Effects of Mechanical Forces and Mediators of Hypertrophy on Remodeling of Gap Junctions in the Heart

Jeffrey E. Saffitz and André G. Kléber

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Role of the Integrins in Endothelial Mechanosensing of Shear Stress
Dance Band on the *Titanic*: Biomechanical Signaling in Cardiac Hypertrophy
Spatial Microstimuli in Endothelial Mechanosignaling

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Peter F. Davies, Guest Editor

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Abstract—This review article focuses on remodeling of gap junctions in response to chemical mediators of ventricular hypertrophy, mechanical forces, and alterations in cell-to-cell adhesion. Signaling mediated by mechanical forces is likely to be involved in the upregulation of cardiac gap junctions during the early phase of cardiac hypertrophy and the subsequent downregulation in cardiac failure. Several signaling pathways involving cAMP, angiotensin II, transforming growth factor-β, vascular endothelial growth factor, and integrin-mediated regulators have been shown to affect expression of gap junction proteins. However, a comprehensive view of regulation of gap junction trafficking, synthesis, and degradation is still lacking. In addition to gap junction regulation by extracellular mechanical forces, there is a close relation between gap junctions and adhesion junctions and their linkage to the cytoskeleton. This can be inferred from experiments on neoformation of cell-to-cell coupling, concomitant upregulation of adherens and gap junctions after mechanical stretch, and human cardiomyopathies caused by genetic defects in cell-cell adhesion junction proteins. The molecular mechanisms responsible for the interaction between mechanical and functional cell-to-cell coupling remain to be elucidated. **(*Circ Res*. 2004;94:585-591.)**

Key Words: gap junctions ■ adhesion junctions ■ mechanical signaling ■ remodeling ■ cardiac hypertrophy and failure

Connexin proteins form intercellular pores in many tissues, such as myocardium, vascular endothelium, and brain.[1] Twenty distinct connexin genes have been identified in the human genome. Three types of connexins, connexin43 (Cx43), Cx40, and Cx45, are expressed in heart.[1] Cx43 is abundant in atrial and ventricular myocardium.[2,3] Cx40 is expressed in atrial tissue and in the atrioventricular conducting system. Cx45 is observed in the sinoatrial and atrioventricular nodes, and small amounts colocalize with Cx43 in adult ventricular myocardium.[4]

Six connexin proteins oligomerize to form a hemichannel (connexon) that migrates to the cell surface membrane and becomes incorporated at the periphery of existing junctional plaques,[5,6] where it combines with a corresponding hemichannel in an adjacent cell to form a complete gap junction channel. Colocalization of different connexin proteins in gap junction plaques, observed immunohistochemically,[7–9] probably reflects formation of heterotypic or heteromeric gap junction channels, as shown in expression systems (oocytes or immortalized cell lines).[10–12]

A major role of gap junctions in the myocardium is to enable rapid and coordinated electrical excitation, a prerequisite for normal rhythmic cardiac function, and probably also to facilitate intercellular exchange of small molecules, such as regulatory proteins. Because diffusion of molecules across gap junctions is possible up to a molecular weight of ≈1000

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From the Center for Cardiovascular Research and the Department of Pathology (J.E.S.), Washington University, St Louis, Mo, and Department of Physiology (A.G.K.), University of Bern, Bern, Switzerland.
Correspondence to André G. Kléber, MD, Department of Physiology, University of Bern, Bühlplatz 5, CH-3012 Bern, Switzerland. E-mail kleber@pyl.unibe.ch

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Da,[13] an important role of gap junctions may involve the intercellular exchange of small regulatory molecules and metabolites.

Over the past years, many of the molecular and biophysical properties of gap junction channels have been described. For example, it has been shown that gap junction proteins are dynamic molecules that turn over rapidly and respond to a variety of regulatory processes. This is astonishing in the light of the fact that a steady state of functional cell-to-cell communication is necessary to assure normal cardiac function. This review focuses on remodeling of gap junctions in response to chemical mediators of ventricular hypertrophy, mechanical forces, and alterations in cell-to-cell adhesion. Changes in expression of functional gap junctions in myocardial hypertrophy and failure may contribute to ventricular arrhythmias and atrial fibrillation often observed in these diseases.

Mediators of Cardiac Hypertrophy and Failure and Mechanical Forces Regulate Gap Junctional Conductance

Regulatory Sites on Connexin Proteins, Connexin Turnover, and Regulation

Regulation of cell-to-cell communication by gap junctions involves either (1) changes that affect the conductance states of a connexin protein or (2) processes that affect the amount of connexin in the junctional plaque through changes in trafficking, synthesis, or degradation. Connexins are composed of four membrane-spanning domains, two extracellular loops, and three intracellular domains, including an intracellular loop that connects the second and third transmembrane domains, and the C- and N-terminal tails. Among the connexins, the C-terminus is the most variable domain in terms of length and amino acid composition. This component contains regulatory sites that confer specificity to channels composed of individual connexins. Changes in phosphorylation in the C-terminus have been associated with connexin assembly into gap junctions, channel closure, disassembly, and degradation.[14,15] The N-terminal domain is important for voltage gating. Specific substitution of amino acids in the N-terminus has been shown to alter the dependence of the gap junctional conductance on the voltage across the junction.[16,17] In contrast to the C-terminal domain, the intracellular N-terminal region contains only two serine residues, and no role for phosphorylation of these residues has been reported.[15]

Importantly, several phosphorylation sites are sensitive to several molecules involved in signaling cascades, although connexin phosphorylation in its totality and integrity has not been fully elucidated. Cx43, the most abundant connexin protein in heart, is phosphorylated primarily at serine residues.[14,15] Enzymes that phosphorylate connexins at serine residues include mitogen-activated protein kinases (MAPKs),[18] protein kinase C,[19] protein kinase A,[20] and casein kinase. Tyrosine phosphorylation occurs in cells that express activated tyrosine kinases and usually causes a decrease in junctional conductance.[21–24] The consequences of connexin phosphorylation for cell-to-cell communication are complex and involve assembly and disassembly of gap junctions in the junctional plaque, connexin channel gating, and, probably, degradation.[14,15]

The importance of these processes is underlined by the observation that the trafficking of gap junctions is very rapid, with half-lives in the plaque of 1 hour up to a few hours.[25–28] New connexons assembled in the endoplasmic reticulum and the Golgi travel in vesicles to the plasma membrane, where they are added to the periphery of existing junctional plaques. Steady state is maintained by the simultaneous removal of connexons from the center of the plaque[5] to be degraded via both lysosomal and proteasomal pathways.[27,28]

Although multiple studies have defined the role of phosphorylation in gap junction channel function and connexin turnover, relatively little is known about transcriptional regulation of connexin expression. In early cardiac development, the Cx40 gene, among others, is regulated by the homeodomain transcription factor Nkx2.5.[29] Recently, overexpression of Nkx2.5 in adult cardiomyocytes has been shown to reduce Cx43 expression,[30] a change that may be responsible for atrioventricular nodal conduction disturbances and bradycardia observed in these pheotypes.[31] Ai et al[32] have shown that Cx43 expression is induced by Wnt1 signaling and that this is mediated transcriptionally, possibly through pathways regulated by β-catenin. However, the exact role of transcriptional regulation of connexin expression in adult cardiomyocytes is not clearly defined.[33]

Mediators of Hypertrophy and Failure Change Gap Junction Expression

The cardiac hypertrophic response is a dynamic continuum in which progressive changes in gene expression first create adaptive structural and functional changes but eventually can lead to increasingly maladaptive changes, culminating in heart failure. Ventricular conduction delay, often reflected by prolongation of the QRS interval in the surface ECG, is a general feature of chronic left ventricular hypertrophy in humans. Electrical propagation velocity first increases in hypertrophied ventricles but then decreases as hypertrophy becomes more severe.[34–36] Decrements in conduction velocity and conduction block may be related to discontinuities in extracellular resistance caused by interstitial fibrosis[37–39] and an increase in intercellular resistance attributable to decreased connexin expression.[40–42] It has been demonstrated immunohistochemically that ventricular Cx43 expression is reduced in patients with chronic ischemic heart disease.[43] These results and others suggest that reduced gap junction channel protein levels occur as a general rule in chronic myocardial disease states, such as healed myocardial infarction,[40–42] chronic hibernation,[43] and end-stage aortic stenosis.[44] The signaling mechanisms leading to connexin downregulation in hypertrophy and failure have not yet been fully defined. An important role in connexin downregulation in cardiac failure may be played by stress activation of c-Jun–activated N-terminal kinase (JNK). Indeed, a rapid and massive decrease of Cx43 expression (up to $\approx$90%) was consistently observed with activation of JNK by either exposure of cultured cells to anisomycin, transfection of cultured cells with a specific JNK activator, or targeted activation of JNK in vivo.[45]

It is difficult to predict the functional consequences of cell-to-cell uncoupling in the setting of chronic heart failure. Cardiac arrhythmogenesis ultimately results from slowing of electrical propagation velocity and from the development of discontinuous conduction or unidirectional block. With respect to the first change, cell-to-cell uncoupling by down-regulation of connexins slows propagation velocity and therefore increases the propensity to arrhythmias. However, cell-to-cell uncoupling can also attenuate the formation of unidirectional block and therefore decrease the likelihood of arrhythmia initiation.[46] Results from genetically engineered mouse models are inconclusive. It is not yet possible to establish a straightforward relationship between a change in gap junction expression produced by genetic manipulation and arrhythmogenesis. For instance, heterozygous deletion of Cx43, the main gap junction protein in ventricle, produces only a moderate or very small insignificant decrease in conduction velocity.[47–49] Yet heterozygous Cx43 deletion increases ventricular arrhythmias after induction of acute regional myocardial ischemia.[50] Mice with conditional deletion of Cx43 die in the first weeks after birth from ventricular arrhythmias,[51] and the incidence of arrhythmias is increased if the reduced gap junction expression is spatially heterogeneous (chimeric mice[51]). This may indicate that arrhythmogenesis is related to a decrease or heterogeneity in gap junction expression.

In contrast to the situation seen in end-stage heart disease, results of studies in vitro and in vivo suggest that compensatory hypertrophic growth may be associated with increased connexin levels, increased number of gap junctions, and enhanced intercellular coupling. For example, long-term exposure (24 hours) of cultured neonatal rat ventricular myocyte to a membrane-permeable form of cAMP increases the tissue content of Cx43 by ≈2-fold and increases the number of gap junctions interconnecting cells.[52] These changes are associated with a significant increase in conduction velocity without apparent changes in active membrane properties.[52] Similarly, cultured neonatal rat ventricular myocytes exposed for 24 hours to angiotensin II (Ang II) also exhibit a 2-fold increase in Cx43 content and an increase in the number of gap junctions interconnecting cells.[53] As described in more detail in the next section, chemical mediators such as Ang II and vascular endothelial growth factor (VEGF) have been shown to play a role in stretch-induced upregulation of Cx43. These observations suggest that chemical signals may function by autocrine or paracrine mechanisms to regulate intercellular coupling in response to mechanical forces.

Responses of Cultured Myocytes to Mechanical Load In Vitro

Numerous studies have characterized responses of cardiac myocytes to mechanical load in vitro. The earliest studies demonstrated that brief intervals of static stretch of neonatal rat myocytes induced features of the hypertrophic response, including increases in protooncogene and contractile protein expression and activation of signal transduction pathways, including those mediated by MAPKs, tyrosine kinases, protein kinase C, and phospholipases C and D.[54–57] More recent experiments in which myocytes have been subjected to pulsatile stretch have also demonstrated activation of numerous signal transduction pathways, including all three members of the MAPK family, focal adhesion kinase (FAK), and the JAK/STAP pathway.[58,59] Mechanical stretch of cultured neonatal rat ventricles induces release of growth-promoting factors, including Ang II, endothelin-1, VEGF, and transforming growth factor-β (TGF-β).[54,60–63] For example, Shyu et al[60] reported a 3-fold increase in Ang II in the culture media of rat neonatal myocytes stretched for 1 hour. Seko et al[61] demonstrated that 5 minutes of pulsatile stretch is sufficient to induce rapid secretion of VEGF and increased expression of both VEGF and VEGF receptor mRNA in cultured cardiac myocytes.

Using a custom-designed and -fabricated in vitro system that produces uniform, unidirectional pulsatile stretch, we have shown that stretching monolayers of neonatal rat ventricular myocytes to 110% of resting cell length at a frequency of 3 Hz caused dramatic upregulation of Cx43 after only 1 hour.[64] An additional increase occurred after 6 hours of stretch (Cx43 signal measured by confocal microscopy increased from 0.73 to 1.86 and 2.02% cell area after 1 and 6 hours, respectively). This was paralleled by significant increases in conduction velocity from 27 to 35 cm/sec after 1 hour and to 37 cm/sec after 6 hours.[64] No significant changes in action potential upstroke velocity or cell size were observed, suggesting that more rapid conduction velocity was related mainly, if not entirely, to enhanced electrical coupling.[64]

To gain insights into chemical signaling mechanisms regulating stretch-induced upregulation of Cx43, we tested the hypothesis that VEGF and TGF-β, both of which are known to be synthesized and secreted by cardiac myocytes,[61] are released in response to pulsatile stretch and stimulate Cx43 expression in cardiac myocytes. We showed, for example, that addition of either exogenous TGF-β (10 ng/mL) or VEGF (100 ng/mL) to unstretched neonatal rat ventricular myocytes for 1 hour increased Cx43 expression by ≈1.8-fold,[65] comparable with that observed in cells subjected to pulsatile stretch for 1 hour.[65] We also observed that stretch-induced upregulation of Cx43 expression could be blocked by either anti-VEGF or anti–TGF-β antibodies.[65] Stretch-induced enhancement of conduction was also blocked by anti-VEGF antibodies. In additional studies, we showed that VEGF was secreted into the culture medium during stretch.[65] Furthermore, stretch-conditioned medium (recovered from cultures stretched for 1 hour) was able to stimulate Cx43 expression when added to nonstretched cells, and this effect was also blocked by the addition of anti-VEGF antibody.[65] Upregulation of Cx43 expression stimulated by exogenous TGF-β was blocked by anti-VEGF antibody, but VEGF stimulation of Cx43 expression was not blocked by anti-TGF-β antibody. These results show that early (within 1 hour) stretch-induced upregulation of Cx43 expression is mediated, at least in part, by VEGF, which acts downstream of TGF-β. In similar studies on Ang II, Shyu et al[60] showed that upregulation of Cx43 after several hours of stretch could be blocked by addition of the AT_1 antagonist losartan. These results suggest that multiple chemical signals, released from cells in response to stretch, may mediate upregulation of

Cx43 and act during different intervals of mechanical stimulation.

Integrin Signaling and Its Potential Role in Stretch-Activated Upregulation of Cx43

It is likely that stretch-activated changes in cardiac myocyte structure and function are mediated by signaling pathways initiated by interactions between integrins and extracellular matrix proteins. Indeed, overexpression of β_1 integrins, by itself, can induce a hypertrophic response in neonatal rat ventricular myocytes in vitro and enhance the effects of α_1 adrenergic stimulation.[66] Inhibition of β_1 integrin function and signaling reduces the hypertrophic response.[66] FAK, a primary mediator of integrin signaling, may also play a role in the hypertrophic and adhesive responses of neonatal rat ventricular myocytes in culture.[67–70] FAK can also be activated by VEGF[67] and recently has been shown to translocate to costameres in cardiac myocytes subjected to stretch.[71] Future research will likely elucidate the complete signaling pathway by which mechanical stimulation of cardiac myocytes leads to altered cell-cell communication at gap junctions.

Dependence of Gap Junction Expression on Cell-to-Cell Adhesion

Cell-to-cell adhesion plays an important role in tissue function.[72] Mechanical junctions between cells are composed of discrete clusters of adhesion molecules that span the membranes of adjacent cells and interact in the extracellular space. Intracellular domains of these adhesion molecules are connected via linker proteins to components of the cytoskeleton to create a continuous network that connects intercellular adhesions junctions across cells. The principal adhesions molecules localized at intercalated disks of cardiac myocytes are the N-cadherins, which form fascia adherens junctions, and the desmosomal cadherins, which make desmosomes. The major linker proteins include members of the catenin and plakin families. In fascia adherens junctions of cardiac myocytes, N-cadherins are linked to actin in sarcomeres by both β-catenin and γ-catenin (plakoglobin). In desmosomes, the desmosomal cadherins desmoglein and desmocollin are linked to intermediate filaments of the myocyte cytoskeleton (composed of desmin) mainly by desmoplakin and plakoglobin.[72]

Several studies in cardiac and noncardiac tissue suggest a close relationship between the number of channels clustered in the gap junction plaque and the function and integrity of adhesion junctions. Epidermal tumors of CA3/7 carcinoma cells, for instance, have fewer gap and adherens junctions than normal mouse epidermal cells of the same type (3PC).[73] Tumor promoters such as phorbol esters and benzoyl peroxide concomitantly diminish connexin and E-cadherin expression in both 3PC and CA3/7 cells. This is consistent with the notion that diminished cell-to-cell adhesion and communication are associated with dysregulation of cell growth.[73]

In the heart, evidence for a close interaction between regulation of cell-to-cell adhesion and functional cell-to-cell coupling can be derived from multiple lines of evidence, including (1) the distinct morphological association between the two types of junctions, (2) the temporal sequence of neoformation of cell-to-cell junctions during experimental cell apposition, and (3) experiments involving mechanical stretch of cardiac myocytes. A close morphological relationship between gap junction plaques and fascia adherens junctions has been observed in a 3D analysis of cell-to-cell coupling in dog ventricular myocardium.[74,75] In the terminal intercalated disks located at the ends of ventricular myocytes, large ribbon-like gap junctions oriented perpendicularly to the long cell axis alternate with interdigitated fascia adherens junctions. This morphological picture[74] is generally interpreted as reflecting mechanical protection of the gap junction plaque (with its high density of clustered channels) against contraction by the adherens junctions.[75] The temporal relationship between neoformation of adherens junctions and gap junctions has been studied by several investigators[76–79] in cell culture models in which dissociated adult rat ventricular myocytes reestablished contact. Shortly after cell seeding, freshly disaggregated myocytes dedifferentiated and membrane regions containing the former intercalated disks became smooth and unstructured. Reformation of intercalated disks with increasing age in culture (culture days 3 to 4) was characterized by initial formation of intercellular fibrillar structures and subsequent appearance of subsarcolemmal plaques. Early intercalated disks consisted of these nascent adhesion junctions clustered in zipper-like arrangements. Immunohistochemical analysis at this early stage showed positive signal for N-cadherin, β-catenin, and plakoglobin but only very minor amounts of connexin. Gap junctions containing Cx43 became evident only once complete adherens junctions had formed, and in all cases, the new gap junctions were immediately adjacent to the adherens junctions (culture days 6 through 12). The authors concluded that the formation of adherens junctions was a prerequisite for subsequent gap junction formation. Several studies have implicated an important role for the zonula occludens-1 protein (ZO-1) and β-catenin in assembly or maintenance of gap junctions and in regulating connexin expression. Toyofuku et al[80] demonstrated a direct association of the C-terminal domain of Cx43 with the N-terminal domain of ZO-1 in cardiac myocytes. Ai et al[32] used immunohistochemical and biochemical approaches to show that Cx43 and β-catenin colocalize in cardiac myocytes and that Cx43–β-catenin complexes could be immunoprecipitated from Triton X-100–soluble lysates. More recently, Wu et al[81] showed that when neonatal rat cardiac myocytes are cultured under low Ca^{2+} conditions, immunoreactive signals for α-catenin, β-catenin, ZO-1, and Cx43 occur intracellularly, but when cells are transferred to physiological Ca^{2+} conditions, signals for α-catenin, β-catenin, and ZO-1 redistribute to the cell surface membrane within 10 minutes at sites of cell-cell contact. However, only after these proteins accumulate at apparent junctions does Cx43 signal occur at the cell surface. The role of scaffolding proteins is an emerging subject of great interest not only regarding gap junction channels but other types of ion channels as well.

A relationship between cell-cell adhesion junctions and gap junctions has been suggested in experiments involving remodeling of cell-to-cell contacts induced by pulsatile

stretch. If neonatal rat ventricular myocytes, grown on a collagen substrate, are exposed to short periods of pulsatile stretch,[64] there is not only rapid and highly significant upregulation of connexin proteins (see the previous section) but also concomitant upregulation of N-cadherin,[64,62] plakoglobin, and desmoplakin[82] at sites of intercellular junctions.

A new and fascinating aspect of the relationship between adhesion junctions and gap junctions has become evident from the analysis of familial cardiomyopathies caused by mutations in plakoglobin and desmoplakin, molecules responsible for linking adhesion junctions to the cytoskeleton. Naxos disease, which is caused by a recessive mutation in plakoglobin,[83] is a cardiocutaneous syndrome that includes woolly hair, palmoplantar keratoderma, and arrhythmogenic right ventricular cardiomyopathy.[84] At the level of the heart, the disease is characterized by progressive loss of right ventricular working myocardium, with replacement by fat and connective tissue. Its clinical manifestations involve life-threatening ventricular arrhythmias and frequent sudden cardiac death.[85,86] The histopathological findings in the right ventricle predominate, but involvement of the left ventricle has also been reported. The mutation in Naxos disease is a deletion of nucleotides 2157 and 2158 in plakoglobin.[83] This defect results in a premature termination of translation and truncates the C-terminus of the plakoglobin protein by 56 amino acids.[83] Most likely this defect interferes with the linkage between the intercellular adhesion molecules and the cytoskeleton. The extent to which this potential defect in mechanical cell-to-cell coupling may lead to altered regulation of connexin expression and resulting arrhythmias has not yet been elucidated. However, in another cardiocutaneous syndrome, caused by a mutation in desmoplakin, such a relationship has been suggested by a recent case report. Carvajal syndrome, described in 1998 by Dr Luis Carvajal-Huerta,[87] includes woolly hair, palmoplantar keratoderma, and dilated cardiomyopathy. The cardiomyopathy presents with an enlarged and poorly contracting left ventricle in the radiograph and echocardiography. Electrocardiographic signs include peripheral low voltage, disturbances of the QRS complex, polymorphic ventricular premature beats, and runs of ventricular tachycardia.[88] In contrast to Naxos disease, the pathoanatomical findings are more generally distributed and not confined to the RV. Carvajal syndrome is caused by a recessive mutation in the gene encoding desmoplakin.[89] The mutation consists of single nucleotide deletion leading to a premature stop codon and a truncation of the tail of the protein.[89] Immunohistochemistry of palm skin from patients with Carvajal syndrome has revealed abnormal distribution of desmoplakin.[89] A recent analysis of the pathology of Carvajal syndrome reported diminished expression of desmoplakin, plakoglobin, and Cx43 at intercalated disks.[90] These observations suggest that abnormal protein-protein interactions at intercellular junctions may contribute to both contractile and electrical dysfunction in Carvajal syndrome. Although several studies have been published on remodeling of cytoskeletal and adherens proteins per se in the setting of heart failure, no reports on the interaction between adherens junctions and gap junctions remodeling are available as yet.[91]

In summary, experimental research during recent years has provided evidence for a high degree of plasticity characterizing cell-to-cell communication. Signaling mediated by mechanical forces is likely responsible for upregulation of cardiac gap junctions during the early phase of cardiac hypertrophy and, perhaps, the subsequent downregulation. Several signaling pathways involving cAMP, angiotensin II, TGF-β, VEGF, and integrin-mediated regulators have shown to affect expression of gap junction proteins. The list of substances involved in gap junction regulation is likely to get longer, and an integrative picture of the complex interactions between the various regulatory pathways is still lacking.

Several experimental studies as well as data obtained in patients with genetic defects suggest a close relation between cell-cell adhesion junctions and gap junctions, not only as described many years ago, in relation to subcellular topology, but also with respect to common regulatory pathways. The molecular mechanisms responsible for the interaction between mechanical and functional cell-to-cell coupling remain to be elucidated.

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