.W. Lo. 1997. Heart and neural tube defects in transgenic mice overexpressing the Cx43 gap junction gene. *Development.* 124:1281-92.

116. Falk, M.M. 2000. Biosynthesis and structural composition of gap junction intercellular membrane channels. *Eur J Cell Biol.* 79:564-74.

117. Falk, M.M., L.K. Buehler, N.M. Kumar, and N.B. Gilula. 1997. Cell-free synthesis and assembly of connexins into functional gap junction membrane channels. *EMBO J.*

16:2703-16.

118. Falk, M.M., and N.B. Gilula. 1998. Connexin membrane protein biosynthesis is influenced by polypeptide positioning within the translocon and signal peptidase access. *J Biol Chem.* 273:7856-64.

119. Falk, M.M., N.M. Kumar, and N.B. Gilula. 1994. Membrane insertion of gap junction connexins: polytopic channel forming membrane proteins. *J Cell Biol.* 127:343-55.

120. Ferrell, J.E., Jr. 1996. MAP kinases in mitogenesis and development. *Curr Top Dev Biol.* 33:1-60.

121. Filson, A.J., R. Azarnia, E.C. Beyer, W.R. Loewenstein, and J.S. Brugge. 1990. Tyrosine phosphorylation of a gap junction protein correlates with inhibition of cell-to-cell communication. *Cell Growth Differ.* 1:661-8.

122. Fishman, G.I., A.P. Moreno, D.C. Spray, and L.A. Leinwand. 1991. Functional analysis of human cardiac gap junction channel mutants. *Proc Natl Acad Sci U S A.* 88:3525-9.

123. Florkiewicz, R.Z., R.A. Majack, R.D. Buechler, and E. Florkiewicz. 1995. Quantitative export of FGF-2 occurs through an alternative, energy- dependent, non-ER/Golgi pathway. *J Cell Physiol.* 162:388-99.

124. Foote, C.I., L. Zhou, X. Zhu, and B.J. Nicholson. 1998. The pattern of disulfide linkages in the extracellular loop regions of connexin 32 suggests a model for the docking interface of gap junctions. *J Cell Biol.* 140:1187-97.

125. Galy, B., A. Maret, A.C. Prats, and H. Prats. 1999. Cell transformation results in the loss of the density-dependent translational regulation of the expression of fibroblast growth factor 2 isoforms. *Cancer Res.* 59:165-71.

126. Galzie, Z., A.R. Kinsella, and J.A. Smith. 1997. Fibroblast growth factors and their receptors. *Biochem Cell Biol.* 75:669-85.

127. George, C.H., P.E. Martin, and W.H. Evans. 1998. Rapid determination of gap junction formation using HeLa cells microinjected with cDNAs encoding wild-type and chimeric connexins. *Biochem Biophys Res Commun.* 247:785-9.

128. Ghoshroy, S., D.A. Goodenough, and G.E. Sosinsky. 1995. Preparation, characterization, and structure of half gap junctional layers split with urea and EGTA. *J Membr Biol.* 146:15-28.

129. Gilmore, T.D. 1999. The Rel/NF-kappaB signal transduction pathway: introduction. *Oncogene.* 18:6842-4.

130. Gilula, N.B., O.R. Reeves, and A. Steinbach. 1972. Metabolic coupling, ionic coupling and cell contacts. *Nature*. 235:262-5.

131. Ginzberg, R.D., and N.B. Gilula. 1979. Modulation of cell junctions during differentiation of the chicken otocyst sensory epithelium. *Dev Biol.* 68:110-29.

132. Goldberg, G.S., P.D. Lampe, and B.J. Nicholson. 1999. Selective transfer of endogenous metabolites through gap junctions composed of different connexins. *Nat Cell Biol.* 1:457-459.

133. Goncalves, L.M. 1998. Fibroblast growth factor-mediated angiogenesis for the treatment of ischemia. Lessons learned from experimental models and early human experience. *Rev Port Cardiol.* 17 Suppl 2:II11-20.

134. Gong, X., E. Li, G. Klier, Q. Huang, Y. Wu, H. Lei, N.M. Kumar, J. Horwitz, and N.B. Gilula. 1997. Disruption of alpha3 connexin gene leads to proteolysis and cataractogenesis in mice. *Cell.* 91:833-43.

135. Gonzalez, F.A., D.L. Raden, and R.J. Davis. 1991. Identification of substrate recognition determinants for human ERK1 and ERK2 protein kinases. *J Biol Chem.* 266:22159-63.

136. Goodenough, D.A. 1992. The crystalline lens. A system networked by gap junctional intercellular communication. *Semin Cell Biol.* 3:49-58.

137. Goodenough, D.A., J.A. Goliger, and D.L. Paul. 1996. Connexins, connexons, and intercellular communication. *Annu Rev Biochem.* 65:475-502.

138. Gospodarowicz, D. 1975. Purification of a fibroblast growth factor from bovine pituitary. *J Biol Chem.* 250:2515-20.

139. Gossen, M., and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline- responsive promoters. *Proc Natl Acad Sci U S A.* 89:5547-51.

140. Gossen, M., S. Freundlieb, G. Bender, G. Muller, W. Hillen, and H. Bujard. 1995. Transcriptional activation by tetracyclines in mammalian cells. *Science.* 268:1766-9.

141. Grassi, F., L. Monaco, G. Fratamico, S. Dolci, E. Iannini, M. Conti, F. Eusebi, and M. Stefanini. 1986. Putative second messengers affect cell coupling in the seminiferous tubules. *Cell Biol Int Rep.* 10:631-9.

142. Gray, M.O., J.S. Karliner, and D. Mochly-Rosen. 1997. A selective epsilon-protein kinase C antagonist inhibits protection of cardiac myocytes from hypoxia-induced cell death. *J Biol Chem.* 272:30945-51.

143. Green, W.N., and N.S. Millar. 1995. Ion-channel assembly. *Trends Neurosci.* 18:280-7.

144. Gros, D., T. Jarry-Guichard, I. Ten Velde, A. de Maziere, M.J. van Kempen, J. Davoust, J.P. Briand, A.F. Moorman, and H.J. Jongsma. 1994. Restricted distribution of connexin40, a gap junctional protein, in mammalian heart. *Circ Res.* 74:839-51.

145. Gros, D.B., and H.J. Jongsma. 1996. Connexins in mammalian heart function. *Bioessays.* 18:719-30.

146. Grummer, R., O. Traub, and E. Winterhager. 1999. Gap junction connexin genes Cx26 and Cx43 are differentially regulated by ovarian steroid hormones in rat endometrium. *Endocrinology.* 140:2509-16.

147. Guerrero, P.A., R.B. Schuessler, L.M. Davis, E.C. Beyer, C.M. Johnson, K.A. Yamada, and J.E. Saffitz. 1997. Slow ventricular conduction in mice heterozygous for a connexin43 null mutation. *J Clin Invest.* 99:1991-8.

148. Guo, H., P. Acevedo, F.D. Parsa, and J.S. Bertram. 1992. Gap-junctional protein connexin 43 is expressed in dermis and epidermis of human skin: differential modulation by retinoids. *J Invest Dermatol.* 99:460-7.

149. Haefliger, J.A., R. Bruzzone, N.A. Jenkins, D.J. Gilbert, N.G. Copeland, and D.L. Paul. 1992. Four novel members of the connexin family of gap junction proteins. Molecular cloning, expression, and chromosome mapping. *J Biol Chem.* 267:2057-64.

150. Hall, D.G., G.E. Morley, D. Vaidya, M. Ard, T.R. Kimball, S.A. Witt, and M.C. Colbert. 2000. Early onset heart failure in transgenic mice with dilated cardiomyopathy . *Pediatr Res.* 48:36-42.

151. Hare, J.F., and K. Taylor. 1991. Mechanisms of plasma membrane protein degradation: recycling proteins are degraded more rapidly than those confined to the cell surface. *Proc Natl Acad Sci U S A.* 88:5902-6.

152. Hasegawa, T., A. Kimura, M. Miyataka, M. Inagaki, and K. Ishikawa. 1999. Basic fibroblast growth factor increases regional myocardial blood flow and salvages myocardium in the infarct border zone in a rabbit model of acute myocardial infarction. *Angiology.* 50:487-95.

153. Haubrich, S., H.J. Schwarz, F. Bukauskas, H. Lichtenberg-Frate, O. Traub, R. Weingart, and K. Willecke. 1996. Incompatibility of connexin 40 and 43 hemichannels in gap junctions between mammalian cells is determined by intracellular domains. *Mol Biol Cell.* 7:1995-2006.

154. He, D.S., and J.M. Burt. 2000. Mechanism and selectivity of the effects of halothane on gap junction channel function. *Circ Res.* 86:E104-9.

155. He, D.S., J.X. Jiang, S.M. Taffet, and J.M. Burt. 1999. Formation of heteromeric gap junction channels by connexins 40 and 43 in vascular smooth muscle cells. *Proc Natl Acad Sci U S A.* 96:6495-500.

156. Hefti, M.A., B.A. Harder, H.M. Eppenberger, and M.C. Schaub. 1997. Signaling pathways in cardiac myocyte hypertrophy. *J Mol Cell Cardiol.* 29:2873-92.

157. Hellmann, P., R. Grummer, K. Schirrmacher, M. Rook, O. Traub, and E. Winterhager. 1999. Transfection with different connexin genes alters growth and differentiation of human choriocarcinoma cells. *Exp Cell Res.* 246:480-90.

158. Hennemann, H., E. Dahl, J.B. White, H.J. Schwarz, P.A. Lalley, S. Chang, B.J. Nicholson, and K. Willecke. 1992a. Two gap junction genes, connexin 31.1 and 30.3, are closely linked on mouse chromosome 4 and preferentially expressed in skin. *J Biol Chem.* 267:17225-33.

159. Hennemann, H., T. Suchyna, H. Lichtenberg-Frate, S. Jungbluth, E. Dahl, J. Schwarz, B.J. Nicholson, and K. Willecke. 1992b. Molecular cloning and functional expression of mouse connexin40, a second gap junction gene preferentially expressed in lung. *J Cell Biol.* 117:1299-310.

160. Hertig, C.M., S. Butz, S. Koch, M. Eppenberger-Eberhardt, R. Kemler, and H.M. Eppenberger. 1996. N-cadherin in adult rat cardiomyocytes in culture. II. Spatio-temporal appearance of proteins involved in cell-cell contact and communication. Formation of two distinct N-cadherin/catenin complexes. *J Cell Sci.* 109:11-20.

161. Hii, C.S., S.Y. Oh, S.A. Schmidt, K.J. Clark, and A.W. Murray. 1994. Lysophosphatidic acid inhibits gap-junctional communication and stimulates phosphorylation of connexin-43 in WB cells: possible involvement of the mitogen-activated protein kinase cascade. *Biochem J.* 303:475-9.

162. Hirschi, K.K., C.E. Xu, T. Tsukamoto, and R. Sager. 1996. Gap junction genes Cx26 and Cx43 individually suppress the cancer phenotype of human mammary carcinoma cells and restore differentiation potential. *Cell Growth Differ.* 7:861-70.

163. Hoh, J.H., S.A. John, and J.P. Revel. 1991. Molecular cloning and characterization of a new member of the gap junction gene family, connexin-31. *J Biol Chem.* 266:6524-31.

164. Holm, I., A. Mikhailov, T. Jillson, and B. Rose. 1999. Dynamics of gap junctions observed in living cells with connexin43-GFP chimeric protein. *Eur J Cell Biol.* 78:856-66.

165. Homma, N., J.L. Alvarado, W. Coombs, K. Stergiopoulos, S.M. Taffet, A.F. Lau, and M. Delmar. 1998. A particle-receptor model for the insulin-induced closure of

connexin43 channels. *Circ Res.* 83:27-32.

166. Horrigan, M.C., J.L. Malycky, S.G. Ellis, E.J. Topol, and F.A. Nicolini. 1999. Reduction in myocardial infarct size by basic fibroblast growth factor following coronary occlusion in a canine model. *Int J Cardiol.* 68 Suppl 1:S85-91.

167. Hossain, M.Z., P. Ao, and A.L. Boynton. 1998. Platelet-derived growth factor-induced disruption of gap junctional communication and phosphorylation of connexin43 involves protein kinase C and mitogen-activated protein kinase. *J Cell Physiol.* 176:332-41.

168. Hossain, M.Z., A.B. Jagdale, P. Ao, and A.L. Boynton. 1999a. Mitogen-activated protein kinase and phosphorylation of connexin43 are not sufficient for the disruption of gap junctional communication by platelet-derived growth factor and tetradecanoylphorbol acetate. *J Cell Physiol.* 179:87-96.

169. Hossain, M.Z., A.B. Jagdale, P. Ao, A. Kazlauskas, and A.L. Boynton. 1999b. Disruption of gap junctional communication by the platelet-derived growth factor is mediated via multiple signaling pathways. *J Biol Chem.* 274:10489-96.

170. Hoyt, R.H., M.L. Cohen, and J.E. Saffitz. 1989. Distribution and three-dimensional structure of intercellular junctions in canine myocardium [see comments]. *Circ Res.* 64:563-74.

171. Hu, X., and G. Dahl. 1999. Exchange of conductance and gating properties between gap junction hemichannels. *FEBS Lett.* 451:113-7.

172. Huang, G.Y., E.S. Cooper, K. Waldo, M.L. Kirby, N.B. Gilula, and C.W. Lo. 1998a. Gap junction-mediated cell-cell communication modulates mouse neural crest migration. *J Cell Biol.* 143:1725-34.

173. Huang, G.Y., A. Wessels, B.R. Smith, K.K. Linask, J.L. Ewart, and C.W. Lo. 1998b. Alteration in connexin 43 gap junction gene dosage impairs conotruncal heart development. *Dev Biol.* 198:32-44.

174. Huang, X.D., G.E. Sandusky, and D.P. Zipes. 1999. Heterogeneous loss of connexin43 protein in ischemic dog hearts. *J Cardiovasc Electrophysiol.* 10:79-91.

175. Hudlicka, O., and M.D. Brown. 1996. Postnatal growth of the heart and its blood vessels. *J Vasc Res.* 33:266-87.

176. Husoy, T., S.O. Mikalsen, and T. Sanner. 1993. Effects of five phorbol esters on gap junctional intercellular communication, morphological transformation and epidermal growth factor binding in Syrian hamster embryo cells. *Carcinogenesis.* 14:73-7.

177. Imagawa, M., R. Chiu, and M. Karin. 1987. Transcription factor AP-2 mediates

induction by two different signal- transduction pathways: protein kinase C and cAMP. *Cell.* 51:251-60.

178. Jaye, M., J. Schlessinger, and C.A. Dionne. 1992. Fibroblast growth factor receptor tyrosine kinases: molecular analysis and signal transduction. *Biochim Biophys Acta.* 1135:185-99.

179. Jiang, J.X., and D.A. Goodenough. 1996. Heteromeric connexons in lens gap junction channels. *Proc Natl Acad Sci U S A.* 93:1287-91.

180. Jin, Y., K.B. Pasumarthi, M.E. Bock, A. Lytras, E. Kardami, and P.A. Cattini. 1994. Cloning and expression of fibroblast growth factor receptor-1 isoforms in the mouse heart: evidence for isoform switching during heart development. *J Mol Cell Cardiol.* 26:1449-59.

181. John, S.A., R. Kondo, S.Y. Wang, J.I. Goldhaber, and J.N. Weiss. 1999. Connexin-43 hemichannels opened by metabolic inhibition. *J Biol Chem.* 274:236-40.

182. Johnson, D.E., and L.T. Williams. 1993. Structural and functional diversity in the FGF receptor multigene family. *Adv Cancer Res.* 60:1-41.

183. Jones, H.W., Jr., V.A. McKusick, P.S. Harper, and K.D. Wuu. 1971. George Otto Gey. (1899-1970). The HeLa cell and a reappraisal of its origin. *Obstet Gynecol.* 38:945-9.

184. Jordan, K., J.L. Solan, M. Dominguez, M. Sia, A. Hand, P.D. Lampe, and D.W. Laird. 1999. Trafficking, assembly, and function of a connexin43-green fluorescent protein chimera in live mammalian cells. *Mol Biol Cell.* 10:2033-50.

185. Jordan, M., A. Schallhorn, and F.M. Wurm. 1996. Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res.* 24:596-601.

186. Kadle, R., J.T. Zhang, and B.J. Nicholson. 1991. Tissue-specific distribution of differentially phosphorylated forms of Cx43. *Mol Cell Biol.* 11:363-9.

187. Kam, Y., D.Y. Kim, S.K. Koo, and C.O. Joe. 1998. Transfer of second messengers through gap junction connexin 43 channels reconstituted in liposomes. *Biochim Biophys Acta.* 1372:384-8.

188. Kane, M.F., M. Loda, G.M. Gaida, J. Lipman, R. Mishra, H. Goldman, J.M. Jessup, and R. Kolodner. 1997. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. *Cancer Res.* 57:808-11.

189. Kanemitsu, M.Y., and A.F. Lau. 1993. Epidermal growth factor stimulates the

disruption of gap junctional communication and connexin43 phosphorylation independent of 12-0- tetradecanoylphorbol 13-acetate-sensitive protein kinase C: the possible involvement of mitogen-activated protein kinase. *Mol Biol Cell.* 4:837-48.

190. Kanemitsu, M.Y., L.W. Loo, S. Simon, A.F. Lau, and W. Eckhart. 1997. Tyrosine phosphorylation of connexin 43 by v-Src is mediated by SH2 and SH3 domain interactions. *J Biol Chem.* 272:22824-31.

191. Kang, K.S., B.C. Kang, B.J. Lee, J.H. Che, G.X. Li, J.E. Trosko, and Y.S. Lee. 2000. Preventive effect of epicatechin and ginsenoside Rb(2) on the inhibition of gap junctional intercellular communication by TPA and H(2)O(2). *Cancer Lett.* 152:97-106.

192. Kanter, H.L., J.G. Laing, S.L. Beau, E.C. Beyer, and J.E. Saffitz. 1993a. Distinct patterns of connexin expression in canine Purkinje fibers and ventricular muscle. *Circ Res.* 72:1124-31.

193. Kanter, H.L., J.G. Laing, E.C. Beyer, K.G. Green, and J.E. Saffitz. 1993b. Multiple connexins colocalize in canine ventricular myocyte gap junctions. *Circ Res.* 73:344-50.

194. Kanter, H.L., J.E. Saffitz, and E.C. Beyer. 1992. Cardiac myocytes express multiple gap junction proteins. *Circ Res.* 70:438-44.

195. Kanter, H.L., J.E. Saffitz, and E.C. Beyer. 1994. Molecular cloning of two human cardiac gap junction proteins, connexin40 and connexin45. *J Mol Cell Cardiol.* 26:861-8.

196. Kaprielian, R.R., M. Gunning, E. Dupont, M.N. Sheppard, S.M. Rothery, R. Underwood, D.J. Pennell, K. Fox, J. Pepper, P.A. Poole-Wilson, and N.J. Severs. 1998. Downregulation of immunodetectable connexin43 and decreased gap junction size in the pathogenesis of chronic hibernation in the human left ventricle [see comments]. *Circulation.* 97:651-60.

197. Kardami, E. 1990. Stimulation and inhibition of cardiac myocyte proliferation in vitro. *Mol Cell Biochem.* 92:129-35.

198. Kardami, E., and B.W. Doble. 1998. Cardiomyocyte gap junctions: A target of growth-promoting signaling. *Trends Cardiovasc Med.* 8:180-87.

199. Kardami, E., and R.R. Fandrich. 1989. Basic fibroblast growth factor in atria and ventricles of the vertebrate heart. *J Cell Biol.* 109:1865-75.

200. Kardami, E., L. Liu, and B.W. Doble. 1991. Basic fibroblast growth factor in cultured cardiac myocytes. *Ann N Y Acad Sci.* 638:244-55.

201. Kardami, E., L. Liu, S. Kishore, B. Pasumarthi, B.W. Doble, and P.A. Cattini. 1995. Regulation of basic fibroblast growth factor (bFGF) and FGF receptors in the heart. *Ann N Y Acad Sci.* 752:353-69.

202. Kardami, E., L.J. Murphy, L. Liu, R.R. Padua, and R.R. Fandrich. 1990. Characterization of two preparations of antibodies to basic fibroblast growth factor which exhibit distinct patterns of immunolocalization. *Growth Factors.* 4:69-80.

203. Karin, M., and Y. Ben-Neriah. 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol.* 18:621-63.

204. Karin, M., Z. Liu, and E. Zandi. 1997. AP-1 function and regulation. *Curr Opin Cell Biol.* 9:240-6.

205. Kashiwagi, Y., T. Haneda, J. Osaki, S. Miyata, and K. Kikuchi. 1998. Mechanical stretch activates a pathway linked to mevalonate metabolism in cultured neonatal rat heart cells. *Hypertens Res.* 21:109-19.

206. Kass, S., C. MacRae, H.L. Graber, E.A. Sparks, D. McNamara, H. Boudoulas, C.T. Basson, P.B. Baker, 3rd, R.J. Cody, M.C. Fishman, et al. 1994. A gene defect that causes conduction system disease and dilated cardiomyopathy maps to chromosome 1p1-1q1. *Nat Genet.* 7:546-51.

207. Kaufmann, R. 1995. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry: a novel analytical tool in molecular biology and biotechnology. *J Biotechnol.* 41:155-75.

208. Kaufmann, U., B. Martin, D. Link, K. Witt, R. Zeitler, S. Reinhard, and A. Starzinski-Powitz. 1999. M-cadherin and its sisters in development of striated muscle. *Cell Tissue Res.* 296:191-8.

209. Kawamura, S., K. Yoshida, T. Miura, Y. Mizukami, and M. Matsuzaki. 1998. Ischemic preconditioning translocates PKC-delta and -epsilon, which mediate functional protection in isolated rat heart. *Am J Physiol.* 275:H2266-71.

210. Kaye, D., D. Pimental, S. Prasad, T. Maki, H.J. Berger, P.L. McNeil, T.W. Smith, and R.A. Kelly. 1996a. Role of transiently altered sarcolemmal membrane permeability and basic fibroblast growth factor release in the hypertrophic response of adult rat ventricular myocytes to increased mechanical activity in vitro. *J Clin Invest.* 97:281-91.

211. Kaye, D.M., R.A. Kelly, and T.W. Smith. 1996b. Cytokines and cardiac hypertrophy: roles of angiotensin II and basic fibroblast growth factor. *Clin Exp Pharmacol Physiol Suppl.* 3:S136-41.

212. Kelsell, D.P., J. Dunlop, H.P. Stevens, N.J. Lench, J.N. Liang, G. Parry, R.F. Mueller, and I.M. Leigh. 1997. Connexin 26 mutations in hereditary non-syndromic sensorineural deafness [see comments]. *Nature.* 387:80-3.

213. Kenne, K., R. Fransson-Steen, S. Honkasalo, and L. Warngard. 1994. Two

inhibitors of gap junctional intercellular communication, TPA and endosulfan: different effects on phosphorylation of connexin 43 in the rat liver epithelial cell line, IAR 20. *Carcinogenesis*. 15:1161-5.

214. Kim, D.Y., Y. Kam, S.K. Koo, and C.O. Joe. 1999. Gating connexin 43 channels reconstituted in lipid vesicles by mitogen- activated protein kinase phosphorylation. *J Biol Chem*. 274:5581-7.

215. King, T.J., L.H. Fukushima, T.A. Donlon, A.D. Hieber, K.A. Shimabukuro, and J.S. Bertram. 2000. Correlation between growth control, neoplastic potential and endogenous connexin43 expression in HeLa cell lines: implications for tumor progression. *Carcinogenesis*. 21:311-5.

216. Kirchhoff, S., J.S. Kim, A. Hagendorff, E. Thonnissen, O. Kruger, W.H. Lamers, and K. Willecke. 2000. Abnormal cardiac conduction and morphogenesis in connexin40 and connexin43 double-deficient mice. *Circ Res*. 87:399-405.

217. Kirchhoff, S., E. Nelles, A. Hagendorff, O. Kruger, O. Traub, and K. Willecke. 1998. Reduced cardiac conduction velocity and predisposition to arrhythmias in connexin40-deficient mice. *Curr Biol*. 8:299-302.

218. Kirshenbaum, L.A., W.R. MacLellan, W. Mazur, B.A. French, and M.D. Schneider. 1993. Highly efficient gene transfer into adult ventricular myocytes by recombinant adenovirus. *J Clin Invest*. 92:381-7.

219. Klint, P., and L. Claesson-Welsh. 1999. Signal transduction by fibroblast growth factor receptors. *Front Biosci*. 4:D165-77.

220. Kondo, R.P., S.Y. Wang, S.A. John, J.N. Weiss, and J.I. Goldhaber. 2000. Metabolic inhibition activates a non-selective current through connexin hemichannels in isolated ventricular myocytes. *J Mol Cell Cardiol*. 32:1859-1872.

221. Konishi, H., M. Tanaka, Y. Takemura, H. Matsuzaki, Y. Ono, U. Kikkawa, and Y. Nishizuka. 1997. Activation of protein kinase C by tyrosine phosphorylation in response to H2O2. *Proc Natl Acad Sci U S A*. 94:11233-7.

222. Koo, S.K., D.Y. Kim, S.D. Park, K.W. Kang, and C.O. Joe. 1997. PKC phosphorylation disrupts gap junctional communication at G0/S phase in clone 9 cells. *Mol Cell Biochem*. 167:41-9.

223. Kouhara, H., Y.R. Hadari, T. Spivak-Kroizman, J. Schilling, D. Bar-Sagi, I. Lax, and J. Schlessinger. 1997. A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway. *Cell*. 89:693-702.

224. Koval, M., S.T. Geist, E.M. Westphale, A.E. Kemendy, R. Civitelli, E.C. Beyer, and T.H. Steinberg. 1995. Transfected connexin45 alters gap junction permeability in

cells expressing endogenous connexin43. *J Cell Biol.* 130:987-95.

225. Kraft, A.S., and W.B. Anderson. 1983. Phorbol esters increase the amount of Ca2+, phospholipid-dependent protein kinase associated with plasma membrane. *Nature.* 301:621-3.

226. Kraft, A.S., W.B. Anderson, H.L. Cooper, and J.J. Sando. 1982. Decrease in cytosolic calcium/phospholipid-dependent protein kinase activity following phorbol ester treatment of EL4 thymoma cells. *J Biol Chem.* 257:13193-6.

227. Kreegipuu, A., N. Blom, and S. Brunak. 1999. PhosphoBase, a database of phosphorylation sites: release 2.0. *Nucleic Acids Res.* 27:237-9.

228. Krutovskikh, V., and H. Yamasaki. 2000. Connexin gene mutations in human genetic diseases. *Mutat Res.* 462:197-207.

229. Krutovskikh, V.A., S.M. Troyanovsky, C. Piccoli, H. Tsuda, M. Asamoto, and H. Yamasaki. 2000. Differential effect of subcellular localization of communication impairing gap junction protein connexin43 on tumor cell growth in vivo. *Oncogene.* 19:505-13.

230. Ku, P.T., and P.A. D'Amore. 1995. Regulation of basic fibroblast growth factor (bFGF) gene and protein expression following its release from sublethally injured endothelial cells. *J Cell Biochem.* 58:328-43.

231. Kumai, M., K. Nishii, K. Nakamura, N. Takeda, M. Suzuki, and Y. Shibata. 2000. Loss of connexin45 causes a cushion defect in early cardiogenesis. *Development.* 127:3501-12.

232. Kumar, N.M., and N.B. Gilula. 1992. Molecular biology and genetics of gap junction channels. *Semin Cell Biol.* 3:3-16.

233. Kurata, W.E., and A.F. Lau. 1994. p130gag-fps disrupts gap junctional communication and induces phosphorylation of connexin43 in a manner similar to that of pp60v-src. *Oncogene.* 9:329-35.

234. Kwak, B.R., and H.J. Jongsma. 1996. Regulation of cardiac gap junction channel permeability and conductance by several phosphorylating conditions. *Mol Cell Biochem.* 157:93-9.

235. Kwak, B.R., T.A. van Veen, L.J. Analbers, and H.J. Jongsma. 1995. TPA increases conductance but decreases permeability in neonatal rat cardiomyocyte gap junction channels. *Exp Cell Res.* 220:456-63.

236. Laird, D.W., M. Castillo, and L. Kasprzak. 1995. Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat

mammary tumor cells. *J Cell Biol.* 131:1193-203.

237. Laird, D.W., K.L. Puranam, and J.P. Revel. 1991. Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochem J.* 273:67-72.

238. Laird, D.W., and J.P. Revel. 1990. Biochemical and immunochemical analysis of the arrangement of connexin43 in rat heart gap junction membranes. *J Cell Sci.* 97:109-17.

239. Lal, R., S.A. John, D.W. Laird, and M.F. Arnsdorf. 1995. Heart gap junction preparations reveal hemiplaques by atomic force microscopy. *Am J Physiol.* 268:C968-77.

240. Lampe, P.D., B.P. Nguyen, S. Gil, M. Usui, J. Olerud, Y. Takada, and W.G. Carter. 1998. Cellular interaction of integrin alpha3beta1 with laminin 5 promotes gap junctional communication. *J Cell Biol.* 143:1735-47.

241. Lampe, P.D., E.M. TenBroek, J.M. Burt, W.E. Kurata, R.G. Johnson, and A.F. Lau. 2000. Phosphorylation of connexin43 on serine368 by protein kinase C regulates gap junctional communication. *J Cell Biol.* 149:1503-12.

242. Landschulz, W.H., P.F. Johnson, and S.L. McKnight. 1988. The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science.* 240:1759-64.

243. Larsen, W.J., H.N. Tung, S.A. Murray, and C.A. Swenson. 1979. Evidence for the participation of actin microfilaments and bristle coats in the internalization of gap junction membrane. *J Cell Biol.* 83:576-87.

244. Lau, A.F., V. Hatch-Pigott, and D.S. Crow. 1991. Evidence that heart connexin43 is a phosphoprotein. *J Mol Cell Cardiol.* 23:659-63.

245. Lau, A.F., M.Y. Kanemitsu, W.E. Kurata, S. Danesh, and A.L. Boynton. 1992. Epidermal growth factor disrupts gap-junctional communication and induces phosphorylation of connexin43 on serine. *Mol Biol Cell.* 3:865-74.

246. Le, S.Y., and J.V. Maizel, Jr. 1997. A common RNA structural motif involved in the internal initiation of translation of cellular mRNAs. *Nucleic Acids Res.* 25:362-9.

247. Lee, J.Y., Y.A. Hannun, and L.M. Obeid. 2000. Functional dichotomy of protein kinase C (PKC) in tumor necrosis factor- alpha (TNF-alpha) signal transduction in L929 cells. Translocation and inactivation of PKC by TNF-alpha . *J Biol Chem.* 275:29290-8.

248. Lee, W., A. Haslinger, M. Karin, and R. Tjian. 1987. Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein

gene and SV40. *Nature*. 325:368-72.

249. Lennon, J.J., and K.A. Walsh. 1999. Locating and identifying posttranslational modifications by in-source decay during MALDI-TOF mass spectrometry. *Protein Sci.* 8:2487-93.

250. Lerner, D.L., K.A. Yamada, R.B. Schuessler, and J.E. Saffitz. 2000. Accelerated onset and increased incidence of ventricular arrhythmias induced by ischemia in Cx43-deficient mice. *Circulation.* 101:547-52.

251. Levine, A.J. 1993. The tumor suppressor genes. *Annu Rev Biochem.* 62:623-51.

252. Lewis, T.S., P.S. Shapiro, and N.G. Ahn. 1998. Signal transduction through MAP kinase cascades. *Adv Cancer Res.* 74:49-139.

253. Li, X., and J.M. Simard. 1999. Multiple connexins form gap junction channels in rat basilar artery smooth muscle cells. *Circ Res.* 84:1277-84.

254. Lin, J.S., R. Eckert, J. Kistler, and P. Donaldson. 1998. Spatial differences in gap junction gating in the lens are a consequence of connexin cleavage. *Eur J Cell Biol.* 76:246-50.

255. Lin, J.S., S. Fitzgerald, Y. Dong, C. Knight, P. Donaldson, and J. Kistler. 1997. Processing of the gap junction protein connexin50 in the ocular lens is accomplished by calpain. *Eur J Cell Biol.* 73:141-9.

256. Liu, L., B.W. Doble, and E. Kardami. 1993a. Perinatal phenotype and hypothyroidism are associated with elevated levels of 21.5- to 22-kDa basic fibroblast growth factor in cardiac ventricles. *Dev Biol.* 157:507-16.

257. Liu, L., K.B. Pasumarthi, R.R. Padua, H. Massaeli, R.R. Fandrich, G.N. Pierce, P.A. Cattini, and E. Kardami. 1995. Adult cardiomyocytes express functional high-affinity receptors for basic fibroblast growth factor. *Am J Physiol.* 268:H1927-38.

258. Liu, S., S. Taffet, L. Stoner, M. Delmar, M.L. Vallano, and J. Jalife. 1993b. A structural basis for the unequal sensitivity of the major cardiac and liver gap junctions to intracellular acidification: the carboxyl tail length. *Biophys J.* 64:1422-33.

259. Liu, W.S., and C.A. Heckman. 1998. The sevenfold way of PKC regulation. *Cell Signal.* 10:529-42.

260. Lo, C.W., M.F. Cohen, G.Y. Huang, B.O. Lazatin, N. Patel, R. Sullivan, C. Pauken, and S.M. Park. 1997. Cx43 gap junction gene expression and gap junctional communication in mouse neural crest cells. *Dev Genet.* 20:119-32.

261. Lo, C.W., K.L. Waldo, and M.L. Kirby. 1999. Gap junction communication and the

modulation of cardiac neural crest cells. *Trends Cardiovasc Med.* 9:63-9.

262. Loewenstein, W.R. 1968. Communication through cell junctions: Implications in growth control and differentiation. *Dev Biol.* 19:151-83.

263. Loewenstein, W.R. 1981. Junctional intercellular communication: the cell-to-cell membrane channel. *Physiol Rev.* 61:829-913.

264. Loewenstein, W.R., and Y. Kanno. 1966. Intercellular communication and the control of tissue growth: lack of communication between cancer cells. *Nature.* 209:1248-9.

265. Long, C.S., K. Kariya, L. Karns, and P.C. Simpson. 1990. Trophic factors for cardiac myocytes. *J Hypertens Suppl.* 8:S219-24.

266. Loo, L.W., J.M. Berestecky, M.Y. Kanemitsu, and A.F. Lau. 1995. pp60src-mediated phosphorylation of connexin 43, a gap junction protein. *J Biol Chem.* 270:12751-61.

267. Loo, L.W., M.Y. Kanemitsu, and A.F. Lau. 1999. In vivo association of pp60v-src and the gap-junction protein connexin 43 in v-src-transformed fibroblasts. *Mol Carcinog.* 25:187-95.

268. Luke, R.A., E.C. Beyer, R.H. Hoyt, and J.E. Saffitz. 1989. Quantitative analysis of intercellular connections by immunohistochemistry of the cardiac gap junction protein connexin43. *Circ Res.* 65:1450-7.

269. Luke, R.A., and J.E. Saffitz. 1991. Remodeling of ventricular conduction pathways in healed canine infarct border zones. *J Clin Invest.* 87:1594-602.

270. Mackay, D., A. Ionides, Z. Kibar, G. Rouleau, V. Berry, A. Moore, A. Shiels, and S. Bhattacharya. 1999. Connexin46 mutations in autosomal dominant congenital cataract. *Am J Hum Genet.* 64:1357-64.

271. Madhukar, B.V., M. Yoneyama, F. Matsumura, J.E. Trosko, and G. Tsushimoto. 1983. Alteration of calcium transport by tumor promoters, 12-O-tetradecanoyl phorbol-13-acetate and p,p'-dichlorodiphenyltrichloroethane, in the Chinese hamster V79 fibroblast cell line. *Cancer Lett.* 18:251-9.

272. Maher, P. 1999. p38 mitogen-activated protein kinase activation is required for fibroblast growth factor-2-stimulated cell proliferation but not differentiation. *J Biol Chem.* 274:17491-8.

273. Makowski, L., D.L. Caspar, W.C. Phillips, and D.A. Goodenough. 1977. Gap junction structures. II. Analysis of the x-ray diffraction data. *J Cell Biol.* 74:629-45.

274. Martin, P.E., J. Steggles, C. Wilson, S. Ahmad, and W.H. Evans. 2000. Targeting motifs and functional parameters governing the assembly of connexins into gap junctions. *Biochem J.* 349:281-7.

275. Martyn, K.D., W.E. Kurata, B.J. Warn-Cramer, J.M. Burt, E. TenBroek, and A.F. Lau. 1997. Immortalized connexin43 knockout cell lines display a subset of biological properties associated with the transformed phenotype. *Cell Growth Differ.* 8:1015-27.

276. Matesic, D.F., H.L. Rupp, W.J. Bonney, R.J. Ruch, and J.E. Trosko. 1994. Changes in gap-junction permeability, phosphorylation, and number mediated by phorbol ester and non-phorbol-ester tumor promoters in rat liver epithelial cells. *Mol Carcinog.* 10:226-36.

277. Matsumoto, H., E.S. Kahn, and N. Komori. 1998. Nonradioactive phosphopeptide assay by matrix-assisted laser desorption ionization time-of-flight mass spectrometry: application to calcium/calmodulin-dependent protein kinase II. *Anal Biochem.* 260:188-94.

278. Mazet, F., B.A. Wittenberg, and D.C. Spray. 1985. Fate of intercellular junctions in isolated adult rat cardiac cells. *Circ Res.* 56:195-204.

279. McKarns, S.C., and D.J. Doolittle. 1992. Limitations of the scrape-loading/dye transfer technique to quantify inhibition of gap junctional intercellular communication. *Cell Biol Toxicol.* 8:89-103.

280. McKeehan, W.L., F. Wang, and M. Kan. 1998. The heparan sulfate-fibroblast growth factor family: diversity of structure and function. *Prog Nucleic Acid Res Mol Biol.* 59:135-76.

281. McNeil, P.L., L. Muthukrishnan, E. Warder, and P.A. D'Amore. 1989. Growth factors are released by mechanically wounded endothelial cells. *J Cell Biol.* 109:811-22.

282. Mehta, P.P., A. Hotz-Wagenblatt, B. Rose, D. Shalloway, and W.R. Loewenstein. 1991. Incorporation of the gene for a cell-cell channel protein into transformed cells leads to normalization of growth. *J Membr Biol.* 124:207-25.

283. Mehta, P.P., B.L. Lokeshwar, P.C. Schiller, M.V. Bendix, R.C. Ostenson, G.A. Howard, and B.A. Roos. 1996. Gap-junctional communication in normal and neoplastic prostate epithelial cells and its regulation by cAMP. *Mol Carcinog.* 15:18-32.

284. Mehta, P.P., C. Perez-Stable, M. Nadji, M. Mian, K. Asotra, and B.A. Roos. 1999. Suppression of human prostate cancer cell growth by forced expression of connexin genes. *Dev Genet.* 24:91-110.

285. Merle, P.L., J.J. Feige, and J. Verdetti. 1995. Basic fibroblast growth factor activates calcium channels in neonatal rat cardiomyocytes. *J Biol Chem.* 270:17361-7.

286. Merle, P.L., Y. Usson, M. Robert-Nicoud, and J. Verdetti. 1997. Basic FGF enhances calcium permeable channel openings in adult rat cardiac myocytes: implication in the bFGF-induced increase of free Ca2+ content. *J Mol Cell Cardiol.* 29:2687-98.

287. Mesnil, M., V. Krutovskikh, C. Piccoli, C. Elfgang, O. Traub, K. Willecke, and H. Yamasaki. 1995. Negative growth control of HeLa cells by connexin genes: connexin species specificity. *Cancer Res.* 55:629-39.

288. Meyer, R.A., D.W. Laird, J.P. Revel, and R.G. Johnson. 1992. Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J Cell Biol.* 119:179-89.

289. Micevych, P.E., and L. Abelson. 1991. Distribution of mRNAs coding for liver and heart gap junction proteins in the rat central nervous system. *J Comp Neurol.* 305:96-118.

290. Mikalsen, S.O., T. Husoy, G. Vikhamar, and T. Sanner. 1997. Induction of phosphotyrosine in the gap junction protein, connexin43 [published erratum appears in FEBS Lett 1997 Jul 14;411(2-3):394]. *FEBS Lett.* 401:271-5.

291. Mikalsen, S.O., and O. Kaalhus. 1996. A characterization of pervanadate, an inducer of cellular tyrosine phosphorylation and inhibitor of gap junctional intercellular communication. *Biochim Biophys Acta.* 1290:308-18.

292. Mikalsen, S.O., and O. Kaalhus. 1997. A characterization of permolybdate and its effect on cellular tyrosine phosphorylation, gap junctional intercellular communication and phosphorylation status of the gap junction protein, connexin43. *Biochim Biophys Acta.* 1356:207-20.

293. Mikalsen, S.O., and O. Kaalhus. 1998. Properties of pervanadate and permolybdate. Connexin43, phosphatase inhibition, and thiol reactivity as model systems. *J Biol Chem.* 273:10036-45.

294. Miyamae, M., M.M. Rodriguez, S.A. Camacho, I. Diamond, D. Mochly-Rosen, and V.M. Figueredo. 1998. Activation of epsilon protein kinase C correlates with a cardioprotective effect of regular ethanol consumption [published erratum appears in Proc Natl Acad Sci U S A 1999 Mar 16;96(6):3330]. *Proc Natl Acad Sci U S A.* 95:8262-7.

295. Miyataka, M., K. Ishikawa, and R. Katori. 1998. Basic fibroblast growth factor increased regional myocardial blood flow and limited infarct size of acutely infarcted myocardium in dogs. *Angiology.* 49:381-90.

296. Mochly-Rosen, D. 1995. Localization of protein kinases by anchoring proteins: a theme in signal transduction. *Science.* 268:247-51.

297. Mochly-Rosen, D., and A.S. Gordon. 1998. Anchoring proteins for protein kinase

C: a means for isozyme selectivity. *Faseb J.* 12:35-42.

298. Mochly-Rosen, D., G. Wu, H. Hahn, H. Osinska, T. Liron, J.N. Lorenz, A. Yatani, J. Robbins, and G.W. Dorn, 2nd. 2000. Cardiotrophic effects of protein kinase C epsilon: analysis by in vivo modulation of PKCepsilon translocation [see comments]. *Circ Res.* 86:1173-9.

299. Moorby, C.D. 2000. A connexin 43 mutant lacking the carboxyl cytoplasmic domain inhibits both growth and motility of mouse 3T3 fibroblasts. *Mol Carcinog.* 28:23-30.

300. Moorby, C.D., and E. Gherardi. 1999. Expression of a Cx43 deletion mutant in 3T3 A31 fibroblasts prevents PDGF-induced inhibition of cell communication and suppresses cell growth. *Exp Cell Res.* 249:367-76.

301. Moreno, A.P., J.C. Saez, G.I. Fishman, and D.C. Spray. 1994. Human connexin43 gap junction channels. Regulation of unitary conductances by phosphorylation. *Circ Res.* 74:1050-7.

302. Morley, G.E., J.F. Ek-Vitorin, S.M. Taffet, and M. Delmar. 1997. Structure of connexin43 and its regulation by pHi. *J Cardiovasc Electrophysiol.* 8:939-51.

303. Morley, G.E., S.M. Taffet, and M. Delmar. 1996. Intramolecular interactions mediate pH regulation of connexin43 channels. *Biophys J.* 70:1294-302.

304. Morris, J.A., A.J. Dorner, C.A. Edwards, L.M. Hendershot, and R.J. Kaufman. 1997. Immunoglobulin binding protein (BiP) function is required to protect cells from endoplasmic reticulum stress but is not required for the secretion of selective proteins. *J Biol Chem.* 272:4327-34.

305. Morriss-Kay, G.M. 1996. Craniofacial defects in AP-2 null mutant mice. *Bioessays.* 18:785-8.

306. Mosser, D.D., and N.C. Bols. 1982. The effect of phorbols on metabolic cooperation between human fibroblasts. *Carcinogenesis.* 3:1207-12.

307. Munster, P.N., and R. Weingart. 1993. Effects of phorbol ester on gap junctions of neonatal rat heart cells. *Pflugers Arch.* 423:181-8.

308. Murray, S.A., S.Y. Williams, C.Y. Dillard, S.K. Narayanan, and J. McCauley. 1997. Relationship of cytoskeletal filaments to annular gap junction expression in human adrenal cortical tumor cells in culture. *Exp Cell Res.* 234:398-404.

309. Musil, L.S., B.A. Cunningham, G.M. Edelman, and D.A. Goodenough. 1990. Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J Cell Biol.* 111:2077-88.

310. Musil, L.S., and D.A. Goodenough. 1991. Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J Cell Biol.* 115:1357-74.

311. Musil, L.S., and D.A. Goodenough. 1993. Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell.* 74:1065-77.

312. Musil, L.S., A.C. Le, J.K. VanSlyke, and L.M. Roberts. 2000. Regulation of connexin degradation as a mechanism to increase gap junction assembly and function. *J Biol Chem.*

313. Nagy, J.I., W.E. Li, C. Roy, B.W. Doble, J.S. Gilchrist, E. Kardami, and E.L. Hertzberg. 1997. Selective monoclonal antibody recognition and cellular localization of an unphosphorylated form of connexin43. *Exp Cell Res.* 236:127-36.

314. Naus, C.C., K. Elisevich, D. Zhu, D.J. Belliveau, and R.F. Del Maestro. 1992. In vivo growth of C6 glioma cells transfected with connexin43 cDNA. *Cancer Res.* 52:4208-13.

315. Newbold, R.F., and J. Amos. 1981. Inhibition of metabolic cooperation between mammalian cells in culture by tumor promoters. *Carcinogenesis.* 2:243-9.

316. Newton, A.C. 1995. Protein kinase C: structure, function, and regulation. *J Biol Chem.* 270:28495-8.

317. Newton, A.C. 1997. Regulation of protein kinase C. *Curr Opin Cell Biol.* 9:161-7.

318. Nicholson, B.J., P.A. Weber, F. Cao, H. Chang, P. Lampe, and G. Goldberg. 2000. The molecular basis of selective permeability of connexins is complex and includes both size and charge. *Braz J Med Biol Res.* 33:369-78.

319. Niessen, H., H. Harz, P. Bedner, K. Kramer, and K. Willecke. 2000. Selective permeability of different connexin channels to the second messenger inositol 1,4,5-trisphosphate. *J Cell Sci.* 113:1365-72.

320. Niessen, H., and K. Willecke. 2000. Strongly decreased gap junctional permeability to inositol 1,4, 5- trisphosphate in connexin32 deficient hepatocytes. *FEBS Lett.* 466:112-4.

321. Nugent, M.A., and R.V. Iozzo. 2000. Fibroblast growth factor-2. *Int J Biochem Cell Biol.* 32:115-20.

322. O'Brien, J., R. Bruzzone, T.W. White, M.R. Al-Ubaidi, and H. Ripps. 1 998. Cloning and expression of two related connexins from the perch retina define a distinct

subgroup of the connexin family. *J Neurosci.* 18:7625-37.

323. Ogawa, H., M. Oyamada, T. Mori, M. Mori, and H. Shimizu. 2000. Relationship of gap junction formation to phosphorylation of connexin43 in mouse preimplantation embryos. *Mol Reprod Dev.* 55:393-8.

324. Oh, S., Y. Ri, M.V. Bennett, E.B. Trexler, V.K. Verselis, and T.A. Bargiello. 1997. Changes in permeability caused by connexin 32 mutations underlie X- linked Charcot-Marie-Tooth disease. *Neuron.* 19:927-38.

325. Oh, S.K., and P. Sarnow. 1993. Gene regulation: translational initiation by internal ribosome binding. *Curr Opin Genet Dev.* 3:295-300.

326. Okada-Ban, M., J.P. Thiery, and J. Jouanneau. 2000. Fibroblast growth factor-2. *Int J Biochem Cell Biol.* 32:263-7.

327. Omens, J.H. 1998. Stress and strain as regulators of myocardial growth. *Prog Biophys Mol Biol.* 69:559-72.

328. Omori, Y., and H. Yamasaki. 1999. Gap junction proteins connexin32 and connexin43 partially acquire growth-suppressive function in HeLa cells by deletion of their C- terminal tails. *Carcinogenesis.* 20:1913-8.

329. Ornitz, D.M. 2000. FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *Bioessays.* 22:108-12.

330. Oyamada, M., H. Kimura, Y. Oyamada, A. Miyamoto, H. Ohshika, and M. Mori. 1994. The expression, phosphorylation, and localization of connexin 43 and gap-junctional intercellular communication during the establishment of a synchronized contraction of cultured neonatal rat cardiac myocytes. *Exp Cell Res.* 212:351-8.

331. Padua, R.R., P.L. Merle, B.W. Doble, C.H. Yu, P. Zahradka, G.N. Pierce, V. Panagia, and E. Kardami. 1998. FGF-2-induced negative inotropism and cardioprotection are inhibited by chelerythrine: involvement of sarcolemmal calcium-independent protein kinase C. *J Mol Cell Cardiol.* 30:2695-709.

332. Padua, R.R., R. Sethi, S.E. Davey-Forgie, L. Liu, N.S. Dhalla, and E. Kardami. 1996. Cardioprotection and basic fibroblast growth factor. *In* Heart Hypertrophy and Failure. N.S. Dhalla, P.K. Singal, and R.E. Beamish, editors. Kluwer Academic Publishers, Boston, Mass. 501-518.

333. Padua, R.R., R. Sethi, N.S. Dhalla, and E. Kardami. 1995. Basic fibroblast growth factor is cardioprotective in ischemia- reperfusion injury. *Mol Cell Biochem.* 143:129-35.

334. Pahl, H.L. 1999. Activators and target genes of Rel/NF-kappaB transcription factors. *Oncogene.* 18:6853-66.

335. Pal, J.D., X. Liu, D. Mackay, A. Shiels, V.M. Berthoud, E.C. Beyer, and L. Ebihara. 2000. Connexin46 mutations linked to congenital cataract show loss of gap junction channel function. *Am J Physiol Cell Physiol.* 279:C596-C602.

336. Parker, T.G. 1995. Molecular biology of myocardial hypertrophy and failure: gene expression and trophic signaling. *New Horiz.* 3:288-300.

337. Parker, T.G., K.L. Chow, R.J. Schwartz, and M.D. Schneider. 1991. TGF-beta 1 and fibroblast growth factors selectively up-regulate tissue- specific fetal genes in cardiac muscle cells. *Ciba Found Symp.* 157:152-60.

338. Partanen, J., S. Vainikka, and K. Alitalo. 1993. Structural and functional specificity of FGF receptors. *Philos Trans R Soc Lond B Biol Sci.* 340:297-303.

339. Pasumarthi, K.B., B.W. Doble, E. Kardami, and P.A. Cattini. 1994. Over-expression of CUG- or AUG-initiated forms of basic fibroblast growth factor in cardiac myocytes results in similar effects on mitosis and protein synthesis but distinct nuclear morphologies. *J Mol Cell Cardiol.* 26:1045-60.

340. Pasumarthi, K.B., E. Kardami, and P.A. Cattini. 1996. High and low molecular weight fibroblast growth factor-2 increase proliferation of neonatal rat cardiac myocytes but have differential effects on binucleation and nuclear morphology. Evidence for both paracrine and intracrine actions of fibroblast growth factor-2. *Circ Res.* 78:126-36.

341. Paul, D.L., L. Ebihara, L.J. Takemoto, K.I. Swenson, and D.A. Goodenough. 1991. Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of Xenopus oocytes. *J Cell Biol.* 115:1077-89.

342. Paulson, A.F., P.D. Lampe, R.A. Meyer, E. TenBroek, M.M. Atkinson, T.F. Walseth, and R.G. Johnson. 2000. Cyclic AMP and LDL trigger a rapid enhancement in gap junction assembly through a stimulation of connexin trafficking. *J Cell Sci.* 113:3037-49.

343. Pawson, T. 1995. Protein modules and signalling networks. *Nature.* 373:573-80.

344. Pelletier, D.B., and A.L. Boynton. 1994. Dissociation of PDGF receptor tyrosine kinase activity from PDGF- mediated inhibition of gap junctional communication. *J Cell Physiol.* 158:427-34.

345. Pepper, M.S., and P. Meda. 1992. Basic fibroblast growth factor increases junctional communication and connexin 43 expression in microvascular endothelial cells. *J Cell Physiol.* 153:196-205.

346. Peracchia, C., A. Sotkis, X.G. Wang, L.L. Peracchia, and A. Persechini. 2000. Calmodulin directly gates gap junction channels. *J Biol Chem.* 275:26220-4.

347. Perkins, G.A., D.A. Goodenough, and G.E. Sosinsky. 1998. Formation of the gap junction intercellular channel requires a 30 degree rotation for interdigitating two apposing connexons. *J Mol Biol.* 277:171-7.

348. Peters, N.S., C.R. Green, P.A. Poole-Wilson, and N.J. Severs. 1993. Reduced content of connexin43 gap junctions in ventricular myocardium from hypertrophied and ischemic human hearts. *Circulation.* 88:864-75.

349. Peters, N.S., N.J. Severs, S.M. Rothery, C. Lincoln, M.H. Yacoub, and C.R. Green. 1994. Spatiotemporal relation between gap junctions and fascia adherens junctions during postnatal development of human ventricular myocardium [see comments]. *Circulation.* 90:713-25.

350. Petrocelli, T., and S.J. Lye. 1993. Regulation of transcripts encoding the myometrial gap junction protein, connexin-43, by estrogen and progesterone. *Endocrinology.* 133:284-90.

351. Piechocki, M.P., R.D. Burk, and R.J. Ruch. 1999. Regulation of connexin32 and connexin43 gene expression by DNA methylation in rat liver cells. *Carcinogenesis.* 20:401-6.

352. Ping, P., J. Zhang, X. Cao, R.C. Li, D. Kong, X.L. Tang, Y. Qiu, S. Manchikalapudi, J.A. Auchampach, R.G. Black, and R. Bolli. 1999. PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia- reperfusion in conscious rabbits. *Am J Physiol.* 276:H1468-81.

353. Ping, P., J. Zhang, Y. Qiu, X.L. Tang, S. Manchikalapudi, X. Cao, and R. Bolli. 1997. Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ Res.* 81:404-14.

354. Piotrowicz, R.S., P.A. Maher, and E.G. Levin. 1999. Dual activities of 22-24 kDa basic fibroblast growth factor: inhibition of migration and stimulation of proliferation. *J Cell Physiol.* 178:144-53.

355. Plum, A., G. Hallas, T. Magin, F. Dombrowski, A. Hagendorff, B. Schumacher, C. Wolpert, J. Kim, W.H. Lamers, M. Evert, P. Meda, O. Traub, and K. Willecke. 2000. Unique and shared functions of different connexins in mice. *Curr Biol.* 10:1083-91.

356. Postma, F.R., T. Hengeveld, J. Alblas, B.N. Giepmans, G.C. Zondag, K. Jalink, and W.H. Moolenaar. 1998. Acute loss of cell-cell communication caused by G protein-coupled receptors: a critical role for c-Src. *J Cell Biol.* 140:1199-209.

357. Prowse, D.M., G.P. Cadwallader, and J.D. Pitts. 1997. E-cadherin expression can alter the specificity of gap junction formation. *Cell Biol Int.* 21:833-43.

358. Puceat, M., R. Hilal-Dandan, B. Strulovici, L.L. Brunton, and J.H. Brown. 1994. Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. *J Biol Chem.* 269:16938-44.

359. Puranam, K.L., D.W. Laird, and J.P. Revel. 1993. Trapping an intermediate form of connexin43 in the Golgi. *Exp Cell Res.* 206:85-92.

360. Quarto, N., and F. Amalric. 1994. Heparan sulfate proteoglycans as transducers of FGF-2 signalling. *J Cell Sci.* 107:3201-12.

361. Quist, A.P., S.K. Rhee, H. Lin, and R. Lal. 2000. Physiological role of gap-junctional hemichannels. Extracellular calcium-dependent isosmotic volume regulation. *J Cell Biol.* 148:1063-74.

362. Rabionet, R., P. Gasparini, and X. Estivill. 2000. Molecular genetics of hearing impairment due to mutations in gap junction genes encoding beta connexins. *Hum Mutat.* 16:190-202.

363. Reaume, A.G., P.A. de Sousa, S. Kulkarni, B.L. Langille, D. Zhu, T.C. Davies, S.C. Juneja, G.M. Kidder, and J. Rossant. 1995. Cardiac malformation in neonatal mice lacking connexin43 [see comments]. *Science.* 267:1831-4.

364. Reuss, B., R. Dermietzel, and K. Unsicker. 1998. Fibroblast growth factor 2 (FGF-2) differentially regulates connexin (Cx) 43 expression and function in astroglial cells from distinct brain regions. *Glia.* 22:19-30.

365. Reuss, B., and K. Unsicker. 1998. Regulation of gap junction communication by growth factors from non- neural cells to astroglia: a brief review. *Glia.* 24:32-8.

366. Revel, J.P., and M.J. Karnovsky. 1967. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. *J Cell Biol.* 33:C7-12.

367. Reynhout, J.K., P.D. Lampe, and R.G. Johnson. 1992. An activator of protein kinase C inhibits gap junction communication between cultured bovine lens cells. *Exp Cell Res.* 198:337-42.

368. Risek, B., S. Guthrie, N. Kumar, and N.B. Gilula. 1990. Modulation of gap junction transcript and protein expression during pregnancy in the rat. *J Cell Biol.* 110:269-82.

369. Rivedal, E., T. Sanner, T. Enomoto, and H. Yamasaki. 1985. Inhibition of intercellular communication and enhancement of morphological transformation of Syrian hamster embryo cells by TPA. Use of TPA-sensitive and TPA-resistant cell lines. *Carcinogenesis.* 6:899-902.

370. Rose, B., P.P. Mehta, and W.R. Loewenstein. 1993. Gap-junction protein gene

suppresses tumorigenicity. *Carcinogenesis*. 14:1073-5.

371. Rothman, J.E., and L. Orci. 1992. Molecular dissection of the secretory pathway. *Nature*. 355:409-15.

372. Rozakis-Adcock, M., J. McGlade, G. Mbamalu, G. Pelicci, R. Daly, W. Li, A. Batzer, S. Thomas, J. Brugge, P.G. Pelicci, et al. 1992. Association of the Shc and Grb2/Sem5 SH2-containing proteins is implicated in activation of the Ras pathway by tyrosine kinases. *Nature*. 360:689-92.

373. Rozental, R., C. Giaume, and D.C. Spray. 2000. Gap junctions in the nervous system. *Brain Res Rev*. 32:11-5.

374. Rubin, J.B., V.K. Verselis, M.V. Bennett, and T.A. Bargiello. 1992. Molecular analysis of voltage dependence of heterotypic gap junctions formed by connexins 26 and 32. *Biophys J*. 62:183-93; discussion 193-5.

375. Ruch, R.J., J.E. Klaunig, and M.A. Pereira. 1987. Inhibition of intercellular communication between mouse hepatocytes by tumor promoters. *Toxicol Appl Pharmacol*. 87:111-20.

376. Rybin, V.O., and S.F. Steinberg. 1994. Protein kinase C isoform expression and regulation in the developing rat heart. *Circ Res*. 74:299-309.

377. Saez, J.C., A.C. Nairn, A.J. Czernik, G.I. Fishman, D.C. Spray, and E.L. Hertzberg. 1997. Phosphorylation of connexin43 and the regulation of neonatal rat cardiac myocyte gap junctions. *J Mol Cell Cardiol*. 29:2131-45.

378. Sasame, A., H. Nakajima, K. Tamura, M. Miyagi, H. Rakue, M. Usui, T. Katoh, Y. Naitoh, and C. Ibukiyama. 1999. A study to determine if basic fibroblast growth factor (bFGF) reduces myocardial infarct size in acute coronary arterial occlusion. *Jpn Heart J*. 40:165-78.

379. Scheinowitz, M., A. Kotlyar, S. Zimand, D. Ohad, I. Leibovitz, N. Bloom, I. Goldberg, D. Nass, S. Engelberg, N. Savion, and M. Eldar. 1998. Basic fibroblast growth factor induces myocardial hypertrophy following acute infarction in rats. *Exp Physiol*. 83:585-93.

380. Schiavi, A., A. Hudder, and R. Werner. 1999. Connexin43 mRNA contains a functional internal ribosome entry site. *FEBS Lett*. 464:118-22.

381. Schneider, M.D., W.R. McLellan, F.M. Black, and T.G. Parker. 1992. Growth factors, growth factor response elements, and the cardiac phenotype. *Basic Res Cardiol*. 87:33-48.

382. Schultz, J.E., S.A. Witt, M.L. Nieman, P.J. Reiser, S.J. Engle, M. Zhou, S.A.

Pawlowski, J.N. Lorenz, T.R. Kimball, and T. Doetschman. 1999. Fibroblast growth factor-2 mediates pressure-induced hypertrophic response. *J Clin Invest.* 104:709-19.

383. Sears, J., T. Nakano, and M. Sears. 1998. Adrenergic-mediated connexin43 phosphorylation in the ocular ciliary epithelium. *Curr Eye Res.* 17:104-7.

384. Severs, N.J. 2000. The cardiac muscle cell. *Bioessays.* 22:188-99.

385. Severs, N.J., K.S. Shovel, A.M. Slade, T. Powell, V.W. Twist, and C.R. Green. 1989. Fate of gap junctions in isolated adult mammalian cardiomyocytes. *Circ Res.* 65:22-42.

386. Shiba, Y., Y. Sasaki, C. Hirono, and Y. Kanno. 1989. Close relationship between modulation of serum-induced stimulation of DNA synthesis and changes in gap-junctional intercellular communication in quiescent 3T3-L1 cells caused by cyclic AMP and the tumor-promoting phorbol ester TPA. *Exp Cell Res.* 185:535-40.

387. Shiels, A., D. Mackay, A. Ionides, V. Berry, A. Moore, and S. Bhattacharya. 1998. A missense mutation in the human connexin50 gene (GJA8) underlies autosomal dominant "zonular pulverulent" cataract, on chromosome 1q. *Am J Hum Genet.* 62:526-32.

388. Shiokawa-Sawada, M., H. Mano, K. Hanada, S. Kakudo, T. Kameda, K. Miyazawa, Y. Nakamaru, T. Yuasa, Y. Mori, M. Kumegawa, and Y. Hakeda. 1997. Down-regulation of gap junctional intercellular communication between osteoblastic MC3T3-E1 cells by basic fibroblast growth factor and a phorbol ester (12-O-tetradecanoylphorbol-13-acetate). *J Bone Miner Res.* 12:1165-73.

389. Shubeita, H.E., J. Thorburn, and K.R. Chien. 1992. Microinjection of antibodies and expression vectors into living myocardial cells. Development of a novel approach to identify candidate genes that regulate cardiac growth and hypertrophy. *Circulation.* 85:2236-46.

390. Simon, A.M., D.A. Goodenough, and D.L. Paul. 1998. Mice lacking connexin40 have cardiac conduction abnormalities characteristic of atrioventricular block and bundle branch block. *Curr Biol.* 8:295-8.

391. Siuzdak, G. 1994. The emergence of mass spectrometry in biochemical research [published erratum appears in Proc Natl Acad Sci U S A 1995 Jan 17;92(2):646]. *Proc Natl Acad Sci U S A.* 91:11290-7.

392. Slavin, J. 1995. Fibroblast growth factors: at the heart of angiogenesis. *Cell Biol Int.* 19:431-44.

393. Speir, E., V. Tanner, A.M. Gonzalez, J. Farris, A. Baird, and W. Casscells. 1992. Acidic and basic fibroblast growth factors in adult rat heart myocytes. Localization,

regulation in culture, and effects on DNA synthesis. *Circ Res.* 71:251-9.

394. Speir, E., Y.F. Zhou, M. Lee, S. Shrivastav, and W. Casscells. 1989. Fibroblast growth factors are present in adult cardiac myocytes, in vivo [corrected and issued with original paging in Biochem Biophys Res Commun 1988 Dec 30;157(3)]. *Biochem Biophys Res Commun.* 159:1336-40.

395. Srinivas, M., R. Rozental, T. Kojima, R. Dermietzel, M. Mehler, D.F. Condorelli, J.A. Kessler, and D.C. Spray. 1999. Functional properties of channels formed by the neuronal gap junction protein connexin36. *J Neurosci.* 19:9848-55.

396. Stagg, R.B., and W.H. Fletcher. 1990. The hormone-induced regulation of contact-dependent cell-cell communication by phosphorylation. *Endocr Rev.* 11:302-25.

397. Staudinger, J., J. Lu, and E.N. Olson. 1997. Specific interaction of the PDZ domain protein PICK1 with the COOH terminus of protein kinase C-alpha. *J Biol Chem.* 272:32019-24.

398. Staudinger, J., J. Zhou, R. Burgess, S.J. Elledge, and E.N. Olson. 1995. PICK1: a perinuclear binding protein and substrate for protein kinase C isolated by the yeast two-hybrid system. *J Cell Biol.* 128:263-71.

399. Steele, E.C., Jr., M.F. Lyon, J. Favor, P.V. Guillot, Y. Boyd, and R.L. Church. 1998. A mutation in the connexin 50 (Cx50) gene is a candidate for the No2 mouse cataract. *Curr Eye Res.* 17:883-9.

400. Stein, I., A. Itin, P. Einat, R. Skaliter, Z. Grossman, and E. Keshet. 1998. Translation of vascular endothelial growth factor mRNA by internal ribosome entry: implications for translation under hypoxia. *Mol Cell Biol.* 18:3112-9.

401. Stergiopoulos, K., J.L. Alvarado, M. Mastroianni, J.F. Ek-Vitorin, S.M. Taffet, and M. Delmar. 1999. Hetero-domain interactions as a mechanism for the regulation of connexin channels. *Circ Res.* 84:1144-55.

402. Stults, J.T. 1995. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI- MS). *Curr Opin Struct Biol.* 5:691-8.

403. Sullivan, R., G.Y. Huang, R.A. Meyer, A. Wessels, K.K. Linask, and C.W. Lo. 1998. Heart malformations in transgenic mice exhibiting dominant negative inhibition of gap junctional communication in neural crest cells. *Dev Biol.* 204:224-34.

404. Sullivan, R., C. Ruangvoravat, D. Joo, J. Morgan, B.L. Wang, X.K. Wang, and C.W. Lo. 1993. Structure, sequence and expression of the mouse Cx43 gene encoding connexin 43. *Gene.* 130:191-9.

405. Suske, G. 1999. The Sp-family of transcription factors. *Gene.* 238:291-300.

406. Swenson, K.I., J.R. Jordan, E.C. Beyer, and D.L. Paul. 1989. Formation of gap junctions by expression of connexins in Xenopus oocyte pairs. *Cell.* 57:145-55.

407. Swenson, K.I., H. Piwnica-Worms, H. McNamee, and D.L. Paul. 1990. Tyrosine phosphorylation of the gap junction protein connexin43 is required for the pp60v-src-induced inhibition of communication. *Cell Regul.* 1:989-1002.

408. Takeishi, Y., P. Ping, R. Bolli, D.L. Kirkpatrick, B.D. Hoit, and R.A. Walsh. 2000. Transgenic overexpression of constitutively active protein kinase C epsilon causes concentric cardiac hypertrophy. *Circ Res.* 86:1218-23.

409. Toyofuku, T., M. Yabuki, K. Otsu, T. Kuzuya, M. Tada, and M. Hori. 1999. Functional role of c-Src in gap junctions of the cardiomyopathic heart [see comments]. *Circ Res.* 85:672-81.

410. Traub, O., R. Eckert, H. Lichtenberg-Frate, C. Elfgang, B. Bastide, K.H. Scheidtmann, D.F. Hulser, and K. Willecke. 1994. Immunochemical and electrophysiological characterization of murine connexin40 and -43 in mouse tissues and transfected human cells. *Eur J Cell Biol.* 64:101-12.

411. Trexler, E.B., M.V. Bennett, T.A. Bargiello, and V.K. Verselis. 1996. Voltage gating and permeation in a gap junction hemichannel. *Proc Natl Acad Sci U S A.* 93:5836-41.

412. Trosko, J.E., C.C. Chang, M.R. Wilson, B. Upham, T. Hayashi, and M. Wade. 2000. Gap junctions and the regulation of cellular functions of stem cells during development and differentiation. *Methods.* 20:245-64.

413. Ueno, H., M.B. Perryman, R. Roberts, and M.D. Schneider. 1988. Differentiation of cardiac myocytes after mitogen withdrawal exhibits three sequential states of the ventricular growth response. *J Cell Biol.* 107:1911-8.

414. Unger, V.M., N.M. Kumar, N.B. Gilula, and M. Yeager. 1999a. Expression, two-dimensional crystallization, and electron cryo- crystallography of recombinant gap junction membrane channels. *J Struct Biol.* 128:98-105.

415. Unger, V.M., N.M. Kumar, N.B. Gilula, and M. Yeager. 1999b. Three-dimensional structure of a recombinant gap junction membrane channel. *Science.* 283:1176-80.

416. Vagner, S., C. Touriol, B. Galy, S. Audigier, M.C. Gensac, F. Amalric, F. Bayard, H. Prats, and A.C. Prats. 1996. Translation of CUG- but not AUG-initiated forms of human fibroblast growth factor 2 is activated in transformed and stressed cells. *J Cell Biol.* 135:1391-402.

417. Valiunas, V., and R. Weingart. 2000. Electrical properties of gap junction

hemichannels identified in transfected HeLa cells. *Pflugers Arch.* 440:366-79.

418. Valiunas, V., R. Weingart, and P.R. Brink. 2000. Formation of heterotypic gap junction channels by connexins 40 and 43. *Circ Res.* 86:E42-9.

419. van Kempen, M.J., C. Fromaget, D. Gros, A.F. Moorman, and W.H. Lamers. 1991. Spatial distribution of connexin43, the major cardiac gap junction protein, in the developing and adult rat heart. *Circ Res.* 68:1638-51.

420. Vaney, D.I., and R. Weiler. 2000. Gap junctions in the eye: evidence for heteromeric, heterotypic and mixed-homotypic interactions. *Brain Res Brain Res Rev.* 32:115-20.

421. Veenstra, R.D. 1996. Size and selectivity of gap junction channels formed from different connexins. *J Bioenerg Biomembr.* 28:327-37.

422. Veenstra, R.D., H.Z. Wang, D.A. Beblo, M.G. Chilton, A.L. Harris, E.C. Beyer, and P.R. Brink. 1995. Selectivity of connexin-specific gap junctions does not correlate with channel conductance. *Circ Res.* 77:1156-65.

423. Verselis, V.K., E.B. Trexler, and F.F. Bukauskas. 2000. Connexin hemichannels and cell-cell channels: comparison of properties. *Braz J Med Biol Res.* 33:379-89.

424. Vikhamar, G., E. Rivedal, S. Mollerup, and T. Sanner. 1998. Role of Cx43 phosphorylation and MAP kinase activation in EGF induced enhancement of cell communication in human kidney epithelial cells. *Cell Adhes Commun.* 5:451-60.

425. Walder, L., and R. Lutzelschwab. 1984. Effects of 12-O-tetradecanoylphorbol-13-acetate (TPA), retinoic acid and diazepam on intercellular communication in a monolayer of rat liver epithelial cells. *Exp Cell Res.* 152:66-76.

426. Waldo, K.L., C.W. Lo, and M.L. Kirby. 1999. Connexin 43 expression reflects neural crest patterns during cardiovascular development. *Dev Biol.* 208:307-23.

427. Waltzer, L., and M. Bienz. 1999. The control of beta-catenin and TCF during embryonic development and cancer. *Cancer Metastasis Rev.* 18:231-46.

428. Wang, X.G., and C. Peracchia. 1998. Chemical gating of heteromeric and heterotypic gap junction channels. *J Membr Biol.* 162:169-76.

429. Wang, Y., and M. Ashraf. 1999. Role of protein kinase C in mitochondrial KATP channel-mediated protection against Ca2+ overload injury in rat myocardium. *Circ Res.* 84:1156-65.

430. Wang, Y., K. Hirai, and M. Ashraf. 1999. Activation of mitochondrial ATP-sensitive K(+) channel for cardiac protection against ischemic injury is dependent on

protein kinase C activity. *Circ Res.* 85:731-41.

431. Wang, Y., and B. Rose. 1995. Clustering of Cx43 cell-to-cell channels into gap junction plaques: regulation by cAMP and microfilaments. *J Cell Sci.* 108:3501-8.

432. Wang, Y., and B. Rose. 1997. An inhibition of gap-junctional communication by cadherins. *J Cell Sci.* 110:301-9.

433. Warn-Cramer, B.J., G.T. Cottrell, J.M. Burt, and A.F. Lau. 1998. Regulation of connexin-43 gap junctional intercellular communication by mitogen-activated protein kinase. *J Biol Chem.* 273:9188-96.

434. Warn-Cramer, B.J., P.D. Lampe, W.E. Kurata, M.Y. Kanemitsu, L.W. Loo, W. Eckhart, and A.F. Lau. 1996. Characterization of the mitogen-activated protein kinase phosphorylation sites on the connexin-43 gap junction protein. *J Biol Chem.* 271:3779-86.

435. Werner, R., E. Levine, C. Rabadan-Diehl, and G. Dahl. 1989. Formation of hybrid cell-cell channels. *Proc Natl Acad Sci U S A.* 86:5380-4.

436. Werner, R., E. Levine, C. Rabadan-Diehl, and G. Dahl. 1991. Gating properties of connexin32 cell-cell channels and their mutants expressed in Xenopus oocytes. *Proc R Soc Lond B Biol Sci.* 243:5-11.

437. White, T.W., R. Bruzzone, D.A. Goodenough, and D.L. Paul. 1992. Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. *Mol Biol Cell.* 3:711-20.

438. White, T.W., R. Bruzzone, S. Wolfram, D.L. Paul, and D.A. Goodenough. 1994. Selective interactions among the multiple connexin proteins expressed in the vertebrate lens: the second extracellular domain is a determinant of compatibility between connexins. *J Cell Biol.* 125:879-92.

439. White, T.W., D.A. Goodenough, and D.L. Paul. 1998. Targeted ablation of connexin50 in mice results in microphthalmia and zonular pulverulent cataracts. *J Cell Biol.* 143:815-25.

440. White, T.W., and D.L. Paul. 1999. Genetic diseases and gene knockouts reveal diverse connexin functions. *Annu Rev Physiol.* 61:283-310.

441. White, T.W., D.L. Paul, D.A. Goodenough, and R. Bruzzone. 1995. Functional analysis of selective interactions among rodent connexins. *Mol Biol Cell.* 6:459-70.

442. Willecke, K., R. Heynkes, E. Dahl, R. Stutenkemper, H. Hennemann, S. Jungbluth, T. Suchyna, and B.J. Nicholson. 1991. Mouse connexin37: cloning and functional expression of a gap junction gene highly expressed in lung. *J Cell Biol.* 114:1049-57.

443. Willis, A.E. 1999. Translational control of growth factor and proto-oncogene expression. *Int J Biochem Cell Biol.* 31:73-86.

444. Windoffer, R., B. Beile, A. Leibold, S. Thomas, U. Wilhelm, and R.E. Leube. 2000. Visualization of gap junction mobility in living cells. *Cell Tissue Res.* 299:347-62.

445. Winston, R.L., and M.C. Fitzgerald. 1997. Mass spectrometry as a readout of protein structure and function. *Mass Spectrom Rev.* 16:165-79.

446. Wolf, M., H.d. LeVine, W.S. May, Jr., P. Cuatrecasas, and N. Sahyoun. 1985. A model for intracellular translocation of protein kinase C involving synergism between Ca2+ and phorbol esters. *Nature.* 317:546-9.

447. Wolosin, J.M., M. Schutte, and S. Chen. 1997. Connexin distribution in the rabbit and rat ciliary body. A case for heterotypic epithelial gap junctions. *Invest Ophthalmol Vis Sci.* 38:341-8.

448. Woodgett, J.R., and T. Hunter. 1987. Isolation and characterization of two distinct forms of protein kinase C. *J Biol Chem.* 262:4836-43.

449. Wu, G., T. Toyokawa, H. Hahn, and G.W. Dorn, 2nd. 2000. epsilon protein kinase C in pathological myocardial hypertrophy. Analysis by combined transgenic expression of translocation modifiers and galpha q. *J Biol Chem.* 275:29927–30.

450. Xie, H., D.W. Laird, T.H. Chang, and V.W. Hu. 1997. A mitosis-specific phosphorylation of the gap junction protein connexin43 in human vascular cells: biochemical characterization and localization. *J Cell Biol.* 137:203-10.

451. Xing, E.P., Y. Nie, L.D. Wang, G.Y. Yang, and C.S. Yang. 1999. Aberrant methylation of p16INK4a and deletion of p15INK4b are frequent events in human esophageal cancer in Linxian, China. *Carcinogenesis.* 20:77-84.

452. Yahuaca, P., J.F. Ek-Vitorin, P. Rush, M. Delmar, and S.M.. Taffet. 2000. Identification of a protein kinase activity that phosphorylates connexin43 in a pH-dependent manner. *Braz J Med Biol Res.* 33:399-406.

453. Yamamoto, T., A. Ochalski, E.L. Hertzberg, and J.I. Nagy. 1990. LM and EM immunolocalization of the gap junctional protein connexin 43 in rat brain. *Brain Res.* 508:313-9.

454. Yamasaki, H., A.M. Aguelon-Pegouries, T. Enomoto, N. Martel, G. Furstenberger, and F. Marks. 1985. Comparative effects of a complete tumor promoter, TPA, and a second- stage tumor promoter, RPA, on intercellular communication, cell differentiation and cell transformation. *Carcinogenesis.* 6:1173-9.

455. Yamasaki, H., T. Enomoto, N. Martel, Y. Shiba, and Y. Kanno. 1983. Tumour promoter-mediated reversible inhibition of cell-cell communication (electrical coupling). Relationship with phorbol ester binding and de novo macromolecule synthesis. *Exp Cell Res.* 146:297-308.

456. Yamasaki, H., V. Krutovskikh, M. Mesnil, T. Tanaka, M.L. Zaidan-Dagli, and Y. Omori. 1999. Role of connexin (gap junction) genes in cell growth control and carcinogenesis. *C R Acad Sci III.* 322:151-9.

457. Yamasaki, H., and C.C. Naus. 1996. Role of connexin genes in growth control. *Carcinogenesis.* 17:1199-213.

458. Yamazaki, T., I. Komuro, and Y. Yazaki. 1998. Signalling pathways for cardiac hypertrophy. *Cell Signal.* 10:693-8.

459. Yanagisawa-Miwa, A., Y. Uchida, F. Nakamura, T. Tomaru, H. Kido, T. Kamijo, T. Sugimoto, K. Kaji, M. Utsuyama, C. Kurashima, and et al. 1992. Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor. *Science.* 257:1401-3.

460. Yates, J.R., 3rd. 1998. Mass spectrometry and the age of the proteome. *J Mass Spectrom.* 33:1-19.

461. Yeager, M. 1998. Structure of cardiac gap junction intercellular channels. *J Struct Biol.* 121:231-45.

462. Yoshida, K., S. Kawamura, Y. Mizukami, and M. Kitakaze. 1997. Implication of protein kinase C-alpha, delta, and epsilon isoforms in ischemic preconditioning in perfused rat hearts. *J Biochem (Tokyo).* 122:506-11.

463. Yu, W., G. Dahl, and R. Werner. 1994. The connexin43 gene is responsive to oestrogen. *Proc R Soc Lond B Biol Sci.* 255:125-32.

464. Zahs, K.R. 1998. Heterotypic coupling between glial cells of the mammalian central nervous system. *Glia.* 24:85-96.

465. Zeng, Y.X., K. Somasundaram, and W.S. el-Deiry. 1997. AP2 inhibits cancer cell growth and activates p21WAF1/CIP1 expression. *Nat Genet.* 15:78-82.

466. Zhang, J.T., M. Chen, C.I. Foote, and B.J. Nicholson. 1996. Membrane integration of in vitro-translated gap junctional proteins: co- and post-translational mechanisms. *Mol Biol Cell.* 7:471-82.

467. Zhang, J.T., and B.J. Nicholson. 1989. Sequence and tissue distribution of a second protein of hepatic gap junctions, Cx26, as deduced from its cDNA. *J Cell Biol.* 109:3391-401.

468. Zhang, X., C.J. Herring, P.R. Romano, J. Szczepanowska, H. Brzeska, A.G. Hinnebusch, and J. Qin. 1998. Identification of phosphorylation sites in proteins separated by polyacrylamide gel electrophoresis. *Anal Chem.* 70:2050-9.

469. Zhang, Y.W., I. Morita, M. Nishida, and S.I. Murota. 1999a. Involvement of tyrosine kinase in the hypoxia/reoxygenation-induced gap junctional intercellular communication abnormality in cultured human umbilical vein endothelial cells. *J Cell Physiol.* 180:305-13.

470. Zhang, Y.W., I. Morita, X.S. Yao, and S. Murota. 1999b. Pretreatment with eicosapentaenoic acid prevented hypoxia/reoxygenation- induced abnormality in endothelial gap junctional intercellular communication through inhibiting the tyrosine kinase activity. *Prostaglandins Leukot Essent Fatty Acids.* 61:33-40.

471. Zhao, H.B., and J. Santos-Sacchi. 2000. Voltage gating of gap junctions in cochlear supporting cells: evidence for nonhomotypic channels. *J Membr Biol.* 175:17-24.

472. Zhou, L., E.M. Kasperek, and B.J. Nicholson. 1999. Dissection of the molecular basis of pp60(v-src) induced gating of connexin 43 gap junction channels. *J Cell Biol.* 144:1033-45.

473. Zhou, X.W., A. Pfahnl, R. Werner, A. Hudder, A. Llanes, A. Luebke, and G. Dahl. 1997. Identification of a pore lining segment in gap junction hemichannels. *Biophys J.* 72:1946-53.

474. Zhu, D., S. Caveney, G.M. Kidder, and C.C. Naus. 1991. Transfection of C6 glioma cells with connexin 43 cDNA: analysis of expression, intercellular coupling, and cell proliferation. *Proc Natl Acad Sci U S A.* 88:1883-7.

475. Zhu, D., G.M. Kidder, S. Caveney, and C.C. Naus. 1992. Growth retardation in glioma cells cocultured with cells overexpressing a gap junction protein. *Proc Natl Acad Sci U S A.* 89:10218-21.

476. Zhu, W., N. Mironov, and H. Yamasaki. 1997. Increased genetic stability of HeLa cells after connexin 43 gene transfection. *Cancer Res.* 57:2148-50.

477. Zuppinger, C., M.C. Schaub, and H.M. Eppenberger. 2000. Dynamics of early contact formation in cultured adult rat cardiomyocytes studied by N-cadherin fused to green fluorescent protein. *J Mol Cell Cardiol.* 32:539-55.