Cardiac gap junction channels: modulation of expression and channel properties

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Abstract

In the heart, intercellular gap junction channels constructed from connexin molecules are crucial for conduction of the electric impulse. Cardiomyocytes can be interconnected by channels composed of three types of connexin proteins: Cx40, Cx43 or Cx45. In mammalian hearts, these three isoforms are regionally differently expressed and even between the species differences exist. Each of these channel-types possesses specific properties and are susceptible to modulation by various mechanisms. In this paper we compare the differences in properties of these channels as deduced from studies on transfected cells and isolated cardiomyocytes and discuss the factors involved in modulation of channel properties. Next, we evaluate the consequences of alterations in expression and modulation of channel properties for cardiac function. Therefore, we have compared reports on genetically engineered animals and discuss this information in relation to various pathophysiological disorders. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Normal growth, development and appropriate functioning of many tissues and organs depend on maintenance and regulation of intercellular communication. This can be mediated by extracellular neurohumoral factors, but most mammalian cells also directly connect by gap junctions. Gap junctions are agglomerates (plaques) of multiple intercellular channels which connect the cytoplasm of adjacent cells. Thereby they provide both electrical and metabolic coupling. Electrical coupling fulfills a key role in excitable tissues (e.g. smooth and cardiac muscle) where propagation of the electric impulse is mediated by passage of ions through gap junction channels. Metabolic coupling, i.e. the transport of small metabolites, nucleotides and second messengers with a molecular weights up to 1.2 kD [1–3], might also play an important role in differentiated tissues (e.g. in electrical synapses of neurons) but certainly has a critical function in embryo- and organogenesis.

As depicted in Fig. 1A, each gap junction channel is composed of 12 connexin (Cx) molecules, assembled from two hexameric hemichannels (connexons), one of each delivered by both participating cells. The individual connexins form a multigene family of highly related but not identical transmembrane proteins (reviewed in [4]). In mammals, at least 15 members, named after their theoretical molecular mass (in kD), have been cloned to date (for review see [4–7]). The overall gene and protein structure is largely conserved among the various isoforms. Fig. 1B shows that all connexin proteins consist of four transmembrane segments (M1–M4), two extracellular loops (E1, E2), one intracellular loop (CL) and a cytoplasmic amino and carboxy terminus (for review see [4]). Strong

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conservation of the amino acid sequence is mainly found in both extracellular loops, the amino terminus and the four transmembrane segments. Three of these transmembrane segments (1, 2 and 4) consist predominantly of hydrophobic amino acids while the third segment has a more amphipathic character suggestive of a role in the inner lining of the aqueous pore [8]. The two extracellular loops (E1 and E2) each contain three conserved cysteines that likely participate in the process of docking of the two hemichannels [9–11]. Disulfide bonds in between the cysteines within E1 and E2 but also crossing the space between E1 and E2 create the β-sheet conformation required for interaction between the two opposing connexons (see [4]). After translation of the mRNA, connexins are inserted in the membranes of the endoplasmic reticulum [12], followed by oligomerization into connexons in the Golgi complex [13]. Once in the sarcolemma, a functional channel is created by head-to-head docking of hemichannels at spots where both plasma membranes are in close apposition, thereby creating an aqueous pore between both cells.

When both connexons are constructed from identical connexins (Fig. 1C), a homotypic channel is formed. The degree of conservation (among different connexin isoforms) of the second extracellular loop [10], provides the opportunity for connexons built from one particular type of connexin (homomeric), to dock with a connexon composed of another isoform thereby creating a heterotypic channel. Additionally, even within one hemichannel, different connexins can be integrated (heteromeric connexon) which can result in both homotypic- and heterotypic channel formation.

The biochemical and electrical characteristics of gap junction channels depend on their constituent connexins. Moreover, the properties of gap junction channels can be modulated by various mechanisms like (de)phosphorylation of the individual connexin proteins, the transjunctional voltage gradient, intracellular hydrogen and free calcium concentration, and extracellular fatty acid composition. Alterations in the amount of channels can be induced by changes in expression level of the connexin proteins, turnover rate, and distribution (presumably affected by the phosphorylation state of the channels [14]). In this review we will focus on modulating factors affecting expression and characteristics of gap junctions in the mammalian heart.
2. Expression of gap junction proteins in the heart

Propagation of the action potential from cell to cell is mediated by current flow through gap junction channels, primarily located in the intercalated disc (ID) at the end-to-end intercellular connections. Large and small IDs are found parallel to the long axis of the cell (interplicate) while only small IDs can be found in regions at right angles to the long axis (plicate) [15].

In mammalian hearts, cardiomyocytes most prominently express gap junctions built of connexin40 (Cx40), connexin43 (Cx43) and connexin45 (Cx45). Therefore, we will restrict this review to these three isoforms. Another three isoforms have been reported; in several species, connexin37 is expressed in the endothelial cells of the endocardium, aorta and coronary vessels [16–18] where it is co-expressed with Cx40 [19]. Connexin46 protein has in addition to other isoforms been demonstrated in the rabbit sino-atrial node [20]. Connexin50 protein has been detected in the atrioventricular valves of rat heart [21].

The expression patterns of Cx40, Cx43 and Cx45 are subject to remarkable spatio-temporal differences [17,22–24]. In adult hearts Cx43 is the major expressed isoform. Fig. 2 depicts the generalized adult expression patterns of Cx40, Cx43 and Cx45. In mammalian hearts, Cx43 is expressed in virtually all myocytes of the working atrial- and ventricular mammalian myocardium regardless the stage of development. No Cx43 is found in the nodal cells of the SA and AV node. In the ventricular conduction system of mouse and rat, Cx43 is absent in the common His-bundle and proximal parts of both bundle branches, but it is expressed in more distal parts of the bundle branches. In larger species as cow, dog, pig and human, Cx43 is expressed in all parts of the ventricular conduction system.

Cx45 is expressed in the rabbit SA node [25]. In rat and mouse hearts, Cx45 is found in the AV node and complete ventricular conduction system but only at very low levels in the surrounding working myocardium of atria and ventricles [26,27].

The third connexin, Cx40, is strongly co-expressed with Cx43 in the working myocardium of the atria (except in rat). During fetal and neonatal development, Cx40 is still present in the ventricles where the intensity of expression gradually declines from endo- to epicard [28]. However, within days of birth, expression levels decrease and in adult stages Cx40 is absent in the ventricular working myocardium. Cx40 remains strongly expressed in the ventricular conduction system where it colocalizes with Cx45. Finally, low levels of Cx40 are present in the SA node and AV node of dog and cow [29], and the rabbit SA node [20,25]. Differences in expression patterns between the hearts of different species have been described in detail by Gros and Jongsma [30] and quoted references.

3. Modulation of gap junction channels built of Cx40, Cx43 and Cx45 in a reduced cell system

Cardiac gap junction channels have been intensively studied in cultured cardiac cardiomyocytes. Although ventricular cardiomyocytes probably exclusively express Cx43, other cardiomyocytes can express more than one type of connexin. The expression of more than one connexin isoform might give rise to heterotypic/heteromeric channel formation, thereby creating a plethora of different channel types.

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**Fig. 2. Generalized expression pattern of Cx40, Cx43 and Cx45 in the different regions of the mammalian heart.** SAN, sinoatrial node; AVN, atrioventricular node; AVB, atrioventricular bundle or His-bundle; BB, bundle branches; PF, Purkinje fibers.
with a high level of complexity. Appropriate assessment of the electrical properties of the individual gap junction isoforms requires a simplified cell system expressing only one isoform.

The genes encoding for the different cardiac connexins have been characterized, cloned and introduced in non-communicating tumor cells or Xenopus oocytes in order to create a reduced cell system overexpressing (exogenous) gap junction channels built of one type of connexin only. Such a simplified system allows for evaluation of single channel properties and some of the modulating factors affecting these properties. We will focus our attention on the genes cloned from humans, mice and rats.

The isoform- but also the species-specific characteristics are particularly determined by variations in the amino acid sequence of the cytoplasmic loop (between transmembrane segments 2 and 3) and the C-terminus where multiple amino acid motifs are found to be susceptible to phosphorylation by different intracellular protein kinases [6,31–33]. Phosphorylation occurs predominantly on serine residues, but also on threonine and tyrosine residues. Interestingly, cyclic nucleotides which activate protein kinases, are also able (in nanomolar concentrations) to modulate channel properties directly [34]. Together with more conserved domains of the protein, the cytoplasmic loop and carboxy-terminus might be involved in different modes of regulation. The C terminus, N terminus, first transmembrane segment, first extracellular loop and a conserved proline residue in the second transmembrane segment, are reported to participate in the voltage gating properties of the channels [35,36], possibly similar to pH gating, according to a ball-and-chain model [37]. In addition, voltage gating might be influenced by the interaction of a connexon with its opposing connexon [38]. Protonation of three conserved histidine residues in the cytoplasmic loop might be required in the process of pH gating [39–41]. In Cx43 channels, these histidines may serve as a receptor for as-yet unidentified parts of the carboxy-terminus in order to facilitate pH gating according to the ball-and-chain model [42,43]. A similar model was postulated for Cx40 but not for Cx45 [44]. A cooperative interaction has been described for Cx40 and Cx43 which increases the pH sensitivity of those channels [45]. Within the physiologically relevant range of intracellular $Ca^{2+}$ and $H^+$ concentrations, it is unlikely that $Ca^{2+}$ or $H^+$ independently strongly affect the conductive properties of Cx43 channels [41,46]. Yet, there might be a (not completely understood) synergistic mechanism between $Ca^{2+}$ and $H^+$ that potentiates their effect on Cx43 channels and could be of importance under conditions of cardiac ischemia when intracellular $Ca^{2+}$ and $H^+$ are elevated [47,48].

Intercellular coupling concerns macroscopic (electrical) conductance ($g_e$) and metabolic coupling. Both are determined by the number of expressed channels ($N$), the single channel conductance ($\gamma$) or permeability and the open probability of a single channel ($P_o$). Single channel properties can be affected by modulation of $\gamma$ but also by changes in $P_o$ of the channels. Electrical coupling of cells can be studied using the double-voltage-clamp technique [49,50]. Metabolic coupling is evaluated by the passage of small fluorescent (dye) metabolites [1]. Most commonly used tracers are Lucifer yellow (LY, 443 Da), 2',7'-dichlorofluorescein (DCF, 401 Da) and 6-carboxyfluorescein (6CF, 376 Da). The following sections summarize the characteristics of homotypic channels built of Cx40, Cx43 and Cx45 as deduced from studies on transfected cells (see also Table 1).

### 3.1. Cx40 channel properties

The Cx40 gene has been characterized and cloned from several species (see [61]). The coding region exists of 358 amino acids in human Cx40 (hCx40) and mouse Cx40 (mCx40) and of 356 in rat Cx40 (rCx40). On Western blot, the protein can be separated in two bands of 40- and 42 kD (mCx40) and of 356 in rat Cx40 (rCx40). On Western blot, the protein can be separated in two bands of 40- and 42 kD (mCx40). The following sections summarize the characteristics of homotypic channels built of Cx40, Cx43 and Cx45 as deduced from studies on transfected cells (see also Table 1).

### Table 1

General properties of Cx40, Cx43 and Cx45 from human, mouse and rat

<table>
<thead>
<tr>
<th>Cx40</th>
<th>Cx43</th>
<th>Cx45</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td>Rat</td>
<td>Mouse</td>
</tr>
<tr>
<td>Coding region (aa)</td>
<td>358</td>
<td>356</td>
</tr>
<tr>
<td>Motifs for PKC</td>
<td>7</td>
<td>12</td>
</tr>
<tr>
<td>PKA</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>PKG</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>MAP kinase</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>$V_g$</td>
<td>N.D.</td>
<td>+/− 50 mV [54]</td>
</tr>
</tbody>
</table>

aa, Amino acids; pS, pico-Siemens; for others see abbreviation list.
Cx40 channels are mildly sensitive to the transjunctional voltage gradient (Vj) with a half-maximal inactivation at +70 mV [53,54]. Depending on the composition of the pipette solution [55], single channel recordings in transfected cell-lines show a variety of large conductance states of 120–200 pS and less frequently, a much smaller substate of –30–40 pS [51,52,54,56]. Although rCx40 transfected in N2A cells appears cation selective, this was not found for mCx40 [54,57]. Both hCx40 and mCx40 are permeant to LY [9,52], while rCx40 is permeant to DCF and 6CF [54]. Sequence analysis of the cytoplasmic loop and the carboxy-terminus has revealed multiple consensus motifs for phosphorylation by several intracellular protein kinases [31,32]. Western analysis showed that Cx43 indeed exists in a phosphorylated and non-phosphorylated configuration [51,52]. The sequences for hCx40, rCx40 and mCx40 differ significantly. The hCx40 protein contains seven putative sites for modulation by protein kinase C (PKC), but rCx40 and mCx40 contain 12 and 15 sites, respectively. The number of putative sites for modulation by PKA and PKG are two and one in human but one and three in rat and mouse. Consensus sites were not found for modulation by MAP-kinase in the three species.

In Hela cells transfected with mCx40, direct activation of both PKC and PKA increased phosphorylation of Cx40 (measured by incorporation of 32P), but did not alter electrophoretic mobility of the 40 kD native protein on Western blot [51]. In contrast, in hCx40 transfected cells, a shift from 40 to 42 kD was found after stimulation of PKA [52]. In addition, γ1 and dye permeability increased significantly [52]. Up till now, no modulatory role for activated PKG has been reported.

3.2. Cx43 channel properties

Characteristics of Cx43 channels, the main cardiac connexin, have been studied extensively (see [6]). The coding region exists of 382 amino acids in human, mouse and rat. Like Cx40 channels, Cx43 channels are not very sensitive to Vj. Conductance starts to decrease when ΔV exceeds +70 mV and half-maximal inactivation occurs at +70–60 mV [58]. Cx43 channels are not ion selective [57]. rCx43 and hCx43 channels exhibit three γ1 values: a major state at 40–60 pS, and two states of minor occurrence of 20–30 pS and 70–100 pS. In mCx43 only the two largest are detected [51,59,60]. Dye permeability has been shown for LY [9,51,61,62]. hCx43 contains ten putative phosphorylation sites [31,32] for PKC, four for PKA, three for PKG and two for MAP kinase. In rCx43 and mCx43, 14 sites for PKC, three for PKA, four for PKG and three for MAP kinase can be identified (see Table 1). In most studies, the protein presents on Western blot as three bands with molecular weights ranging from 41 to 46 kD. They represent the non-phosphorylated state (NP), a phosphorylated state (P1) and a highly phosphorylated state (P2) of gradually larger molecular size [60,63–65]. Phosphorylation of Cx43 has been described mainly on serine but also on tyrosine residues and seems to be involved in trafficking and insertion into the membrane [14]. Direct stimulation of PKC with TPA (a phorbol ester) rapidly (hyper)-phosphorylates Cx43 on Ser368 [66]. After prolonged stimulation, down-regulation of Cx43 and trafficking failure are observed (see [4]). Additionally, phosphorylation of rCx43 on Ser255 accelerates internalization and degradation [67].

Serine phosphorylation, mediated by PKA, PKC, PKG and MAP kinase, merely stimulates the prevalence of the smallest γ1. In contrast, treatment of cells with agents promoting dephosphorylation, shifts γ1 to the largest state [60]. Permeation to dye molecules seems closely related to the size of γ1. However, changes in γ1 appear not exclusively related to changes in g1 because phosphorylation might also affect Pγ. Stimulation of PKG (in rCx43 transfected cells) and PKC (in rCx43 and hCx43 transfected cells), reduces both dye permeability and γ1 [59,65,68]. Surprisingly, PKG stimulation causes a reduction in g1, whereas stimulation of PKC increases γ1 although both decrease γ1. This can only be explained by a different effect on Pγ assuming that time limits a substantial change in the number of channels (the effect of phosphorylation occurs within minutes while half-life of Cx43 is about 1.5 h [69]). hCx43 channels are insensitive to modulation by PKG [65]. The substitution of Ala at position 257 in hCx43 instead of Ser in rCx43 explains why rCx43 is susceptible to phosphorylation by PKG and hCx43 is not. PKA activation using cAMP and forskolin reduces γ1 in hCx43 channels [68], but has no effect on rCx43 channels [59].

Finally, tyrosine phosphorylation reduces g1, which can be induced by inhibition of tyrosine-phosphatase, or by activation of viral tyrosine kinases as p130bag-fps and v-Src [70,71]. This is illustrated by a reduction of g1 when Cx43 is phosphorylated by v-Src on tyr265 [72]. Besides the tyrosine kinase activity of v-Src, downstream activation of MAP-kinase induces phosphorylation on serine residues [73]. In rat liver epithelial cells, activation of MAP kinase by epidermal growth factor and of PKC and MAP kinase by platelet-derived growth factor, rapidly decreases g1 by phosphorylation of Cx43 on Ser255, 279 and 282 [74–76].

3.3. Cx45 channel properties

Cx45 is the least extensively studied of the three connexins expressed in cardiomyocytes. The gene has been cloned from mouse and human (see [6]), and also from rat (personal communication, Dr. M.F.A. Bierhuizen). The coding region of the gene consists of 396 amino acids in human and rat, and 395 in mouse. Most investigations concern hCx45 endogenously expressed in SkHePl cells [41,77,78] or mCx45 transfected in Hela cells [9,79]. Cx45 channels are selective for cations [80]. They are steeply
voltage dependent compared to Cx40 and Cx43, with half maximal activation at \( +/− 20 \text{ mV} \) [56,78]. Cx45 channels are more sensitive to changes in intracellular pH than Cx43 channels, with a complete block of junctional communication at pH 6.3 [41]. Unitary conductances are small with values of about 20 and 40 pS [59,79,80]. mCx45 but not hCx45 channels appear moderately permeable for LY [9,59,62]. Veenstra et al. showed that hCx45 channels are permeant to DCF but not to 6CF which depends on the charge of the molecules [80].

There are 15 putative phosphorylation sites [31,32] for PKC in hCx45 and 17 in rCx45 and mCx45. In all three species one site is found for modulation by PKA but no consensus sites were found for modulation by PKG or MAP-kinase. As with Cx40 and Cx43, Cx45 is phosphorylated mainly on serine, but also on tyrosine residues [77,79,81,82]. Phosphorylation of serine residues in the carboxy-tail of mCx45 stabilizes the protein [83]. There is debate about the electrophoretic mobility of the protein. Some groups have separated Cx45 as a single band of 45 kD [26,83], or of 48 kD [78], while others claim to have detected two bands of 46 kD and 48 kD of which the 46-kD band was proposed to be either a proteolytic product [77], or the unphosphorylated state of the native protein [79]. Kwak et al. have reported that \( \gamma \) of hCx45 channels increases upon stimulation of PKC, but not of PKA and PKG. Phosphorylation by PKC elicits a third conductance state of 16 pS [59]. \( \gamma \) of mCx45 channels is not affected by stimulation of PKC, PKA, PKG nor by tyrosine phosphatase inhibition mediated by pervanadate. However, \( \gamma \) decreases on stimulation of PKA and inhibition of tyrosine phosphatase, but increases on stimulation of PKC. Changes in \( g_\text{i} \) on stimulation of PKA and phosphatase inhibition were accompanied by an increase in phosphorylation as shown on Western blot [79].

### 3.4. From transfected cells back to the heart

Even in a simplified cell system there is complexity in regulation, because of differences in properties of homotypic channels of Cx40, Cx43 and Cx45 with their differential modulation by protein kinases. Additionally, at least in transfected cells, heterotypic channels exist for Cx40/Cx45 and Cx43/Cx45 [9,84]. Recently, two studies suggested the formation of both heterotypic and heteromeric Cx40/Cx43 channels [85,86] which is in contrast with previous data [11,53,87]. Heterotypic- and heteromeric channel properties differ from those of the two participating connexins when expressed as a homotypic channel [85,86,88]. In transfected cells the connexins are under the control of an artificial promoter. Therefore, modulation of properties is restricted to short-term processes. In cardiomyocytes prolonged stimulation of protein kinases and exposure to signaling molecules may provoke additional effects on expression and distribution of the channels. In Section 4 we will discuss the regulation of channel properties in cardiomyocytes.

### 4. Modulatory mechanisms of gap junction channels in cardiac myocytes

In cardiomyocytes, most studies regarding short-term regulation of gap junction channels (affecting single channel properties) are performed using cultured neonatal myocytes or pairs/clusters of freshly isolated adult myocytes derived from incomplete dissociation of cardiac tissue. Studies on changes in expression/distribution of connexins induced by prolonged stimulation of protein kinases or by application of growth factors, are generally performed using cultured neonatal (1–2 days old) rat ventricular myocytes. These cells are relatively easy to isolate in large amounts and can be cultured in monolayers for more than a week while retaining their electrical activity. In contrast, adult myocytes lose their expression of connexins very rapidly on isolation. Also, they do not attach in confluent monolayers.

Cultured neonatal ventricular myocytes express Cx40 and Cx43 and a small amount of Cx45 [28,89]. In culture, similar to the intact neonatal ventricle, the heterogenic expression of Cx40 declines in time and is susceptible to culture conditions [28]. It is unknown whether the heterogeneity of Cx40 expression (including regulation) in culture is a reflection of the original expression in the heart.

In neonatal cardiomyocytes, gap junctional coupling is characterized by channels with a main \( \gamma \) of 40–45 pS, but also a substate of 20 pS (using K-glucuronate as charge carrier) [90–92]. Dephosphorylation of Cx43 channels increases \( \gamma \) to 70 pS [91]. Dye permeability seems related to the size of \( \gamma \). Although channels of other connexins might be present, the measured \( \gamma \) values closely resemble those described for Cx43 channels in transfected cells.

In adult myocytes, \( \gamma \) values have been measured both in ventricular and atrial cells. Both guinea-pig and rabbit ventricular cells express channels insensitive to \( V_\text{j} \) with a main \( \gamma \) value of \( \sim 100 \text{ pS} \) (using CsCl as charge carrier), presumably reflecting Cx43 channels [18,93]. In rabbit atrial cells, next to the 100 pS conductance a larger conductance of 185 pS was measured which likely represents the gating of Cx40 channels [18].

Several studies on modulation of channel properties show that especially under conditions of ischemia, \( g_\text{i} \) might be synergistically modulated by \( H^+ \), \( Ca^{2+} \) and amphiphatic lipid metabolites [47,48,94–96]. The effect of phosphorylation of connexin molecules on modulation of channel properties can be studied using membrane permeant drugs which directly activate the protein kinases. In addition, application of several extracellular factors has been used to study receptor mediated activation of kinases (see Fig. 3).

Acute stimulation of PKG, as described for rCx43 channels in transfected cells, reduces \( g_\text{i} \), \( \gamma \) and dye permeability [90,97]. In many (but not all [90]) reports an increase of \( \gamma \) is found upon application of cAMP [97–99], isoproterenol [99,100], forskolin [99], norepinephrine
[101], or by dialyzing the catalytic subunit of PKA into the cells [102]. The modulating role of PKC is debated. Stimulation of PKC increased phosphorylation of Cx43 on seryl residues [103]. Reports describing modulation mediated by PKC show a decrease of $\gamma$ and dye coupling but an overall increase in $g_I$ [90], or a decrease in $g_I$ without changes in $\gamma$ [104].

Cardiomyocytes possesses a local renin–angiotensin system in which angiotensin-I (AngI) is converted by angiotensin converting enzyme (ACE) to angiotensin-II (AngII). AngII, the effective metabolite of the system, exerts its action through binding at AT-1- and AT-2 receptors, thereby activating PKC and MAP kinases Erk1 and Erk2, respectively. De Mello and co-workers have shown that administration of renin, Ang-I or Ang-II to adult rat myocytes rapidly reduces $g_I$ due to activation of PKC [105–108]. In contrast, inhibition of ACE increases $\gamma$ [107]. Phenylephrine binding to $\alpha_1$-adrenergic receptors also activates PKC and, similarly to Ang-II, decreases $g_I$ [109]. Additionally, basic FGF (bFGF) reduced metabolic coupling as assessed by dye-transfer while phosphorylation of Cx43 was strongly increased mainly on serine residues but also on tyrosine residues [110].

These short-term effects (within 5–10 min) on gap junctional conductance are probably caused by connexin phosphorylation because within this limited period no changes in expression or translocation are expected. When the stimulation period is prolonged to 6–24 h, additional changes have been reported in expression level of the connexin proteins in rat neonatal cardiomyocytes.

Myotrophin, strongly increased the amount of Cx43 mRNA [111] while cAMP significantly increased conduction velocity in strands of cultured myocytes [82]. This effect was accompanied by an increased expression (gap junctions increased in number and size) of Cx43 and Cx45 which resulted from an increase in Cx43 mRNA and an enhanced translation of Cx45 mRNA. A similar increase in Cx43 expression, functionally shown by more and larger gap junctions, was observed in an AT-I receptor mediated response after stimulating cells with Ang-II [112]. Wnt-1 stimulation of neonatal rat cardiomyocytes increased Cx43 mRNA and protein which resulted in an enhanced dye permeability and propagation of calcium [113]. In non-myocyte cardiac cells like fibroblasts, bFGF increased both Cx43 mRNA and protein thereby increasing dye transfer [114]. Additionally, potentially interesting factors as epidermal growth factor (EGF), platelet derived growth factor (PDGF), transforming growth factor $\beta$ (TGF$\beta$), tumor necrosis factor $\alpha$ (TNF$\alpha$) and interleukin 1$\beta$ (IL-1$\beta$), affect Cx43 channel properties in non-cardiac cells [75,76,115–117]. Finally, mechanical stretch which activates multiple signaling pathways has been reported to strongly increase expression of Cx43 but not of Cx40 in rat neonatal cardiomyocytes [118,119]. Increased expression of Cx43 resulted both from an increase in mRNA and an decrease in turnover of the protein [119].

5. Implications of gap junction modulation for cardiac function

Results from studies on transfected cells and cardiomyocytes are reasonably comparable. The question remains to what extent modulation of intercellular coupling affects cardiac performance. In only one study on patients suffering from complex congenital hearts defects, a Ser364Pro mutation was detected which in transfected cells appeared to induce abnormal behavior of Cx43 channels [120] but this was not confirmed by others [121]. The spatial differences in expression of Cx40, Cx43 and
Cx45 channels (Section 2) combined with differences in connexin properties and modulation (Sections 3 and 4), appear to be reflected into specific conductive properties in different regions of the heart. The most direct approach to unraveling the specific role of a given connexin is targeted deletion of the gene involved. Using this strategy, Cx40, Cx43 and Cx45 knock-out (KO) mice have been generated.

Another approach is to study the function of gap junction channels in multiple forms of cardiac disease where changes in expression, distribution and phosphorylation during the progression of the underlying pathophysiology have been reported.

5.1. Connexin knock-out mice

Cx43 KO mice die perinatally due to an obstruction of the right outflow tract caused by cardiac malformations [122,123]. Isolated and perfused neonatal Cx43 KO hearts were completely unexcitable [124]. In contrast, heterozygous mice are viable and reproductive. The heterozygous hearts have a normal morphology and show a 50% reduction in expression of Cx43 mRNA and protein [125]. The consequences of this severe reduction in Cx43 protein for ventricular conduction are debated. Initial reports mention a 30–40% reduced ventricular conduction velocity and a prolongation of the QRS complex [125,126]. However, detailed optical mapping did not reveal differences [127]. During ischemia an increased susceptibility to ventricular arrhythmias was found [128]. A similar 50% reduction in Cx43 expression was found in the atria but this did not affect conduction velocity [126]. Transgenic mice overexpressing a dominant-negative form of Cx43 (leading to non-functional channels) are phenotypically similar to Cx43 KO mice [129].

Electrophysiological characteristics of mice heterozygous for Cx40 deletion are comparable to wild-type mice [130], but Cx40 KO mice show multiple aberrations: prolongation of PQ interval [130,131], reduced atrial conduction velocity with a prolonged P-wave, prolonged sinus-node-recovery time, prolonged Wenckebach period, atrial tachyarrhythmias following burst pacing, and reduced A–V conduction; [132,133], impaired left bundle branch conduction and right bundle branch block [134]. No differences were found between WT and KO mice regarding ventricular conductive properties which is explained by the absence of Cx40 in the ventricle. A prolongation of QRS duration was described [130,133], but not when the ventricle was paced from the apex [133] which indicates that the prolongation is due to impairment of the conduction system [134].

Two groups have reported the generation of a Cx45 KO mouse [135,136]. Heterozygous mice are comparable with WT animals as with heterozygous Cx40 KO mice. However, Cx45 KO mice die in utero on day 10.5 pc. Embryonic hearts were dilated due to defective vascular development [136]. Interestingly, within 24 h of the first contractions of these developing hearts in utero, severe impairment of atrial contraction develops and atrio–ventricular conduction block appears [135].

Obviously, gap junctions play an important role in growth and development. Thus, deletion of connexin genes not only affects their specific cardiac function but may also induce developmental alterations. To date, inducible KO mice are constructed in which the connexin gene is surrounded by loxP sites. With this approach, an inducible system activates a recombinase protein (Cre) which connect both loxP sites by deletion of the DNA (connexin gene) in between [137]. In this way, deletion of the genes can be induced at any stage of development which may result in clearer distinction between the role of gap junctions in development and cardiac function.

5.2. Ischemic heart disease and cardiac hypertrophy

Most myocardial diseases are marked by alterations at the expression level of the connexins and/or the anisotropic distribution of gap junction plaques [138–140]. A decrease in expression level of ventricular Cx43 is often detected by Western (protein)- or Northern (mRNA) analysis [141,142]. Obviously, changes in cell and tissue homeostasis and alterations in (circulating) levels of second messengers and growth factors (see Section 4) have an impact on the alteration in expression pattern. In hearts of hypertensive rats (spontaneous or renin transgenic) where the renin–angiotensin signaling is forced, a threefold increase in Cx40 expression and a threefold decrease in Cx43 expression was reported [143]. Regardless the nature of cardiac disease, one of the most commonly found alterations is a shift in distribution of gap junction plaques from the end-to-end IDs to lateral cell borders (see [144]). In healthy myocardium, the anisotropic distribution of gap junctions contributes to differences between conduction velocity in longitudinal and transverse directions. A redistribution of Cx43 gap junctions has been reported in the borderzone of infarcts [142] where it was associated with the localization of reentrant circuits [145], but also in hibernating myocardium [146], in hypertrophied hearts [139], in ischemic hearts [141] and in hypertrophic cardiomyopathy [138]. Often, the redistribution is accompanied by a decrease in size of the gap junction plaques [146] or in the total amount of plaques per ID [139]. In the study of Uzzaman et al. this change was associated with a 30% reduction in longitudinal conduction velocity [139].

Despite the fact that in most cardiomyopathies an increased propensity to arrhythmias has been reported, the functional contribution of both type of alterations, i.e. change in density and distribution, are not completely understood. The significance of redistribution to lateral cell-borders is debatable because lateral gap junctions may prove non-functional if located in intracellular invagina-
tions of the sarcolemma [147]. However, both redistribution and a 20–40% reduction in gap junctions per se will decrease the anisotropy in the tissue which is often considered as antiarrhythmic [148], but by some as proarhythmic because it decreases the ‘wavelength’ of the myocardium [149]. Despite these significant alterations, Jongsmaj and Wilders computed only small to moderate changes in conduction velocity and anisotropy ratio [150]. Both and Jongsmaj and Wilders and Spach et al. showed that the contribution of changes in cytoplasmic resistivity (cellular geometry) might be equally important especially for longitudinal conduction [150,151]. In addition, Spach et al. showed that discontinuity in anisotropy, which results in nonlinear conduction, increases the propensity for arrhythmias far more than uniform changes in anisotropy ratio in a continuous system. This implies that conduction block in a discontinuous system can be induced by minor alterations in intercellular coupling compared to the extensive uncoupling required in a continuous system [152]. Modulation by phosphorylation of the connexin proteins has been introduced as an important factor for remodeling of gap junctions in diseased hearts. In normal hearts, Cx43 exists in a phosphorylated state. Beardslee et al. suggested that unphosphorylated Cx43 is principally degraded by the proteosomal pathway and phosphorylated Cx43 by the lysosomal pathway [69]. Recently, the same group showed in a model of acute ischemia that uncoupling of cardiac tissue is associated with dephosphorylation of Cx43 and accumulation of unphosphorylated Cx43 in the gap junction plaques [153]. In hereditary cardiomyopathic hamsters, endstage congestive heart failure correlates with a c-Src mediated increase in phosphorylation of Cx43 on tyrosine residues. As a consequence, $g_j$ between pairs of myocytes decreased [154]. These studies suggest that in the intact heart phosphorylation serves as an important modulator of cardiac conduction.

Studies on remodeling of gap junctions in atrium are scarce. In dog atrium, atrial fibrillation induced by pacing increased the expression of Cx43. In chronic atrial fibrillation in the goat, Cx40 expression is reduced. Moreover, this reduction is heterogeneous. In contrast to the dog, in the goat atrium no changes in the amount of Cx43 mRNA and protein have been detected, but the prevalence of unphosphorylated Cx43 seems increased [140,155,156].

6. Concluding remarks

A vast amount of information regarding the specific properties of different gap junction channels, their expression in the heart and the mechanisms to modulate their properties, has been acquired during the past decade. Extrapolation of this knowledge obtained from experiments with transfected cells or isolated and cultured cardiomyocytes to the intact heart is a challenge for the near future. Deletion of connexin genes affects cardiac conduction only to a moderate extent. This is illustrated by heterozygous Cx40- and Cx43 KO mice in which conduction properties are comparable to WT hearts. These observations were supported by a theoretical study [150]. In cardiomyopathic hearts, alterations in gap junctions expression/distribution may be involved in the increased propensity to arrhythmogenesis, but changes in cellular dimensions following the pathophysiological process, may be relevant as well [151]. Inducible KO mice will be an adequate tool in the near future. Generation of mice with (inducible) forced expression, or targeted deletion of potentially involved genes, allows to focus the attention on specific factors in the variety of involved signaling processes. For example, Nguyen-Tran et al. showed that mice deficient in HF-1b, a transcription factor preferentially expressed in the cardiac conduction system, display sudden cardiac death. This was associated with a reduced expression, and redistribution of Cx40 in the cardiac conduction system [157]. Furthermore, in another mouse model, a constitutive active retinoic acid receptor provokes cardiac hypertrophy accompanied with a heterogeneous reduction of Cx43 expression in the ventricles [158]. These are examples of new models that in the near future may advance our knowledge on the relevance of gap junctions in cardiovascular disease.

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