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Gap Junctions

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Abstract

Gap junctions are essential to the function of multicellular animals, which require a high degree of coordination between cells. In vertebrates, gap junctions comprise connexins and currently 21 connexins are known in humans. The functions of gap junctions are highly diverse and include exchange of metabolites and electrical signals between cells, as well as functions, which are apparently unrelated to intercellular communication. Given the diversity of gap junction physiology, regulation of gap junction activity is complex. The structure of the various connexins is known to some extent; and structural rearrangements and intramolecular interactions are important for regulation of channel function. Intercellular coupling is further regulated by the number and activity of channels present in gap junctional plaques. The number of connexins in cell-cell channels is regulated by controlling transcription, translation, trafficking, and degradation; and all of these processes are under strict control. Once in the membrane, channel activity is determined by the conductive properties of the connexin involved, which can be regulated by voltage and chemical gating, as well as a large number of posttranslational modifications. The aim of the present article is to review our current knowledge on the structure, regulation, function, and pharmacology of gap junctions. This will be supported by examples of how different connexins and their regulation act in concert to achieve appropriate physiological control, and how disturbances of connexin function can lead to disease. © 2012 American Physiological Society. *Compr Physiol* 2:1981-2035, 2012.

Introduction

Gap junctions are integral membrane proteins that enable the direct cytoplasmic exchange of ions and lowmolecular weight metabolites between adjacent cells. These connections are essential in many aspects of animal physiology including propagation of electrical signals and coordination of cell signaling by transfer of second messengers. The proteins that form gap junctions differ between vertebrates and nonvertebrates. In vertebrates, gap junctions are formed by connexins, whereas the gap junction proteins of nonvertebrate animals are termed

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innexins (517). Connexins share structural features with innexins though there is no amino acid sequence homology between the two. Proteins with amino acid sequence homology to innexins have been identified in vertebrates (500). These proteins, called pannexins, are able to form intercellular channels when overexpressed, but studies show that native pannexins are likely glycosylated in their extracellular domains and do not form cell-cell channels (507). Therefore, it is suggested that they act as hemichannels (114).

The aim of this article is solely to review our current knowledge of connexins. A simple search on PubMed on "gap junction OR connexin" yields more than 15,000 hits. This shows that a huge amount of scientific work has been conducted in this field, and obviously it is impossible to cover every detail in this article. The article aims to cover the basic principles of connexin structure, how connexin expression, transcription, turnover, and trafficking are regulated, as well as how mature gap junction channels are affected by gating and posttranslational modification. Finally, some insight to the physiological function of gap junctions and their involvement in various diseases is given, along with a status of experimental pharmacological treatments targeting gap junctions.

Basic Properties of Connexins

Connexin structure

Gap junctions are formed by the apposition of connexons from adjacent cells, where each connexon is formed by six connexin proteins (Fig. 1A). There are 21 different connexin genes in the human genome and 20 in mice. Connexins are tetraspan transmembrane (TM) domain proteins with intracellular N- and C-termini (Fig. 1B). Two extracellular loops (E1 and E2) provide continuity between TM domains TM1-TM2 and TM3-TM4, while the region between TM2 and TM3 (the "cytoplasmic loop;" CL) is located in the intracellular space. Connexins are expressed in all tissues except differentiated skeletal muscle, erythrocytes, and mature sperm cells. Each of the connexins can form gap junctions by themselves; however, studies have demonstrated that many cells coexpress more than one connexin isoform, giving rise to heteromeric connexons (more than two different connexins in a connexon) and heterotypic channels (a gap junction channel with different connexons), conferring further diversity in their composition and function. Connexins are named based on their predicted molecular weight (e.g., Cx43 is ~43 kD in size) and this nomenclature continues to be widely used. Of note, this nomenclature does give rise to confusion in some cases where homologous connexins have different names (i.e., molecular weights) in different species (Table 1; h-human; m-mouse). In the present review, we have adapted to the nomenclature decided upon at the International Gap Junction Meeting in Denmark 2007. Non-human connexins are named after their human ortholog when differing, but to ease the linking to the original literature the species dependent name is given in parenthesis. For example, mouse Cx30.2 is referred to as Cx31.9 (mCx30.2).

Connexins can be divided into five subgroups (α, β, γ, δ, or ε) with respect to their extent of sequence identity and length of the CL (Table 1). Connexins are then abbreviated with "GJ" for gap junction and numbered according to the order of discovery. For example, Cx43 was the first connexin of the α-group (GJα1) and Cx32 was the first connexin of the β-group (GJβ1) (http://www.genenames.org/genefamily/gj.php).

Many studies have aimed at characterizing the high-order structure of the gap junction channel. Early work showed that a connexin hemichannel has 6-fold symmetry, with a pore in the center (413,699). The initial observations were confirmed and expanded by several authors [e.g., references (275, 276, 683)]. A major step forward came from the work of Unger et al. (698). These authors used electron crystallography to solve the three-dimensional structure of gap junction channels formed by Cx43 subunits. The assembled

gap junction channels were observed at a resolution of 7.5 Å in the membrane plane and 21 Å in the vertical direction. The study clearly showed the electron densities corresponding to 24 TM domains in α-helical order. As shown in Figure 2, they are organized as six repeats of four TM domains (labeled A-D), consistent with the notion that a connexon is formed by the oligomerization of six connexins, each with four TM domains. The images further revealed that the pore narrows from 40 to 15 Å at the boundary with the extracellular gap. This narrowing [initially proposed by the low-angle x-ray diffraction analysis of Makowski et al. (413), as well as data of Unwin and Zampighi (699)] is due to the tilting of the channel-lining TM domain (labeled "C" in the right panel of Fig. 3). Whether this structure corresponds to that of an open or a closed channel remains to be determined. The data of Unger et al. further showed that the C α-helix extends beyond the putative boundary of the bilayer and into the cytoplasm. If the "C" domain corresponds to the third TM domain this may suggest that the second half of the CL is also an α-helix, which conforms part of the vestibule of the pore. The latter would be consistent with observations of this domain using NMR (155) and with the functional mapping of this area (620). Unger et al. provided a fundamental framework for understanding the gap junction pore from a structural point of view. However, it is worth noting that the investigators needed to truncate the carboxyl terminus of Cx43 to improve the diffraction quality of the two-dimensional crystals. Hence, with the exception of a small fragment of the CL, intracellular regions were not characterized within this structure.

Structural analysis using x-ray crystallography has recently provided the gap junction field with the highest resolution of a gap junction channel (409). At a resolution of 3.5 Å, the overall structure of the Cx26 channel was determined as follows; length of the channel, approximately 155 Å ; the extracellular gap, approximately 40 Å; the outer diameter of the channel at the cytoplasmic end, approximately 90 Å, which decreases to approximately 50 Å in the extracellular portion; the inner diameter of the channel, approximately 40 Å at the channel entrance, and narrows to approximately 14 Å around the midpoint of the membrane region (Fig. 3A/B). The channel, which includes the 24 TM spanning domains, contains approximately 60% α-helical structure. The Cx26 monomer is a four-helical bundle in which each pair of adjacent helices is antiparallel (Fig. 3C). TM1 and TM2 face the luminal side of the pore; however, the TM2 and the cytoplasmic half of the TM1 are not exposed to the lumen because they are covered by a short α-helix from the N-terminus. TM3 and TM4 are on the outside of the channel facing the lipid environment. The extracellular loop E1 contains a 3_{10} helix at the beginning and a short α-helix in its carboxyl-terminal half. E2, together with E1, form a short antiparallel β-sheet. Six conserved cysteine residues, three in each loop, form intramolecular disulphide bonds between E1 and E2. The intermolecular interactions between two adjoining connexons involve both the E1 and E2 domains and numerous hydrogen bonds and salt bridges help create a tight seal that isolates the channel interior to form the connection between adjacent cells. Similar to Cx43 structure (698), the CL and cytoplasmic tail (CT) domains were not visualized in the Cx26 electron density map. This led the authors to suggest that the channel is in an open conformation, since no obstructions through the pore were identified combined with their crystallization conditions.

Although, neither crystallographic technique was able to address the structure of the CT or CL because of the dynamic nature of these domains, these same characteristics that interfere with crystallographic techniques make NMR an ideal tool for studying them. Previous NMR studies have focused on identifying regions of structural order and binding between the CT and CL of Cx43 because these domains play a fundamental role in gap junction regulation (155,274,647). For example, the CT may mediate pH gating in a "particle-receptor" interaction, modeled after the "ball-and-chain" mechanism of voltage-dependent inactivation (Fig. 4A) (332). A direct pH-dependent interaction between a soluble version of the Cx43CT (S255-I382) and a peptide corresponding to the second half of the CL (D119-K144) (155)

was identified. This interaction was dependent on the CL peptide forming an α-helical structure in response to the acidification (Fig. 4B). It was proposed that the second half of the CL acts as the "receptor" for the CT domain (155, 606). In a separate study, the soluble version of the CT domain was identified by NMR as being highly flexible and predominately disordered in structure (Fig. 4C) (648). Moreover, changes in the pH of the solvent caused dimerization of the Cx43CT (649), which may be a relevant step in the regulation of Cx43 by intracellular factors such as low pH.

Connexin structure during gap junction regulation

Although there have been some spectacular successes in solving the structures of integral membrane proteins by xray crystallography, especially for the gap junction proteins, these structures only provide a snapshot of the protein in a single conformation. Electron crystallography, cryo-electron microscopy, and atomic force microscopy (AFM) offer alternative, albeit lower resolution, approaches to membrane protein structure determination that are not dependent on the generation of three-dimensional crystals. Over the past few years, the Sosinsky and Lal laboratories have taken advantage of these non x-ray crystallographic methods to provide novel insight into how chemical mediators of gap junction regulation (e.g., Ca^{2+} and pH) affect the structure of Cx43 and Cx26 channels and hemichannels.

The Sosinsky laboratory used AFM to image the conformational changes of the cytoplasmic and extracellular surfaces of native Cx26 gap junction plaques in response to changes in calcium concentration (456). Calcium has long been postulated to play a crucial role in the gating of the gap junction intercellular communication by decreasing or suppressing electrical coupling (508). In the absence of calcium, the cytoplasmic surface formed a hexameric pore (2.8 nm inner diameter) protruding 1.7 nm from the membrane bilayer and exhibited a high degree of structural flexibility. Similar to the cytoplasmic surface, the extracellular connexon surface formed a hexameric pore exhibiting an inner diameter of 1.5 nm and protruded 1.6 nm from the lipid bilayer (Fig. 5A). When the extracellular hemichannel surface was imaged in the presence of calcium (0.5 mmol/L), the entrance narrowed significantly as evident in the reduced pore size (from 1.5 to 0.6 nm) (Fig. 5B). The conformational change was fully reversible and specific amongst other divalent cations tested (e.g., magnesium). The conformational change did not alter the height nor cause a rotation of the extracellular domains. Calcium also had a profound, but different effect on the cytoplasmic surface, inducing the formation of microdomains and an increase in the plaque height. However, the cytoplasmic surface appeared too flexible to be imaged at sufficiently high resolution to assign structural changes. These observations, together with the differences in intracellular and extracellular hemichannel sensitivity to calcium concentrations (intracellular, μmol/L range; extracellular, mmol/L range), support the idea that a different gating mechanism for channel closure exists at the extracellular surface from the one that occurs at the cytoplasmic surface.

A different mechanism underlying Cx26 channel closure was observed in response to alterations in pH (772). High-resolution imaging by AFM of Cx26 hemichannels revealed that the pore was closed in response to acidification, but only in the presence of an aminosulfonate buffer (e.g., HEPES). Aminosulfonate compounds acts as modulators of the Cx26 channel (41). The overall diameter of the hexameric hemichannel did not change, yet the depth of the channel decreased concomitant with an increase in the width of the connexon lobes under the more acidic conditions. For example, at pH 7.6, the channel entrance diameter was 1.7 nm, while at pH 6.0, the diameter was 0.6 nm and there was an approximately 6.5° rotation in the hemichannel lobes. These results support the model of a physical gate close to the extracellular surface, not only because the pore diameter decreases upon acidification, but also because its depth is shallower.

More recently, the Sosinsky laboratory has reported the electron crystallographic structure of a human Cx26 mutant (M34A) (490) previously shown to cause prelingual nonsyndromic hereditary deafness [M34T (308)]. The mutation expressed higher quantities of protein than wild-type (wt) Cx26. Interestingly, the Cx26 mutant was purified as hemichannels; however, during the crystallization process, full Cx26 gap junction channels were formed. This indicates that hydrophobic interactions at the extracellular domains may drive hemichannels to redock into dodecameric channels. The three-dimensional structure was determined to a resolution of 10 Å in the x-y plane. A comparison was made between the Cx26 M34A mutant (β-subgroup) and the Cx43 263T truncation mutant (α-subgroup) and the structure of these two connexin isoforms were very similar (698). Also, similar to the Cx43 263T truncation mutant and Cx26 crystal structure, the resolution of the cytoplasmic and extracellular domains was insufficient to assign these segments and the polypeptide boundary to specific sequences within the TM domain. The three-dimensional structure also displays a prominent density in the pore of each hemichannel, suggesting that a plug physically blocks the channel within the membrane. The likely candidates for the plug are the cytoplasmic N-terminal, CT, or CL domains.

AFM has also been utilized to examine the threedimensional molecular surface topology of nontruncated Cx43 hemichannels reconstituted in lipid membranes (677). The images showed that the hemichannels fell within two groups that differed in height. The groups were identified as the extracellular surface facing-up, with a height of 1.3 nm from the membrane surface and the intracellular surface facing-up, with a height of 4.7 nm. The latter is consistent with the longer protruding CT domain and allowed for a clear distinction of the sidedness of the images because each hemichannel has an equal probability of facing-up or down. A detailed analysis of the calcium-sensitive conformational changes was performed with the Cx43 hemichannels. In calcium-free buffer, AFM images of the extracellular face showed a well-defined donut-like structure consisting of six subunits and a central pore-like depression. The pore diameter was 2.5 nm with a depth of 0.8 nm. This pore size was not changed in the presence of magnesium or nickel; however, calcium was able to close the channels in a concentrationdependent manner. The channels were completely closed at 1.8 mmol/L calcium (pore diameter −1.8 nm), while 73% and 26% of the channels were closed at 1.4 and 1 mmol/L, respectively. Only two-states (2.5 and 1.8 nm) were observed, suggesting that a single hemichannel switches between open and closed states in an all-or-none manner, and the open/closed probability is calcium concentration dependent. This is consistent with previous functional studies of hemichannels and their physiological role (159, 280, 514).

The open and closed channel diameters for Cx43 (2.5 and 1.8 nm) are similar to those predicted from the electron microscopy study by Unger et al. (698), but quite different when compared with the open and closed diameters (1.5 and 0.6 nm) for isolated Cx26 gap junction channels (456). Also, Muller et al. (456) showed that Cx26 hemichannels close at a lower calcium concentration than Cx43 hemichannels (0.5 and 1.8 mmol/L, respectively), suggesting this difference in calcium sensitivity may be related to the functional differences between the two hemichannels.

Interfacial energy maps were used to determine the hydrophobic versus hydrophilic surfaces, both on the Cx43 extracellular loops, as well as the pore mouth (677). In the closed state, the extracellular loops are tightly packed and in the open state, the extracellular loops are at least partly unfolded (a larger pore, a slightly larger outer diameter, and well-resolved extracellular loops). The hemichannel opening is associated with a significant increase in the interfacial energy in the pore mouth region (0.118 J/m^2 for the closed and 0.163 J/m^2 for the open channels). Such an increase would indicate the presence of hydrophobic surfaces exposed at the pore vestibule. The data indicate that refolding of the extracellular loops

exposes their hydrophobic domains in a calcium-dependent manner and that the extracellular loops in nonjunctional hemichannels are structurally different when compared with those in the gap junctional hemichannels.

Based on the "particle-receptor" model for Cx43 channel gating, movement of the CT domain controls channel permeability by interacting with the CL domain to physically occlude the pore (155,274,454). To test this hypothesis, the Lal laboratory used AFM-based single-molecule spectroscopy with antibody modified AFM tips and connexin mimetic peptide-modified tips to examine the flexibility of Cx43 CT domain and extracellular loop domains, and to estimate the energetics of their movements (387). For these experiments, AFM tips were linked via a flexible spacer to either a CT specific antibody (anti-$CT_{252-270}$ or anti-$CT_{360-382}$) or to a connexin mimetic peptide (called GAP26, which binds to the extracellular loop), and subsequently binding to Cx43 hemichannels reconstituted in a lipid bilayer was measured. The observed GAP26-Cx43 interaction showed very little, if any, molecular stretching of the Cx43 extracellular loop. However, a significantly larger extension was observed with the anti-$CT_{360-382}$-Cx43 interaction, showing that the CT has a large degree of flexibility. As expected, the anti-$CT_{252-270}$-Cx43 interaction caused a shorter extension because its interaction site is located in the middle portion of the flexible Cx43CT domain and thus closer to the membrane. As previously observed, 1.8 mmol/L calcium closes Cx43 hemichannels (677). To further examine the role of the Cx43 CT domain, calcium was added into the anti-$CT_{360-382}$-Cx43 force measurement medium. The specific rupture events (which indicate binding between the antibody and Cx43 CT) frequently detected under calcium-free conditions were significantly diminished in the presence of 1.8 mmol/L calcium, a process that was fully reversible. Hemichannel closing at 1.8 mmol/L calcium is believed to introduce conformational changes in the Cx43 CT domain, which results in steric hindrance preventing binding. Altogether, the stretch length, the presence of antibody-CT domain interaction at 0 mmol/L calcium, and the lack of one at 1.8 mmol/L calcium, strongly support calciumdependent conformational changes of the CT domain and the "ball and chain" model of hemichannel gating.

Permeability of gap junction channels

Traditionally, gap junctions have been characterized as nonspecific pores between cells passing molecules up to 1 kDa in molecular mass, and expectation was that the pore diameter would be the primary selectivity determinant among the different connexin isoforms. Since then, a variety of approaches have been used to investigate permeability properties of connexin channels. Studies involving measurement of unitary conductance, ion selectivity, and permeability to fluorescent tracers of various sizes, charges, and chemistries, have revealed a wide variety of pore properties. No atomic ions are large enough to be excluded and homotypic gap junctions of Cx43, Cx40, Cx37, and Cx45 shows similar selectivity to monovalent cations (e.g., K^+ and Na^+) (32, 716, 717, 733); however, the charge selectivities range from a slight anion preference (Cx32) to a high cation selectivity (Cx40 and Cx43) (32,667,733). The electrophysiological properties of macroscopic coupling are determined by the biophysical properties of the underlying channels. Channel behavior is complex usually with a main open state, as well as states of lower conductance. The unitary conductance of gap junction channels depends on the connexin isotypes; Cx36 channels display a unitary conductance of 14 pS (674), whereas Cx37 form channels with a very high electrical conductance of 300 pS (718). The conductance of cell-to-cell channels can in most cases be calculated from the conductance of the individual hemichannels under the assumption that the channels work as two resistors in series.

The initial use of a range of fluorescent tracers and uncharged molecules to study gap junction permeation provided significant information regarding the pore diameter limitation of Cx channels (suggested ranking: Cx43>Cx32>Cx26>Cx37>Cx46 and Cx43>Cx40)

(248). Though, fluorescent tracers became useful to report the existence and extent of junctional communication (e.g., use of Lucifer Yellow), they provide limited information regarding the nature of the permeability pathway itself. Important as the use of nonbiologic tracers have been in the estimation of pore diameters, these tracers convey little information on the ability of connexins to transfer biological molecules such as metabolites and second messengers. Although the literature on permeation of biological molecules is limited, important efforts have focused on the characterization of the permeability of connexin channels to endogenous cytoplasmic molecules. This is not a simple task due to the existence of more than 20 connexin isoforms, with different pore properties, that can almost all form heteromeric channels. Additionally, permeability properties of a given channel are expected to be modulated by reversible posttranslational modification (e.g., phosphorylation, see section on posttranslational modifications) or protein-protein interactions. Each answer will therefore be specific to a particular structural and biochemical channel, at a specific time. Measuring the flux of biological molecules poses many problems that make a reliable quantification difficult. Often the molecules in question are subject to cellular metabolism and in the case of second messengers, the messengers can affect coupling by activation of downstream cascades. In both cases, transfer can be grossly overor underestimated. Recent reviews summarize extensive information regarding the permeation by cytoplasmic molecules through connexin channels (248, 249). As previously established, most of the studied gap junction channels (homomeric Cx26, Cx32, and Cx43 and heteromeric Cx26/Cx30 or Cx32) are permeable to second messengers and metabolites, such as ATP or ADP, Ca^{2+}, cAMP, IP_3, glutamate, glutathione, and of interest to siRNA. However, connexin isoforms can influence the gap junctional permeability. For example, Cx43 channels have a 100- to 300-fold higher selectivity for ATP over those of Cx32, while glutamate, glutathione, and ADP show a 10- to 20-fold preferential permeability through Cx43 channels, whereas adenosine is 10-fold more permeable through Cx32 (219). These studies demonstrate the differences in intercellular transfer of natural permeants among the different connexins. However, the physical basis of this selectivity remains unclear, as there are no evident correlations between metabolite properties and their permeability through different connexin channels.

Despite the limited information on permeation of endogenous molecules, it is quite clear that large differences occur between different connexin species. These differences may explain the need for multiple connexin types to regulate communication during development and signaling events. That the differences in permeation are of physiological importance are exemplified in mouse models where connexin genes have been exchanged with another connexin. In the case of Cx43 exchange to Cx31 or Cx26 leads to functional and anatomical defects (748, 782), whereas exchange to Cx32 or Cx40 has little or no consequence (524). The effects may be very tissue specific as shown in a recent study, where neuronal specific exchange of Cx45 with Cx36 did not affect signaling in the retina. However, cardiac or general exchange did lead to both enlarged hearts and conduction abnormalities and were embryonic lethal (185). Thus, the specific differences in connexin properties can have profound physiological consequences.

Compatibility of connexin types in channel formation

Connexins are somewhat promiscuous in the sense that different connexins can combine to form channels with unique properties. Channels with diverse connexin content are classified on the basis of how the connexins are mixed (Fig. 6). Individual connexons (hemichannels) are termed homomeric when they contain only one connexin type and heteromeric if they contain different connexins. When identical connexons form cell-cell channels they are termed homotypic. In some cases, cells with connexons of different connexin composition form channels, which are then termed heterotypic. This gives rise to four possible

combinations of homo/heteromeric and homo/heterotypic channels. Given the 21 connexins in humans and the fact that connexons contain six connexins, this leads to an explosion of possible combinations. However, not all connexins are compatible in forming functional channels and secondly not all combinations of connexins are expressed in the same or neighboring cells.

Heterotypic combinations of homomeric channels have been studied extensively and are physiologically relevant when cells expressing different connexins couple. Connexin of the α- and β-groups are more likely to form channels with other members of their own group. For example, in the α group, Cx37 forms heterotypic channels with Cx40 (747) and Cx43 (64), but not with theβconnexins Cx26 and Cx32 (747). On the other hand, Cx37 does not form channels with the α-connexins Cx46 and Cx50 (747), so any given combination of interest needs to be tested. A more exhaustive review of possible combinations can be found in reference (763). The functional properties of heterotypic channels are often similar to that expected of two connexons in series and will be dealt with in the section on voltage gating.

Coexpression of different connexins occurs in many cell types as, for example, in the skin, where Cx31, Cx26, Cx43, Cx32, Cx40, Cx30, and Cx45 are expressed at the protein level (144). The study of heteromeric channels is complicated because even if coexpression results in channels/coupling with altered properties, it is difficult to prove that connexins actually incorporate into the same channel. Conversely, even if heteromeric channels are formed, they will exist together with homomeric channels. The relative proportions of hetero- and homomeric connexons will be determined by the probability of hetero- and homomeric oligomerization. In the atrium of the heart, Cx40 and Cx43 are coexpressed and studies show that they most likely form heteromeric channels (256, 702). However, the alterations in macroscopic coupling properties can largely be described by the behavior of homo- and heterotypic channels and it has been concluded that the heteromeric channels are of little importance (702). In contrast, coexpression of Cx40 and Cx43 significantly increases the sensitivity to pH-dependent gating (233). Furthermore, it has been shown that the CTs of Cx40 and Cx43 can interact with each other and with each other's CL, suggesting that interactions within connexins in heteromeric channels are responsible for the shift in pH dependence (57).

Gating of Connexins

The term gating is often used to describe changes in channel opening and closing, that is, a conducting channel becomes either physically available or unavailable (271). Electrical coupling via gap junctions, expressed by the macroscopic conductance (Gj), is determined by three factors: the number of channels present at the plasma membrane (*N*), the open probability (Po), and the unitary conductance of the channel (γ), where $G_j = N*P_o*\gamma$. The number of channels residing in gap junctional plaques is determined by the expression, trafficking, and degradation of connexins, which is covered in a later section. Here, regulation of P_o and γ of channels residing in gap junctional plaques will be discussed. Availability is determined by the P_o of the channels but connexin channels also exhibit several conductance levels, the size of which can be regulated, thence unitary conductance must be taken into consideration. As we shall discuss, channel activity is tightly regulated in response to changes in voltage, calcium concentration, pH, phosphorylation, and protein interactions.

Voltage gating

Gating by the transjunctional voltage—Gap junction conductance can be regulated by transjunctional voltage (V_j) and for some connexins also by the transmembrane voltage (V_m) (for reviews see references 72 and 227). There are 21 different connexins in humans

and each has distinct gating properties. When gap junctional currents are investigated by imposing a voltage gradient, the elicited current inactivates over time in a voltage-dependent manner (see Fig. 7). The initial current is largely independent of voltage, whereas the steady-state current decreases with increasing voltage gradients. The inactivation can be described by the fractional maximum conductance G_{max}, which expresses the conductance relative to the conductance at $V_j = 0$; the fractional minimum conductance G_{min}, which expresses the inactivation at large V_j; and the V_j at which half maximal inactivation occurs ($V_{1/2}$). The parameters can be determined by fitting a Boltzmann function to the steady-state current divided by the initial current as a function of V_j (250). This type of voltage-dependent behavior is reported for all connexins investigated with variation in the degree, speed, and sensitivity of inactivation. $V_{1/2}$ indicates the sensitivity to V_j and varies from around 100 mV in Cx36 (5) to around 10 mV in Cx45 (24), the most sensitive connexin. The effect of voltage-dependent inactivation is also very variable with a G_{min} of 0.5 in perch Cx35 (5) to 0.1 in Cx45 (24). Table 2 shows values for selected connexins, an exhaustive list can be found in reference 227.

The physiological role of voltage gating is largely unexplored, but may for some connexins like Cx43 overlap with the chemical gate, since truncation of the CT in this connexin eliminates both sensitivity to uncoupling at low pH and voltage gating (455, 559). For many connexins the voltage gradient required to close the channels is unlikely to occur under physiological conditions even in excitable tissue, but for connexins that are highly sensitive to voltage, functional roles have been proposed. Cx45 is the most sensitive connexin and is expressed in Purkinje fibers in the heart. These fibers are responsible for conducting action potentials from the conduction system to the working myocardium, whereas the myocytes of the myocardium express only Cx43, which is less sensitive. Cx45 closes when its cytoplasmic face becomes negative relative to the neighbor cell and thus it has been proposed that this prevents retrograde conduction from the myocardium to the conduction system. More recently, it has also been shown that even relative small stable voltage gradients between cells may increase the sensitivity of the gate (498).

Measurements on gap junctional channels in insect cells have shed light on the mechanisms underlying V_j gating. Gap junction channel recordings in insect cells provided evidence that connexins have several conductance states. In these studies, cells were patch-clamped and pushed into contact to observe the formation of electrical coupling. The opening of the first channel from the closed to the main open state was slow and appeared to be composed of a number of smaller transitions. The open channel exhibited fast transitions from the main open state to a substate with a conductance approximately 20% of the main open state. This transition was voltage dependent, and in contrast to the slow opening to the main state, the transitions to and from the substate (often referred to as the residual state) were much faster (73, 74). Although the previous observations were made in insect cells that express innexins rather than connexins, similar conductance steps have been detected in the mammalian connexins, for example, Cx37 (718), Cx43 (451), and Cx40 (71).

Based on the speed of transition, the movement to and from the closed state is termed slow gating (or loop gating), and the faster transition between the main open and residual states is called fast gating. Each hemichannel of a cell to cell channel contains its own gates, which operate in series and determine whether the channel is closed, full open, or in its residual state. For a number of connexins, macroscopic V_j gating is probably related to channel closure by the fast gate, because the fraction of the residual current correspond to the relation between the conductance of the full open and the residual state (72). However, during prolonged large V_j gradients the slow gate may also close (70) in which case the concerted voltage dependence will be affected by both gates.

Each hemichannel contains voltage gates, which may be closed or open depending on the relative polarity (see Fig. 7). In homotypic channels, the relative polarity, at which a channel closes, cannot be determined, but by pairing channels with different gating properties, it is possible to determine the polarity of gating. In the following, gating at negative polarity means that the gate closes when the cytosolic side of the hemichannel becomes negative, and positive gating occurs at relatively positive potentials. In all cases, it seems that slow gating occurs at negative polarity (72). Fast gating on the other hand may occur at either polarity depending on the connexin type, for example, Cx26 closes at positive polarity and Cx32 at negative (25). Gating at positive polarity has been demonstrated for Cx26, Cx30, Cx37, Cx40, Cx46, and Cx50, whereas Cx31, Cx31.9 (mCx30.2), Cx32, Cx43, and Cx45 gate at negative polarity (227).

Determinants of V_j gating—The V_j gating of connexins involves several parts of the molecule and so far the NT, TM1, CL, and CT have been implicated. Cx26 and Cx32 have opposite gating polarity and therefore heterotypic channels only close at one polarity (when Cx26 is relatively positive the cytoplasmic side of Cx32 will be negative and therefore both gates will close), in contrast homotypic Cx26 or Cx32 channels gate symmetrically. Verselis et al. showed that exchange of the NT and TM1 of Cx26 with that of Cx32, resulted in channels that gated asymmetrically with Cx26 channels and symmetrically with Cx32 channels. In other words, the NT and TM1 completely determined the gating (725). The polarity of gating was entirely determined by the second amino acid of the NT, which is uncharged in Cx32 (N2) and negatively charged in Cx26 (D2). Mutation of N2 in Cx32 to a negatively charged amino acid reversed the polarity of gating to positive, whereas a positive amino acid had no effect. Conversely, changing D2 of Cx26 to either an un- or positively charged amino acid, changed the gating polarity of Cx26 to negative (725). In accordance with this, connexins with positive polarity gating such as Cx26, Cx30, Cx37, Cx40, Cx46, and Cx50 are negatively charged at the NT. For channels gating at negative polarity the picture is less clear; Cx45 conforms to the rule by having a relatively positive NT. In contrast, the charge pattern of Cx31 and Cx43 resemble that of Cx26, but they gate at opposite polarity showing that other amino acids must interfere in the determination of gating polarity. For a thorough review of the effects of amino acid substitutions in the NT and TM1, see reference 227.

Voltage gating also depends on the CT and its truncation eliminates fast V_j gating of Cx32 (559), Cx40 (9), and Cx43 (449, 559) (see Fig. 8). Furthermore, interference with CT structure by attaching aequorin or green fluorescent protein (GFP) also prevents fast gating (70, 417). However, the rule of CT involvement is not universal since CT truncation of Cx50 (756) or the attachment of EGFP to Cx47 (675) does not affect gating. It has been proposed that the CT closes the channel by a “particle-receptor” mechanism similar to the “ball and chain” mechanism suggested for fast inactivation of Na channels (11). In the case of connexins, the CT acts as a gating particle, which upon binding to its receptor closes the channel to its residual state. Truncation of the CT of Cx43 eliminates fast gating without affecting slow gating and in support of the receptor-particle model, expression of the Cx43-CT as a separate entity restores fast gating (449). Similar findings were reported for Cx40 and interestingly concatenation of the Cx43-CT to the truncated Cx40 restored gating to the residual state (9).

The receptor to which the CT binds is probably located in the second half of the CL, also referred to as the L2 region. Duffy et al. showed that the CT binds the L2 region in a pH-dependent manner (155) and supporting its role in fast gating, addition of L2 peptide to the cell interior via the pipette solution prevented fast V_j gating (606); also mutation of histidine to glutamate at position 142 reduced the probability of the residual state and inhibited fast gating (620).

Gating by transmembrane voltage—V_m is sensed by connexin channels across the pore in the membrane and especially in the extracellular part of the channel. V_m dependence is pronounced in innexin expressing insect cells, but less common in connexins (72). Cx43 was shown to be gated by V_m with depolarization closing the channels. The gating depended on a region at the border of the TM4 and the CT, since truncation at amino acid 242 abolished Vm gating, whereas truncation at 257 did not (558). In the case of Cx43, uncoupling by cell membrane depolarization may be a mechanism to protect neighboring cells from the electrotonic influence of pathologically depolarized cells.

Chemical gating

Connexins are also gated by the chemical composition of the intracellular milieu, especially by pH and Ca^{2+}. This type of gating has profound physiological implications and uncoupling by these agents have been proposed to have both beneficiary/protective effects (196) and deleterious effects (13). Chemical gating may also occur due to application of exogenous chemicals and pharmacological agents, which is covered in the section on pharmacology of gap junctions.

pH-dependent gating—Intracellular acidification uncouples gap junction channels composed of Cx26 (41), Cx32 (742), Cx38 (735), Cx43 (391), Cx46 (162), Cx50 (ovine Cx49)(162), and Cx62 (mCx57) (499). The set point for half maximal uncoupling by pH is variable and the number of channels active at physiological pH depends highly on the connexin type involved. In the case of Cx46 and Cx50 most channels will be active at pH 7.2 (162), whereas only 1% of all Cx62 (mCx57) channels are open at pH 7.2 (499). Therefore, the effect of acidification or alkalinization on intercellular coupling will differ between tissues relative to the connexin expressed. Cx43 is the most well-studied connexin with regard to pH and much is known about its regulation and the mechanisms involved. Cx43 is mainly in the open configuration at pH 7.2 (163). As described for V_j-dependent gating, there is good evidence that pH gates Cx43 by a particle-receptor mechanism and truncation of the CT makes the channels insensitive to changes in pH (454). The sensitivity to pH is reintroduced by coexpression of the CT with the truncated Cx43 and furthermore Cx43-CT expression also increases the pH sensitivity of Cx32 (454). The receptor-binding site of the CT is located in the second half of the CL (amino acids 119-144, termed L2) (155) and increased binding is induced by structural changes (for references see section on structure of connexins). Another piece of evidence comes from the identification of peptides with the ability to bind the CT. One such peptide, RXP-E, binds to the Cx43-CT and partially prevents uncoupling by acidification and heptanol (621). The section on pharmacology contains a detailed description of the further development of these compounds.

Ca^{2+}-dependent gating—Increasing intracellular Ca^{2+} uncouples gap junctions in a number of tissues. This is often considered a protective mechanism, which will prevent dying cells from destroying their neighbors. However, the range of Ca^{2+} concentration needed for uncoupling varies greatly between studies and there is little consensus on the amount of Ca^{2+} needed for uncoupling (508). Synergistic effects between Ca^{2+} and pH are found in some studies (76, 477). Examining the effects of changes in Ca^{2+} and pH, the effects of pH on intracellular Ca^{2+} and vice versa, as well as the fact that the buffer capacity of the often used Ca^{2+} buffer EGTA is very sensitive to changes in pH (363) may be a confounding factor. The effect of Ca^{2+} is suggested to act via calmodulin and inhibitors of calmodulin prevents uncoupling in a number of cell types (for review see reference 508). The data is supported by expression of calmodulin mutants with increased affinity for Ca^{2+}, showing that increased affinity of calmodulin also increases Ca^{2+} sensitivity of Cx32 (509). Cx32 has been shown to contain two calmodulin binding domains (685). With an increase in

Ca^{2+}, calmodulin is believed to either physically block the channel or close the channel through a conformational change in Cx32. This may be mediated by transdomain bridging of calmodulin, where calmodulin interacts with the NT and the CT binding sites simultaneously (150). A role for calmodulin in gating has also been suggested for Cx36 (75), Cx43 (402), Cx44 (783), and Cx45 (510). In the case of Cx43, calmodulin is believed to bind the CL in a region overlapping with the L2 binding site for the CT (784), which may augment channel closure by the particle-receptor mechanism.

Control of Connexin Expression in the Cell Membrane

Besides direct regulation of gap junction channel function, the functional intercellular coupling is determined by the type of connexin and the number of channels present within gap junction plaques. Different cell types express different connexins, and both the composition and quantities may vary over time. Tissue and time specific control of connexins is also achieved by regulating mRNA levels, protein synthesis, trafficking, and degradation. In the following, mechanisms, which regulate the expression of connexins and gap junctions in the cell membrane, are reviewed and selected examples of physiological functions are given.

Connexin transcription

Transcription factor control—The expression of connexins is determined by the transcription factors, which govern the transcription of their genes. The gene structure of connexins consists of two or more exons. The entire coding region is generally placed in the last exon, which is preceded by uncoding exons (496). Exceptions to the rule are Cx36, Cx40.1 (mCx39), and Cx62 (mCx57), in which the coding region spans two exons (642). The connexin transcripts can vary in their 5′ untranslated region (5′UTR) due to differences in the transcription of the preceding non-coding exons. In some cases, these differences are linked to the activation of different promoter regions. For example, rat and human Cx32 contains two 5′UTR exons (435), each of which are preceded by a separate promoter region (263). In liver cells, the first promoter region is activated by hepatocyte nuclear factor-1 resulting in one transcript (324), whereas in Schwann cells, activation of the second promoter by Sox-10 yields a different transcript (54). In the case of mouse Cx43, the situation is considerably more complex since three promoter regions and alterative splicing produce nine different transcripts. The distribution of transcripts varies between and within tissues and since the translation efficacy of the transcripts varies greatly, it is likely that differences in protein levels are partly governed by the nature of the transcript 5′UTR (515).

Transcription of most connexins is regulated by a combination of ubiquitous and tissue specific transcription factors. An interesting example is the heart, where Cx40, Cx43, and Cx45 are differentially expressed in the chambers and conduction system. In the atria Cx40 and Cx43 are co-expressed, whereas Cx43 is the sole connexin of the ventricular myocardium (613). Expression of Cx40 is driven by the cardiac specific transcription factors Tbx5 (65) and GATA4 (385) together with the ubiquitous Sp1 (45). In early development, Cx40 is expressed in the developing ventricles up to 14 days postcoitum, but absent from the ventricle on day 19 (139). A possible explanation for the disappearance of Cx40 is that the ventricle specific transcription factor HRT2 downregulates Cx40 by repressing the transcription of Tbx5 (325) and GATA4 (304). In accordance with this hypothesis, knockout of HRT2 results in left ventricular expression of atrial genes including Cx40 (325). In the conduction system, Cx40 and to a lesser extent Cx43 are expressed only in the more distal parts (613). The reason may partly be that Cx40 and Cx43 transcription is repressed by Tbx2 (90), Tbx3 (55, 278) and in the case of Cx43 by Nkx2.5 (676). Thus, it seems that much of the specificity of connexin expression in the heart is governed by a combination of tissue specific transcription activators and repressors.

Connexins are also under the influence of ubiquitous transcription factors and may be regulated dramatically within very short time frames in important physiological functions. An example is the Cx43 upregulation in the uterus during labor, which is considered essential for the synchronization of forceful coordinated contractions (203). The expression level of Cx43 is low during pregnancy, possibly due to the action of progesterone, which inhibits Cx43 transcription (491) by downregulating AP-1 (438) and upregulating the transcription repressors p54nrb (151) and ZEB1 (551). At the time of labor, a combination of hormonal activation and stretch of the myometrium increases the expression AP-1 proteins of the Fos and Jun families and together they form an AP-1 transcription factor that activates the Cx43 promoter (513, 521, 628, 754). This increases the Cx43 protein level and the formation of gap junction plaques. Once coupling is high and contraction is stimulated, synchronous contraction occurs. The importance of Cx43 upregulation is demonstrated by the delayed parturition in mice with a smooth muscle specific knockout of Cx43 (152) and in mice carrying a dominant negative oculodentodigital dysplasia mutation in Cx43 (684). Cx43 is also upregulated in the human uterus at term (89, 95). One study has addressed the role of Cx43 in human parturition, where the Cx43 mRNA and protein levels was reduced in uterine biopsies from women experiencing prolonged labor compared to women giving normal birth (99).

Epigenetic regulation—Besides direct regulation via transcription factors, connexin mRNA/expression levels are also regulated by histone acetylation, DNA methylation, and micro-RNAs. Much of the evidence for the epigenetic regulation arises from broad inhibition of the enzymes involved, with subsequent investigation of mRNA levels, protein levels, and/or functional coupling. When interpreting the results, it is important to remember that general inhibition of epigenetic mechanisms may regulate connexin expression and function by changing the expression of other proteins. An example of this is the direct acetylation of connexins (728).

Histone acetylation: DNA is wrapped around histones in a structure called the nucleosome. The nucleosomes are involved in the packing of DNA and the tightness of this packing regulates gene transcription. Histone function is regulated by covalent modifications such as acetylation. Histone acetylation reduces the interaction between nucleosomes, loosens the chromatin structure, and promotes transcription. Acetylation is mediated by histone acetyltransferases (HATs) and deacetylation by histone deacetylases (HDACs). Histone acetylation is reported to affect several connexins in different cell types (728). Inhibition of HDACs was first shown to increase the amount of Cx43 (481) and later demonstrated for Cx36 (416) and Cx32 (729).

HDAC inhibition increases the transcription of genes that are repressed due to acetylation. Cx36 is dominantly expressed in neuronal tissue (351). Transcription of neuronal genes is repressed in other cell types by RE-1 silencing transcription factor (REST)-mediated recruitment of HDACs to the target genes. Haefliger and coworkers showed that the regulatory region of the *Cx36* gene contains a REST binding element, which recruits REST and silences Cx36 transcription in HeLa cells (416). Another study showed that HDAC inhibition or expression of a REST mutant that activates transcription, upregulated Cx36 in non-neuronal cells but not in neuronal (277), consistent with the idea that Cx36 is expressed in neurons mainly due to the lack of REST-mediated repression.

HDAC inhibitors also inhibit deacetylation of cytosolic proteins and may thereby affect gap junction function independently of transcription. This is seen in mdx mice, a genetic cardiomyopathy model, where Cx43 is redistributed from the intercalated disc to the lateral membranes (102). This condition is rescued by acetylase inhibition, whereas HDAC inhibitors induce the lateralized phenotype in control mice (102). The changes are related to

Cx43 localization rather than to its expression and demonstrate that changes in coupling after interference with acetylation are not necessarily related to the regulation of connexin gene transcription.

DNA methylation: Methylation of cytosines is a mechanism of gene regulation and hypermethylation represses gene expression. The Cx32 promoter is methylated in liver epithelium, which expresses Cx43 but not Cx32, whereas hepatocytes, which express Cx32 but not Cx43, are methylated in the Cx43 promoter (519). These results suggest that methylation plays a role in normal physiological regulation of connexin expression; however, most of our knowledge about regulation by methylation arises from studies of cancer. Cancer is often associated with reduced levels of connexin expression, which is believed to play a role in the lack of growth regulation. In HeLa cells, clones that do not express Cx43 have higher growth rates and treatment with a demethylating agent leads to reexpression of Cx43 and increased growth control (313). Methylation of the Cx43 promoter reduces Cx43 levels in nonsmall cell lung cancer by interfering with AP-1 binding (87) and downregulation of Cx43 levels in nasopharyngeal carcinoma cells is also related to promoter methylation (765). The promoter region of Cx32 is methylated and Cx32 expression low in renal carcinomas, where demethylation leads to reexpression of Cx32, indicating a causal relation (272, 761). Reduced Cx26 expression involving DNA methylation has been demonstrated for lung cancer (622), hepatocellular carcinomas (695), and breast cancer (88,672). In contrast, a study on breast cancer showed methylation of the *Cx26* gene but no correlation to Cx26 expression (634). This was also found to be the case in esophageal tumors (397). The Cx36 promoter is methylated in colon cancer (56); however, increased methylation of the Cx36 promoter does not seem to have any effect on Cx36 expression (637). In fact, when analyzing both methylation and expression data, methylation of the promoter regions of Cx36, Cx30, Cx37, and Cx45 was only found to decrease the expression of Cx45 (637). This study indicates, that although promoter methylation occurs, it does not necessarily translate into corresponding alterations in expression. Therefore, it can be concluded that DNA methylation may be involved in both physiological and pathophysiological changes in connexin expression, but care must be taken when considering determination of methylation in the absence of expression data.

Micro-RNA: The stability and translation of mRNA can be regulated by micro-RNAs and some of these regulate the expression of connexins. Micro-RNAs act by binding to the 3′-UTR of the target mRNA, and genomic analysis of the *Cx36*, *Cx43*, and *Cx45* genes indicate that multiple binding sites exist (544). A clear physiological function of micro-RNA-mediated regulation has only been established for Cx43. During skeletal muscle development, myoblasts fuse to form muscle fibers and during this process expression of Cx43 is lost. During differentiation the micro-RNAs miR1 and miR-206 are upregulated and both reduce expression of Cx43 (6, 311). The reverse is observed in osteoblasts, where differentiation is dependent on Cx43 expression. miR-206 expression in osteoblasts reduces Cx43 levels and inhibits differentiation, which can be prevented by reexpression of Cx43 even in the presence of miR-206 (286). In contrast to skeletal muscle, high expression levels of Cx43 are needed for normal conduction in the heart. Patients with coronary artery disease often have heterogeneous and reduced levels of Cx43 and they are at risk of arrhythmia. A study shows that miR-1 is upregulated in these patients, which possibly reduces Cx43 expression. When miR-1 is overexpressed in normal and infarcted rat hearts, conduction is slowed and arrhythmia susceptibility increased. These effects correlate with Cx43 repression and elimination of miR-1 reverses the effects (760). Subsequently, it was shown that miR-1 upregulation and its downstream effects are reversed by the β-adrenergic agonist propranolol. This suggests that sympathetic stimulation may regulate this pathway (400). The effects of micro-RNAs are also regulated by binding of RNA-binding proteins and in

the case of miR-1 and miR-206, it was shown that binding of Dnd1 prevented repression of Cx43 (306).

Connexin turnover and trafficking

Translation and ER sorting—Connexins are believed to be cotranslationally inserted into the ER membrane; however, the experimental evidence for this assumption is limited. It has been reported that Cx26 and Cx32 are cotranslated into ER microsomes *in vitro* and that somewhat surprisingly Cx26, but not Cx32, can be posttranslationally incorporated into ER microsomes in their native orientation (778). The extent to which this occurs *in vivo* is unknown and remains an unexplored possibility. In the ER, folding into mature connexins occurs and intramolecular disulfide bonds are formed between the extracellular loops (294, 541).

Once translated, connexins have a very high turnover rate in most cases, with half-lives of a few hours in both cultured cells and native tissues (30,177,353,691). In some cases such as chicken lens cultures, Cx45.6 and Cx56 (human orthologs: Cx50 and Cx46, respectively) turnover is much lower, around 1.5 days (291), showing that rates differ between connexins and probably also between cell types.

In certain cells with high turnover, the connexins are partly turned over by ER-associated degradation (ERAD) (460), which is responsible for removing immature, damaged, and/or misfolded proteins by retrotranslocation to the cytoplasm, polyubiquitination, and degradation by the proteasome. Monitoring of protein folding is achieved in part by chaperones; however, for connexins it has also been reported that proteolytic cleavage of the NT of misfolded connexins, can target them for degradation after overexpression or prevention of disulfide bond formation (175, 176, 713). ERAD is often initiated by recognition proteins and Lau and co-workers reported that Cx43 interacts with CIP75 (a ubiquitin-like and ubiquitin-associated domain containing protein) in the ER and that CIP75 expression determines the rate of Cx43 degradation independent of ubiquitination (378, 666). Although it is normally assumed that ERAD removes damaged/misfolded and thus dysfunctional proteins, this may not hold true for connexins, where ERAD inhibition increases both the number and functional coupling of gap junctions (713). Therefore, regulation of ERAD may be a physiological way of adjusting gap junctional communication. For example, mild heat shock and oxidative stress reduces ERAD of connexins (713), which may represent a protective mechanism whereby cells under stressful conditions can receive, for example, reducing agents such as glutathione from neighboring healthy cells (460).

The Golgi complex and oligomerization—From the ER Cx26 and Cx43 are transported by COPIImediated transport to the Golgi and depend on the small GTPase Sar1 (411, 681). *En route* to the plasmamembrane, connexins are transported through the Golgi complex, where their presence has been demonstrated (259,463,532,540,625). One exception may be Cx26, which has been proposed to bypass the Golgi (208) although this findingwas later challenged (681). Unlike most membrane proteins, connexins do not oligomerize in the ER but do so after transport into the trans-Golgi network. Connexins are found as monomers when they are prevented from exiting ER (328, 463) and fractionation studies show that Cx26 and Cx32 hexamers are exclusively present in the Golgi of liver tissue (145). Multimers have been demonstrated in ER but probably represent aberrant oligomerization when protective mechanisms become saturated. Along the same lines, low expression levels of Cx43 linked to an ER retention signal produces only monomers, whereas high expression results in substantial oligomerization (591). This feature is shared by several other connexins, where high expression also promotes oligomerization of Cx26, Cx32, and Cx43 in the ER (714).

When oligomerization occurs in the ER after overexpression of connexins, gap junction like plaques form between folds of the ER (337), which is probably an unwanted result and a possible reason that mechanisms to prevent oligomerization in the ER have evolved. The exact nature of these mechanisms is unknown but Koval and co-workers showed that ER oligomerization of Cx43 is prevented by the chaperone ERp29 (123). Possibly, such a mechanism may also exist for other connexins and chaperones.

Membrane insertion and gap junction plaque formation—Trafficking of connexins from the Golgi to the plasma membrane depends on an intact cytoskeleton. For Cx43, the budding of secretory vesicles from the Golgi requires the action of the small GTPase Arf1 (411), and the vesicles are subsequently transported to the plasma membrane. Most studies show that Cx43 transport depends on microtubules (213, 238, 360, 680), whereas transport of Cx26 (680), Cx30 (537), and Cx31 (257) do not. Cx43-GFP has been demonstrated to traffic via microtubules from the Golgi to the plasma membrane, where connexons diffuse freely and are inserted at the edges of existing gap junction plaques (360). Using an elegant approach of Cx43 pulse labeling, Gaietta et al. showed that new Cx43 was added at plaque edges, surrounding older Cx43 in the center (193). Once in the plaque, the movement of Cx43 is highly restricted without mixing of new and old Cx43 (193,360), but this feature is probably not universal to all connexins since the fluorescence of bleached centers in Cx26 plaques were rapidly repopulated by diffusion of Cx26 from the unbleached plaque perimeter (681). However, the concept of free diffusion and recruitment to the outer perimeter of existing plaques has been challenged as a universal mechanism. Shaw and co-workers showed that transport of Cx43 via microtubules depends on the microtubule plus end tracking protein EB1, which connects microtubules to adherens junctions and selectively guides Cx43 to these sites. The study also showed that repopulation of plaques after photobleaching occurred only at the plaque sites and not in neighboring areas, indicating a more direct insertion in the plaque area (617). Such transport mechanisms may be particularly important in tissues with highly organized gap junction structure such as cardiomyocytes, and a subsequent study showed that EB1 is displaced from adherens junctions in stressed myocardium, resulting in reduced insertion of Cx43 at the intercalated discs (639).

Incorporation into plaques renders Cx43 insoluble in the mild detergent Triton X-100, an event that coincides with the phosphorylation into the P2 isoform (462). Both phosphorylation and the acquisition of Triton insolubility is considered a hallmark of communication competent gap junctions and although the exact overall phosphorylation pattern of the P2 form may vary, it is clear that Cx43 phosphorylated at S325/328/330 or S328/330 migrate as the P2 form (355) (for further information on connexin phosphorylation, please see the section on posttranslational regulation).

Internalization—Double membrane gap junction vesicles (termed annular gap junctions) were early on demonstrated by electron microscopy (217, 359). These annular gap junctions were later demonstrated to arise when one cell internalizes not only its own connexins but also those from a neighboring cell to which they are docked (296,468). It has been suggested that annular gap junctions arise when whole plaques or large parts thereof are internalized, but for Cx26 and Cx32, it was shown that plaques are also internalized as smaller aggregates (191).

In 1979, Larsen et al. reported that annular gap junctions are associated with clathrin (359). Indeed Cx43 is internalized by a clathrin-dependent mechanism (283, 468, 520) with clathrin being exclusively located at the cytoplasmic surface of the junction (474). Furthermore, inhibition of clathrinmediated endocytosis prevents internalization in response to epidermal growth factor (EGF) (367) and the carcinogen DDT (180). Some components

of the machinery were later identified, among these were the adaptor proteins Dab2 and AP-2, and the GTPase dynamin (237, 520). Dynamin is responsible for the scission of vesicles from the plasma membrane, and dynamin complexes with Cx43 and the nonreceptor tyrosine kinase c-Src. Dynamin is subsequently activated by c-Src, which induces internalization of Cx43 (215). Clathrindependent endocytosis is also regulated by the small GTPase Arf6 whilst expression of a GTP restricted Arf6 mutant promotes internalization of Cx43 (411), supporting the importance of clathrin-mediated mechanisms.

Calveolin-mediated endocytosis has been implied by the colocalization of calveolins with connexins (358, 380, 599); however, data suggest that the role of calveolin may be to guide connexins from the Golgi to the plasmamembrane or to stabilize them at the membrane, since calveolin knockdown inhibits intercellular communication (358).

Given the short half-life of connexins, internalization appears to occur continuously, and we know now of several stimuli and physiological processes, which regulate this internalization. Most of these also stimulate degradation and will be discussed later.

Degradation and recycling—Connexin degradation depends on the action of both the lysosomes and the proteasome Targeting for internalization and lysosomal degradation is obtained by monoubiquitination, whereas polyubiquitination targets for proteasomal degradation (317). Proteasomal involvement in connexin breakdown was first described by Laing et al., who showed that a proteasomal inhibitor increased the Cx43 content 6-fold (348). Since then, it has been demonstrated that Cx43 is subject to ubiquitination, endocytosis, and degradation in response to EGF (367), phorbol esters (368), TNF-α (312), and Gαqcoupled receptors (442). Also, in lens epithelial cells where Cx43 and Cx46 are reciprocally expressed, the expression of Cx46 downregulates Cx43 by ubiquitination and subsequent degradation of Cx43 (19).

The proteasome only degrades unfolded proteins and since connexins are internalized as annular gap junctions, the role of the proteasome has been unclear. Combinations of lysosomal and proteasomal inhibitors suggest that degradation is sensitive to both in an nonadditive manner, but there is little evidence to support a sequential mode of action (460).

Several studies show that proteasomal inhibitors prevent the internalization of Cx43 (348,350,535). This finding led to the proposal that proteasomes regulate endocytosis of Cx43 independent of its actual degradation. One possible mechanism is that inhibition of the proteasome prevents deubiquitination of its substrates and thus leads to a depletion of free ubiquitin. This hypothesis is supported by thework of Rivedal and co-workers, who showed that proteasomal inhibition prevents ubiquitination of Cx43 (368) and depletes free ubiquitin (366). Likewise, proteasomal inhibition reduces free ubiquitin and prevents ubiquitination of Cx43 after stimulation of Gαq-coupled receptors in rat neonatal cardiomyocytes (442).

In the final step of ubiquitination, ubiquitin is transferred to its target by E3 ubiquitin ligases, many of which recognize PY-motifs in their targets. Such a motif is found in Cx43 and its mutation increased the amount of Cx43 by decreasing its degradation (678). Subsequently, it was shown that the ubiquitin ligase Nedd4 binds the C-terminus of Cx43 and although knockdown of Nedd4 in rat liver epithelial cells did not alter the total amount of Cx43, membrane localization of Cx43 was increased (372). Since then interaction with Nedd4 has been verified in both Cos-cells (218), corneal fibroblasts (312), and in cardiomyocytes (442). Furthermore, Cx43 was found to be monoubiquitinated (368) suggesting that ubiquitin is essential in targeting Cx43 to lysosomal degradation. The role of ubiquitination is summarized in Figure 9.

Annular gap junctions are detected in lysosomes by EM (217). Consistent with the importance of lysosomal breakdown, several studies show that inhibition of lysosomal activity increases the level of Cx43 protein (30, 348, 349, 350, 442, 464, 535, 678). In a number of cell types, this is associated with the accumulation of connexin positive intracellular vesicles (464, 535, 678). In contrast, lysosomal inhibition in cardiac cells slows the rate of Cx43 degradation but increases the amount of Cx43 in gap junction plaques (30,349) and prevents uncoupling after stimulation of Gαq-coupled receptors (442) (442).

Gap junctions may also be degraded by autophagy, and annular gap junctions sequestrated by double membrane structures have been reported (516). Such structures are found in cardiac cells, where some Cx43 colocalizes with the autophagosomal marker LC3. The association of both Cx43 and LC3 with lipid rafts is increased in heart failure, suggesting that autophagy plays a role in degrading Cx43 in this condition (268). Autophagy also degrades Cx43 and Cx50 in normal rat kidney (NRK) cells during starvation. Intracellular connexins were found to be enclosed in a structure containing LC3 and colocalized with p62, a protein that targets ubiquitinated proteins for autophagy. Furthermore, knockdown of Atg5, which is required for autophagy, prevented starvation induced connexin degradation (379).

Instead of being degraded, some internalized connexin may be recycled back to the plasmamembrane, as suggested by the colocalization of internalized Cx43 with rab4 and rab11, which are markers of recycling endosomes (215). This phenomenon may be particularly important during mitosis, where Cx43 is endocytosed and recycled to the plasmamembrane in the final stages of mitosis (48).

Posttranslational Regulation of Connexins

Chemical modification of connexins after their translation is an important tool for the regulation of gap junction channels in all types of tissue. Gap junctions are known to be highly regulated by changes in connexin phosphorylation and ubiquitination, but acetylation, hydroxylation, glutamate γ-carboxylation, methylation, palmitoylation, oxidation, deamidation, *S*-nitrosylation, and truncation have also been described (393, 394, 552, 736). Some posttranslational modifications are involved in the gating of electrical and metabolic coupling of mature gap junction channels, whilst others are involved in regulation of the connexin “lifecycle” such as trafficking, assembling, disassembling, and degradation. With the exception of ubiquitination (which was covered in the section on connexin turnover and trafficking), the following section will give an overview of the current knowledge of posttranslational modification of the different connexin family members, how these modifications are mediated, and what the functional consequences are.

Phosphorylation of connexins

The most extensively studied posttranslational modification of connexins is undoubtedly phopshorylation. That connexins are phosphoproteins was first discovered in the mid 1980s (578, 669). Since then a variety of phosphorylation sites have been identified. Classically, connexin phosphorylation is analyzed by studying their electrophoretic mobility, by direct incorporation of P^{32}-phosphate, or by the use of phosphospecific antibodies. When analyzed by SDS-PAGE most connexins display multiple electrophoretic isoforms. For Cx43, this includes a fast migrating “nonphosphorylated” (P0 or NP) band (P0 is not necessarily completely dephosphorylated) and at least two slower migrating bands, usually called P1 and P2 (461). Following alkaline phosphoatase treatment both P1 and P2 end up comigrating with P0 (461). This indicates that phosphorylation is the main posttranslational modification, which determines electrophoretic mobility of Cx43. Incorporation of radio-labeled phosphate into protein species is one of the oldest biochemical methods for

phosphorylation analysis and it is also used to study connexin phosphorylation. However, this technique is mostly used for in vitro studies. Finally, phosphor-specific antibodies are also useful to study connexin phosphorylation. With this technique, it is important to note that p-Ser and p-Thr antibodies tend to cross react with other negative charges, which may produce a high number of false positives. Also, the high number and proximity of phosphorylation sites, which are known for most connexins, makes it difficult to develop highly site-specific phosphoantibodies for the use in gap junction phosphorylation analysis.

More recently, mass spectrometry (MS) has revolutionized the area of phospho-proteomics, including the phosphorylation analysis of connexins. This technique enables sitespecific phosphorylation analysis of the entire protein and is able to identify and sequence phosphopeptides even at picomolar level. Furthermore, tandem MS is capable of assigning phosphorylation sites in multiphosphorylated proteins such as connexins, to their specific amino acids.

Using the different available techniques, posttranslational regulation by phosphorylation has been described for all the known connexin family members. With the exception of Cx26, all known connexins contain phosphorylation sites located in their CT tail. In addition, Cx26 (393) as well as Cx36 (700), Cx50 (736), and Cx56 (40) contain phosphorylation sites in the CL, and both Cx26 and Cx32 are reported to be phosphorylated in the NT tail when expressed in HeLa cells, but not when purified from mouse liver (394). Furthermore, several phosphorylation sites are indicated in the extracellular domain E2 of Cx26 (393). It should be noted that not all connexins have been subject to systematic MS analysis of phosphorylation sites. Therefore, it is reasonable to assume that additional phosphorylation sites may be identified in the future.

Phosphorylation events are essential for the correct oligomerization of connexins to connexons and also for the correct composition and function of complete dodecameric gap junction channels. The intracellular CT domain, which contains the majority of identified phosphorylation sites, is highly variable amongst different connexin family members. Therefore, phosphorylation of the CT tail influences the function of gap junction channels in a connexin-specific manner.

Phosphorylation of Connexin43—Cx43 is the most abundant and ubiquitously expressed connexin in mammalian cells and also the most extensively studied connexin. The immature Cx43 protein is initially translated as a 40 kDa protein chain, which is phosphorylated soon after synthesis. The phosphorylation status of the protein then changes as it traffics through the endoplasmatic reticulum and Golgi to the plasma membrane, ultimately forming a gap junction channel. (For further information on connexin synthesis and trafficking, see previous section). To date, 21 putative phosphorylation sites have been identified in the CT domain of Cx43 (Table 3).

As seen in Table 3, the phosphorylation sites in Cx43 are primarily serine amino acids (for references see Table 3); however, phosphorylation of Tyr247 and Tyr265 (93, 356, 357, 383, 645, 687), as well as threonine phosphorylation (122) has also been reported for Cx43. Some of the specific phosphorylation sites in Cx43 are implicated in the regulation of electrical and metabolic coupling of mature gap junction channels; whereas other sites seem to regulate the various stages of the connexin “life cycle” including hemichannel oligomerization, export of the protein to the plasma membrane, gap junction assembly, and connexin degradation (356, 357).

Moreno and colleagues were the first to show that gap junction channels composed of Cx43 exhibit distinct unitary conductance values, which are correlated with the phosphorylation

state of the protein (450, 452). Shortly after, Kwak et al. found that activation of protein kinase G (PKG) by 8-bromoguanosine 3′:5′-cyclicmonophosphate (8Br-cGMP) increased incorporation of P^{32} into rat Cx43 (but not human Cx43) expressed in SKHep1 cells, which was associated with a decreased gap junctional conductance (344). Most likely, PKG phosphorylates Ser257 of rat Cx43. In the human Cx43 sequence, amino acid residue 257 is replaced by an alanine, which cannot be phosphorylated. Later on, dephosphorylation of Ser368 in Cx43 was shown to occur with a time course similar to that of electrical uncoupling of cardiomyocytes in the ischemic heart, with no concomitant changes in the total amount of Cx43 (31). Finally, a MS study conducted on Cx43 purified from Langendorff perfused rat hearts exposed to different time frames of ischemia showed various time dependent changes in Cx43 phosphorylation (14). Specifically, this study showed that Ser306 become fully dephosphorylated within the first 7 min of ischemia, whereas Ser330 become phosphorylated. Between 15 and 30 min of ischemia, the critical time interval where gap junction uncoupling occurs, Ser297 and Ser368 also become fully dephosphorylated. In another study, ischemic preconditioning of isolated rat hearts was found to cause a 34% decrease in the maximal rate of uncoupling together with a diminished overall dephosphorylation of Cx43 during a subsequent period of prolonged ischemia (287). Based on this observation, the authors concluded that preservation of phosphorylated Cx43 in gap junctions is responsible for delayed uncoupling induced by preconditioning. Taken together, these findings shows that site specific phosphorylation of Cx43 is a key mechanism for the regulation of electrical coupling through gap junction channels composed of Cx43. Furthermore, the gating of Cx43 channels seem to be based on an interplay between various phosphorylation sites.

To further address the functional consequences of the site specific phosphorylations involved in gap junction uncoupling during ischemia, Procida and co-workers (530) conducted a study examining the effect of serine to alanine substitutions of position 296, 297, and 306 of Cx43. (An alanine substitution mimics a constitutively dephosphorylated form of a serine residue.) Alanine substitution of Ser296 or Ser297 in Cx43 had no significant effect on either macroscopic electrical coupling or single-channel conductance in HeLa cells. However, an alanine substitution of Ser306 was found to reduce electrical coupling to 57% (530). The reduced coupling occurred in the absence of changes in the total number of gap junction channels. Instead the change in electrical coupling was mediated by a reduced single-channel conductance (530). Based on these data, it seems reasonable to conclude that Ser306 is an important regulatory phosphorylation site, which is involved in electrical uncoupling during cardiac ischemia.

When Ser306 was first discovered as a phosphorylation site, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and other kinases were tested for their ability to phosphorylate small synthetic peptides containing this specific phosphorylation site. However, the responsible kinase was not identified. In contrast, a recent study using a peptide containing the full length of the CT tail of Cx43, showed that Ser306 is a target for *in vitro* phosphorylation by CaMKII (282). CaMKII is known to play a central role in the regulation of a variety of cellular functions in the heart such as Ca^{2+} homeostasis, transcription, and apoptosis (61,785). In addition, increased expression and activation of CaMKII occurs in cardiac disease states such as hypertrophy, heart failure, myocardial ischemia, and infarction (410, 769). However, the specific role of CaMKII in the regulation of Ser306 during ischemia remains to be further investigated.

Another specific phosphorylation site of Cx43, which seems to be highly involved in the gating of mature gap junction channels, is Ser368. As already mentioned, Ser368 becomes dephosphorylated during cardiac ischemia with a time course similar to that of electrical uncoupling (14,31). Studies have also shown that 12-O-tetradecanoyl-phorbol 13-acetate

(TPA)-induced protein kinase C (PKC) phosphorylation of Cx43 is associated with reduced single-channel conductance (346, 354), which can be prevented by substitution of Ser368 to an alanine (354). However, it should be noted that PKC may not only phosphorylate Ser368 but also Ser365, Ser369, Ser372, and Ser373 of Cx43 (616). Despite the reduction in single-channel conductance following TPA-induced Cx43 phosphorylation, Kwak and co-workers found that the macroscopic electrical coupling was increased (346). This indicates that PKC-induced phosphorylation influences the open probability of Cx43 gap junction channels.

The effect of Ser368 phosphorylation on dye coupling has been examined in cardiomyocytes, HeLa cells, and fibroblasts, but with contradicting results. In HeLa cells, it was found that mutation of Ser368 to an alanine results in a slight (statically insignificant) decrease in dye coupling compared to wt Cx43 (767). However, when alanine substitutions were introduced into Ser365, Ser368, Ser369, and Ser373 all at once, dye coupling was severely reduced (767). This implies that phosphorylation of Ser368 itself is not crucial for Cx43-mediated dye coupling in HeLa cells, whereas simultaneous dephosphorylation of all of the phosphorylation sites located in the extreme CT region causes a marked drop in dye transfer. Contradictory to these findings, TPA-induced PKC phosphorylation of Cx43 has been shown to contribute to a decrease in dye coupling in both neonatal cardiomyocytes (346) and fibroblasts (354), which is probably mediated by a conformational change of Cx43 (22). Further studies are needed to determine the reason for the different findings on the effect of Ser368 and PKC phosphorylation on dye coupling, but one potential explanation could simply be the differences in cell systems used. All together, these data shows that PKC and the phosphorylation sites (including Ser368) located in the extreme CT region of Cx43 play a key role in the regulation of both electrical and metabolic coupling of gap junction channels composed of Cx43.

Compared to PKC, Cx43 has been found to be a relative poor substrate for cAMP-dependent protein kinase (PKA). However, PKA is able to phosphorylate both Ser364 and Ser365 in Cx43 and phosphorylation of Ser364 and/or Ser365 seems to accelerate a subsequent PKC-induced phosphorylation of other serines in the 362 to 376 region (616). In support of this, H89 (a PKA inhibitor) was found to inhibit follicle stimulating hormone (FSH)-induced phosphorylation of Ser365, Ser368, Ser369, and Ser373 of Cx43 in granulosa cells (768). However, another study found that Ser365 phosphorylation or a mimicked phosphorylation (mutation of Ser365 to an Asp) reduces PKC-induced phosphorylation of Ser368 during ischemia (646). Though contradictory, these findings illustrate that different phosphorylation sites may interact with each other and add to the complexity of Cx43 phosphorylation.

Tyrosine phosphorylation is also a subject for contradicting results regarding its role in the regulation of Cx43 gap junction channels. Src kinase-mediated phosphorylation of Tyr265 has been found to reduce electric conductance between fibroblasts without affecting the expression of mature gap junction channels (212, 527). On the other hand, Tyr265 has also been implicated in the regulation of an interaction between Cx43 and the cytoskeleton protein zonula occludens-1 (ZO-1), which is important for the localization of Cx43 at the intercalated discs of cardiomyocytes (687). Thereby, in contrast to the findings by Giepmans and co-workers, phosphorylation of Tyr265 would lead to a reduction in the amount of Cx43 present at the cell surface.

In addition to phosphorylating tyrosine residues in Cx43, Src kinase may also induce mitogen-activated protein kinase (MAPK) to phosphorylate Cx43. MAPK is able to phosphorylate Ser255, Ser279, and Ser282 of Cx43 (739). Phosphorylation of these MAPK sites is not required for normal gap junction assembly in HeLa cells, but phosphorylation of Ser279 and/or Ser282 is correlated with a decrease in both dye coupling and electrical conductance (738). Inhibition of dye coupling by EGF has also been linked to direct

phosphorylation of Cx43 by MAPK (565). Studies have indicated that TPA-induced uncoupling of Cx43 gap junction channels is not only mediated by PKC phosphorylation, but rather by interplay between PKC and the MAPK pathway (565, 638).

Ser325, Ser328, and/or Ser330 are closely involved in the trafficking of Cx43. Cooper and Lampe found that casein kinase 1 (CK1)-induced phosphorylation of Ser325, Ser328, or Ser330 regulate Cx43 gap junction assembly in normal rat kidney cells (109). Also, using an antibody specific for phosphorylation at Ser325, Ser328, and Ser330, it was shown that these sites are phosphorylated in the gap junction plaqueassociated isoform of Cx43 (643). Furthermore, during cardiac ischemia, relocation of Cx43 from the intercalated disk to the lateral edges of the myocytes, is correlated with dephosphorylation of Ser325, Ser328, and/or Ser330 (355). Also, mouse fibroblasts expressing Cx43 in which Ser325, Ser328, or Ser330 is mutated to alanine show decreased dye coupling and significantly delayed development of electrical coupling compared to cells expressing wt Cx43 (355). Taken together, these data demonstrate a role for phosphorylation of Ser235, Ser328, and Ser330 in the regulation of efficient gap junction assembly and Cx43 lateralization.

As already indicated, some of the contradicting findings regarding the effect of site-specific Cx43 phosphorylation may be explained by the differences in test systems, that is, different cell types or studies performed on either cell cultures or tissue. A study has shown that most Cx43 exists in a semiphosphorylated or dephosphorylated form when expressed in HeLa cells (644), whereas cardiomyocytes have been shown to contain mainly phosphorylated Cx43 (353). Importantly, it must also be noted that electric and metabolic coupling may be conversely affected. Studies by Kwak and co-workers have found that TPA-induced PKC activity in cardiomyocytes leads to increased gap junction conductance accompanied by a fall in dye coupling (342,346). Also, another study evaluating several Cx43 expressing cell lines showed no correlation between electrical and metabolic coupling (164). These observations are important since some studies only measure either electrical conductance or dye transfer and uses the result as a measure for changes in gap junction communication. In addition, the ability to phosphorylate and/or the effects of site-specific changes in Cx43 phosphorylation may depend on the existing phosphorylation state of Cx43 (616, 768). Finally, is seems that several kinases are able to phosphorylate some of the specific phosphorylation sites located in Cx43 (Table 3). All of this underlines the extremely complex nature of Cx43 phosphorylation.

Dephosphorylation of Connexin43 by protein phosphatases—Compared to the knowledge we have about protein kinases involved in phosphorylation of connexins, far less is currently known about the protein phosphatases (PPs), which are responsible for dephosphorylation of connexins.

Based on their amino acid sequences and crystal structures, phosphoserine and phosphothreonine PPs are divided into three different gene families: phospho-proteinphosphatases (PPPs), metal-dependent protein phosphatases (PPM), and aspartate-based phosphatases such as FCP [TFIIF-associating component of RNA polymerase II carboxyterminal domain (CTD) phosphatase] and small CTD phosphatase (SCP) (619). PPPs are the largest class of PPs and they are further divided into the subgroups PP type 1, 2A, 2B, 4, 5, 6, and 7 based on their sequence (101, 619).

Currently, PP1, PP2A, and PP2B have been connected to dephosphorylation of Cx43 (267). Chronic activation of PP2B is associated with decreased expression levels and redistribution of Cx43 in mouse hearts. Furthermore, the present Cx43 is found mainly in its dephopshorylated form (91). Okadaic acid and calyculin A (potent inhibitors of PP1 and PP2A) both delay electrical uncoupling of gap junctions during ATPdeprived conditions in

neonatal cardiomyocytes (158). However, an even better preservation of conductance was obtained in the presence of the selective PP1 inhibitors heparin and PP inhibitor 2 (I2) (158). Furthermore, in the presence of ATP, addition of *p*-nitrophenyl phosphate (a specific PP1 stimulator) led to gradual gap junction uncoupling, unless I2 was added simultaneously (158). Together, data clearly indicate a role for PP1 in the regulation of gap junctions during ATP depletion. This conclusion is further supported by findings in isolated perfused rat hearts and adult cardiomyocytes, where the presence of okadaic acid and calyculin A, but not fostriecin (a selective PP2A inhibitor) was found to decrease Cx43 dephosphorylation during simulated ischemia (290). In contrast to these findings, it was previously shown that fostriecin mimics the effect of ischemic preconditioning in ischemic adult cardiomyocytes from both pigs and rabbits (12). Furthermore, inhibition of PP2A was also found to reduce infarct size following myocardial infarction in isolated rabbit hearts, even when administrated after the onset of ischemia (740). This indicates a role for Cx43 dephosphorylation in infarct development. The authors further found that the effect of PP2A inhibition on infarct size was similar to that of ischemic preconditioning, even though ischemic preconditioning had no effect on the activities of either PP1 or PP2A (740).

At normal physiological conditions, both PP1 and PP2A have been found to colocalize with Cx43 in the left ventricle of rabbit hearts (3). Ai and Pogwizd further found that heart failure induced by aortic constriction caused an increase in the proportion of nonphosphorylated Cx43, which was accompanied by an increased level (over 2.5-fold) of colocalized PP2A, whereas colocalized PP1 remained unchanged (3). PP1 and PP2A are also present in the left ventricle of minipigs; however, only PP2A was found to colocalize with Cx43 in this species (686). When isolated hearts were exposed to 90 min of low flow ischemia, it induced dephosphorylation of Cx43 along with an increase in total PP2A levels. However, the amount of PP2A, which coprecipitated with Cx43, was not affected during ischemia. Furthermore, Totzeck et al. found that although ischemic preconditioning preserved Cx43 phosphorylation during ischemia, ischemic precondition did not affect PP2A levels or activity (686). Since the lack of effect of ischemic preconditioning on PP2A as well as PP1 level and activity was also shown for rat hearts (740), it seems that preservation of Cx43 phosphorylation by ischemic preconditioning may not be directly mediated by changes in phosphatase activity. However, the presented data leave no doubt that PPs are involved in the regulation of Cx43 phosphorylation during different pathophysiological conditions.

Posttranslational modifications of Cx26 and Cx32—Posttranslational modifications of Cx26 and Cx32 (the major liver gap junction proteins) immunopurified from HeLa cells and mouse liver have been examined by matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF-MS) (394). This study was the first to identify posttranslational modifications of Cx26, which include hydroxylation and/or phosphorylation in the NT domain, as well as γ-carboxylation in the CL. Importantly, they also found that the postranslational modifications differ depending on whether Cx26 is expressed in liver tissue or HeLa cells. Specifically, the NT of Cx26 was hydroxylated (in position Asp2 or Asn14) when expressed in liver cells, whereas the same peptide fragment was phosphorylated (in position Asp2, Thr5, or Ser8) when expressed in HeLa cells. Furthermore, the CL of Cx26 purified from mouse liver contained γ-carboxylations (on Glu114, Glu119, or Glu120), which was not detected in Cx26 from HeLa cells (394). Later on, acetylation of the NT domain and at several lysine residues of the CT tail have been identified, along with additional hydroxylation sites in the CL, γ-carboxylation sites in the extracellular domain E1, methylation sites in E1 and the CT domain, as well as several phosphorylation sites in E2 (393). Many of the identified posttranslational modification sites of Cx26 have also been connected to disease-causing mutations. However, the exact regulatory and functional role of the posttranslational modifications of these sites remains to be established.

The posttranslational modifications identified for Cx32 from mouse liver are phosphorylation of the NT domain (Thr4, Tyr7, Thr8, or Ser11), γ-carboxylation of the CL (two of Glu109, Glu118, or Glu119), as well as palmitoylation of the CT (either Cys280 or Cys283). γ-carboxylation of the CL was also detected in Cx32 from HeLa cells. However, in contrast to the posttranslational modifications identified in liver cells, phosphorylation or hydroxylation of the NT domain (Asn2 or Asn14) and phosphorylation of the CT tail (His237, Ser233, or Ser240) was identified for Cx32 from HeLa cells (394). In addition to the posttranslational modification sites identified for Cx32 by MS, studies in hepatocytes have shown that Ser233 can be phosphorylated by PKA and PKC (580). Furthermore, CaMKII was also found to phosphorylate unspecified serine and threonine residues, but not Ser233 in Cx32 (580).

As for Cx43, Cx32 has also been identified as a target for EGF receptor tyrosine kinase (146). Itwas shown that calmodulin binds to Cx32 in the absence of calcium, which thereby prevents phosphorylation of Cx32 by EGF-receptor tyrosine kinase. This led the authors to conclude that the phosphorylation site(s) for EGF-receptor tyrosine kinase is located in a calmodulin-binding site of Cx32. Both residue 1-27 in the NT domain and residue 216 to 230 of the CT tail of Cx32 have been identified as calmodulin binding sites in Cx32 (685). Since Tyr7 was identified as a potential phosphorylation site in Cx32 purified form both mouse liver and HeLa cells (394), this could, potentially, be the target for EGF-receptor tyrosine kinase. However, this remains to be experimentally verified.

The available data on different posttranslational modifications of Cx26 and Cx32 indicate that in addition to the well-described phosphorylation of connexins, many other posttranslational modifications are also involved in the regulation of gap junction channels. Furthermore, each connexin seem to be differently processed depending on the cellular background, which is important when comparing studies conducted in different cell or tissue systems.

Phosphorylation of Connexin40 and Connexin45—The posttranslational regulation of Cx40 and Cx45 has been studied less extensively than other connexins. Activation of both PKA and PKC have been found to increase P^{32} incorporation in to Cx40 in transfected human cells (689) and administration of 8-Br-cAMP causes a mobility shift of Cx40 when examined by western blotting (710). When expressed in SKHep1 cells, PKA-induced phosphorylation of Cx40 results in increased macroscopic gap junction conductance and Lucifer Yellow permeability of Cx40 gap junction channels (710). Cx40 is also phosphorylated in neural layers of the mammalian retina (421) and dephosphorylation of Cx40 was recently found to be a key mechanism in electrical uncoupling between microvascular endothelial cells (ECs) during sepsis (52). Specifically, Bolen and co-workers found that reduced serine phosphorylation of Cx40 is responsible for reduced electrical coupling following exposure to lipopolysaccharide (LPS) during hypoxia and reoxygenation. Furthermore, they found that this uncoupling could be prevented by PKA activation and mimicked in control cells by PKA inhibition (52).

In addition to the vasculature and retina, Cx40 is also highly expressed in the atria and conducting system of the myocardium (231). Changes in Cx40 expression and distribution, as well as *Cx40* gene mutations and polymorphisms all play a role in development and maintenance of atrial fibrillation (AF) (84). Currently, the role of posttranslational modifications of Cx40 in AF remains unknown. However, based on the important role phosphorylation of Cx43 has in ventricular arrhythmias, it seems reasonable to assume that phosphorylation or other posttranslational modifications of Cx40 may also play a significant role in AF.

Serine phosphorylation of Cx45 has been shown in a variety of cell types (79, 122). Western blots show that Cx45 migrate as a doublet including a 46 and a 48 kD band, where the 48 kDa band represents the phosphorylated form (711). The specific phosphorylation sites of Cx45 remain to be determined, but the relative intensity of the 48 kDa band in a western blot increases upon activation of PKA and MAPK (711). Furthermore, PKC activation has been reported to decrease Cx45 single-channel conductance (341) and increase the macroscopic conductance (711) of Cx45 gap junction channels. However, the effect on macroscopic conductance occurred in the absence of any visible changes in Cx45 phosphorylation when examined by western blot (711). In contrast, activation of PKA or MAPK, which increases Cx45 phosphorylation, decreases macroscopic junctional conductance (711). These studies demonstrate that various protein kinases are differentially involved in phosphorylation and regulation of electrical coupling of gap junction channels composed of Cx45.

Posttranslational modifications of Connexin46 and Connexin50—Cx46 and Cx50 combine to form the gap junctions in ocular lens fiber cells. These gap junctions play an important role in normal lens growth and maintenance of lens transparency (225, 569, 607) and mutations in the *Cx46* and *Cx50* genes are associated with development of cataracts (405, 661). (For further information on the physiological role of Cx46 and Cx50 in the eye, see Section “Physiological Function in Cellto-Cell Communication.”)

In addition to posttranslational regulation by phosphorylation, CT cleavage of lens fiber connexins has also been proposed to occur naturally during the maturation of lens fiber cells (382, 766). Calpain was identified as the enzyme that removes a 32-kDa portion of the CT tail of bovine Cx50 and the same study identified Glu290 and Ser300 as cleavage sites (382). In contrast, two other studies have identified His284, Phe286, and Glu290 (618, 736) as targets for truncation of bovine Cx50 and yet another study has identified residue Glu365 as a target for caspase-3-like protease induced truncation in chicken Cx45.6 (the chicken homologue of Cx50) (766). Yin and co-workers further showed that the cleavage of chicken Cx45.6 was inhibited by casein kinase II (CKII)-induced phosphorylation of Ser363 (766), indicating that chicken Cx45.6 truncation is regulated by phosphorylation. A recent MS study identified a total of 18 and 11 phosphorylation sites in bovine Cx50 and Cx46, respectively (736). For Cx46, all of the identified phosphorylation sites are located in the CT domain, whereas three of the identified phosphorylation sites in Cx50 are located in the CL (736). In addition, five truncation sites, all located in the CT region, were identified for Cx50, whereas seventeen truncation sites, located in the NT, the CT, and in the CL, were observed in Cx46. Furthermore, the distribution of truncations at the various sites for both Cx50 and Cx46was highly variable between samples obtained from the nucleus and from the cortex of the lens. Some truncations are specific to either nucleus or cortex samples, whereas other sites are detected in both samples (736). Based on these data, it seems reasonable to conclude that truncation of Cx46 and Cx50 is a highly regulated mechanism, which could (at least partly) be controlled through the phosphorylation status of the protein.

The physiological consequences of truncation and phosphorylation status of lens fiber connexins have not been completely elucidated. However, the CT regions of Cx50 and Cx46, which contains most of the identified phosphorylation and truncation sites, seem to play important roles in channel gating and permeability. Several studies have shown that cleavage of the CT does not abolish the ability to form functional channels (140,162,756). Some studies have shown that truncated lens connexins retain their sensitivity to cytosolic acidification (140, 162, 662), whereas others have found that cleavage of the CT of Cx50 abolishes pH gating *in vitro* (756). However, differences in experimental design may again underlie these conflicting results. The greatest physiological consequence of Cx50 truncation is a significant reduction in macroscopic coupling, which is found to occur without affecting unitary conductance (140, 656). However, the specific effect of decreased

macroscopic conductance in lens fiber cells and how this may contribute to the development of cataracts still remains to be established.

S-nitrosylation of connexins

S-nitrosylation, the covalent attachment of a nitrogen monoxide group to the thiol side chain of cysteine, has emerged as an important mechanism for dynamic, posttranslational regulation of many proteins, in a way comparable to that of phosphorylation. *S*-nitrosylation represents a form of redox modulation, which may occur during oxidative stress, and it is believed to be a common mediator of nitric oxide (NO) effects (269,419). In support of a role of cysteine residue nitrosylation in Cx43 hemichannel opening, Retamal et al. found that a brief application of dithiothreitol (DTT) to metabolically inhibited astrocytes decreases dye uptake and *S*-nitrosylation of surface Cx43 without any apparent effect on the degree of dephosphorylation (556). Moreover, they demonstrated that dye uptake induced by NO donors is greatly reduced by DTT and by hemichannel blockers. Notably, NO donors had little effect on the amount of surface Cx43 or its phosphorylation state (556).

Another study have also emphasized a critical role for *S*-nitrosylation/denitrosylation of Cx43 in the regulation of communication between smooth muscle cells and ECs in the blood vessel wall (664). They found that Cx43 is constitutively *S*-nitrosylated on Cys271 by active endothelial NO synthase compartmentalized at the myoendothelial junction, whereby the permeability of the gap junction channel is enhanced. Conversely, stimulation of smooth muscle cells with the constrictor phenylephrine caused Cx43 to become denitrosylated by *S*-nitrosoglutathione reductase, which thereby attenuated channel permeability (664).

A single study on Cx46 *S*-nitrosylation has also been conducted. Retamal and co-workers examined the effects of the NO donor *S*-nitrosoglutathione (GSNO) on the electrical properties and fluorescent-dye permeability of Cx46 hemichannels expressed in *Xenopus laevis* oocytes (557). GSNO was found to enhance voltage sensitivity, increase tailcurrent amplitude, and change activation and closing kinetics of hemichannels composed of both wt Cx46 and a Cx46 mutant, in which the CT domain was replaced with that of Cx43 (557). However, the effect of GSNO vanished when hemichannels was composed of mutated Cx46, in which the intracellular and TM helix cysteines were mutated to alanine. Therefore, they concluded that Cx46 hemichannels are sensitive to NO and that the NO effects are mediated by *S*-nitrosylation of one or more of the intracellular cysteines. However, at the same time they also found that NO had no major effects on Cx46 hemichannel permeability at normal resting potential (557). Therefore, it seems unlikely that NO induced *S*-nitrosylation of Cx46 is involved in the development of cataract.

Final considerations

Although phosphorylation is the most extensively studied form of posttranslational modification of connexins, the recent progress in the quality of MS studies have made it possible to identify other forms of posttranslational modifications. Therefore, it is now clear that although phosphorylation of connexins is very important in the regulation of connexins, phosphorylations may regulate and interact with other forms of posttranslational modifications to regulate generation, gating, and degradation of gap junction channels. The experimental research needed to evaluate the biological effects and relevance of the different forms of chemical modifications have only just begun, and there is still a long way to go before we completely understand the complexity of posttranslational regulation of connexins in health and disease.

Physiological Function in Cell-to-Cell Communication

Virtually all cells in solid tissues are joined by gap junctions that allow transport of small molecules such as amino acids, sugars, small peptides, and intracellular messengers between cells, and in addition provides electrical coupling between cells by allowing the transfer of electrical charge carried by various ion species, for example, K^+, Na^+, Cl^-, and Ca^{2+} (574). Gap junctions therefore provide a universal function as intercellular channels of communication that form the basis of direct cellular interactions. Given the relatively low specificity of gap junctions that allows passage of a wide variety of molecules, it is not surprising that they are involved in a wide variety of physiological functions in different cell types. In many cases our knowledge stems from the effects of human mutations in connexin genes and the effects of targeted gene disruptions in mice (149,630,787). In general, the association of genetic and acquired disturbance of connexin function with human diseases have emphasized the importance of proper intercellular communication and shed new light on the physiological role of gap junctions for recent reviews see (351, 787). It is interesting that in some cases a specific function is directly related to a specific connexin isoform, whereas elsewhere several isoforms may be substituted for one another without any apparent functional consequences. In addition, there are examples where the function of gap junctions are unrelated to their function as intercellular channels (167) (see also section on noncoupling functions of connexins), emphasizing the great complexity of the field.

Cardiovascular system

Heart—Gap junctions are present in most cells of the cardiovascular system. The heart expresses several connexin isoforms with Cx43, Cx40, and Cx45 being the dominant isoforms (127, 128, 335). Gap junctions play a critical role by allowing the passage of current between the cells of the myocardium, which is a requirement for the propagation of the action potential across the heart. In working myocardium, gap junctions are preferentially present in the region of the intercalated disks providing an “end-to-end” coupling of the cardiomyocytes (581,610). The uneven distribution of cardiac gap junctions in atrial and ventricular cardiomyocytes partly determines the anisotropy of cardiac tissue, with the relative paucity of “side-to-side” gap junctions leading to preferential conduction of the action potential along the long axis of the cardiomyocytes (581). The connexins have different distributions in the specialized cardiac tissues. Cx43 is the major connexin in the working myocardium of the ventricles (581, 610, 722), whereas the atrial cardiomyocytes express a mixture of Cx40, Cx43, and Cx45 (128, 231, 706). Mice that are heterozygous for the Cx43 null mutation ($Cx43^{+/-}$) are reported to have either slowed epicardial conduction in the ventricles, and a widening of the QRS complex (235, 679), or to have no apparent changes in conduction (455,708). Apparently, reductions larger than 50% are needed to get robust effects on conduction (121, 708), but during ischemia $Cx43^{+/-}$ mice are more prone to arrhythmias (369). The specialized structure of the conduction system, that is, the sinoatrial- and AV-nodes, and the Hiss-Purkinje system, has been found to express Cx40, whereas Cx43 are expressed to a lesser extent (229, 347, 486, 487, 581, 610, 707). In agreement with this, Cx40 null mice have cardiac conduction abnormalities like atrioventricular and bundle branch block (42, 632). Cx45 and Cx31.9 (mCx30.2) are expressed in the AV node and in the center of the sinoatrial node (58). Recent data from mice indicates that Cx31.9 (mCx30.2), which has the lowest unitary conductance among the connexins, may play a significant role in the slow propagation of the action potential through the AV node (334, 335, 598). The relevance of these findings for humans remains controversial as a recent study has failed to find any expression of Cx31.9, which is the human ortholog of mouse Cx30.2, in the conduction system (333).

Cx43 also plays a role in cardiac development. Mice that lack Cx43 survive to birth and are born with beating hearts. However, they die shortly after birth due to obstruction of the right ventricular outflow tract, an abnormality that prevents normal perfusion of the lungs (549). In humans, there also appears to be a link between mutations in Cx43 and cardiac malformations in that mutations has been associated with the hypoplastic left heart syndrome and atrioventral canal defects in pediatric patients (124).

Alterations in both the amount and cellular distribution of gap junctions have been reported in many types of cardiac disease, and it has been suggested that these changes may cause arrhythmias and/or sudden cardiac death (156, 221, 611, 612, 613, 614, 615). Cardiac diseases are often associated with a reduced and/or heterogeneous expression of connexins (288). As a result, there will be slowing of conduction and enhancement of discontinuities in cardiac anisotropy, both of which is believed to predispose to reentrant arrhythmias (322). It also appears as if chronic arrhythmias by themselves result in electrical remodeling of the heart with an altered expression and distribution of gap junctions (613). Attempts to design drugs that enhance gap junction conductance have shown some promise in treating and/or preventing reentrant arrhythmias (13, 245, 261, 318, 319, 320, 538, 659, 755) (see section on pharmacology for further information).

Besides current, cardiomyocytes may also exchange biologically important molecules through gap junctions. This may have significance with regard to the progression and spread of cell injury and death during myocardial ischemia. Nonselective pharmacological uncoupling of gap junctions has been found to reduce infarct size following acute ischemia (196,583), and mice heterozygous for the Cx43 null mutation ($Cx43^{+/-}$) developed smaller infarcts compared to wt mice following coronary artery ligation (302). The issue remains controversial, since studies using enhancers of gap junction activity also reported reduced infarct size following chronic infarction (252) and acute ischemia reperfusion (262). The discrepancies may be the result of the use of relatively unspecific pharmacologic agents, different species of experimental animals, and different experimental protocols in the studies.

Blood vessels—In the vascular system, gap junctions couple the vascular smooth muscle cells (VSMC), the ECs, and also connect ECs to underlying smooth muscle cells via myoendothelial gap junctions (244). The major connexins found in blood vessels are Cx37, Cx40, Cx43, and Cx45. The intercellular communication in the vasculature contributes to the regulation of resistance in different vascular beds according to metabolic needs, and therefore ultimately participates in the regulation of blood pressure. Pathological conditions, such as atherosclerosis, diabetes, and hypertension are associated with changes in connexin regulation and expression (46, 243, 246, 343, 777).

In atherosclerosis, expression of Cx37, Cx40, and Cx43 varies throughout the progression of the disease (343) and the connexins play different roles in plaque development (Fig. 10). Heterozygous Cx43 knockout mice present with reduced and more stable atherosclerotic plaques compared to homozygous mice (345, 750), which indicates a promoting role of Cx43 in the development of atherosclerosis. In contrast, both Cx40 (83) and Cx37 (752) protect against plaque development. Cx37 exerts its atheroprotective effect through regulation of monocyte and macrophage recruitment (752), whereas Cx40 protects the vessels by preventing leukocyte adhesion (83). That Cx37 plays an essential role in atherosclerosis is further supported by the findings that a polymorphism of the *Cx37* gene is related to both coronary artery disease (273, 764) and myocardial infarction (386, 751).

Gap junctions between ECs and, to a lesser degree, between the smooth muscle cells facilitates longitudinal signaling along the blood vessels, thereby mediating socalled

conducted vascular responses (240). When vessels are stimulated by agents that modulate the membrane potential, the change in membrane potential is spread via gap junctions along the vessel (604). Depending on whether the applied drug hyper- or depolarizes the membrane, the result is conducted vasodilation or constriction. The response travels over a considerable length of the vessel, and may be observed more than 2 mm from the local stimulation site (240, 605). Although both the VSMCs and the ECs express gap junctions, the ECs, which primarily express Cx40, provides the main path for the conduction process (133).

The presence of myoendothelial gap junctions allows for the passage of current between the smooth muscle cells and ECs, and these two cell layers are tightly coupled electrically (758). Besides their role in conducted responses, myoendothelial gap junctions may play an important role in endothelialmediated vasodilation. Agonist-induced hyperpolarization of ECs may spread through myoendothelial gap junctions to the vascular smooth muscle layer causing hyperpolarization and smooth muscle relaxation (133). Thus, myoendothelial gap junctions may contribute to the endothelium-derived hyperpolarizing factor (EDHF) phenomenon, although other mechanisms also contribute to endothelial-derived hyperpolarization (588).

Vasoconstriction is associated with increases in the intracellular Ca^{2+}, due, at least in part, to IP3-mediated release of Ca^{2+} from intracellular stores. It is becoming evident that IP3 may diffuse across the myoendothelial gap junctions also causing Ca^{2+} release in the ECs (588). This will result in increased production of NO and EDHF, which will counteract the agonist-induced vasoconstriction (588).

Knockout of Cx45 in mice results in a lethal phenotype where the animals die *in utero* due to abnormalities of vascular development (336). Differentiation and positioning of ECs are normal, but subsequent development of the vascular system is impaired in several tissues (336). Cx40 deficient mice have impaired conducted vasodilator responses and are hypertensive (134). The hypertension is not due to impaired conducted responses *per se*, but rather due to dysregulation of the renin-angiotensin system (340,730,731). Neither Cx40 nor Cx37 deficient mice show any abnormalities of vascular development.

Digestive system

The gastrointestinal tract—Gap junctions are highly expressed especially in the inner circular layer of intestinal smooth muscle, forming a syncytium that coordinates the contractile activity of the gut (120). Interestingly, both the circular and the longitudinal layers have been found to be both electrically and dye coupled, although it has not been possible to demonstrate the presence of gap junctions in the outer longitudinal layer of smooth muscle (120,165,775). It has been suggested that the interstitial cells of Cajal (ICC) may indirectly mediate the coupling in the outer muscular layer (390). The ICC are the pacemaker cells in gastrointestinal muscles, and they also mediate or transduce inputs from enteric motor nerves to the smooth muscle syncytium (585, 586, 737). The ICC are interconnected by gap junctions, and, in addition, are electrically and dye coupled to the smooth muscle cells (36, 37, 390). Surprisingly, the current evidence suggests a paucity of gap junctions between the ICC and the smooth muscle cells, and the issue of how electrical and dye coupling are achieved in the gastrointestinal network of ICC and smooth muscle cells remains controversial (119). In Hirschprung's disease part of the gut is aganglionic and lacks normal motility. The expression of Cx43 is severely reduced or absent in ICC and smooth muscle cells in the aganglionic part of the gut, which suggests that decreased levels of gap junctions may be partly responsible for the disturbances of colon motility characteristic of the disease (472).

Salivary glands—Little is known about the role of gap junctions in salivary gland function. The acinar cells express Cx26 and Cx32 suggesting that gap junctions may play a role in the secretory function (339, 457, 623). In contrast, the myoepithelial cells that surround the acini express Cx43, which imply a role for gap junctions in coordinating the contractile function of these cells (457).

Pancreas—Like in the salivary glands, the exocrine part of the pancreas also express Cx26 and Cx32 in the acinar cells and, like in the salivary glands, it is assumed that gap junctions coordinates the secretory response of the cells within an acini (428, 660). However, the function of gap junctions do not appear to be critical for the secretory function, since isolated acinar cells has an intact secretory function (81).

In the endocrine islets of the pancreas, the β-cells are coupled by gap junctions made of Cx36 (608, 609). Several lines of evidence suggest that cell-cell coupling is essential for the normal release of insulin. Isolated islets show a normal response to stimulation by glucose whereas isolated β-cells show an increased basal release of insulin, lack of response to glucose stimulation, decreased basal expression of the insulin gene, decreased proinsulin biosynthesis, and a blunted increase in intracellular Ca^{2+} in response to glucose stimulation, and these effects are partially reversed by reaggregation of the cells (476). A very similar phenotype is seen in Cx36-deficient mice (548). The general pattern is disruption of normal Cx36 function, which results in an increased basal release of insulin, a lack of further insulin secretion following physiological levels of glucose stimulation, lack of synchronization of the normal intercellular Ca^{2+} transients following glucose stimulation, and disappearance of the normal pulsatile release of insulin despite the presence of morphologically normal islets (29, 548). Although the molecular mechanism whereby gap junction-mediated coupling influences islet function and insulin secretion is unknown, there is strong experimental evidence in favor of a central role of Cx36 in the control of islet function and insulin release in rodents (29, 476). In a recent study in humans, Cx36 mRNA was found to correlate with the expression of the insulin gene in the islets of both control and type 2 diabetics. This suggests that Cx36 contributes to the control of β-cell function by modulating gene expression (608).

Cx36 may also play a role in the pathogenesis of diabetes. The gene coding for Cx36 (GJA9) is located at chromosome 15 q14, which is a locus found to be associated with type 2 diabetes in humans (34,110,453). Furthermore, there are data to suggest that at least in mice, Cx36 may protect pancreatic β-cells against cytotoxic insults (29, 476).

Liver—Hepatocytes are both electrically and dye coupled due to the presence of Cx26 and Cx32 in the cells (129, 364, 473, 690). The electrical coupling synchronizes the electrical responses in hepatocytes to agonists such as glucagon (364). Gap junctions are also required for the propagation of agonist induced Ca^{2+} waves across populations of hepatocytes. The propagation appears to be mediated by IP3 diffusing across the gap junctions, releasing intracellular Ca^{2+}, and activating phospholipase C in the neighboring cells (96, 157, 566, 575). The latter functions as a regenerative mechanism, allowing the Ca^{2+} wave to propagate across an entire hepatic acinus. The functional importance of the gap junctional coupling is illustrated by the observation that hepatocytes from Cx32 deficient mice show a strong reduction in IP3 permeability, which is associated with drastic reductions in glucose release in response to nerve stimulation and to agonists like glucagon and norepinephrine (471, 475, 665).

Reproductive system

Female reproductive system—Gap junctions are abundantly present in many tissues of the female reproductive system. Connexins are present in the smooth muscle cells of the oviduct and in the uterus, where they play a role in determining the hormonal-dependent motor activity in these tissues (43,202,264,560). The amount of gap junctions in the myometrium increases at the time of labor, and their appearance are necessary for coordinating the contractile activity of the uterine smooth muscle required for the expulsion of the fetus (197, 198, 200,201,202,203, 440, 633). The expression of gap junctions in the uterine smooth muscle is controlled by endocrine factors with estrogens enhancing the expression and progesterone suppressing the expression of gap junctions (199, 232, 260, 406,407,408, 533, 563) (for more information see section on expression and trafficking). A very similar mechanism operates in the oviduct, where the number of gap junctions in the smooth muscle cells and the motor activity are increased by estrogens and decreased by progesterone (264,560). These effects are likely to play an important role in propelling the ovum toward the uterine cavity following ovulation.

Gap junctions play an intriguing role in the communication between the maturing oocyte and the surrounding granulosa cells, an interaction that is required for normal follicular development (309). The granulosa cells surrounding the oocyte are interconnected by gap junctions, where the main connexin is Cx43 (230). The presence of Cx43 is required for normal development and maturation of the follicle. Follicles from Cx43-deficient mice are morphologically abnormal, they fail to achieve meiotic competence, and they cannot be fertilized *in vitro* (1). The ovarian phenotype in Cx43 deficient mice is very similar to that seen in animals lacking oocyte-specific protein growth differentiation factor 9 (GDF9), a paracrine factor necessary for normal follicular growth and maturation (214, 309). It has been suggested that, as the follicle grows, the outer layers of the follicle will be at an increasing distance from the oocyte and thus be exposed to lower concentrations of GDF9. Gap junctions may be necessary for relaying GDF9-induced signaling molecules to the most distal granulosa cells, coordinating follicular growth (309). The oocyte is coupled to the granulosa cells via homotypic Cx37 containing gap junctions (631, 719). Mice lacking Cx37 are infertile, and their follicles do not mature normally (631). The granulosa-oocyte coupling allows transfer of metabolites and signaling molecules between the granulosa cells and the oocyte necessary for the normal development of the follicle. The amount of Cx43 is increased prior to ovulation due to stimulation by FSH (230). It is thought that the efficient metabolic coupling in the follicle allows the transfer of cGMP from the granulosa cells to the oocyte, and that this is essential for preventing the oocyte from completing meiosis (478,592). Just prior to ovulation there is a surge of LH, which elicits a MAP kinase dependent phosphorylation of Cx43 and a decreased Cx43 expression, which disrupts the gap junctional coupling in the wall of the follicle and thus stops the flow of cGMP to the oocyte allowing it to complete meiosis at the time of ovulation (478,592). It is of interest to note that the immature oocyte lacks mechanisms for pH regulation, and apparently relies on transport mechanisms in the surrounding granulosa cells for this purpose, with gap junctions establishing the necessary coupling between the two cell types (181, 182). There is no direct evidence linking gap junctional dysfunction to infertility in humans. It has, however, been reported that in granulosa cells from patients undergoing *in vitro* fertilization, Cx43 levels and intercellular conductances are positively correlated with embryo quality as judged by cleavage rate and morphology, and Cx43 levels are significantly higher in patients who becomes pregnant than in those who do not (732).

The male reproductive system—Various connexins are expressed throughout the tissues of the male reproductive system, with Cx43 as the predominant isoform (525, 526). The androgen producing Leydig cells, which are present in the interstitium of the testis,

show both electrical and dye coupling mediated by Cx43-based gap junctions (62, 111, 511, 564, 715). There is only limited information available regarding the hormonal regulation of gap junction expression in Leydig cells, but is has been reported that human chorionic gonadotropin reduced Cx43 mRNA and protein levels (770). In addition, in the Leydig cell line TM3 stimulation of testosterone secretion by LH was associated with a decreased coupling, an effect that was mimicked by activators of PKA and PKC (220). Together these data suggest that gap junction coupling may play a role as modulators of hormone secretion.

Gap junctions composed of Cx43 connect both Sertoli cells and germ cells in the seminiferous epithelium. These junctions are seen between Sertoli cells and between Sertoli cells and germ cells, primarily spermatogonia, and early and late spermatocytes (137). Although the underlying molecular mechanisms are unresolved, it appears as if Cx43 is necessary for normal spermatogenesis (526). Thus, Cx43 deficient mice has a 50% reduction in primordial germ cells in the foetal testis (299) and testicular tissue from these mice show a persistent reduction in germ cells when grafted under the kidney capsule of adult male mice (570). Recently, similar observations have been made in Sertoli cell specific conditional Cx43 knockout mice (63, 651, 652). The male mice showed an arrest of spermatogenesis at the level of spermatogonia or a Sertoli cell only syndrome with absence of germ cells. The function of Cx43 is specific, since other connexins are unable to compensate for the loss of Cx43 function (524, 748). As in the Leydig cell, the expression of Cx43 in the Sertoli cells is subject to hormonal regulation. Androgens and estrogens have been found to disrupt gap junctions and reduce the coupling in Sertoli cells (266, 523, 526), whereas thyroid hormones increases the levels of Cx43 in Sertoli cells (216, 658). Retinoids are of importance for Sertoli cell function and spermatogenesis, and it is possible that this may, at least partly, be due to its effects on Cx43 expression in Sertoli cells (28, 92, 401).

Nervous system

The central nervous system—Gap junctions are present in neurons and glia cells throughout the nervous system (103, 104, 192, 465, 466, 489, 545, 546, 547, 625, 757). In mammalians, Cx36 is expressed in neurons throughout the brain, and it appears to be present exclusively in neuron-neuron gap junctions, and not in gap junctions between glial cells (103,546,547). Other connexins have also been described in neurons, for example, Cx45 and Cx62 (mCx57), although they all have a much more limited distribution compared to that of Cx36 (194, 641). Although gap junctions between neurons and between glia cells are common, gap junctions between neurons and glia cells are uncommon (547). Gap junctions provide an additional form of contact between neurons in addition to the well-known chemical synapse. They form an electrical synapse, which in contrast to the chemical synapse, allows bidirectional signaling between neurons. A striking finding is that not all neurons have electrical synapses; rather they are present in specialized subpopulations, for example, inhibitory interneurons (192). The precise function of electrical synapses is not known, but they may play an important role in generating or modulating synchronous activity in groups of neurons (69, 136, 189, 279, 398, 692, 786). The dynamics of electrical synapses results in a function resembling a first-order lowpass filter, and as a result they will reduce high-frequency noise, and thus, improve the signal-to-noise ratio in neural signaling pathways (47,104). In addition to providing electrical coupling, gap junctions may also be important for specific neuron-to-neuron passage of small molecules, although little evidence exists for this in the mature brain. In view of the widespread occurrence of gap junctions in neurons, it is noteworthy that only limited neurological dysfunction has been observed in knockout animals. In $Cx36^{-/-}$ mice there appears to be a subtle effect on hippocampal gamma oscillations, but apart from the visual defects (see later), there is little or no neurological phenotype (69, 316). A very similar pattern was seen in mice with a conditional neuron directed knockout of Cx45. The major neurophysiological effect was a disruption of

kainite induced gamma oscillations in the hippocampus (786). The behavioral phenotype was minimal, although an effect on novel object recognition was reported (786).

Among glial cells there are abundant gap junctions between the astrocytes themselves, fewer between the astrocytes and oligodendrocytes, and very few or none between the oligodendrocytes (300, 420, 465, 547). Astrocytes and oligodendrocytes express a distinct collection of connexins, with Cx30.2 (mCx29), Cx32 and Cx47 being present in oligodendrocytes and Cx30 and Cx43 being present in astrocytes (489). Consequently, gap junctions between oligodendrocytes and astrocytes will always be heterotypic. Mutations or knockout of Cx32 and Cx47 results in disorders of myelination. Whereas loss of Cx32 predominantly results in demyelination of peripheral nerves (see later), loss of function of Cx47 leads to a devastating dysmyelinating disease in humans, Pelizaeus-Merzbacher-like disease (697). In mice, knockout of Cx47 also results in demyelination in the CNS, but the demyelination is less severe in mice compared to humans (431, 480). Mouse with a double knockout (Cx32 and Cx47) develops a severe dysmyelinating phenotype (431, 480). It is therefore clear that connexins and gap junctions are of importance for the function of myelinated fibers, although the exact mechanisms leading to loss of myelination remains unknown. Neuronal activity is associated with the release of K^+ and an increase in the local K^+ concentration (105). One function of the panglial syncytium may be the disposal of extracellular K^+; so-called potassium siphoning (489, 543). This is necessary for maintaining a low extracellular K^+ concentration, and thus, normal neuronal activity. By spatially buffering the released K^+, and by providing a restricted pathway whereby K^+ can reach the blood vessels, the panglial network may play an important role in maintaining brain K^+ homeostasis. Myelinated fibers release K^+ into the space between the axon and the oligodendrocytes and it has been suggested that disturbances in the function of the panglial syncytium may lead to accumulation of K^+ andwater in the oligodendrocytes. The intracellular accumulation of ions andwater may be an important mechanism in the demyelinating process (543). Like in other tissues (see previous text), connexins are involved in the propagation of calcium waves across glial cells, but the physiological role of these waves in the panglial syncytium remains to be fully established (593).

The peripheral nervous system—Charcot-Marie-Tooth disease is a demyelinating condition with progressive degeneration of peripheral nerves caused by mutations in Cx32 (323). In peripheral nerves, the sheaths of myelin are formed by Schwann cells wrapping their cytoplasm several times around the nerve fiber. Cx32-based gap junctions form intracellular connections between adjacent loops in one cell, thereby providing a radial diffusion pathway that allows metabolic and nutritional support of the most distal parts of the cytoplasm (18,746). Disruption of this pathway is apparently associated with Schwann cell dysfunction, leading to loss of the myelin sheath, and peripheral nerve dysfunction.

The eye—The lens is an avascular organ with an epithelial layer covering its anterior surface, and a large mass of fiber cells that forms the bulk of the organ. The lens fibers are formed from the epithelial cells at the equator of the lens, and as they move inward they gradually lose their organelles to become mature lens fibers. The differentiating fibers extend from the surface and approximately 15% of the distance into the lens; and the mature fibers forms the core of the lens. The entire structure is a functional syncytium due to the presence of gap junctions coupling all cells in the lens (425). The epithelial cells contain Cx43 and Cx50 (43, 116), whereas the lens fiber cells contain Cx46 and Cx50 (315, 504), and the presence of dye and electrical coupling between all cells of the lens have been demonstrated (423, 539). Gap junctions are essential to lens function by allowing a flow of current and fluid that transports nutrients into the central part of the lens (422). The driving force is created by the action of the Na-K-ATPase, which together with the passive Na^+ permeability of the lens fibers creates an intracellular voltage and concentration gradient

from the inside to the outside of the lens. The net result is an inward flow along the extracellular space carrying nutrients into the center of the lens, together with an intracellular outward flow removing Na^+ and water from the core of the lens (422). In the outer differentiating fibers, both Cx46 and Cx50 contribute to the coupling, whereas in the mature fibers located in the center of the lens Cx46 is solely responsible for the coupling (16, 224). In mature fibers, both Cx46 and Cx50 are truncated by removal of their CT tail by calpain (382); a modification that leads to closure of Cx50-based gap junctions, but not of Cx46-based gap junctions (140, 162). So although both connexins are present in the mature fibers, only Cx46-based gap junctions contribute to the coupling. (For further information of Cx46 and Cx50 truncation, see section on posttranslational regulation.)

The coupling conductance is regulated by pH in the outer differentiating fibers, with a decrease in pH rapidly closing all gap junctions in this layer (424). In contrast, gap junctions in the mature fibers lack pH sensitivity and are not closed by cytoplasmic acidification (17, 424, 436, 600). This could be necessary for maintaining adequate coupling, since the core of the lens is acidic (26). There is an interaction between Cx46- and Cx50-based gap junctions in the lens with respect to pH sensitivity. In wt mice, acidification nearly abolishes cell-cell coupling (16). In contrast, in Cx50 knockout mice, the initial coupling is reduced by approximately 50%, corresponding to the loss of Cx50-based gap junctions, but the remaining coupling is nearly unchanged following acidification (16). This suggests that the pH sensitivity is provided by Cx50 and that, somehow, there is an interaction between Cx50 and Cx46 with respect to pH sensitivity (425).

The importance of gap junctions in normal lens function is amply illustrated by the fact that mutations in Cx46 and Cx50 are one of the common causes for hereditary cataracts in humans and mice (425). In addition, Cx50 may also be of importance for the normal development of the eyes, as Cx50 knockout mice have micropthalmia (569, 745).

In the vertebrate retina electrical synapses, i.e. gap junctions, are found in all the five neuronal cell types present (47). Thus, there are electrical synapses between rods, between cones, between rods and cones, between horizontal cells, between bipolar and amacrine cells, between amacrine cells, between amacrine cells and ganglion cells, and between ganglion cells. They establish bidirectional electrical coupling, and their function is similar to that seen in neurons in the rest of CNS (see previous text). The predominant connexin is Cx36, but Cx45, Cx50, and Cx62 (mCx57) are also present in the retinal neurons (47). Only Cx36 knockout mice are known to have a visual phenotype. Although the retina showed no morphological defects, recordings showed a reduced scotopic function, consistent with reduced vision during low light conditions (236). This is consistent with the observation that electrical synapses are involved in the rod signaling pathway (236).

The inner ear—Gap junctions play a central role in hearing, as evidenced by the fact that nearly 50% of all cases of inherited neurosensory deafness is due to mutations in the genes coding for Cx26, Cx30, Cx31, Cx32, and possibly Cx43 (418). In the vast majority of these cases, the cause is a mutation in the gene coding for Cx26 (307, 365, 743). Connexins are expressed in the epithelia and connective tissues, but not in the sensory cells (the hair cells) of the cochlea. They establish a functional syncytium among these cells (183, 188). In the cochlea, Cx26 and Cx32 may be involved in buffering and recycling of K^+ (183,418). The sensory cells of the cochlea, the hair cells, are exposed to the endolymph through their cilia, which has a high concentration of K^+ (~150 mmol/L), while their cell bodies are surrounded by the perilymph, which has an ionic composition similar to the extracellular fluid (270). Upon stimulation by sound K^+ enters the hair cells due to the large electrochemical gradient, causing depolarization of the cells, a step essential for the sensation of sound. K^+ then exits the hair cells into the perilymph, where it is taken up by the surrounding epithelial cells and

supporting cells and transported to the lateral wall of the cochlea via the "epithelial gap junction network." The K^+ is then taken up by the fibrocytes of the spiral ligament, and, through the "connective tissue gap junction network," transported back to the stria vascularis, and then finally secreted back into the endolymph (270). When this recirculation is disrupted, the result is cochlear dysfunction and hearing loss. It is of interest to note that some connexin mutants that cause deafness, form channels that conduct ionic current similar to the wt, suggesting the intercellular transport of other compounds, for example, IP_3, may also be important for normal hearing (35, 418, 779).

Immune system

Despite the fact that most cells of the immune system express connexins, very little is known about the role of gap junctions in these cells (470). Immune stem cells establish gap junctions with the surrounding stromal cells in the bone marrow and these are necessary for the terminal differentiation of primary T and B cells (82, 330, 444, 445, 528). Likewise, gap junctions are necessary for the further maturation of T and B cells in the thymus and in the peripheral lymph nodes, but very little information is available regarding the underlying molecular events (444, 445, 470). B cells and antigen presenting dendritic cells may establish heterocellular gap junctions, and treatment with connexin mimetic peptides or 18α-glycyrrhetinic acid reduces the secretion of immunoglobulins (331, 493). It has been suggested that gap junctions may be involved in antigen presentation by allowing cell-to-cell transfer of antigens, so that small peptides generated in one cell may be transported via gap junctions to neighboring cells, where they then are presented on the cell surface by MHC class 1 molecules (469, 502). Recently, it was reported that activation of T lymphocytes (CD4+) is associated with an upregulation of Cx43 expression, and that T lymphocytes (Th1) establishes heterocellular contact with macrophages in the tissues; and that gap junctions are of significance for clonal expansion (39, 494).

Gap junctions may be involved in the transmigration of leukocytes across the endothelium, but the exact nature of the gap junction-mediated interaction between leukocytes and the endothelium remains unknown (170,492,590,774). An effect on leukocyte recruitment would be in agreement with observations suggesting a role for gap junction in the inflammatory response; see reference 596 for a recent review.

Cell growth, migration, and gene expression

Connexins have complex functions in controlling cell growth, migration, and gene regulation (303). For example, mutations in Cx43 lead to the complex phenotype of oculodentodigital dysplasia, a condition characterized by multiple developmental disturbances including dental anomalies, fused fingers, and abnormalities in the development of the eye, nervous system, bones, and many other abnormalities (505).

The mechanisms through which connexins exerts these effects are highly diverse. In some cases it is directly related to channel formation with transfer of growth and gene regulatory factors. Possible candidates are Ca^{2+}, cAMP, and inositol triphosphate (495, 575). Recent data also suggest that small oligonucleotides like siRNA can pass through gap junctions and provide a direct pathway whereby one cell can influence gene activity in neighboring cells (704,749). In addition to its role in forming gap junctions, connexins may also contribute directly to cell-cell adhesion independent of channel function. This may have an important role with regard to growth control. Connexins may also contribute to contact inhibition, and loss of connexins may play a role in oncogenesis (432). In some cases this effect is independent of channel formation or function (118, 314, 485). Instead, the effect may be mediated through direct interactions between the intracellular C-terminal part of connexins and proteins of the cytoskeleton like zona occludens-1 (ZO-1), caveolin, and drebrin, in

addition to various kinases and phosphatases (94, 153, 211, 265). Also, normal migration of cells during, for example, neurogenesis appear to depend on connexin mediated adhesion rather than direct channel formation (94, 167). Binding of growth promoting substances like β-catenin may be an alternative mechanism whereby Cx43 could influence cell growth. The growth promoting effect of β-catenin depends on its subcellular localization; binding to Cx43 may prevent it from reaching the nucleus and thus preventing its interaction with transcription factors (4). In cardiac myocytes, there is evidence for the presence of Cx43 in the mitochondrial membrane, where it may contribute to the release of apoptosis-promoting factors (228).

It is possible that connexin genes may act as “hubs” in gene expression networks. Data from gene array studies show that perturbation of the gene coding for Cx43 may have effects on the expression of hundreds of other genes in the network (285, 650). Clearly, such an effect has important implications for the role of connexins in the normal development of the organism.

Noncoupling Roles of Connexins

Besides their classical role as mediators of intercellular coupling, connexins also play roles that are independent of their function in cell-cell channels. The most studied example of this is the role of unpaired hemichannels in the plasma membrane, which upon opening may exert different physiological effects ranging from paracrine signaling to loss of homeostasis-induced cell death. More surprisingly, connexins have also been assigned to roles exerted in the nucleus and mitochondria as well as some channel-independent effects, which involve connexin-binding proteins. The current knowledge is reviewed in the later section.

Hemichannels

Undocked connexons in unopposed parts of the cell membrane are referred to as hemichannels. Like proper gap junction channels they may be formed by all the known connexins, and they exist in both homo- and heteromeric conformations (172). In addition to connexins, it appears likely that the recently discovered family of pannexins forms similar hexameric hemichannels in the plasma membrane (20, 66, 395). Pannexins have a membrane topology similar to the connexins, although they have no sequence homology (500, 501). Pannexin hemichannels will not be dealt with further in the present text.

When arriving at the plasma membrane hemichannels may diffuse laterally in the plasma membrane and become incorporated into gap junction plaques, but it appears likely that the plasma membrane at any given time contains a significant number of undocked hemichannels. They form large pores in the cell membrane, which upon opening allows the exchange of ions and small molecules between the intra- and the extracellular compartment. Current evidence suggests that the synthesis and intracellular trafficking of undocked hemichannels closely mimics that of the connexons, which later ends up in intercellular channels.

The amount of unopposed hemichannels in the membrane can be assessed by biotinylation of surface membrane proteins followed by “pull down” of the labeled proteins (552,584,595). In Cx32-transfected HeLa cells approximately 4% of surface connexin was in unopposed hemichannels (584), whereas in cultured astrocytes approximately 15% of surface Cx43 was found in unopposed hemichannels (552). Metabolic inhibition, treatment with a Ca^{2+} ionophore or FGF-1 all resulted in increased levels of hemichannels in the surface membrane (552, 584, 595). An increase in intracellular Ca^{2+} appeared to be necessary for the response and since it was not associated with an increase in the total connexin levels, it was primarily due to a redistribution of connexons in the surface

membrane (552, 584, 595). In HeLa transfectants, the increased levels of Cx43 hemichannels following stimulation with FGF-1 involved activation of p38 MAP kinase, and the data suggests that the activation of this kinase is a downstream response to the Ca^{2+} elevation (595).

The conductance and permeability characteristics of hemichannels are very similar to that of the full gap junction channels. Since a hemichannel represents one half of the full cell-cell channel it is to be expected that it will have twice the conductance and permeability of the full channel, and indeed this appears to be the case (576). Thus, like the cell-cell channels, an open hemichannel would be permeable to rather large molecules (up to ~1 kDa), and it is clear that if a substantial amount of hemichannels were open under resting conditions, the cell would undergo severe derangements in electrolyte balance, membrane depolarization, and loss of small intracellular molecules like ATP. Because of these potentially fatal consequences it is generally accepted that most hemichannels are closed during resting conditions (23, 38). A possible exception could be Cx26 hemichannels. Expression of either human or sheep Cx26 in oocytes or Neuro2a cells, resulted in hemichannels that were open under resting conditions apparently without compromising cell viability (226,562). Interestingly, transfection with rat Cx26 did not result in expression of open hemichannels indicating significant species differences (226).

While most hemichannels are closed during resting conditions, there is now good evidence to suggest that opening of hemichannels for short periods allows exchange of molecules between the intra- and extracellular compartment, and that this exchange plays a significant role in many physiological and pathophysiological processes (23, 38, 172, 576, 577).

The controversy surrounding the existence of hemichannel mediated transmembraneous fluxes stems from the fact that their presence mostly has been inferred from indirect evidence. The first evidence came from studies showing that expression of connexins in cells lead to the appearance of membrane permeabilities or currents not present in the nontransfected cells (161, 504). The observations that (1) such currents or permeabilities could be inhibited by gap junction blockers, (2) the induced pathways were permeable to larger molecules (up to 1 kDa), and 3) the currents had reversal potentials around 0 mV indicative of a pathway with low selectivity gave further support for the presence of open hemichannels (107, 293, 376, 522, 663). However, because of the poor specificity of gap junction blockers together with the fact that other ion channels may allow the transmembraneous passage of larger molecules and metabolites (135,143,434) it could not be ruled out that other ion channels mediated the observed fluxes. The possibility that hemichannels may under some circumstances carry transmembraneous currents have been strengthened by single channel recordings in transfected cells. These studies have demonstrated both conductances and substates compatible with what is known from intercellular channels (106, 694, 701, 705, 727).

Hemichannel gating properties

Although most hemichannels appears to be closed in the resting state, several factors may trigger their opening. Among the first to be described was a decrease in the extracellular Ca^{2+} concentration (161), but later it has been shown that also changes in phosphorylation status, the extracellular ion composition, membrane potential, intracellular pH and redox potential, and mechanical stimulation may cause opening of hemichannels (553, 576, 577).

Extracellular cations—Reducing the extracellular Ca^{2+} concentration increases the open probability of all investigated connexin hemichannels (514, 576, 577). Despite this uniform effect of lowering extracellular Ca^{2+}, the mechanism seems to differ between hemichannels made from different connexins (223, 456, 531, 677, 726). Although most hemichannels

appear to be closed when Ca^{2+} is above 1 mmol/L, this mechanism may be of pathophysiological significance, for example, during cerebral ischemia, where the extracellular Ca^{2+} level may be as low as 0.1 mmol/L (251). The extracellular levels of other divalent cations like Zn^{2+}, Cd^{2+}, Co^{2+}, Ba^{2+}, and Mg^{2+} also affects the gating properties, with the general picture being inhibition with increasing concentrations (85, 86, 160, 668). Replacing extracellular Na^{+} by K^{+}, Cs^{+}, or Rb^{+} causes an increase in the open probability of Cx46 and Cx50 hemichannels, suggesting that monovalent cations may interact with Ca^{2+} in determining the activity of hemichannels (653).

Phosphorylation—Cx43 hemichannels reconstituted in liposomes are permeable to Lucifer Yellow, and treatment with alkaline phosphates before or after the reconstitution increases the permeability whereas treatment with MAP kinase or PKC decreases it (22, 310). Phosphorylation of serine 368 may be of particular significance in this respect, since hemichannels formed by a mutated Cx43 lacking this residue (Cx43 S368A) remain in the open state (22). These data strongly suggest that phosphorylation may directly regulate Cx43 hemichannel activity.

Membrane potential—The available studies suggest that depolarizing the cell membrane increases the open probability of hemichannels (141,161,289,514,703,776). However, as activation requires substantial depolarization of the membrane, the physiological importance of hemichannel activation by voltage remains to be demonstrated. One possibility is that interactions exist between activation by depolarization and reduced extracellular Ca^{2+}. This has been shown for Cx46, where lowering of extracellular Ca^{2+} results in a hyperpolarizing shift in the activation curve (514, 726).

Chemical factors—Hemichannels composed of Cx26, Cx35, Cx38, Cx43, Cx45, and Cx46 have all been found to be sensitive to the intracellular pH, with acidification leading to closure of the channels (184, 289, 562, 693, 701, 744). In addition to pH, some hemichannels may also be sensitive to the intracellular redox potential. The data are less clear since both oxidation and reduction appears to be able to increase the open probability of the hemichannels. Oxidative stress increases the activity of Cx43 hemichannels, and since the effect occurs within a few seconds, it is most likely due to activation of hemichannels already present in the membrane (488, 552, 553). The effect could be blocked by reducing agents like DTT or intracellular glutathione, or scavengers of free radicals, suggesting that it is due to oxidation of cysteine residues in the C-terminal part of Cx43 (107, 552, 553). In contrast, it has also been reported that DTT increases the open probability of Cx43 hemichannels under normoxic conditions (554). This apparent paradox has been suggested to be explained by a modulation of the sensitivity to the redox potential by progressive changes in phosphorylation status (555).

Mechanical stimulation—Various forms of mechanical stress such as direct mechanical stimulation, hyperosmotic stress, and shear stress have all been reported to increase the activity of connexin hemichannels (21, 195, 292, 568, 629). Whether it is a direct effect or whether it is mediated by other mechanisms like phosphorylation remains to be determined.

Hemichannel pharmacology

Hemichannels are blocked by the same compounds that blocks intercellular channels. Thus the unspecific gap junction inhibitors heptanol, octanol, halothane, carbenoxolone, 18-glycyrrhetinic, various fatty acids (e.g., arachidonic acid) and fenamate derivatives [e.g., flufenamic acid (FFA)] also all block hemichannels. All these agents have a low specificity since they block other ion channels as well. Consequently, results obtained from pharmacologic manipulation of hemichannels by these agents should be interpreted with

caution. Lanthanides such as La^{3+} and Gd^{3+} appear to block hemichannels by entering the pore region of the channel. Like the previous agents it is unspecific in the sense that it may also block other ion channels, but in contrast to the other agents it is without effect on intact intercellular channels when added to the extracellular medium. In contrast, the connexin mimetic peptides are considered to be more specific blockers of hemichannels. They are designed to mimic parts of the extracellular loop of the various connexins, and they were originally intended for inhibiting cell-cell communication by blocking formation of new cell-cell channels, but they appear to be useful tools for inhibiting connexin hemichannels as well (59, 60, 171, 173, 371). However, these results may have to be interpreted with caution since a recent study failed to show any inhibitory action of the peptides on connexin hemichannel activity (113). Instead, membrane channels formed by the unrelated pannexin1 were inhibited. The connexin mimetic peptides may therefore be less specific than previously thought, since they may also have effects on other channels, in particular pannexin hemichannels.

Quinine has been reported to activate a series of connexin hemichannels found in different vertebrates (147, 414, 415, 561, 744), although there appears to be no reports of such an effect in mammalian systems.

Physiological and pathophysiological roles

Hemichannels have been suggested to be involved in several physiological and pathophysiological processes. Most of the experimental data on hemichannels have however been obtained *in vitro*, and often in transfected cells that normally does not express connexins. The scarcity of data from *in vivo* studies calls for considerable caution when extrapolating results obtained in cell systems to processes occurring in the normal or diseased organism.

Release of ATP, NAD^+, and glutamate—Hemichannels have been suggested to play an important role in the release of signaling molecules to the extracellular medium. Several studies have shown that hemichannels may be an important pathway for releasing ATP to the extracellular compartment (7, 8, 59, 60, 100, 169, 174, 195, 204, 207, 222, 242, 301, 305, 371, 373, 412, 458, 483, 503, 506, 568, 597, 636, 663, 781). The released ATP can bind to purinergic receptors on the secreting or neighboring cells, and thus act as a signaling molecule in auto- and paracrine responses. One well described effect is the initiation of Ca^{2+} waves, where the released ATP diffuses and acts extracellularly to cause an increase in the intracellular Ca^{2+} concentration, which spreads across the neighboring cells. Ca^{2+} waves have been described in many different cell types, for example, epithelia, nervous tissue, liver and smooth muscle cells (166, 572, 587, 741, 771). The spread of Ca^{2+} waves across cell populations may among other things play important roles in embryogenesis by coordination the migration and differentiation of progenitor cells (166, 741).

Interestingly, Cx30 is present in the luminal membrane of renal tubular epithelial cells in the distal part of the nephron (427). Being an unopposed membrane, they must be present as hemichannels and appear to play a role in pressure natriuresis by allowing secretion of ATP into the tubular lumen with subsequent inhibition of sodium reabsorption in response to acute increases in the arterial blood pressure (636).

Hemichannels may also play an important role in the regulation of the intracellular Ca^{2+} concentration. Cyclic ADP ribose (cADPR) is an endogenous ligand for Ryanodine receptors and causes release of Ca^{2+} from intracellular stores (179). It is synthesized from NAD^+ by the ectoenzyme CD38 and transported back into the cell through active or facilitated transport mechanisms (298). The precursor NAD^+ appears to reach the ectoenzyme by release via Cx43 hemichannels (67, 68).

Hemichannels have also been proposed as a pathway for the release of glutamate from astrocytes (762). The release was especially prominent under conditions of low extracellular Ca^{2+} concentrations; levels that may well occur during ischemia, seizures or spreading depression (251). Since glutamate mediated excitotoxicity is one of the major mechanisms underlying ischemic brain injury, blockade of hemichannels may be a new therapeutic target for preventing brain injury. However, these data have been challenged by more recent studies that have failed to find glutamate release through hemichannels (388).

Hemichannels and cell death—Many of the factors that regulate connexin hemichannels are found during metabolic inhibition and/or ischemiareperfusion, that is, ATP depletion, protein dephosphorylation, decrease in extracellular Ca^{2+} and Mg^{2+} concentrations, increases in intracellular Ca^{2+} concentration, and changes in redox potential. It is therefore likely that hemichannels may be activated during metabolic inhibition and/or ischemiareperfusion (23, 108, 293, 327, 374, 488, 555, 584, 624, 682, 721). Opening of hemichannels may lead to collapse of the membrane potential due to disturbances in ionic homeostasis including intracellular Na^+ and Ca^{2+} gain and loss of K^+. Loss of metabolically relevant compounds such as glucose, ATP,NAD^+, and free radical scavengers like glutathione and ascorbic acid may contribute to cellular injury and death (2, 68, 542, 553). In addition, the extracellular release of K^+, glutamate, and metabolites of arachidonic acid may cause depolarization and excitotoxicity and thus paracrine mediated cell death in neighboring cells (555, 629, 671, 762). In agreement with such a role, acute knockdown of connexins or application of certain pharmacological blockers have been found to have a protective effect under ischemic conditions suggesting that opening of connexin hemichannels may contribute to cell death (131, 178, 186, 187, 255, 624, 682). This issue is complicated by the fact that other studies indicate a protective effect of gap junctions and hemichannels during ischemic conditions (98,329,439,597). Thus, Cx43 hemichannels has been implicated in heart pre- and postconditioning (602), and data suggest that ATP secreted via Cx36 and Cx43 hemichannels may act via purinergic receptors to induce neuroprotection during ischemia (381, 597). Preconditioning has been shown to reduce the degradation of Cx43 hemichannels, which leads to an increase in the amount surface hemichannels (381). Supporting the involvement of Cx43 in the preconditioning response, Cx43 null mice were insensitive to hypoxic preconditioning (381). Further studies are clearly needed to define the specific circumstances under which hemichannels may have either protective or detrimental roles.

Hemichannels in hereditary diseases—Mutations in different connexins have been implicated in several human genetic disorders, and in some cases it has been speculated that defective hemichannel function could play a role in the pathogenesis; for a recent review see reference 594.

Hemichannels in acquired human diseases—Besides their role in ischemia, which is reviewed previously, hemichannels may be involved in the pathogenesis of such diverse conditions as atherosclerosis, inflammation, and bacterial infections (241, 553, 594, 688, 720). However, most of the data were obtained *in vitro*, and there is very little data from studies in the integrated organism. This makes it difficult to judge the clinical relevance of the observations, and it is clear that additional research is required to define the possible roles of hemichannels in human diseases.

The gap junction proteome

Several connexin-binding proteins have been identified and are collectively referred to as the gap junction proteome (for recent reviews see references 210, 352). In particular, the CT domains of most connexins contain multiple sites for protein-protein interactions. Some

connexin-binding proteins involved in trafficking, gating, or posttranslational modification of connexins are discussed in other sections of this article. In addition, connexins also interact with proteins involved in cell growth (such as the CCN3-Cx43 (190, 206) and Discs Large homolog 1 (Dlgh1)-Cx32 interaction (154)) as well as receptor and channel proteins such as the acetylcholine receptor (AChR) (773) and aquaporin-0 (AQP0) (389). The variety of proteins that interact with connexins, leaves no doubt that connexins have several channel-independent effects. However, the gap junction proteome is not yet complete and as more connexin-binding proteins may be identified in the future, additional channel-independent effects of gap junctions may also be described.

Connexins regulate cell growth independent of coupling

Connexin expression is capable of regulating cell growth (759) and connexins have been hypothesized to play a role in cancer development (for review see (reference 112)). The link between gap junctions and cell growthwas originally proposed by Loewenstein (396,429) and the idea is supported by many subsequent studies. Suppression of cell growth can be obtained by regulation of gene expression and several genes involved in the cell cycle or cell cycle repression have been implicated (112). The effect of connexins on cell growth is probably mediated by both coupling-dependent and couplingindependent components (112). However, reintroduction of connexin in tumor cells can suppress growth without increasing coupling (281, 534). For example, growth suppression is observed in cultured cells without cell-cell contacts (446), cells cultured with GJ inhibitors (446), cells expressing connexins that are incapable of forming functional channels (484), and cells expressing only CT-fragments of connexins (117,446,780). Therefore, it seems that coupling-independent effects are of major importance.

Cx43 is by far the most studied connexin with regard to growth suppression, but other connexins, such as Cx26 (433), Cx30 (529), Cx32 (53, 430), have been shown to have similar effects; whereas other studies show that Cx32 (433) and Cx40 (205, 433) do not. The overlap for Cx32 suggests that the effect may depend on both cell type and setting. This holds true also for Cx43, which can be highly expressed even in very tumorigenic cells (635).

In the case of Cx43, growth suppression is likely caused by the CT, since expression of Cx43-CT is equally efficient in suppressing growth compared to full length channels and since Cx43 lacking the CT does not affect cell growth even though they form functional channels (446, 780). The inhibitory effect is reported to depend on phosphorylation, and mimicking constitutive phosphorylation of S262 prevents growth suppression (118). The basis of the inhibitory effect is possibly linked to a nuclear localization of connexins. Expressing the Cx43-CT in cardiomyocytes and HeLa cells results in a strong nuclear localization (117), but notably this does not occur for the full length Cx43. However, the Cx43-CT may play a physiological role under certain circumstances. Taffet and co-workers identified a 20 kDa CT fragment in several cell lines that accounted for up to 65% of the total Cx43 content (full length plus CT fragment) (297). The authors could not detect the fragment in native rat tissue; however, this does not rule out a role for the CT fragment under special circumstances. It is certainly tempting to speculate that induced cleavage of Cx43 could regulate cell growth, though the CT may also exert its effect while part of the full length protein. For example, Cx43 regulates the transcription factor NOV. Increased Cx43 expression not only upregulates the transcription factor NOV but also mediate NOVs translocation from the nucleus to the cell membrane by direct binding to the CT (190, 205, 239). Expression of CT-truncated Cx43 does not affect NOV expression or localization, showing that the effect is CT specific (190, 205).

Future studies are needed to elucidate the role of connexins in growth regulation and determine whether their CTs act as transcription factors, alter transcription by binding factors like NOV, initiate signal transduction, or something entirely different.

Mitochondrial connexin

The mitochondrial localization of Cx43 was originally reported in cultured ECs by a combination of immunostaining and western blot (375). Treatment with homocysteine increased the amount of Cx43 in mitochondria without increasing intercellular coupling. Homocysteine treatment also reduced the EDHF-mediated vasodilatory response to acetylcholine and the authors hypothesized that sequestering of Cx43 from gap junctional plaques reduced EDHF signaling between the endothelium and smooth muscle cells.

Around the same time, it was shown that the cardioprotective effect obtained by ischemic preconditioning was lost in mice with a heterozygous knockout of Cx43 ($Cx43^{+/-}$) (603). The mice have a 50% reduction in Cx43 expression, but exhibit normal action potential propagation due to overcapacity in coupling known as the conduction reserve (709). Therefore, it was deemed unlikely that reduced intercellular coupling *per se* eliminates preconditioning, which is supported by the finding that preconditioning is also lost in isolated cardiomyocytes (no cell-cell contact) from $Cx43^{(+/-)}$ mice (377). Preconditioning is induced by short intermittent episodes of ischemia and reduces infarct size during subsequent prolonged ischemia (459). Many processes and signaling mechanisms have been implied in preconditioning and mitochondria play a central role. Cell death by necrosis is often associated with mitochondrial swelling and rupture, which deprives the cell of energy production and exposes it to Ca^{2+} overload, which in the case of the cardiomyocyte leads to disruptive hypercontracture. Apparently, preconditioning primes the mitochondria and makes them resistant to ischemia. So if Cx43 plays a noncoupling role in preconditioning, they could do so from the mitochondria. Schulz and co-workers demonstrated that Cx43 is indeed found in mitochondria in both rats, mice, pigs, and humans (49), but apparently only in mitochondria located just below the sarcolemma and not in those lying deeper between the myofibrils (51). Mitochondrial Cx43 (mito-Cx43) have been reported to reside in both the outer (228) and inner (49, 567) membranes of mitochondria, and several lines of evidence show that the Cx43-CT is most likely located in the intermembrane space (51). Cardiac mito-Cx43 content is acutely increased by preconditioning (49, 399) and homocysteine (696), whereas hyperglycemia reduces mito-Cx43 in the retina (441). Some information has come forth about the route of Cx43 to the mitochondria. Cx43 does not contain mitochondria-targeting sequences as such, but enters the mitochondria via the translocase of the outer membrane (TOM) by binding to chaperones. Cx43 interacts with the chaperone Hsp90 and Tom20, and inhibition of Hsp90 by geldanamycin prevents Cx43 translocation to the mitochondria (567).

The role of mito-Cx43 in preconditioning is not entirely clear but is thought to involve formation of reactive oxygen species (ROSs) and activation of mitochondrial K_{ATP} channels (mito-K_{ATP}). Preconditioning involves ROS formation, which can be stimulated by the K_{ATP} opener diazoxide (497). Diazoxide-induced ROS formation is reduced in $Cx43^{(+/-)}$ myocytes, whereas direct application of ROS formation still offers protection, indicating that Cx43 is working upstream of ROS formation (258). Consistent with this, inhibition of Cx43 translocation to the mitochondria by geldanamycin also prevents diazoxide-induced protection (567). However, geldanamycin does not prevent preconditioning induced by short pulses of ischemia, indicating that parallel Cx43-dependent signaling mechanisms are involved and that these mechanisms may have different thresholds regarding the amount of mito-Cx43 needed.

Diazoxide activates the mito-K_{ATP} channel that also resides in the inner mitochondrial membrane. Using patch clamp on isolated preparations of inner mitochondrial membranes, it was shown that the protective effect of diazoxide was mediated by PKC-ε activation and that this activation was entirely dependent on the presence of Cx43 because mito-K_{ATP} activation was reduced or abolished by hetero and homozygous KO of Cx43 (573). Interestingly, the same study also showed that several blockers of Cx43 hemiand gap junctional channels also prevented PKC activation of mito-K_{ATP} channels, implying that Cx43 permeation may be needed.

Several lines of evidence indicate that the protective role of Cx43 in the mitochondria may be unique to this connexin. Mitochondrial Cx43 has not been investigated in many noncardiac tissues, but the gap junction inhibitor carbenoxolone inhibits opening of the permeability transition pore in mitochondria isolated from astrocytes (Cx43 expressing) but not in mitochondria from liver cells (expressing Cx26 and Cx32) (15). The reason for the lack of protection by Cx32 could be either that it does not offer protection *per se* and/or because it simply does not translocate to the mitochondria. In favor of the latter, mice in which the Cx43 coding sequence is exchanged for that of Cx32 express Cx32 in the myocardium, but Cx32 is not present in mitochondria as Cx43 is in wt mice (437).

Even though the exact mechanisms are currently being unraveled there is good evidence that ischemic stress promotes the transport of Cx43 to the mitochondria and that mito-Cx43 protect the mitochondria from rupturing and killing the cardiomyocytes. Interestingly, this ability seems to be lost with age, where the amount of both Cx43 and mito-Cx43 is reduced (50) and possibly the mechanism is only for protection in the young. However, the mechanism is not universal to all tissues expressing Cx43 and as described in the section on hemichannels, Cx43 may protect neurons in an auto/paracrine manner by acting as hemichannels. It will also be interesting to see if other connexins than Cx43 translocate to the mitochondria and offer similar protection.

Pharmacology of Gap Junctions

Pharmacological agents aimed at modifying (in fact, impairing) the function of ion channels have existed for a number of years. Their use has improved the life of many and perhaps, impaired the life of some. In the case of antiarrhythmic therapy, for example, pharmacological development has been based on making a disrupted electrophysiological substrate, worse. For the most part, agents that seek to treat cardiac arrhythmias are designed to block, rather than improve, ion channel function. Interestingly, drugs exist to interfere with currents mediated by various ion channel proteins: sodium, calcium, or potassium of various kinds; but for reasons that combine luck and technical difficulties (reasons that are found throughout the history of therapeutics), gap junctions have been spared from clinical application as pharmacological targets. At present time, there are no drugs in clinical use that selectively modify gap junction-mediated intercellular communication. However, there is active basic and translational research that seeks to develop such an application. In this section, we briefly review the current status of gap junction pharmacology.

Chemical agents that induce gap junction closure

Long-chain alcohols, fatty acids, and general anesthetics—In an elegant paper published in Nature, 1980 (295), Johnston, Simon, and Ramon first reported that anesthetics, and in particular long-chain alcohols, would cause closure of gap junction channels. These early experiments were followed by others, showing that myristoleic acid, decanoic acid, and palmitoleic acid are all capable of uncoupling cardiac gap junction channels, concurrent with a decrease in open channel probability (77, 670). Similarly, halothane and isoflurane are two general anesthetics that reversibly close gap junctions at concentrations used in

clinical practice (78). Follow-up experiments indicated that, though these agents close gap junctions, they also impair function of several other ionic currents (78, 512, 626). It has been proposed that the mechanism of action of these agents is rather nonspecific, perhaps by disrupting the fluidity of the plasma membrane, and/or affecting the hydrophobic lipid-protein interface (27). Yet, studies in other ion channels suggest that general anesthetics interact directly with the channel protein, likely within selected binding pockets in pore-forming domains that are, on the other hand, shared by various molecules, thus explaining their lack of selectivity for the connexin channels, in particular (392, 589).

Glycyrrhizic acid metabolites—Davidson et al. used a metabolic cooperativity assay in human fibroblasts to determine that the glycyrrhizic acid metabolites 18-α-glycyrrhetinic acid, 18-β-glycyrrhetinic acid (α- and βGA), and carbenoxolone reversibly inhibited coupling in concentrations as low as 2 μmol/L (125). These compounds require a longer exposure time than alkanols and general anesthetics. Their mechanism of action remains unclear, though it has been proposed that they may affect the phosphorylation state of the protein, or modify the ability of connexin subunits to aggregate within the cellular environment (125, 126). A study by Sagar et al. further investigated the effects of carbenoxolone on junctional dye transfer in bovine aortic ECs (BAECs), which express Cx43 (582). The authors found that staurosporine inhibited the carbenoxolone-induced increase in Cx43 content. This result suggested a role for kinases in the mechanism of carbenoxolone. As with other compounds, the effect of these metabolites is not selective for gap junctions (582).

The phosphorylation state of connexin as a pharmacological target—Gap junctions are regulated by a variety of kinases, as well as by activation of pertinent phosphatases (for additional information, see section on Cx phosphorylation). In the particular case of Cx43, it has been postulated that modification of its phosphorylation state may affect its ability to associate with partner molecules and/or to function as a channel-forming protein (see, e.g., references 44, 550, 712). Kinase activity could then be seen as a potential path for modifying intercellular communication. In fact, recent studies have suggested that some of the peptides said to preserve the open state of gap junctions, do so via alteration of kinase pathways (see reference 14). Yet, it is important to emphasize that kinases are, in general, highly promiscuous enzymes that catalyze the phosphorylation of multiple substrates. From this perspective, it is difficult to conceive that molecules that alter kinase activity could be developed as selective modifiers of gap junctionmediated intercellular communication.

Antimalarials—The effects of the antimalarials on gap junction coupling, particularly quinine and quinine-derivatives-like mefloquine, are interesting because they seem to be connexin-subtype specific. A study by Srinivas et al. showed that quinine dramatically blocks channels formed by Cx36 and Cx50, and moderately blocks Cx45 channels (shown by a 50% decrease in gap junction conductance), whereas the other connexin subtypes were insensitive to quinine exposure (654). This study represented the first demonstration of connexin-specific gap junction block by a pharmacological agent.

Fenamates—These nonsteroidal drugs are generally known for their antiinflammatory effects at nanomolar concentrations, but at higher concentrations (10-100 μmol/L), they were shown to block membrane ion channels (247, 601). These studies were followed by others, showing that these compounds have uncoupling effects in monolayer cultured cells expressing gap junction channels (247). Harks et al. showed that flufenamic acid (FFA), niflumic acid (NFA), and meclofenamic acid (MFA) caused uncoupling in Cx43-expressing NRK cells. The block was rapid, but not entirely reversible. The order of potency was

MFA>NFA>FFA for block of electrical and dye coupling (247). The work was followed and expanded by the elegant studies of Srinivas and Spray demonstrating that FFA does not greatly discriminate between connexins, and that the effect does not seem to be mediated by direct binding of the drug to the pore of the gap junction channel. Single channel recordings indicated that FFA reduced channel open probability without modifying current amplitude, a fact consistent with other gap junction blockers (655).

Cardiac glycosides—The observation that cardiac glycosides strophanthidin, ouabain, and digitoxin decrease intercellular coupling attracted a great deal of attention at a time when the medical use of digitalis derivatives was still quite extensive (130). Reports that followed the early studies suggested that the effect likely involved an increase in the concentration of intracellular Ca^{2+} (426). These studies have not been pursued further in recent years, perhaps given the decreasing use of these agents in the medical practice. Yet, it remains possible that the structural characteristics of the cardiac glycoside molecules could bring some clues as to the structure of a pharmacophore with selective action on the gap junction molecules.

Molecular approaches to reduction of cell-cell coupling—While the agents described previously reduce coupling in a rather nonselective manner, some investigators have specifically targeted Cx43 RNA by antisense-directed strategies. Small oligonucleotides (typically 20 bases or fewer) have been shown to effectively decrease the amount of gap junction channel protein expressed. In an initial study, Qiu et al. correlated the level of local Cx43 expression with the time course of skin wound healing in mouse (536). These authors showed that a single topical application of Cx43 antisense gel brought about a transient downregulation of Cx43 protein levels, ultimately resulting in a dramatic increase in the rate of skin wound closure. The results showed a dampened inflammatory response and a simultaneously enhanced rate of reepithelization. Microscopically, a significant reduction in neutrophil numbers was seen in the tissue around the wound. The added therapeutic benefits included a significant reduction in the extent of granulation tissue deposition and the subsequent formation of a smaller, less distorted, scar. The studies of Law et al. are consistent with these observations (362). Whether reduction in Cx43 levels could change the time course and formation of scar in other tissues (such as heart) remains to be determined. It is worth noting that decreased Cx43 expression (in this case by gene haplodeficiency) also decreased the progression of atherosclerotic plaque formation in a murine model of the disease (345).

Peptide strategies for reduction of gap junction-mediated intercellular communication—In 1994, Dahl et al. (115) and Monaghan et al. (443) showed that extracellular application of peptides corresponding to relevant extracellular domains of connexins decreased the extent of cell-cell coupling. Specifically, synthetic peptides representing the extracellular loop sequences of Cx32 inhibited cell-cell channel formation (115). The advantages and limitations of the use of these “connexin mimetic peptides” were reviewed by Evans et al. (172). These authors pointed to the fact that these molecules could block not only Cx37, Cx40, and Cx43 channels but also connexin hemichannels. Various groups have shown these peptides to effectively inhibit gap junctional transfer of fluorescent dyes, electrical coupling, and synchronized Ca^{2+} oscillations in smooth muscle cells (172). The two most widely used peptides include Gap 26 and Gap 27, which correspond respectively to sequences on the first and second extracellular loops of Cx43. These two peptides have also been shown to inhibit the intercellular propagation of Ca^{2+} waves in monolayer cell cultures (172). Yet, additional studies suggest that these peptides may not be as specific as originally thought. Indeed, in examining the effects of connexin and pannexin mimetic peptides, Wang et al. (734) showed an attenuation of channel currents that was not

consistent with sequence-specific actions of the peptides. They found that connexin mimetic peptides inhibited pannexin channel currents, but did not inhibit the connexin currents of the channels from which the sequence was derived. Similarly, pannexin mimetic peptides did inhibit pannexin channel currents, but also extended promiscuously to Cx46 channels (734).

Agents that increase gap junction coupling

In the previous sections, we have described agents known to decrease coupling (albeit with limited specificity). Less is known about molecules that could increase electrical communication in the heart. Though some kinases can improve gap junction-mediated coupling, their action can be hardly argued as specific, as mentioned before. Indeed, all of these agents have nonconnexin molecules as targets, and act on complex intracellular signaling pathways that affect a number of cellular processes. Specificity of targeting remains an important challenge in connexin pharmacology today. As with the use of connexin-mimetic molecules, peptide-based strategies have been developed to identify molecules that can enhance intercellular communication in the heart. The use of peptides as pharmaceutical agents has become widely accepted over the past several years and numerous trademarked products are examples of successful peptide-based pharmaceuticals in the US market. In the following paragraphs, we review recent progress and the current status of peptidic sequences that enhance or preserve gap junction function.

Peptide-based strategies to increase or preserve gap junctional communication

Antiarrhythmic peptide-10 (AAP-10) and its derivatives: In the early 1980s, Aonuma et al. characterized the ability of a series of natural peptides derived from bovine atria to synchronize the beating of cultured chick cardiomyocytes (10). Given that these compounds were shown to have an effect on the synchronization of pacemaker activity of cardiac cells, they were dubbed “anti-arrhythmic peptides” (AAP). A series of followup studies led to the characterization of AAP10, and its d-amino acid analogue ZP123 (later referred to as “rotigaptide”) as potential antiarrhythmic agents (142, 755). The experiments of Eloff et al. suggested that ZP123 (rotigaptide) may act by preserving intercellular communication under conditions of metabolic stress (168). Separate experiments indicated that this peptide does not modify the morphology of the cardiac action potential; moreover, the peptide was shown to not interact with a panel of integral membrane proteins (254, 321, 755). Treatment with rotigaptide was shown to increase atrial conduction velocity—with no proarrhythmogenic effect—in animal models of AF and congestive heart failure-related AF. More specifically, in dog models of both atrial ischemia-induced AF and chronic atrial dilatation, rotigaptide treatment was associated with a significant antiarrhythmic effect. Additionally, it was shown that rotigaptide prevented conduction slowing and reverted established conduction slowing in the isolated rat atrium in a concentration-dependent manner. The effects of rotigaptide were also examined in rabbit atria with chronic atrial dilatation using high-resolution optical mapping on isolated Langendorff perfused rabbit hearts, as well as in cellular studies of gap junctions in ventricular myocytes (132, 168, 234, 254, 318, 321, 384, 627, 755). Additional animal studies showed that pretreatment with rotigaptide prevented both ventricular and atrial arrhythmias during conditions of acute cardiac ischemia (261) and partially reversed the loss of Cx43 in a canine model of healing infarct (404). Of note, rotigaptide did not prevent conduction slowing or arrhythmia inducibility in this model (404). Rotigaptide was the first drug described as acting primarily by enhancing—or preserving—gap junctional communication (although additional cellular/molecular work would seem justified, and a clear molecular mechanism of action remains lacking). Recent reports have described a new compound (GAP134) as a potential gap junction opener that can be orally administered (518). This compound has been shown to improve conduction and reduce AF/atrial flutter in a canine sterile pericarditis model (571), and to prevent spontaneous ventricular arrhythmias

and reduce infarct size during myocardial ischemia/reperfusion injury in open chest dogs (262). A modest infarct size reduction was also observed in rats subjected to chronic infarction and treated with rotigaptide (252). Additional promising results in an experimental model of AF in dogs have been reported (361). Future studies will define the efficacy and selectivity of this compound, and its potential use as a therapeutic agent.

While rotigaptide and GAP-134 seem to enhance gap junction communication, their cellular/molecular target is unknown. Attempts to demonstrate binding of rotigaptide to the Cx43 molecule have been unsuccessful. In fact, it is believed that rotigaptide does not exert its effect directly on the connexin molecule, but indirectly via activation of yet unidentified kinases [it is known that rotigaptide modifies the phosphorylation state of Cx43 (14, 318)]. The latter carries a high risk of connexin-unrelated effects on cell function, as kinases are likely to interact with a variety of substrates, not only Cx43. The cumulated evidence for rotigaptide-induced effects on Cx43-mediated junctional conductance, and/or Cx43 abundance is substantial see, for example, references 168,253,321,404,627,755, see also reference 132. Dye transfer studies suggest that rotigaptide stimulates Cx43-mediated intercellular communication but it is devoid of activity on Cx26 and Cx32, that is, connexins with short C-terminal tail (CT) that lacks several of the regulatory domains of Cx43 and Cx40 (97). Additional data further suggest that connexinregulatory peptides can influence ATP release in cardiac myocytes (98), and improve cell migration rates of human epidermal keratinocytes and dermal fibroblasts (753). Finally, it is interesting to note that an agent that acts by preserving gap junction communication under conditions of stress would, on the other hand, decrease infarct size, given separate evidence indicating that failure of the Cx43 molecule to chemically gate leads to an increase in the size of the infarct (403).

Structure-based peptides to modulate Cx43 function: αCT-1: A peptide-based strategy, grounded on a structure-activity relation, was also used by Hunter et al. to characterize the interaction of Cx43CT with the scaffolding protein ZO-1 (284). The interaction between these two proteins occurs specifically between the second PDZ domain of ZO-1 and the last nine amino acids of Cx43 [the “PDZbinding domain” (284); see also reference 648]. Accordingly, introduction of a peptide corresponding to the PDZ-binding sequence of Cx43 (dubbed αCT-1) into cells prevented the Cx43-ZO-1 interaction. With this model, the authors demonstrated that prevention of the interaction of Cx43 with ZO-1 leads to a reduction of peripherally associated ZO-1, accompanied by a significant increase in plaque size. Further analysis indicated that this increased plaque size was not due to increased protein expression or decreased turnover, but rather to unregulated accumulation of gap junctional channels from nonjunctional pools. In a recent study, O’Quinn et al. utilized a myocardial cryoinjury model to demonstrate that local delivery of αCT-1 reduced arrhythmia inducibility, prevented Cx43 remodeling, and promoted phosphorylation of Cx43 on serine 368 by PKC-ε. The authors concluded that αCT-1-mediated increase in Cx43-S368 phosphorylation may contribute to reduction in inducible arrhythmia following injury (479). The potential for αCT-1 as a coadjuvant, or as a primary drug in wound healing may extend to other systems. Indeed, Soder and colleagues have demonstrated that of αCT-1 modulates the biological response to silicone implants (640) and can reduce scar progenitors and promote regenerative healing following skinwounding (209). Overall, these results support the notion that the CT domain of Cx43 can be an important target for the development of gap junction-based pharmacology. The results described previously, from the Gourdie laboratory, offer the strongest argument in favor of the notion that Cx43 regulation (mediated by its CT domain) is a valuable target in the prevention of scar formation, and that agents interfering with the molecular mechanisms of CT-mediated Cx43 regulation can have therapeutic applications not only in the heart, but in other systems as well. Additional studies aimed at developing a Cx43CT-based pharmacology for manipulation of gap junction-mediated intercellular communication, are described later.

Structure-based peptides to modulate Cx43 function: RXP-E and its derivatives: An alternative strategy in the search for gap junction modifiers was based on the idea that chemical gating of Cx43 results from the interaction between the CT domain, acting as a gating particle, and a region of the CL that acts as a receptor (155, 454, 606). Using phage display, the Delmar laboratory identified a series of peptides containing the sequence "RXP" (arginine, any amino acid, proline) as a consensus Cx43CT binding motif, and reported that a particular 34-amino acid peptide within this RXP series (dubbed RXP-E) binds to Cx43, prevents heptanol- and low pH-induced gap junction closure, and prevents action potential propagation block (370, 621) (Fig. 11).

While those studies showed significant and promising results, further applications of RXP-E as a drug candidate were hampered because of the molecular size and low membrane permeability of this peptide, as well as the metabolic instability and poor oral bioavailability of peptides in general. Peptide mimetics, on the other hand, can be developed to retain the desired biological properties of a peptide. Steps in the design of mimetic molecules include identification of the essential active components (or amino acids) of the peptide sequence (the pharmacophores), determination of their structure/conformation in aqueous solution and finally, development of a corresponding pharmacophore model. Thus, in a separate study, the same laboratory applied a combination of molecular modeling and experimental methods to identify the first group of pharmacophores with Cx43-binding activity (724). Molecular modeling, based on analysis of the RXP series of peptides, led to the identification of both cyclized and linear peptides, 6 to 8 amino acids long, with the ability to bind to the CT of Cx43 and prevent the closure of Cx43 channels triggered by octanol superfusion. These data also provided the first three-dimensional imprint of a potential site in the CT region of Cx43 that could be used for binding of exogenous molecules. Yet, a more precise assignment of the active components of these peptides still remained necessary, to address the question of whether a three-amino acid model can serve as a platform for small molecule design with potential application in living organisms. Thus, a separate study reported a combination of biochemical, spectroscopic, and electrophysiological techniques to demonstrate that the primary sequence RR(X)Y ("X" being a linker amino acid) is a Cx43 binding motif. *In silico* analysis, validated experimentally, further showed that the triangular secondary structure formed by the two arginines and the tyrosine side chains (a "pharmacophore triangle") serves as platform for the design of synthetic molecules that target cardiac gap junction channels. These experiments led to the first peptidomimetic compound capable of preventing chemical gating of Cx43 (compound "ZP2519") (723) and demonstrated that rational, structure-based drug design can be applied to cardiac gap junction pharmacology. These molecules can then serve as tools to determine the role of gap junction regulation in cardiac arrhythmogenesis and, potentially, serve as pharmacological agents for treatment of a selected subset of cardiac arrhythmias.

Final considerations

Development of a connexin-based pharmacology has made a significant impact in various areas, and perhaps more prominently, in wound healing, as well as in cardiac arrhythmias. As for the latter, at present, pharmacological intervention of cardiac arrhythmias relies, largely, on the use of molecules that impair the function of ion channel proteins. Moreover, while a number of drugs are available to affect sodium, calcium, or potassium currents, gap junctions have eluded the action of exogenous compounds. Whether cardiac gap junctions can be successfully targeted in antiarrhythmic therapy remains unclear. One of the reasons why this question remains unanswered is the lack of compounds that can, selectively and effectively, interfere with gap junction function. The studies described previously seek to develop small molecules that can restore or preserve the function of the major cardiac gap junction channel, Cx43. In the course of studies from various laboratories, a small arsenal of

natural and synthetic peptides, as well as pharmacophores, has been generated. Yet, the utility of these molecules in therapeutics remain to be defined. Various authors have proposed that closure of gap junctions during pathologic conditions such as ischemia could lead to malignant ventricular arrhythmias.Yet, loss of coupling under ischemic conditions could also be seen as cardioprotective, as it could preserve the integrity of tissue that is neighbor to an area of damage. Indeed, if gap junctions were to remain open at the ischemic border zone, apoptosis-triggering signals could move through gap junctions, thus extending the area of damage beyond the one that lacks oxygen supply. Studies on brain ischemia have shown that this "bystander effect" may be responsible, at least in part, for the area of "penumbra" that surrounds ischemic tissue (467). (Interestingly, penumbra is not observed in cardiac ischemia and, on the other hand, healing over is well demonstrated.) Opposite to this view is the argument that, while cell-death signals can move through gap junctions, so do molecular signals that can preserve cell integrity, thus helping cell survival within the area at risk. What is important to acknowledge in these contradictory arguments is that our lack of a clear understanding of the beneficial or deleterious effect of gap junction regulation on pathophysiology is, in part, consequent to the lack of experimental tools that can selectively modulate gap junction channels. Much has been learned, for example, from the use of tetrodotoxin to validate the relevance of sodium channels in health and disease. Yet, while the list of pharmacological tools to modulate sodium, potassium, or calcium channels (among others) is extensive, the pharmacology of gap junctions is in its infancy. Future studies are likely to generate more selective and effective gap junction modifiers to assess their importance in medical therapeutics.

Conclusion

As outlined in this article, our knowledge on the physiology of connexin expression, regulation, and function in both health and disease has increased tremendously over more than three decades of research. However, the article also demonstrates that there is still much to be discovered. The many possible interactions between different connexins and the involvement of an ever increasing list of regulatory binding partners outline the complexity of gap junction physiology. The gap junction proteome may influence both trafficking and function of connexins, and especially the intramolecular rearrangement and binding have emerged as important in the regulation of channel function. Therefore, despite our increasing understanding of the field, several aspects are still subject of debate and contradicting findings remain to be explained.

One of the most heavily debated fields is the role of connexin hemichannels, and although most researchers agree that hemichannels may open under *in vitro* conditions, the extent to which this happens *in vivo* remains to be proven. But other surprising noncoupling roles have been established such as the presence of Cx43 in mitochondria, which regulate mitochondrial function. The potential role of other connexins in mitochondria and/or other intracellular locations, remain an interesting subject of future research.

Many breakthroughs in the gap junction field stem from the development of genetically modified mouse models, where the specific knockout or modification of connexins has allowed us to elucidate their role in different organ systems. Some of these models have confirmed important hypotheses, whereas others have brought up new and unexpected findings and spurred further discoveries. In addition to genetically modified models, the recent progress in the quality of MS studies have increased the possibilities to further explore the chemical modifications that different connexins undergoes during both normal and pathophysiological conditions. Given the important role that posttranslational modification plays in the regulation of connexins, it seems obvious that this is an area, which will also be subject for new interesting findings in the years to come.

Finally, the involvement of gap junctions in development of various diseases implies the need for further research in this area. Hopefully, basic research regarding the general regulation of gap junctions throughout the different organ systems in the body, as well as further research in pharmacological modulation of gap junctions may bring life saving discoveries in the future. Even though the first pharmacological agent targeting gap junctions remains to find its way to the clinic, the key role of gap junctions in the development of patophysiological conditions such as cardiac arrhythmias, atherosclerosis, cataract, and cancer makes gap junctions a promising pharmacological target.

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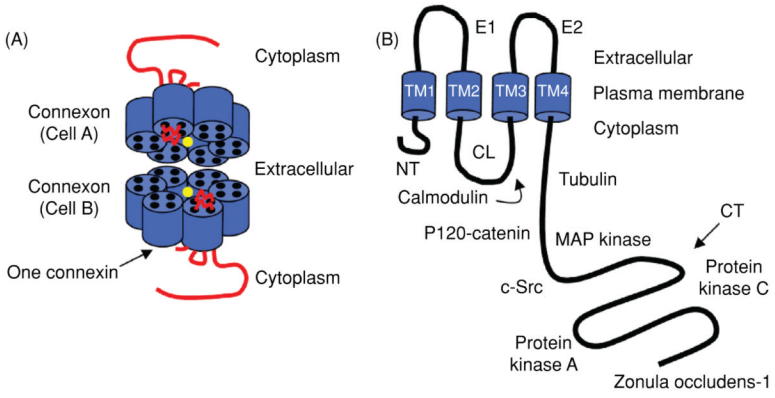

Figure 1.

Model of a Cx43 gap junction channel and monomer. (A) The channel pore location has been indicated by the yellow circle. (B) The Cx43 monomer with protein partners. The abbreviations are as follows: NT, N-terminus; CL, cytoplasmic loop; CT, C-terminus; E1 and E2, extracellular loops 1 and 2; TM1-4, transmembrane segments 1-4.

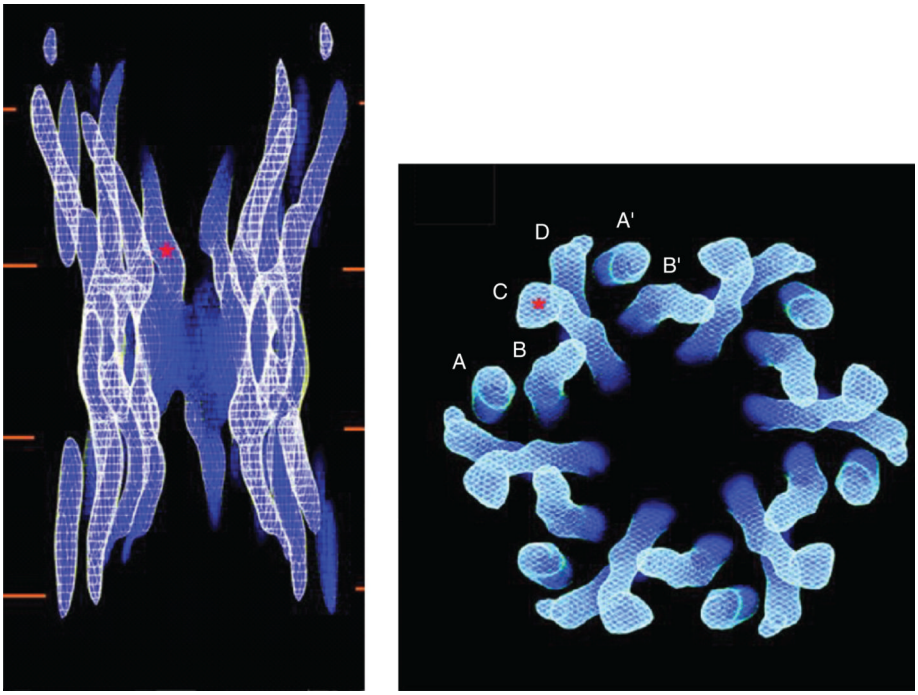

Figure 2.
Cx43 channel structure obtained by electron crystallography. The panel on the left shows a side view of the entire channel. The red lines represent the lipid bilayers. The red asterisk indicates the point at which the pore diameter is estimated to be the smallest. The panel on the right is a view from the cytoplasmic side. The channel is formed by six repeats of four identifiable densities (A-D), each density corresponding to one transmembrane domain. Modified, with permission, from Unger VM, Kumar NM, Gilula NB, Yeager M. Science 283: 1176-1180, 1999 (698). Reprinted, with permission, from AAAS.

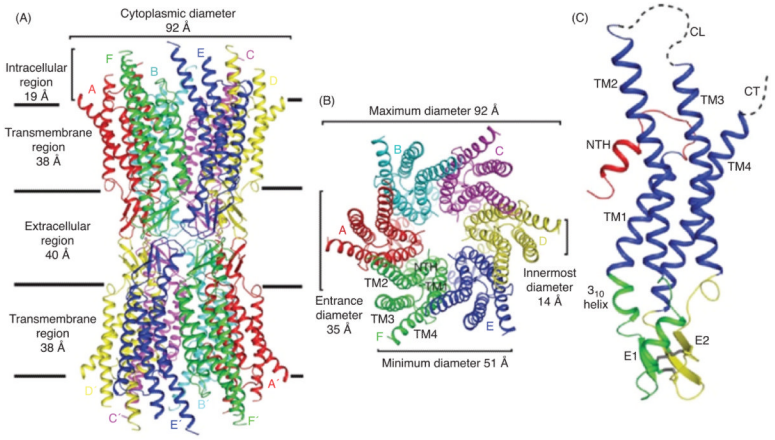

Figure 3.

Structure of the Cx26 gap junction channel and Cx26 protomer in ribbon representation. (A) Side view of the Cx26 gap junction channel. (B) Top view of the Cx26 gap junction channel showing the arrangement of the transmembrane helices TM1 to TM4. (C) Side view of the Cx26 protomer. Color code: red, NT; blue, TM1-TM4; green, E1; yellow, E2; gray, disulphide bonds; dashed lines, CL and CT, which were not visible in the map. E1 and E2 are the loops connecting TM1 and TM2, and TM3 and TM4, respectively. Modified, with permission, from Maeda et al. 2009 (409). Reprinted by permission from Macmillan Publishers Ltd: Nature (458: 597-602), copyright [2009].

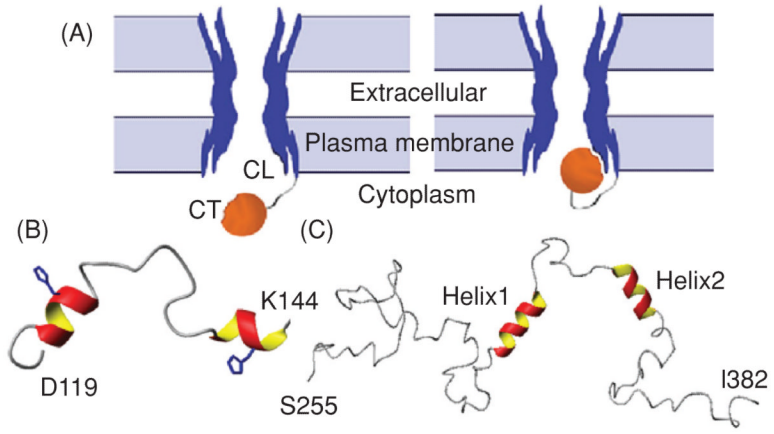

Figure 4.

"Ball-and-chain" model of Cx43 regulation. (A) Under normal conditions, the gate [cytoplasmic tail (CT)] is away from the pore. Under the appropriate stimulus, the gate swings toward the mouth of the channel, binds to a receptor [cytoplasmic loop (CL)] affiliated with the pore, and closes the channel. Lowest energy structure of the (B) Cx43CL and (C) Cx43CT domains; α-helices colored red and yellow. Figure is modified, with permission, from Delmar M, Coombs W, Sorgen P, Duffy HS, Taffet SM, Structural bases for the chemical regulation of Connexin43 channels, Cardiovasc.Res., 2003, 62(2): 268-275, (138) by permission of Oxford University Press, Duffy et al. 2002 (155), and Sorgen et al. 2004 (648), with permission.

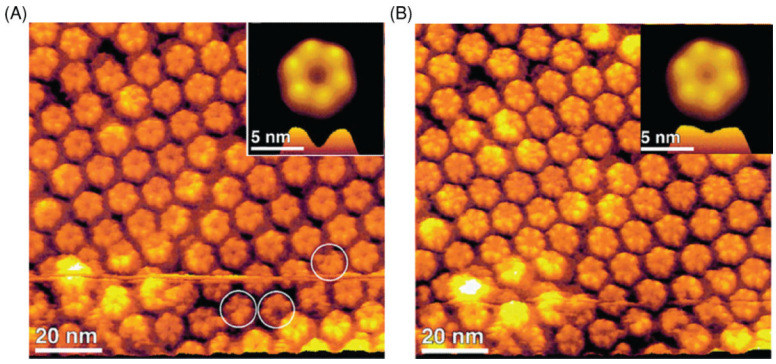

Figure 5.

Conformational changes in Cx26 hemichannels observed in low and high calcium buffers by atomic force microscopy (AFM). (A) AFM topograph showing the extracellular connexon surface imaged in a calcium-free buffer solution. Individual connexons exhibit defects in the number of subunits, as indicated by the circles. (B) Same connexon surface imaged in (A), but in the presence of 0.5 mmol/L calcium. The channel diameter has changed significantly as seen in the correlation averaged top view (inset) and the profile at the bottom of the inset. All images were displayed as relief tilted by 5°. (Modified, with permission, from Müller et al. 2002 (456). Reprinted by permission from Macmillan Publishers Ltd: EMBO J (21: 3598-3607), copyright [2002]).

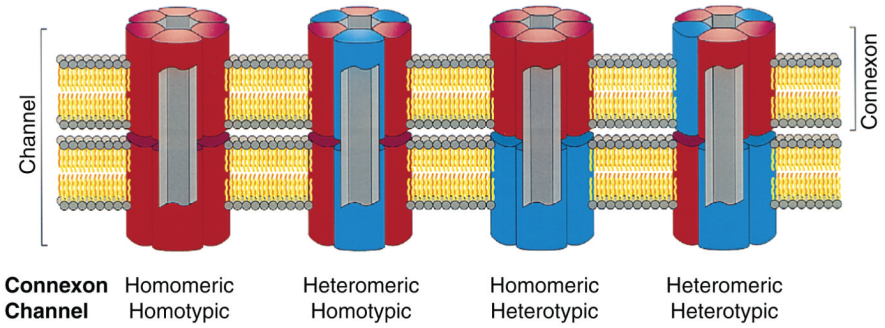

Figure 6.

Possible channels formed by multiple connexins. The figure shows gap junctional channels of different composition. Homomeric connexons are formed by a single connexin type whereas connexons containing more than one connexin type is heteromeric. When connexons of the same composition form a cell-cell channel it is homotypic and if the connexons differ in composition it is heterotypic. Reprinted from Cell, 84(3), Kumar NM, Gilula NB, The gap junction communication channel, 381-8, Copyright [1996], (338) with permission from Elsevier.

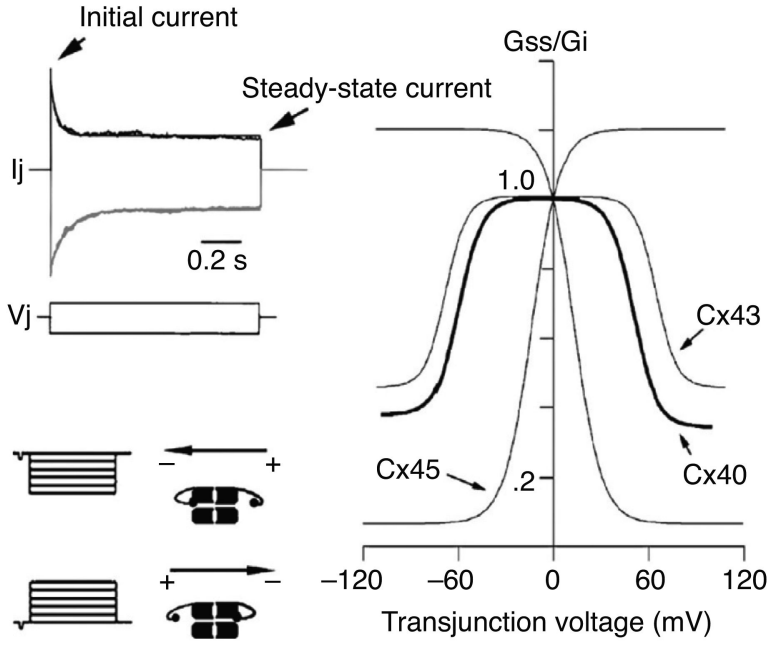

Figure 7.

Voltage dependence of connexin channels. Upper left panel shows the current elicited by imposing a plus or minus 100 mV gradient across Cx43 channels. The initial current that decays over time until it reaches a lower steady-state current. Right panel shows a plot of the fractional conductance (steady-state conductance (Gss) divided by the initial conductance (Gi)] as a function of transjunctional voltage for Cx40, Cx43, and Cx45. Lower left panel demonstrates the concept of gating polarity of Cx43 (negative gating polarity). If the voltage gradient is sufficiently large the gating particle will close the connexion that is relatively negative on the cytoplasmatic side. Figure adapted from Moreno AP, Biophysical properties of homomeric and heteromultimeric channels formed by cardiac connexins, Cardiovasc.Res., 2002, 62(2):276-86, (448) by permission of Oxford University Press.

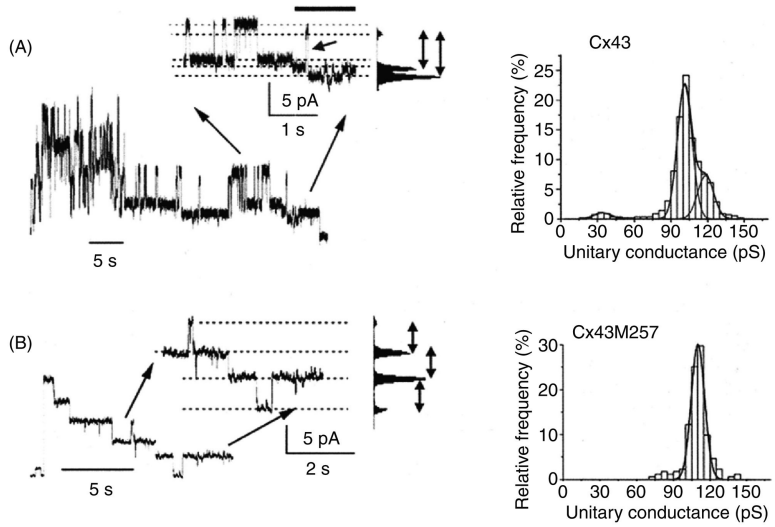

Figure 8.

Effect of cytoplasmic tail (CT) truncation on fast V_j gating in Cx43. (A) Left: trace of channel activity recorded at a transjunctional voltage of −60 mV. In the expanded inset, shows clear transitions between the main open and residual state (fast gating). Right: all-events histogram showing the distribution of the observed conductance events. (B) Recording of activity by CT-truncated Cx43 channels at V_j = −60 mV. The trace and all-events histogram shows that only gating between the main open and closed state was observed. Figure adapted from Moreno AP, Chanson M, Elenes S, Anumonwo J, Scerri I, Gu H, Taffet SM, Delmar M, Role of the carboxyl terminal of connexin43 in transjunctional fast voltage gating, Circ.Res., 90(4):450-7, 2002 (449), with permission.

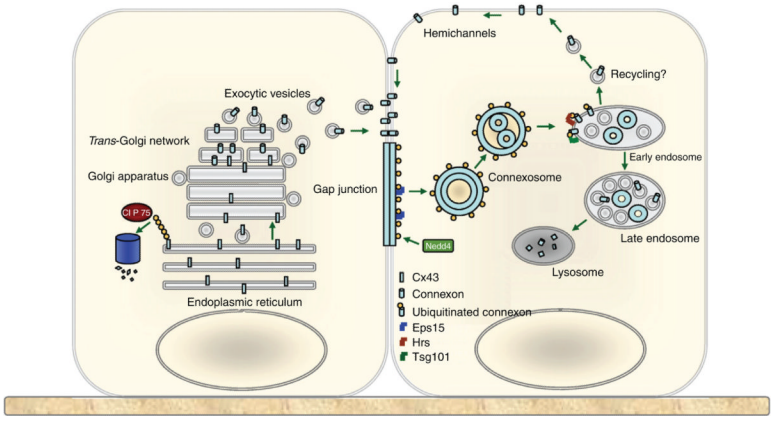

Figure 9.

Role of ubiquitination in trafficking and degradation of Cx43. The left cell summarizes the events of polyubiquitination followed by ERAD. The right cells summarizes internalization and lysosomal degradation after Cx43 monoubiquitination. Reprinted from Cell Signal., Vol 22, Kjenseth A, Fykerud T, Rivedal E, Leithe E, Regulation of gap junction intercellular communication by the ubiquitin system, 1267-73, Copyright [2010] (317), with permission from Elsevier.

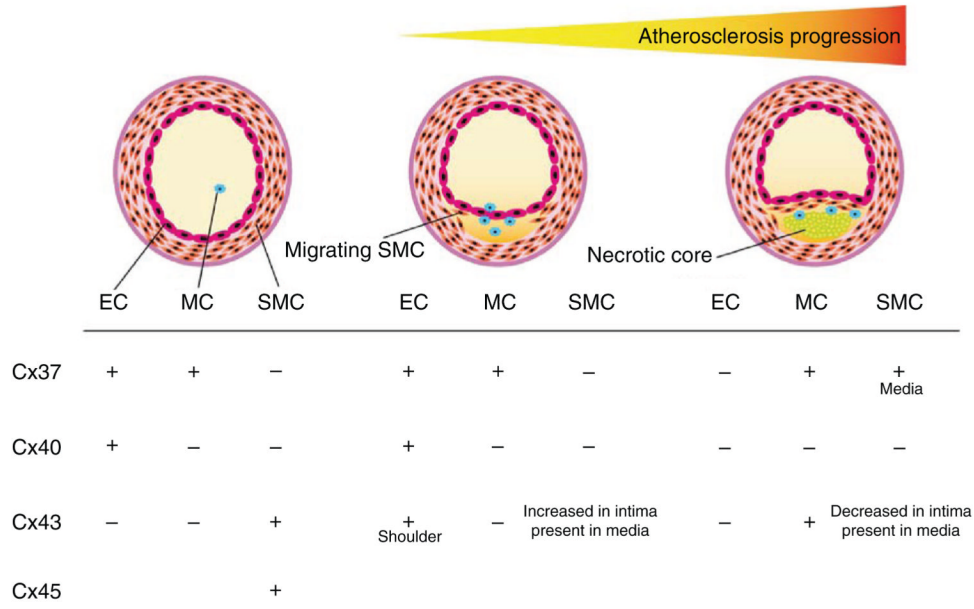

Figure 10.

Expression of connexins in normal vessels and during progression of artherosclerosis. The expression of Cx37, Cx40, Cx43, and Cx45 is indicated for the different cell types involved [endothelial cells (ECs), smooth muscle cells (SMCs), and monocytes (MCs)]. Adapted with kind permission from Springer Science+Business Media: Semin.Immunopathol., Connexins participate in the initiation and progression of atherosclerosis, 31, 2009, 49-61, Morel S, Burnier L, Kwak BR, figure 3, (447).

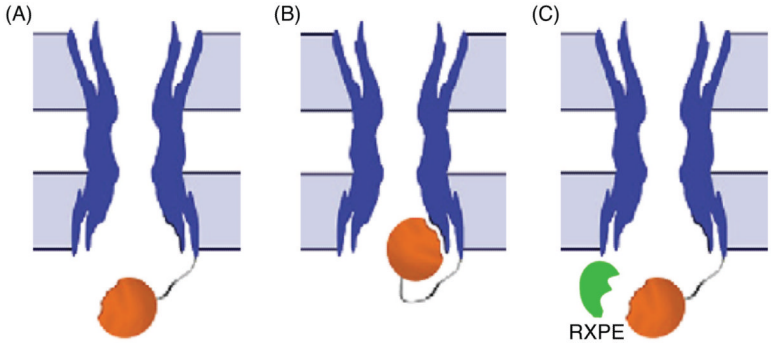

Figure 11.

RXP-E prevents particle-receptor interaction and channel closure. (A) Open channel. (B) Channel closed by particle-receptor interaction. (C) RXP-E binds the cytoplasmic tail (CT) and prevents closure by interrupting binding to the receptor. Modified from Delmar M, Coombs W, Sorgen P, Duffy HS, Taffet SM, Structural bases for the chemical regulation of Connexin43 channels, Cardiovasc.Res., 2004, 62(2): 268-75, (138), by permission of Oxford University Press.

Table 1

Human and Mouse Connexin Family Members

GJ	hCx	mCx
GJε1	hCx23	mCx23
GJβ7	hCx25	–
GJβ2	hCx26	mCx26
GJγ3	hCx30.2	mCx29
GJβ6	hCx30	mCx30
GJβ4	hCx30.3	mCx30.3
GJβ3	hCx31	mCx31
GJβ5	hCx31.1	mCx31.1
GJ δ3	hCx31.9	mCx30.2
GJβ1	hCx32	mCx32
–	–	mCx33
GJδ2	hCx36	mCx36
GJα4	hCx37	mCx37
GJα5	hCx40	mCx40
GJδ4	hCx40.1	mCx39
GJα1	hCx43	mCx43
GJγ1	hCx45	mCx45
GJα3	hCx46	mCx46
GJγ2	hCx47	mCx47
GJα8	hCx50	mCx50
GJα9	hCx59	–
GJα10	hCx62	mCx57

Table 2

Voltage Dependence of Selected Connexins

Connexin	$V_{1/2}$ (half-inactivation voltage, mV)	G_{max}	G_{min}	Single channel conductance, γ, pS	References
26	95	0.94/1.01	0.19/0.19	115-150	(35, 226, 326)
32	60/63	1.02/1/01	0.25/0.27	70	(482, 559)
40	35	1/1	0.19/0.19	158-198	(9, 33)
43	61/61	1/1	0.29/0.29	90-110	(70, 449)
45	23/21	1.16/1.21	0.08/0.07	30	(24)

The voltage dependence can be described by the V_j at which half maximal inactivation occurs ($V_{1/2}$), the fractional maximum conductance relative to the conductance at $V_j = 0$ (G_{max}), and the fractional minimum conductance during inactivation by a large V_j (G_{min}).

Table 3

Cx43 Phosphorylation Sites

Residue	Kinase(s)	Reference(s)
S244	CaMKII	(282)
Y247	v-Src	(383, 645)
S255	MAPK/CaMKII	(80, 282, 739)
S257*	PKG/CaMKII	(282, 342, 344)
S262	PKCε	(14, 148, 657)
Y265	v-Src	(383, 645, 687)
S279	MAPK	(739)
S282	MAPK	(739)
S296	CaMKII	(14, 282)
S297	CaMKII	(14, 282)
S306	CaMKII	(14, 282)
S314	CaMKII	(282)
S325	CK1/CaMKII	(109, 282)
S328	CK1/CaMKII	(109, 282)
S330	CK1/CaMKII	(109, 282)
S364	PKA/CaMKII	(282, 616, 673)
S365	PKC/PKA/CaMKII	(282, 616)
S368	PKC	(22, 356, 579, 616)
S369	PKC/CaMKII	(282, 616)
S372	PKC/CaMKII	(282, 579, 616)
S373	PKC/CaMKII	(282, 616)

*Amino acid residue 257 of rat Cx43 is a serine. In the human Cx43 sequence, serine 257 is replaced by alanine.