Available online at www.sciencedirect.com

SCIENCE DIRECT

Biochimica et Biophysica Acta 1711 (2005) 126–141

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

http://www.elsevier.com/locate/bba

Review

Functional consequences of heterogeneous gap junction channel formation and its influence in health and disease

G. Trevor Cottrell[a], Janis M. Burt[b,*]

[a]*Department of Physiology, Queen's University, Kingston, ON, Canada K7L 3N6*
[b]*Department of Physiology, Arizona Health Sciences Center, PO Box 245051, University of Arizona, Tucson, AZ 85724-245051, USA*

Received 6 May 2004; received in revised form 17 November 2004; accepted 23 November 2004
Available online 8 December 2004

Abstract

The capacity of multiple connexins to hetero-oligomerize into functional heterogeneous gap junction channels has been demonstrated in vivo[1], in vitro[2], and in nonmammalian expression systems. These heterogeneous channels display gating activity, channel conductances, selectivity and regulatory behaviors that are sometimes not predicted by the behaviors of the corresponding homogeneous channels. Such observations suggest that heteromerization of gap junction proteins offers an efficient cellular strategy for finely regulating cell-to-cell communication. The available evidence strongly indicates that heterogeneous gap junction assembly is important to normal growth and differentiation, and may influence the appearance of several disease states. Definitive evidence that heterogeneous gap junction channels differentially regulate electrical conduction in excitable cells is absent. This review examines the prevalence, regulation, and implications of gap junction channel hetero-oligomerization.

Keywords: Gap junction; Connexin; Connexon; Heteromeric; Heterotypic; Junctional conductance; Unitary conductance; Selective permeability

Contents

* Corresponding author. Tel.: +1 520 626 6833; fax: +1 520 626 2383.
E-mail address: jburt@u.arizona.edu (J.M. Burt).

[1] Used herein to denote in an animal.

[2] Used herein to denote in cultured cells.

0005-2736/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2004.11.013

1. Introduction

The ability of different connexin (Cx) isoforms to oligomerize into single gap junction (GJ) channels is both intriguing and disconcerting. The complexity of GJ function created by heterogeneous (mixed Cx composition) channels is fascinating to consider, yet our limited understanding of the physiological roles of homogeneous (single Cx composition) channels makes contemplation of the consequences of heterogeneous assembly daunting.

Six Cxs oligomerize to form a connexon (GJ hemichannel), and two connexons from adjacent cells dock to form a functional GJ channel (Fig. 1A). Connexons composed of only one Cx isoform are termed homomeric (HoM), whereas connexons formed from more than one Cx isoform are labeled heteromeric (HeM). When two identical connexons dock, they form a homotypic (HoT) channel, whereas the channel formed by the docking of nonidentical connexons is heterotypic (HeT) (Fig. 1B). For the purposes of this review, we will distinguish the various combinations of connexons as shown in Fig. 1B. Notably, there are two types of heterogeneous channels: ones containing HoM and HeM connexons, which we refer to as HoM/HeM channels (e.g., Cx40/Cx40–Cx43, connexons isolated by /) and those containing two HeM connexons, which we refer to as HeM/HeM channels (e.g., Cx40–Cx43/Cx40–Cx43). Collectively, these heterogeneous channels are referred to as HeM/HeT channels.

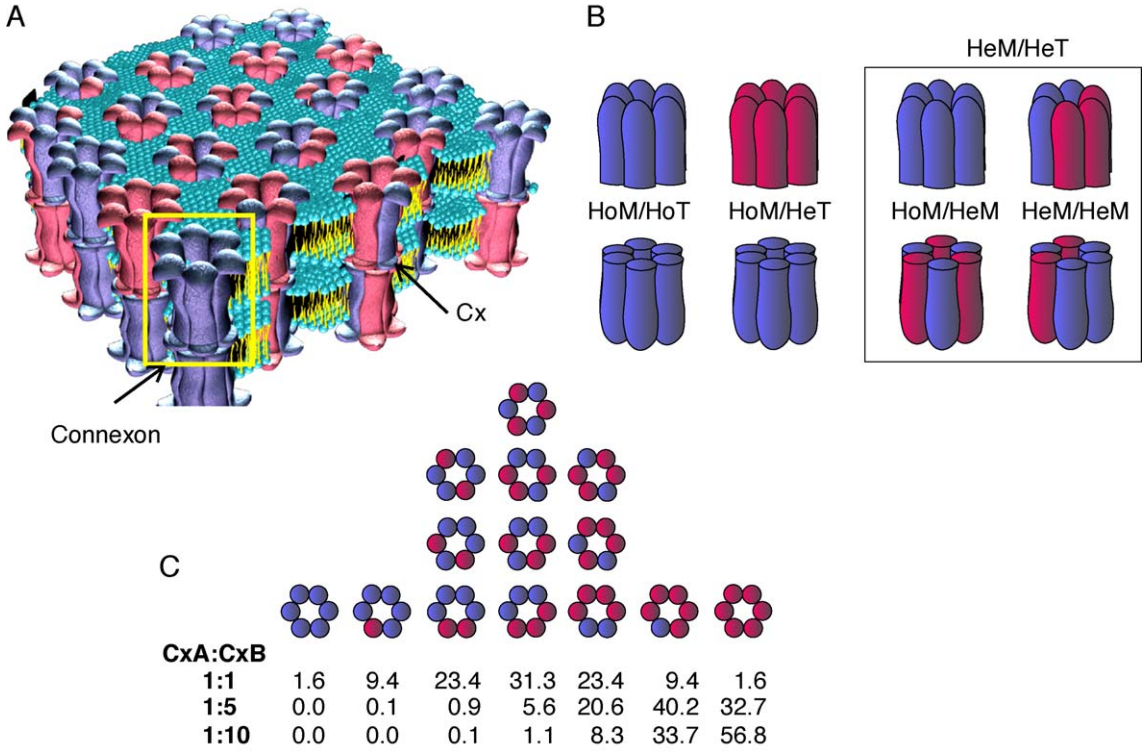

Fig. 1. Heteromeric/heterotypic GJ channel formation. (A) Cartoon depiction of numerous possible GJ channel assembly patterns between cells coexpressing two Cxs (CxA, blue; CxB, red). (B) Connexons composed of only one Cx isoform are termed homomeric (HoM), whereas connexons formed from more than one Cx isoform are labeled heteromeric (HeM). When two identical connexons dock, they form a homotypic (HoT) channel, whereas the channel formed by the docking of nonidentical connexons is heterotypic (HeT). There are two types of HeM/HeT channels: ones containing HoM and HeM connexons (HoM/HeM), and those containing two HeM connexons (HeM/HeM). (C) Schematic representation of the 14 possible connexon conformations in a cell coexpressing two Cxs. The table presents the predicted frequency of each of the conformations as a function of the CxA:CxB expression ratio.

If a cell expresses two Cx isoforms that are free to associate in a random fashion, then it could contain up to 14 differentially composed connexons, 12 HeM and 2 HoM. If each of these 14 connexons were able to dock with all of the others, then pairing two such coexpressing cells could result in as many as 196 (14×14) differentially composed channel types, the proportions of which (assuming random assembly) would depend on the relative expression levels of Cx isoforms (Fig. 1C). Probability theory predicts that small changes in expression ratio could result in substantial changes in the connexin composition of expressed channels (see, e.g., the predictions for a 10:1 vs. 1:1 expression ratio in Fig. 1C).

The prevalence of heterogeneous GJ channel assembly in nature has not been established. It is clear that such channels can and do form (see, e.g., Ref. [1]); however, the extent to which they alter intercellular communication in physiologically necessary ways is not clear in part because unique functional properties that specifically characterize these channel types have not been identified. In the absence of identified unique functional properties, definitive evidence for their (functional) presence in cells or tissues requires a combination of biochemical, imaging, and electrophysiological data, which can be challenging to obtain, especially in vivo.

Based on available data, it seems clear that heterogeneous GJ channel assembly affords cells, and the tissues they comprise, an efficient and sensitive strategy for acute and long-term regulation of intercellular communication. Given what is already known about Cx-specific differences in selectivity and regulation of HoM/HoT channels, the capacity for heterogeneous assembly could benefit cells in the following ways: (1) increase (or limit) the diversity of exchanged molecules between cells; (2) increase the options for regulation of intercellular molecular exchange by intracellular signaling cascades; and (3) provide control over the assembly and trafficking of hemichannels and formation of functional GJ channels and plaques.

To achieve a greater appreciation for the functional consequences of heterogeneous GJ assembly, it will be necessary to (1) determine the favorability of Cx interactions in a tissue- and cell-specific manner; (2) characterize the effects of heterogeneous channels on gating and permselectivity; (3) develop relationships between Cx stoichiometry and function; and (4) discover in vivo models of heteromerization in healthy and diseased tissues. The purpose of this review will be to examine the advancements that have been made in progressing each of these goals.

2. Evidence for heterogeneous gap junction channels

2.1. Evidence in vivo

As mentioned earlier, it is difficult to verify the presence of functional heterogeneous channels in cell and tissue samples. There are numerous settings in which two or more Cxs have been demonstrated to co-localize to the same GJ [2–7]. To demonstrate that these Cxs form HeM connexons requires isolation of connexons from GJ plaques, immunoprecipitation of those connexons with an antibody specific to one of the comprising Cxs and subsequent detection of another Cx isoform in the immunoprecipitate [8,9]. To demonstrate that the HeM connexons contribute to functional heterogeneous channels, either unique channel behaviors or dominant-negative effects must be observed. Such data are not easily obtained and as a consequence evidence for functional heterogeneous channels in vivo is incomplete.

That mixed composition GJs might be functional was initially explored in an indirect fashion by determining whether communication occurred between pairs of cells originating from different tissues and organisms [10–12]; these studies predated the molecular characterization of gap junction genes and consequently the mixed composition of junctions was unproven. The first definitive evidence for in vivo colocalization of two Cxs to the same GJ plaque came in 1987 [5]. These authors used immunohistochemistry and immunoprecipitation techniques to demonstrate in hepatocytes the colocalization of what would later be defined as Cx26 and Cx32 to the same GJ plaque, although they concluded that HeM connexons either did not form or were not common. Subsequent studies by numerous authors using a variety of experimental approaches in hepatocytes and cell expression systems clearly demonstrated that Cx26 and Cx32 can form HeM connexons [13–15] and HeT [16–19] channels. Reconstitution of connexons isolated from liver into liposomes further demonstrated that HeM connexons are present in vivo [20].

The cells of the lens express three gap junction proteins: Cxs 43, 46, and 50. Cx46 and Cx50 are coexpressed and colocalize in fiber cells where they form HeM connexons [9,21]; Cx50 is expressed without Cx46 in the lens epithelium. That animals deficient for either of these proteins develop cataracts indicates that the roles of these Cxs are not completely redundant [22,23]. Supporting this conclusion, transfected cell studies show unique functional properties for HoM/HeT as well as HeM/HeT Cx46 and Cx50 mixed channels [24–26]. A recent study in which Cx46 was knocked into the Cx50 locus such that both proteins are expressed in the lens epithelium demonstrated that the lenses of heterozygous animals ($Cx50^{+/46}$) developed dense cataracts (regardless of whether the animals had Cx46 in the Cx46 locus). These studies all support the conclusion that HeM/HeT channels display unique properties necessary for normal development and function of the mammalian lens.

Cells of the cardiovascular system express Cxs 37, 40, 43 and 45 in a tissue- and developmental stage-specific manner. Dual whole-cell voltage clamp recordings from canine atrial cells, which coexpress Cxs40 and 43, demonstrate single channel conductances (γ_j) that are not observed in cells that

express only one of these proteins [27], thus providing evidence that HeM/HeT Cx40 and Cx43 channels function in the atrium. In a mammalian smooth muscle cell line, A7r5, it was demonstrated using biochemical and electrophysiological techniques that HeM Cx40 and Cx43 connexons form and contribute to functional channels that display properties distinct from either HoM/HoT counterpart [8]. That Cxs 40 and 43 form functional HeM/HeM and HeM/HoM channels has been verified by pairing communication-deficient cell lines made to express one or both Cxs [28–31]. Comparable studies using communication-deficient cell lines have demonstrated Cxs 37 and 43 [32], and Cxs 43 and 45 [33–35] form functional HeM/HeM channels, while Cxs 40 and 45 [36], and Cxs 40 and 37 [37] form functional HoM/HeT channels. Whether Cx40 and Cx45 and Cx40 and Cx37 form HeM/HeM channels has not yet been explored (or reported).

The prevalence of heterogeneous GJ channel assembly and function in vivo is not yet clear; however, the studies described above indicate that it does occur. The extensive literature documenting tissue-specific changes in patterns of Cx coexpression and colocalization during development and in response to disease and injury, in combination with the limited functional data discussed above, highlight the potential of such heterogeneous channels to significantly affect the physiology of coexpressing cells and tissues.

2.2. Evidence from cell expression systems

The use of communication-deficient expression systems for the assessment of heterogeneous GJ communication has been critical in the advancement of our understanding and has led to the mapping of many of the Cx compatibilities depicted in Table 1. Werner et al. [38] demonstrated that a *Xenopus laevis* oocyte made to express Cx43 would form a functional junction with oocytes expressing their endogenous Cx, Cx38, and with oocytes expressing Cx32 (and Cx38). The Cx43/Cx38 junction displayed asymmetric voltage-dependent gating properties that were consistent with each hemichannel retaining its unique gating characteristics. Furthermore, the level of coupling observed in such pairs was considerably larger than that observed between pairs expressing only the endogenous Cx, suggesting that expression of Cx43 in one oocyte somehow favors the development of a junction in the paired oocytes. Swenson et al. [39] confirmed these results and further demonstrated that Cx32-expressing oocytes would not form functional junctions with oocytes that expressed only the endogenous Cx38. More recent work demonstrating that HeT Cx43/Cx32 channels do not form [36,40] leads to the simple conclusion that the HeT channels observed in the Werner and Swenson studies were between Cx43 and the endogenous Cx38 rather than between Cx43 and Cx32.

In subsequent studies, the ability of various Cxs to form functional HoM/HeT channels was extensively examined in both *X. laevis* oocyte [16,26,37,40] and communication-deficient mammalian cell [36] expression systems. A summary of much of this work can found in the review by White and Bruzzone [41]. Collectively, these studies demonstrated that not all connexons dock successfully with all others to form HoM/HeT channels. Cxs have been phylogenetically classified into α, β, γ, and ε subgroups. Initially, it was hypothesized that HoM connexons composed of either an α Cx or a β Cx would dock with any member of the same phylogenetic group, but would not dock with members of another group. These restrictions were thought to reflect differences in the primary amino acid sequences, specifically in the E1 and E2 loops. Despite providing convincing evidence supporting this premise, White et al. [40] downplayed the role of the E1 and E2 loops in defining compatibility because HoM Cx40 did not interact successfully with several other α group Cxs including HoM Cx43, Cx46 and Cx50 (see also Ref. [26]) and β group Cxs32 and 26 were both compatible with α group Cxs46 and 50.

Using HeLa cells as a mammalian expression system and Lucifer yellow dye coupling to assess communication, Elfgang et al. [36] also concluded that docking between α and β HoM connexons is not favored. They also reported that some α combinations were not favored; for example, Cxs 40 and 43 failed to form HeT channels. Through use of chimeric constructs in which portions of Cx43 replaced Cx40, Haubrich et al. [42] concluded that both the E1 and E2 loops as well as the C-terminus participated in determining docking compatibility.

Some of the HoM/HeT combinations found incompatible in the oocyte expression system have subsequently been demonstrated compatible in mammalian expression systems; and some combinations in mammalian expression systems found incompatible by dye coupling assays have subsequently been demonstrated as compatible by electrical coupling assays. The most prominent example is the successful HeT interaction of HoM Cx40 with HoM Cx43 [28,31]. These findings indicate that conclusions regarding Cx compatibility derived from the *Xenopus* oocyte expression system or mammalian expression systems using dye transfer assays should be treated with caution. Discrepancies between oocyte and mammalian expression systems are particularly interesting for they prompt consideration of the roles of accessory proteins (e.g., adhesion proteins) or system-specific post-translational modifications (e.g., phosphorylation) in guiding the docking process.

Cell expression systems have also been used to explore promiscuity in HeM connexon formation. Early work by Stauffer [15] used a biochemical approach to demonstrate that Cx32 and Cx26 could form HeM connexons in transfected insect cells, although there were no functional data to demonstrate that these connexons participated in functional GJ channels. Functional confirmation of HeM/HeT channels requires that these channels display gating or conductance properties distinct from the HoM/HoT and

Table 1
Heterotypic and heteromeric compatibility among mammalian Cxs

	Cx26	Cx30	Cx30.3	Cx32	Cx37	Cx40	Cx43	Cx45	Cx46	Cx50
Cx50	+			+		-[b]	-[b]		+	+
Cx46	+			+		-[b]	+		+	+
Cx45	-[c]	-	-	-[b,c]	+	+		+		
Cx43[e]	-[b,c]	-	+[a]	-[b,c]	+	+	+	+	+	+[d]
Cx40	-[b,c]	+	+	-[b,c]	+	+	+			
Cx37[e]	-[c]	-	+[a]	-[b,c]	+		+			
Cx32	+	+[a]	-	+						
Cx30.3[e]		+	+							
Cx30	+[a]	+								
Cx26	+	+		+						

Yellow squares represent HeT Cx compatibility. Green squares represent HeM Cx compatibility. + indicates a compatibility between Cxs. − indicates that the Cxs are incompatible. A blank cell indicates that the relevant experiments determining compatibility have not been performed. These data were obtained from Refs. [6,7,11–13,18–20,23,27,29,31–36,38,77].

[a] Unpublished data as reported in Ref. [36].

[b] HeT compatibility was assessed in oocyte pairs; therefore, it may not apply in other systems.

[c] HeT compatibility was assessed with dye coupling; therefore, it may not apply to electrical coupling.

[d] The chicken homolog of human Cx50 was used (cCx56).

HoM/HeT properties of their constituent Cxs. If distinct properties are not observed, the presence of HeM/HeT channels could be falsely rejected. Despite this risk, several groups have confirmed functional HeM/HeT configurations in transfected mammalian cells [24,28,29,32,33], although others have failed to show that there are differences in voltage gating and conductance properties where HeM connexons have been biochemically identified [25,30,43].

Cell expression systems have proven valuable for the assessment of Cx compatibility in both HoM/HeT and HeM/HeT GJ channel formations. They have also provided us with insights into the behaviors of such channels. Despite this, their usefulness in predicting HeM/HeT channel formation and function in vivo is not clear. In part, this reflects our incomplete knowledge of the extent to which the cellular trafficking and assembly machinery differs in a cell-specific manner and how this machinery is regulated spatially and temporally.

2.3. Trafficking and assembly

The complexities of GJ assembly and trafficking are still being unraveled for HoM hemichannels (see reviews in Refs. [44,45]), with HeM connexons furthering the detail of this puzzle [46]. Ahmad et al. [47] used an in vitro transcription protocol to assess the preferred assembly compartments for HoM and HeM Cx26 and Cx32 hemichannels. They found that the efficiency of HeM connexon formation in microsomes was greatly increased by the addition of Golgi membranes. This observation implies that machinery supplied by the Golgi facilitates the formation of HeM channels, and that regulation of HeM connexon assembly can occur independently of HoM connexon assembly.

Cx26 and Cx32 hetero-oligomerization was also examined in guinea pig liver membrane fractions [14]. Cx32 was found in all membrane fractions (endoplasmic reticulum, Golgi, sinusoidal plasma membrane, lateral plasma membrane) with the highest levels in the lateral plasma membrane. Cx26 was found in these same fractions; however, the ratio of Cx26:Cx32 varied with each fraction. Although their methodology did not allow for direct comparison of protein levels, the Cx26:Cx32 ratio was much lower in the Golgi, endoplasmic reticulum-Golgi intermediate compartment, and microsomal fractions. The authors speculated that this might be the result of Cx26 taking an alternative route to the membrane, bypassing the Golgi complex. This concept was supported by the observations of Ahmad et al. [47], where it was described that Cx26 can assemble into membranes post-translationally. Cxs do not contain conventional signal sequences for trafficking and assembly, therefore opening the option of multiple routes for trafficking and assembly [14]. So, although Cx32 and Cx26 are capable of forming HeM connexons, the proportions of Cxs in these hemichannels may not be representative of the relative expression levels of the two Cxs in functional GJs.

Insights into HeM channel assembly can be gained from the use of dominant-negative mutant Cxs to knock down GJ formation. Das Sarma et al. [48] exploited this phenomenon (observed in Refs. [49,50]) by using a dominant-negative Cx consisting of bacterial β-galactosidase (β-gal) fused to the C-terminus of Cx43. This Cx43/β-gal mutant was capable of forming heteromers with wild type (wt) Cx43 and wtCx46, but the heteromers were retained in intracellular compartments. The Cx43/β-gal mutant did not oligomerize with wtCx32 implying incompatibility of these Cxs or assembly in different compartments. Importantly, the Das Sarma study provided evidence that some cells have the capacity to regulate whether compatible Cxs intermix. Thus, in HeLa and alveolar epithelial cells, Cx43 and Cx46 coassemble into HeM connexons, but in ROS osteoblastic cells, despite expression of both proteins, only Cx43 traffics to the cell membrane. Similar observations were made for Cx46 and Cx43 assembly and trafficking in ROS, lens, and HeLa cells [51]. These observations further indicate that connexon assembly may not be a random process but rather can be regulated in a cell-specific manner.

3. Effects of heterogeneous GJ channel assembly on channel function

3.1. Gating behavior

3.1.1. Voltage-dependent gating

Relative to HoM/HoT junctions, mixed composition junctions frequently display significant differences in transjunctional voltage-dependent gating (V_j-gating; for review, see Ref. [52]). For HoM/HeT junctions, these differences arise, at least in part, from differences in the voltage polarity and magnitude that result in “fast” gating, but protein–protein interactions within and between connexons also influence voltage-dependent gating, sometimes in unexpected ways [19]. An illustration of the basis for polarity-dependent differences in voltage-dependent gating can be demonstrated in the following example. If Cell 1 of a HoM/HeT junction expresses a Cx that gates in response to a positively oriented voltage and Cell 2 expresses a Cx that gates in response to a negatively oriented voltage, then when Cell 1 is held at a positive voltage relative to Cell 2, both connexons experience a voltage polarity that results in their gating. In contrast, when Cell 1 is held at a negative voltage relative to Cell 2, neither connexon experiences the voltage polarity necessary for gating and junctional conductance is independent of V_j. Werner et al. [38] were the first to show this type of asymmetry in V_j-gating. They studied HoM/HeT Cx38/Cx43 channels in oocytes and found that as the transjunctional voltage difference increased junctional conductance decreased, but only when the Cx43 oocyte was negative relative to the Cx38 oocyte;

voltages of the opposite polarity had no effect on conductance. Many others have since reported asymmetric V_j-gating for this and other Cx pairs [16,17,28,31–34], although for most connexon pairs complete absence of voltage sensitivity with one polarity of voltage difference is rare (see, e.g., Fig. 2). The variable degree of asymmetry displayed by different HoM/HeT combinations may be explained by multiple voltage-sensitive gates and/or modification of gating behavior through connexon–connexon interactions.

V_j-gating of GJs involves both fast and slow components; the fast component results in closure of the full open channel to a residual state whereas the slow component involves complete closure of the channel. The fast gate (in Cx43 and Cx32) resides at the channel's entrance and likely involves interaction of the N-terminal [53,54] and C-terminal domains [54,55]; the location of the slow gate remains uncertain but is likely deep within the pore rather than at its entrance. Although for some Cxs, the voltage polarity that influences the fast and slow gates is the same, e.g., Cxs 32, 43, and 45, for others it differs, e.g., Cx46 and 50 [52,56]. Sensitivity of the fast vs. slow gates to oppositely oriented voltage polarity likely contributes to the asymmetry of some HoM/HeT combinations, like Cx40/Cx43 (Fig. 2).

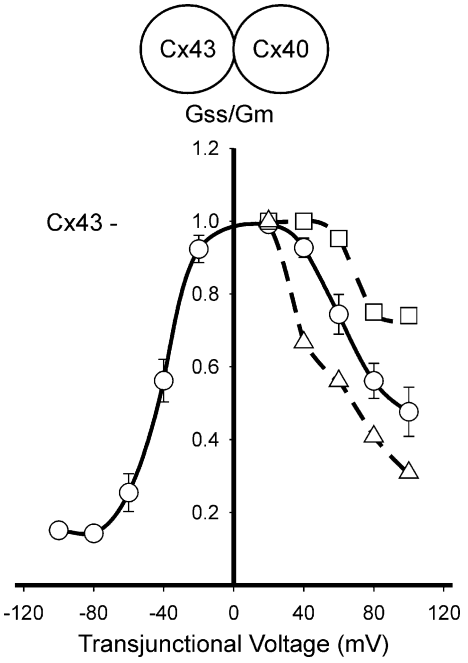

Fig. 2. Heterotypic GJ channels often display asymmetric V_j-dependent gating behavior. Mean (±S.E.) normalized conductance (circles, solid line, n=16) derived from a series of transjunctional voltage steps between cell pairs expressing Cx43 in one cell and Cx40 in the other. All data are reported relative to the Cx43-expressing cell. The dotted lines represent two individual experiments that demonstrate the variability in V_j-gating in the absence of a gating charge, with the squares representing a relatively low voltage sensitivity and the triangles representing a relatively high voltage sensitivity that does not reach a minimum conductance state over the transjunctional voltage steps applied. Data adapted from previous publications [28,29].

Docking of dissimilar connexons can modify various aspects of V_j-gating, including fast- and slow-gate functionality, fast-gating rectification, and V_j sensitivity. Fast V_j-gating of Cx43 is typically triggered when the Cx43-expressing cell is negative relative to the other cell. However, when Cx43 docks with Cx45, this V_j-gating response of the Cx43 connexon is lost [33,34]; as a result, when the Cx45 expressing cell is held positive (Cx43 cell negative) there is only a gradual, prolonged closure of a slow gate [33,34,57]. Slow gating activity of Cx32 is lost when it pairs with Cx26 and the heterotypic channel displays fast V_j-dependent rectification [19]. V_j sensitivity of HoM/HeT Cx40/Cx43 GJs differs from that observed for either HoM/HoT counterpart (Fig. 2) [28,29]. When the polarity of the voltage difference favors fast gating (Cx40 positive, Cx43 negative), the voltage sensitivity is enhanced with a lower G_{min} (the voltage-insensitive residual conductance) and V_0 (the transjunctional potential difference at which half the voltage sensitive current is lost). When the polarity of the voltage difference for fast gating is not favorable (Cx40 negative, Cx43 positive), junctional conductance (g_j) still declines with increasing V_j (perhaps through similarly oriented slow gates), but the magnitude of the decline is variable (Fig. 2) [28]. The mechanistic bases for these deviations in predicted behavior are not understood. It has been suggested that the fast-gate may act as a prop to maintain the slow gate open (possibly by decreasing the voltage field across the slow gate), thereby inducing an apparent decrease in the V_j sensitivity of the slow gate. HeT docking can result in the functional removal of the fast gate, which then permits full closure of the channel via the slow gate [57]. Variability in the gating response of HoM/HeT junctions may suggest that the slow V_j gate is only modestly sensitive to V_j, is sensitive to the opposite (compared to the fast gate) gating polarity, or is sensitive to V_js of either polarity. In summary, V_j-gating of HoM/HeT junctions demonstrates that interactions between the docked connexons can alter the capacity of the fast and slow gates to act as well as their voltage sensitivity.

V_j-dependent gating is further complicated for HeM/HeT channel configurations. Again, the easiest prediction to make regarding the V_j-gating behavior of these channels would be that the relative contribution of the different Cxs to the channel would determine the channel's gating behavior. In some studies, this prediction has been confirmed [25,30,43]; however, other studies have demonstrated a large variety of V_j-gating behaviors in HeM/HeT channels that are not predicted by the (approximate) Cx composition of the comprising connexons [24,28,29,32,33]. From data generated in our laboratory, it appears that specific Cxs may have dominance over the V_j-gating activity of HeM/HeM and HeM/HoM channels [28,29]. When a cell that coexpresses Cx40 and Cx43 is paired with a Cx40-expressing cell (Cx40–Cx43/Cx40 GJ), the

observed V_j-gating behavior is very similar to that observed in HoM/HoT Cx40 GJs, regardless of the Cx40:Cx43 expression ratio in the coexpressing cell and regardless of V_j polarity. However, when these coexpressing cells are paired with a Cx43-expressing cell (Cx40–Cx43/Cx43 GJ), V_j-gating behavior depends on V_j polarity. When the Cx43 cell is hyperpolarized, gating of the HeM/HoM junction resembles that of HoM/HoT Cx43 GJs although with greater V_j variability; when the Cx43 cell is depolarized, the gating behavior depends heavily on the Cx40:Cx43 expression ratio in the HeM cell (see Fig. 5A). The V_j sensitivity of Cx40 exceeds that of Cx43 in HoM/HoT configuration. In HeM/HoM configuration, it appears that the connexon with the highest Cx40 content dictates the voltage response. Similar conclusions were drawn for Cx43–Cx45/Cx43 vs. Cx43–Cx45/Cx45 junctions [33]. A mechanistic understanding of V_j-gating in HeM/HeT GJ channels will be challenging to tease apart; however, the important consideration for the purpose of this review is that protein–protein interactions within and between connexons can cause significant changes in V_j-dependent gating, changes that are not readily predicted by the behavior of the participating Cxs in HoM/HoT or even HoM/HeT channel configurations.

3.1.2. Chemical gating

Perhaps a more physiologically relevant aspect of GJ gating is that induced by cellular acidification (pH-dependent gating, or pH-gating). The mechanistic basis for pH-gating has been studied extensively with the consensus being that it occurs through a ball-and-chain-type interaction between the C-terminus (the ball) and intracellular loop [58]. Once the ball and chain mechanism was described, questions arose as to how Cxs within a hemichannel may cooperate to cause the observed gating effect. Two potential hypotheses have been advanced: (1) each Cx acts independently—therefore pH-gating of a HeM connexon will be determined by its most sensitive Cx constituent; or (2) the Cxs in a hemichannel interact cooperatively creating pH-gating that is a unique function of the Cx constituents and (possibly) distinct from the HoM/HoT channels formed by either Cx constituent. The first hypothesis implies that a single ball is sufficient to close the channel; the second hypothesis implies that multiple balls might be necessary for channel closure or that Cx interactions within the HeM connexon influence the sensitivity of the pH gate.

The first study to determine the impact of hetero-oligomerization on pH-gating utilized Cx32 and a mutant of Cx32 (Cx32*5R/N) in which five arginines (R) in the C-terminus were replaced with asparagines (N) to create an increased sensitivity to pH [59]. If each Cx acts independently, then coexpression of these Cxs in an oocyte model would be expected to result in pH-gating indistinguishable from that observed for the HoM/HoT configuration of the most sensitive Cx (Cx32*5R/N). The data did not support this possibility. When a coexpressing cell was paired with either another coexpressor or with a Cx32-only expressing cell, the pH sensitivity was very similar to a HoM/HoT Cx32 GJ. In contrast, when a HoM Cx32*5R/N oocyte was HeT paired with either a coexpressor or Cx32, pH-gating was similar to the Cx32*5R/N HoM/HoT GJ, but with slowed kinetics. The data suggest that (cooperative) interactions both between and within connexons influence (negatively or positively) the sensitivity and kinetics of the Cx-specific pH-gating response.

Working with α-group Cxs, additional studies lent further support for the presence of cooperative interactions within connexons contributing to pH-gating [60,61]. In these studies, HeM/HeM Cx40–Cx43 channels were shown to be more sensitive to pH than either of the HoM/HoT counterparts. They further showed that coexpression of the C-terminus of Cx43 with a pH-insensitive truncated Cx40 restored pH sensitivity and rendered the junction more sensitive than HoM/HoT Cx40 channels alone. Such observations imply that not only does cooperativity exist in pH-gating of these Cxs, but that this cooperativity involves an enhanced interaction between the C-terminus and other Cx regions (intracellular loop?).

Increased gating sensitivity was also found for Cx40 and Cx43 HeM/HeM junctions responding to halothane [62]. The mechanism of halothane-induced gating is not clear; therefore, it is not known whether the C-terminus plays a functional role in this increased sensitivity. It is interesting that the increase in pH and halothane sensitivity is not correlated with increased V_j-gating sensitivity of HeM/HeM Cx40 and Cx43 GJ channels [29]. The greater impact of heteromerization on pH- and halothane-gating than V_j-gating likely reflects a diversity of structural mechanisms contributing to the gating process and highlight the complexity of gating regulation offered by HeM/HeT channels.

3.2. Channel conductance

Single-channel properties of HeM/HeT GJ channels are also quite complicated. A resistors-in-series model predicts that single channel conductance (γ_j) will be determined by the sum of the resistances contributed by each hemichannel. This expectation holds true for most HoM/HeT channel formations. Studies of Cx43/Cx45, Cx43/Cx40, and Cx43/Cx37 HoM/HeT channels show γ_j variability that generally falls within the range predicted from the corresponding HoM/HoT channels [28,32,43]. However, two caveats are worth noting. First, when HoM/HeT channels are formed between Cxs with opposite fast-V_j-gating polarity, there is significant rectification in the measured γ_j [17,28,31,57]. When V_j polarity favors gating, a full open state conductance predicted by a resistors-in-series model can be measured as well as a V_j-induced residual state. When the V_j polarity does not favor gating, the observed full open state conductance can be much smaller than that predicted by a resistors-in-series model and transitions to a residual

state are not observed. Second, as suggested by channel events not predicted by a resistors-in-series model, it may be that additional conductance states can occur in some HoM/HeT pairings [28,29], although these states could reflect alternative phosphorylation states (see below).

For HeM/HeM channels, rules for predicting the conductances of the 196 possible channel configurations have yet to be elucidated. It might be reasonable to expect that γ_j would reflect the relative contributions of the component Cxs, such that (1) all conductances would lie between those of the corresponding HoM/HoT channels and (2) as the ratio of expression increases the population of channel events would shift towards HoM/HoT behavior of the favored Cx. To evaluate whether this might be the case, the Burt lab generated cell lines in which the Cx40:Cx43 expression ratio increased from 1.5:1 to 10:1 [29,63]. Comparison of event frequency histograms obtained from pairs of 10:1 expressing cells vs. 3:1 expressing cells revealed a clear shift towards HoM/HoT Cx40-like channels (Fig. 3), which suggests that the relative contribution of Cx40 to the population was a significant determinant of channel amplitude. Interestingly, when these coexpressing cells were paired with HoM Cx43, the prevalence of smaller γ_j states increased (Fig. 5B) as the Cx40:Cx43 ratio in the coexpressing cell increased [29]; however, when paired with HoM Cx40, all γ_j conductance profiles looked very similar to that of HoM/HoT Cx40 irrespective of the Cx40:Cx43 ratio in the coexpressing cell. These data

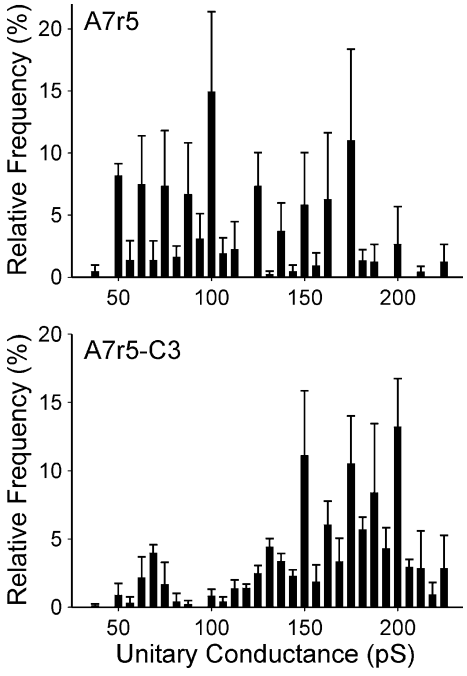

Fig. 3. Connexin expression ratio influences the relative frequency of observed unitary conductances. A7r5 cells express Cx40 and Cx43 at a ratio of ~3:1 whereas A7r5-C3 cells express these proteins at a ratio of 10:1. Note that relative to the A7r5 cells, the channel amplitudes observed between A7r5-C3 cells more closely resemble the channel amplitudes observed for Cx40 HoM/HoT channels, 175-200pS.

indicate that the conductance of HeM/HoM GJ channels reflects interactions within and between connexons and specific Cxs may play a dominant, controlling effect.

3.3. Changes in phosphorylation-dependent regulation

All Cxs, except Cx26, are phosphoproteins; however, whether HoM/HoT channels containing phosphorylated Cxs function differently from nonphosphorylated channels is largely unexplored. Where examined, it is apparent that phosphorylation can alter channel gating and/or conductance [64–67]. Comparatively little is known about the effects of hetero-oligomerization on phosphorylation-dependent regulation. A critical issue in this context is whether or not the sensitivity to phosphorylation-dependent regulation shows cooperativity.

Using the oocyte expression system, Stergiopoulos et al. [61] found that the C-terminus of the phosphoprotein Cx43 is capable of interacting with Cx26 and Cx32 and in so doing confer on these channels sensitivity to insulin-activated signaling cascades. The recent, still preliminary, work of Burt and Steele [64] shows that the conductance of Cx40 HoM/HoT GJs is not reduced by PDGF whereas conductance of Cx43 HoM/HoT GJs is. The conductance of GJs formed by several cell lines that coexpress these proteins was also regulated by PDGF, even when the ratio of Cx40/Cx43 expression was high. These observations offer insight to the physiologic significance of coexpression-HeM/HeT GJs may be regulated by a broader array of strategies than the corresponding HoM/HoT GJs. Clearly, this possibility requires a great deal more attention.

3.4. Changes in GJ channel permselectivity

Permeability and selectivity (permselectivity) of a channel are determined predominantly by the size (cross-sectional area) of the channel's pore and the electrostatic charges that line the pore and vestibule regions. Originally, GJs were regarded as simple, nonselective pores. In recent years, however, it has become abundantly clear that the HoM/HoT GJs formed by various Cx isoforms display distinct permselectivity properties [68–73]. Four studies are notable in this context. Elfgang et al. [36] compared the extent of the dye coupling (using Lucifer Yellow (LY; 2−, 9.5 Å), propidium iodide (PI; 2+, 9.3 Å), ethidium bromide (EB; 1+, 9.3 Å) and DAPI; 1+, 6 Å) as mediated by HoM/HoT junctions composed of Cx26, Cx31, Cx32, Cx37, Cx40, Cx43, or Cx45. All of these Cxs formed junctions that were reasonably well permeated by LY and DAPI; PI and EB coupling was Cx-specific and typically the level of coupling with these dyes was less than with LY or DAPI. Although the study did not estimate the number of channels contributing to coupling in each setting, the data clearly suggest that the degree of Cx-specific selectivity is not extreme. In more recent studies, permeation rates (molecules/s/channel) for various HoM/HoT junctions have been

made and size selectivity evaluated. Valiunas et al. [72,74] estimated the flux rate for LY as 1530, 300, and 213 molecules/s/channel, a 7-fold difference, through Cx43, Cx40, and Cx45 HoM/HoT junctions, respectively. Weber et al. [73] compared the size selectivity of HoM/HoT GJs composed of Cxs 26, 32, 37, 40, 43, or 45 using the anionic Alexa dyes 350, 488, and 594. For the smallest dye (Alexa 350), channel permeability decreased 6-fold as follows: Cx45>Cx40>Cx32>Cx26≈Cx43>Cx37. For the largest dye (Alexa 594), the sequence changed to Cx32>Cx43>> Cx45>Cx26>Cx40 and Cx37 was not detectably permeated. The magnitude of the difference between Cx32 and Cx40 was 16-fold. Importantly, the Lucifer Yellow flux values obtained by Valiunas et al. for Cx40, Cx43, and Cx45 channels were ~200-fold lower than the Alexa 488 values obtained by Weber et al. for these same channels, despite the comparable size and charge of the dyes. The basis for the differences in the two studies is not yet clear, but in both studies Cx43 was much better permeated than Cx40 channels by small, negatively charged molecules. These data speak to significant Cx-specific selectivity differences.

Given the Cx-specific selectivity differences described above, it seems clear that the range of permselective properties defined by HoM/HoT channel configurations could be greatly expanded by HoM/HeT and HeM/HeT configurations. In early studies addressing this possibility (e.g., Refs. [75,76]), the extent of dye coupling in heterocellular settings in vivo was examined and directional dye transfer was observed. Although the Cx composition of the junctions tested in these studies was not rigorously determined, the authors suggested that heterotypic junctions might be responsible for directional transfer—an intriguing conclusion. Elfgang et al. [36] examined the permeability to Lucifer yellow of HoM/HeT junctions formed by Cx26, Cx31, Cx32, Cx37, Cx40, Cx43, or Cx45. It was determined that some HeT combinations were not permeated by the dye, whereas others were—in no case was directional transfer observed. Although the authors concluded that failure of dye coupling resulted from incompatibility of the partnered connexons, subsequent studies have revealed notable exceptions to this conclusion. For example, Cx40 and Cx43 were concluded to be incompatible for GJ formation because Lucifer-Yellow-permeable junctions were not detected when these connexons were paired. Several labs have subsequently shown that these connexons do form functional HoM/HeT channels [28,30], although this pairing is not as favorable as HoM/HoT channel formation [29] and results in poor dye coupling (possibly due to low numbers of channels). Valiunas et al. quantified the Lucifer Yellow flux rate/channel for Cx40/Cx43 and found it was intermediate (550 molecules/sec/channel) to that measured for the corresponding HoM/HoT forms (see above and Ref. [72]). Weber et al. examined the flux rates for Cx32/Cx26 GJs and Cx37/Cx43 GJs; for Alexa 350, permeability was determined either by the most restrictive partner (Cx26) or was intermediate to the partners (Cx37/Cx43). As dye size increased, the more restrictive partner had a larger and larger impact on permeability. Several conclusions can be drawn based on these results. First, permeability of HeT channels for small molecules that are not near the size cutoff are reasonably predicted by the properties of the HoT channels. As the permeating molecule approaches the size cutoff for the channel, the more restrictive connexon partner dominates the permeability properties of the channel. Finally, for the pairs thus far tested, directional transfer of dye has not been observed.

The impact of hetero-oligomerization on junctional permselectivity has also been examined, although conclusions are still largely qualitative in nature. Early work by Brissette et al. [3] showed a correlation between developmentally regulated changes in Cx expression in keratinocytes and selective alterations in the junctional transfer of cytidine triphosphate and methionine. Koval et al. [77] introduced Cx45 into cells that naturally expressed Cx43 and showed that a consequence of coexpression was a reduction in the extent of Lucifer yellow and calcein dye coupling. Since these Cxs are now known to form HeM connexons, it seems likely that Cx45 and Cx43 HeM/HeM channels are less well permeated by Lucifer yellow than HoM/HoT Cx43 channels. This conclusion has since

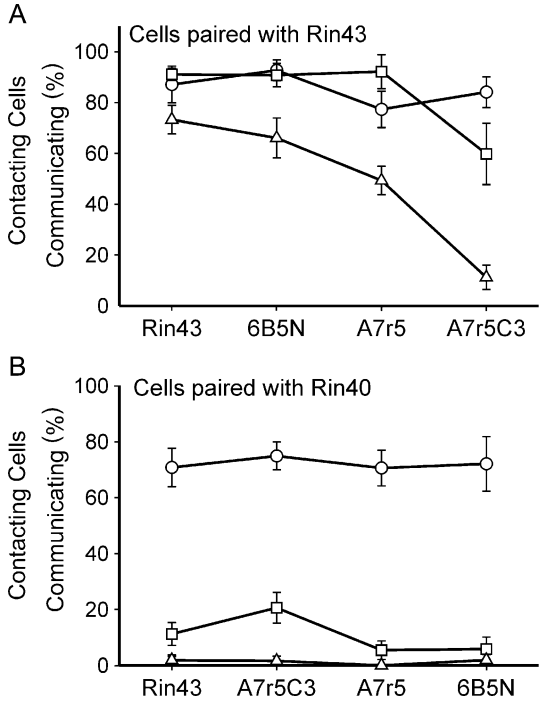

Fig. 4. Dye coupling is limited by the dominant Cx. (A) Cx43-expressing cells (Rin43) cocultured with cells coexpressing Cx40 and Cx43 at Cx40:Cx43 ratios of approximately 1.5:1 (6B5n), 3:1 (A7r5), and 10:1 (A7r5C3) were injected with dyes of various size and charge; NBD-TMA (+1 charge, 280 Da, circles), Alexa 350 (−1 charge, 326 Da, squares), and Alexa 594 (−2 charge, 736 Da, triangles). (B) Experimental conditions were identical to A except that Cx40-expressing cells (Rin40) were cocultured with coexpressing cells. In both A and B, the relative contribution of the less permeable Cx (Cx40) determines the extent of dye transfer. Data adapted from Ref. [29].

gained further support [43]. Bevans et al. [20] reconstituted HoM Cx32 and HeM Cx32 and Cx26 connexons into liposomes and compared permeability to cAMP and cGMP. They found that HoM Cx32 connexons were equally well permeated by cAMP and cGMP but HeM Cx32 and Cx26 connexons, while comparably permeated by cAMP, were poorly permeated by cGMP. Because the permeability of HoM Cx26 connexons was not determined in these studies, the impact of the HeM configuration on selectivity cannot be conclusively stated. However, these studies certainly indicate differences between HeM and HoM forms.

The only published studies addressing ratio-dependent changes in permselectivity of HeM/HeM and HeM/HoM GJs derive from the Burt laboratory. Their data support the conclusion that permselectivity of such junctions is reasonably well predicted by the relative contribution of the Cx constituents (see Figs. 4A, and 5C). In an initial study, Burt et al. [63] found that as the Cx40:Cx43 expression ratio increased in coexpressing cells, the permeability to Lucifer yellow decreased despite comparable levels of electrical coupling. Cottrell et al. [29] explored the impact of expression ratio on permselectivity more thoroughly by comparing results obtained with dyes of differing size and charge. They found that HoM/HoT Cx43 junctions were equally well permeated by positively and negatively charged dyes with only a slight decrease in extent of coupling as dye size increased (Fig. 4A). In contrast, Cx40 junctions were poorly permeated by negatively charged dyes but were comparably permeated by positively charged dye (Fig. 4B). When cells of increasing Cx40:Cx43 expression ratio were tested, GJ permeability shifted from that resembling HoM/HoT Cx43 to that of HoM/HoT Cx40. Of particular interest here were their observations of HoM/HeM junctions. When Cx43-expressing cells were cocultured with cells that coexpressed Cx40 and Cx43 (Cx43/Cx43–Cx40 GJs), they found that as the Cx40:Cx43 expression ratio increased, the GJ permeability again shifted towards that of HoM/HoT Cx40 (Fig. 4A). However, when Cx40-expressing cells were cocultured with these same coexpressing cell lines (Cx40/Cx43–Cx40 GJs), the permeability did not shift with increasing expression ratio; instead, permeability resembled that of HoM/HoT Cx40 junctions irrespective of the Cx40:Cx43 expression ratio in the HeM connexon (Fig. 4B). These observations suggest that the relative contribution of a "dominant" Cx to HeM/HeM channels (Cx40 in this case) can be used to predict the permselectivity characteristics of that channel. If the mechanistic basis for the restricted permselectivity of Cx40 channels is

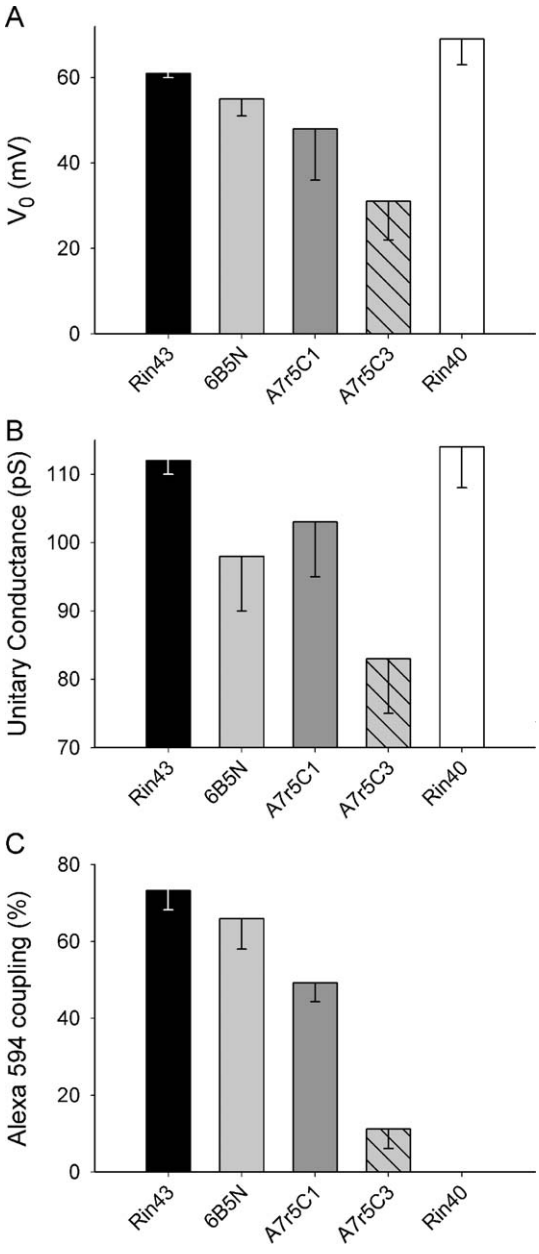

Fig. 5. Alteration of Cx expression ratios can result in progressive shifts in V_j-gating, γ_j, and permselective properties of GJ channels. Functional properties of Rin43 cells paired homotypically (black bar), heterotypically with coexpressing cells (Cx40:Cx43 ratio in 6B5N cells is ~1.5:1, in A7r5-C1 cells is 5:1, and in A7r5-C3 cells is 10:1; gray bars), or heterotypically with Rin40 cells (white bar) can be compared to observe the influence of changing Cx expression ratio on HeM channel behavior. (A) The V_j resulting in a 50% reduction of the voltage-sensitive conductance (V_0) is plotted for each type of junction. In this case, the Cx43-expressing cell is held positive relative to the coexpressing cell such that the gating properties of the HeM connexons is revealed. Note that as Cx40:Cx43 expression ratio increases, the V_0 decreases. (B) The mean conductance of the single-channel events observed in each cell pair was determined and the mean ± S.E. for all pairs in each group plotted. Note the high degree of variability observed in the HoM/HeM pairs (gray bars) and the reduced values therein relative to both HoM/HoT and HoM/HeT junctions. (C) The permeability to a relatively large anionic dye (Alexa 594) is plotted for each type of junction. Note that as the Cx40 content of the coexpressing cell increases, the permeability to the dye decreases to undetectable levels. In all cases, electrical coupling was present between the cells, although coupling in the HoM/HeT cells was reduced relative to the others. Data adapted from Ref. [29].

pore diameter and charge, and if these properties are retained by Cx40 connexons, then the presence of a HoM Cx40 connexon in HeM/HoM or HoM/HeT channels also containing Cx43 would logically be expected to be the principle determinant of permselectivity. In the absence of a HoM Cx40 connexon, the Cx40 content of the HeM connexon might reasonably be expected to be a primary determinant of selectivity properties.

3.5. Stoichiometry and channel function

As stated previously, there are 14 possible hemichannel configurations in a cell that coexpresses two Cxs and 196 possible channel configurations in a pair of such cells. Are there rules governing the assembly of connexons and GJ channels? Are there preferred combinations? The mechanisms that regulate assembly of connexons, trafficking of these connexons to the membrane, docking of connexons to form channels, removal of channels from the membrane and their degradation were recently review in this publication [46]; consequently, they are not reviewed here. With regard to HeM connexon and channel formation, it is worth noting, however, the results of Das Sarma et al. [48], who clearly demonstrated that regulation of HeM connexon assembly can be cell-specific, with preferential assembly in one cell type and random assembly of the same Cxs in another.

To determine whether channel assembly is random, cell lines induced to express two Cxs at different molar ratios are necessary. A comparison of expression ratio in total and plaque protein must then be performed in these cell lines. Finally, because not all channels in plaques are (necessarily) functional, parameters of junctional function must be determined and correlated, if possible, to expression ratio. If assembly of channels is a random function of Cx expression levels, then measured parameters should correlate strongly with the Cx expression ratio. Some progress has been made in this area by the Burt laboratory. They generated A7r5 cell lines with different stable expression ratios (in total protein) of Cx40 and Cx43 and characterized multiple parameters of junctional function in these lines. The data derived from these cell lines strongly support the contention that assembly of functional channels is well described by the assumptions of random assembly. V_j-gating, single-channel conductances, and junctional selectivity differed [29] in cell pairs wherein a cell that coexpressed Cx40:Cx43 at ratios ranging from ~1:1 to 10:1 was paired with a cell expressing only Cx43 (Fig. 5). When single-channel events were examined in pairs of these coexpressing cells, the channel population shifted towards HoM/HoT Cx40 behaviors as the Cx40:Cx43 ratio increased (Fig. 3). If there were no functional parameters that correlated with expression ratio, then there would be little doubt that preferential assembly of functional channels occurs in the A7r5 cell line; in contrast, since there are several functional parameters that correlate with expression ratio, it seems reasonable to conclude that assembly of these Cxs into connexons and channels in the A7r5 cell line is largely dictated by random processes with the consequence that probability theory provides considerable insight to the channel populations formed by these cells. Eventually, through the use of similar strategies, the behavior of junctions with known Cx and connexon composition will be discovered.

4. Influence of heterogeneous assembly on health and disease

4.1. Regulation of cellular growth control

Acute response to growth factors commonly involves the activation of intracellular signaling cascades that phosphorylate Cx phosphoproteins to induce transient decreases in GJ communication. In chronically proliferating cells, e.g., cancerous cells, GJ communication is often down-regulated [78]. It has been proposed that decreased cell-to-cell communication results in concentrating growth signals in the responding cells, thereby facilitating (or even permitting) the growth response [79]. HeM/HeT channel assembly has the potential to significantly alter both acute and chronic growth signaling responses.

We have previously discussed the phosphorylation-mediated gating behavior of HeM/HeT channels. Chronic exposure of the parental line, A7r5, to growth factors (serum) results in a loss of Lucifer yellow dye transfer when compared to growth-arrested cells [80] despite maintenance of electrical coupling. The results of later studies indicated that the observed changes in permselectivity were likely due to an elevated Cx40:Cx43 expression ratio in proliferating vs. growth-arrested cells [81]. The subsequent development of cell lines that expressed these proteins at ratios similar to that observed in the differing growth conditions validated the original observations of decreased permselectivity in growing cells, and demonstrated that Cx40 had the capacity to significantly limit the transfer of anionic dyes in HeM/HeT GJ channels [29]. These observations suggest that a growth stimulus that increases Cx40:Cx43 expression ratio will cause shifts in GJ channel populations that affect a progressive loss of permeability to anions in a precise and graded fashion.

It is not clear what anionic molecules would not be allowed to transfer between cells in the model above, but others have shown that heteromerization of GJ channels can result in an altered ability to share signaling molecules such as cAMP and cGMP [20]. Coexpression of Cx26 with Cx32, which results in the formation of HeM connexons, results in a loss of cGMP permeability while cAMP permeability is relatively unchanged. It would be easy to visualize that the controlled communication of these two ubiquitous signaling molecules could have significant effects on cellular growth control.

4.2. Effects on conduction in excitable tissues

Many excitable cells are known to express multiple Cxs. Cells of the myocardium can express Cx40, Cx45, and Cx43 [82]. Smooth muscle cells can express Cx37, Cx40, Cx43, and Cx45 [4,83,84]. Neurons can express Cx36, Cx43, and Cx45 [2]. Although in each of these systems the Cx expression pattern varies spatially and temporally, the potential for HeM/HeT channel formation is significant. Early studies demonstrating rectifying electrical synapses [85] provided evidence that HeT channels may promote orthodromic conduction of electrical current in neurons and other excitable cells. The prevalence of asymmetric V_j-gating in GJs composed of the cardiovascular Cxs was also suggested by many to account for orthodromic conduction in these excitable tissues [31,33,86]. However, in neither neuronal nor cardiovascular tissues has the existence of HoM/HeT GJ channels been proven. Furthermore, if such HeT channels existed, it is difficult to comprehend how their V_j sensitivity could contribute to directional propagation of electrical current, for the kinetics of V_j-gating are orders of magnitude slower than the rate of conduction of the action potential (however, see the studies of Veenstra et al. [87,88]).

Evidence from canine atrial cells suggests that HeM/HeT channels are prevalent in these excitable cells [27]. V_j and γ_j data from numerous labs have demonstrated that the gating of these types of channels is variable [29,32,33]. In most cases, V_j-gating sensitivity was reduced in the HeM/HeT setting. Taken together, it is not at all clear from the available data that HeM/HeT channels play a significant role in the conduction properties in excitable cells.

4.3. Dominant inhibition in disease states

The extent to which heterogeneous GJ formation affects tissue health and disease is not clear. However, for mutations of Cx26, heterogeneous channel formation has been shown to contribute to disease phenotypes for the inner ear and skin. Mutations of Cx26 have been identified as a causal factor in nonsyndromic sensorineural deafness [89] and syndromic deafness associated with palmoplantar keratoderma (PPK). Using the *Xenopus* oocyte expression system, Rouan et al. [1] evaluated dominant mutations of Cx26 associated with these phenotypes for their capacity to interfere with the assembly of wtCx26 and wtCx43, which colocalize in skin but not the inner ear. None of the mutant forms of Cx26 formed functional channels and all had a dominant inhibitory effect when coexpressed with wtCx26. However, only those mutants associated with PPK inhibited channel formation by coexpressed wtCx43. This result suggests that the PPK phenotype results from dominant interference of wtCx43 function by mutant Cx26. Dominant-negative effects of Cx26 on HeM channels formed with Cx31 may contribute to other skin disorders [90,91]. Using the HeLa expression system, Marziano et al. [92] tested four mutations of Cx26 (including two from the Rouan study) for their capacity to interfere with the assembly of wtCx26 and wtCx30, which co-localize in the inner ear. All the mutant forms of Cx26 failed to form functional channels, which for two of the mutants reflected failure to assemble and traffic normally to the plasma membrane. These intracellular retention mutants were rescued when coexpressed with wtCx26 or wtCx30, which suggests HeM connexon assembly between Cx26 and Cx30. Although all mutants caused hearing loss and were dominantly inherited, none completely eliminated intercellular communication in the coexpression setting, which suggests a more subtle mechanism than complete loss of communication underlies the disease phenotype. These studies indicate that (1) Cx26 hetero-oligomerizes with both Cx43 and Cx30 and (2) that some mutations selectively alter oligomerization with one of these Cxs without affecting the other. The latter observation lends further support to regulated assembly of connexons.

5. Conclusion

The capacity for heterogeneous GJ channel formation has been demonstrated in vivo, in vitro (cell lines and in various mammalian expression systems), and in nonmammalian expression systems. Heterogeneous channels display gating activity, channel conductances, selectivity, and regulatory behaviors that are sometimes not predicted from the behaviors of the corresponding homogeneous channels. Assembly and trafficking of heteromeric connexons can be regulated, and it would not be surprising if docking were also regulated. The available evidence strongly suggests an important role for heterogeneous GJ channels in growth control and in several disease states. Definitive evidence that heterogeneous channels regulate electrical conduction in excitable cells is absent, although clearly GJ channels are essential in this function.

In studies to date, the focus has been on HeM/HeT channels composed of only two Cxs; however, there is no evidence to suggest that this number is limited to two Cxs. With 20 human Cxs identified to date, there are 190 possible HeT channel conformations and potentially thousands of different HeM channel conformations. Ultimately, to appreciate whether HeM/HeT channels play a significant role in the physiology and/or pathophysiology of cells and tissues, it will be necessary to discover the “rules” governing their assembly and function (gating, conductance, selectivity behaviors). Expression systems in which the stoichiometry of assembled channels is known will be critical for elucidation of these rules and ultimately recognition of their contribution to cell and tissue function. Progress in these areas will not only help us to better understand the gap junction’s role in health and disease, but will facilitate development of any therapeutic potential.

Acknowledgements

Work originating in the author's laboratory was supported by the National Institutes of Health, HL58732, and the American Heart Association, 0050020N.

References

[1] F. Rouan, T.W. White, N. Brown, A.M. Taylor, T.W. Lucke, D.L. Paul, C.S. Munro, J. Uitto, M.B. Hodgins, G. Richard, Trans-dominant inhibition of connexin-43 by mutant connexin-26: implications for dominant connexin disorders affecting epidermal differentiation, J. Cell. Sci. 114 (2001) 2105–2113.

[2] M.V. Bennett, R.S. Zukin, Electrical coupling and neuronal synchronization in the mammalian brain, Neuron 41 (2004) 495–511.

[3] J.L. Brissette, N.M. Kumar, N.B. Gilula, J.E. Hall, G.P. Dotto, Switch in gap junction protein expression is associated with selective changes in junctional permeability during keratinocyte differentiation, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 6453–6457.

[4] J.A. Haefliger, P. Nicod, P. Meda, Contribution of connexins to the function of the vascular wall, Cardiovasc. Res. 62 (2004) 345–356.

[5] B.J. Nicholson, T. Dermietzel, D. Teplow, O. Traub, K. Willecke, J.P. Revel, Two homologous protein components of hepatic gap junctions, Nature 329 (1987) 732–734.

[6] N.S. Peters, New insights into myocardial arrhythmogenesis: distribution of gap-junctional coupling in normal, ischaemic and hypertrophied human hearts, Clin. Sci. 90 (1996) 447–452.

[7] B. Risek, Developmental regulation and structural organization of connexins in the epidermal gap junction, Dev. Biol. 164 (1994) 183–196.

[8] D.S. He, J.X. Jiang, S. Taffet, J.M. Burt, Formation of heteromeric gap junction channels by connexins 40 and 43 in vascular smooth muscle cells, Proc. Natl. Acad. Sci. U. S. A. 96 (1999) 6495–6500.

[9] J.X. Jiang, D.A. Goodenough, Heteromeric connexons in lens gap junction channels, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 1287–1291.

[10] I. Fentiman, J. Taylor-Papadimitriou, M. Stoker, Selective contact-dependent cell communication, Nature 264 (1976) 760–762.

[11] J.D. Pitts, J.W. Simms, Permeability of junctions between animal cells, Exp. Cell Res. 104 (1977) 153–163.

[12] M.L. Epstein, N.B. Gilula, A study of communication specificity between cells in culture, J. Cell Biol. 75 (1977) 769–787.

[13] M.L. Dagli, H. Yamasaki, V. Krutovskikh, Y. Omori, Delayed liver regeneration and increased susceptibility to chemical hepatocarcinogenesis in transgenic mice expressing a dominant-negative mutant of connexin32 only in the liver, Carcinogenesis 25 (2004) 483–492.

[14] J.A. Diez, S. Ahmad, W.H. Evans, Assembly of heteromeric connexons in guinea-pig liver en route to the Golgi apparatus, plasma membrane and gap junctions, Eur. J. Biochem. 262 (1999) 142–148.

[15] K.A. Stauffer, The gap junction proteins β_1-connexin (connexin-32) and β_2-connexin (connexin-26) can form heteromeric hemichannels, J. Biol. Chem. 270 (1995) 6768–6772.

[16] L.C. Barrio, T. Suchyna, T. Bargiello, L.X. Xu, R.S. Roginski, M.V.L. Bennett, B.J. Nicholson, Gap junctions formed by connexins 26 and 32 alone and in combination are differently affected by applied voltage, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 8410–8414.

[17] F.F. Bukauskas, C. Elfgang, K. Willecke, R. Weingart, Heterotypic gap junction channels (connexin 26–connexin32) violate the paradigm of unitary conductance, Pflugers Arch. 429 (1995) 870–872.

[18] S. Oh, J.B. Rubin, M.V.L. Bennett, V.K. Verselis, T.A. Bargiello, Molecular determinants of electrical rectification of single channel conductance in gap junctions formed by connexins 26 and 32 [in process citation], J. Gen. Physiol. 114 (1999) 339–364.

[19] J.B. Rubin, V.K. Verselis, M.V.L. Bennett, T.A. Bargiello, Molecular analysis of voltage dependence of heterotypic gap junctions formed by connexins 26 and 32, Biophys. J. 62 (1992) 183–195.

[20] C.G. Bevans, M. Kordel, S.K. Rhee, A.L. Harris, Isoform composition of connexin channels determines selectivity among second messengers and uncharged molecules, J. Biol. Chem. 273 (1998) 2808–2816.

[21] D.I. Vaney, R. Weiler, Gap junctions in the eye: evidence for heteromeric, heterotypic and mixed-homotypic interactions, Brain Res. Rev. 32 (2000) 115–120.

[22] X. Gong, E. Li, G. Klier, Q. Huang, Y. Wu, H. Lei, N.M. Kumar, J. Horwitz, N.B. Gilula, Disruption of alpha3 connexin gene leads to proteolysis and cataractogenesis in mice, Cell 91 (1997) 833–843.

[23] T.W. White, D.A. Goodenough, D.L. Paul, Targeted ablation of connexin50 in mice results in microphthalmia and zonular pulverulent cataracts, J. Cell Biol. 143 (1998) 815–825.

[24] V.M. Berthoud, E.A. Montegna, N. Atal, N.H. Aithal, P.R. Brink, E.C. Beyer, Heteromeric connexons formed by the lens connexins, connexin43 and connexin56, Eur. J. Cell Biol. 80 (2001) 11–19.

[25] M.G. Hopperstad, M. Srinivas, D.C. Spray, Properties of gap junction channels formed by Cx46 alone and in combination with Cx50, Biophys. J. 79 (2000) 1954–1966.

[26] T.W. White, R. Bruzzone, S. Wolfram, D.L. Paul, D.A. Goodenough, Selective interactions among the multiple connexin proteins expressed in the vertebrate lens: the second extracellular domain is a determinant of compatibility between connexins, J. Cell Biol. 125 (1994) 879–892.

[27] S. Elenes, M. Rubart, A.P. Moreno, Junctional communication between isolated pairs of canine atrial cells is mediated by homogeneous and heterogeneous gap junction channels, J. Cardiovasc. Electrophysiol. 10 (1999) 990–1004.

[28] G.T. Cottrell, J.M. Burt, Heterotypic gap junction channel formation between heteromeric and homomeric Cx40 and Cx43 connexons, Am. J. Physiol., Cell Physiol. 281 (2001) C1559–C1567.

[29] G.T. Cottrell, Y. Wu, J.M. Burt, Cx40 and Cx43 expression ratio influences heteromeric/heterotypic gap junction channel properties, Am. J. Physiol., Cell Physiol. 282 (2002) C1469–C1482.

[30] V. Valiunas, J. Gemel, P.R. Brink, E.C. Beyer, Gap junction channels formed by coexpressed connexin40 and connexin43, Am. J. Physiol., Heart Circ. Physiol. 281 (2001) H1675–H1689.

[31] V. Valiunas, R. Weingart, P.R. Brink, Formation of heterotypic gap junction channels by connexins 40 and 43, Circ. Res. 86 (2000) E42–E49.

[32] P.R. Brink, K. Cronin, K. Banach, E. Peterson, E.M. Westphale, K.H. Seul, S.V. Ramanan, E.C. Beyer, Evidence for heteromeric gap junction channels formed from rat connexin43 and human connexin37, Am. J. Physiol., Cell Physiol. 273 (1997) C1386–C1396.

[33] T. Desplantez, D. Halliday, E. Dupont, R. Weingart, Cardiac connexins Cx43 and Cx45: formation of diverse gap junction channels with diverse electrical properties, Pflugers Arch. 448 (2004) 363–375.

[34] S. Elenes, A.D. Martinez, M. Delmar, E.C. Beyer, A.P. Moreno, Heterotypic docking of Cx43 and Cx45 connexons blocks fast voltage gating of Cx43, Biophys. J. 81 (2001) 1406–1418.

[35] A.P. Moreno, G.I. Fishman, E.C. Beyer, D.C. Spray, Voltage dependent gating and single channel analysis of heterotypic gap junction channels formed of Cx45 and Cx43, in: Y. Kanno, K. Kataoka, Y. Shiba, Y. Shibata, T. Shimazu (Eds.), Intercellular Communication through Gap Junctions, vol. 4, Elsevier, Amsterdam, 1995, pp. 405–408.

[36] C. Elfgang, R. Eckert, H. Lichtenberg-Frate, A. Butterweck, O. Traub, R.A. Klein, D. Hulser, K. Willecke, Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells, J. Cell Biol. 129 (1995) 805–817.

[37] R. Bruzzone, J.A. Haefliger, R.L. Gimlich, D.L. Paul, Connexin40, a component of gap junctions in vascular endothelium, is restricted in its ability to interact with other connexins, Mol. Biol. Cell 4 (1993) 7–20.

[38] R. Werner, E. Levine, C. Rabadan-Diehl, G. Dahl, Formation of hybrid cell–cell channels, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 5380–5384.
[39] K.I. Swenson, J.R. Jordan, E.C. Beyer, D.L. Paul, Formation of gap junctions by expression of connexins in *Xenopus* oocyte pairs, Cell Regul. 57 (1989) 145–155.
[40] T.W. White, D.L. Paul, D.A. Goodenough, R. Bruzzone, Functional analysis of selective interactions among rodent connexins, Mol. Biol. Cell 6 (1995) 459–470.
[41] T.W. White, R. Bruzzone, Multiple connexin proteins in single intercellular channels: connexin compatibility and functional consequences, J. Bioenerg. Biomembr. 28 (1996) 339–350.
[42] S. Haubrich, H.J. Schwarz, F. Bukauskas, H. Lichtenberg-Frate, O. Traub, R. Weingart, K. Willecke, Incompatibility of connexin 40 and 43 Hemichannels in gap junctions between mammalian cells is determined by intracellular domains, Mol. Biol. Cell 7 (1996) 1995–2006.
[43] A.D. Martinez, V. Hayrapetyan, A.P. Moreno, E.C. Beyer, Connexin43 and connexin45 form heteromeric gap junction channels in which individual components determine permeability and regulation, Circ. Res. 90 (2002) 1100–1107.
[44] J.C. Saez, V.M. Berthoud, M.C. Branes, A.D. Martinez, E.C. Beyer, Plasma membrane channels formed by connexins: their regulation and functions, Physiol. Rev. 83 (2003) 1359–1400.
[45] M. Yeager, V.M. Unger, M.M. Falk, Synthesis, assembly and structure of gap junction intercellular channels, Curr. Opin. Struct. Biol. 8 (1998) 517–524.
[46] D. Segretain, M.M. Falk, Regulation of connexin biosynthesis, assembly, gap junction formation, and removal, Biochim. Biophys. Acta 1662 (2004) 3–21.
[47] S. Ahmad, J.A. Diez, C.H. George, W.H. Evans, Synthesis and assembly of connexins in vitro into homomeric and heteromeric functional gap junction hemichannels, Biochem. J. 339 (1999) 247–253.
[48] S.J. Das, R.A. Meyer, F. Wang, V. Abraham, C.W. Lo, M. Koval, Multimeric connexin interactions prior to the trans-Golgi network, J. Cell. Sci. 114 (2001) 4013–4024.
[49] D.L. Paul, K. Yu, R. Bruzzone, R.L. Gimlich, D.A. Goodenough, Expression of a dominant negative inhibitor of intercellular communication in the early *Xenopus* embryo causes delamination and extrusion of cells, Development 121 (1995) 371–381.
[50] R. Sullivan, C.W. Lo, Expression of a connexin 43/β-galactosidase fusion protein inhibits gap junctional communication in NIH3T3 cells, J. Cell Biol. 130 (1995) 419–429.
[51] M. Koval, J.E. Harley, E. Hick, T.H. Steinberg, Connexin46 is retained as monomers in a trans-Golgi compartment of osteoblastic cells, J. Cell Biol. 137 (1997) 847–857.
[52] F.F. Bukauskas, V.K. Verselis, Gap junction channel gating, Biochim. Biophys. Acta 1662 (2004) 42–60.
[53] F.F. Bukauskas, A. Bukauskiene, V.K. Verselis, Conductance and permeability of the residual state of connexin43 gap junction channels, J. Gen. Physiol. 119 (2002) 171–186.
[54] V.K. Verselis, C.S. Ginter, T.A. Bargiello, Opposite voltage gating polarities of two closely related connexins, Nature 368 (1994) 348–351.
[55] A.P. Moreno, M. Chanson, S. Elenes, J. Anumonwo, I. Scerri, H. Gu, S.M. Taffet, M. Delmar, Role of the carboxyl terminal of connexin43 in transjunctional fast voltage gating, Circ. Res. 90 (2002) 450–457.
[56] A.L. Harris, Emerging issues of connexin channels: biophysics fills the gap, Q. Rev. Biophys. 34 (2001) 325–472.
[57] F.F. Bukauskas, A.B. Angele, V.K. Verselis, M.V. Bennett, Coupling asymmetry of heterotypic connexin 45/connexin 43-EGFP gap junctions: properties of fast and slow gating mechanisms, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 7113–7118.
[58] G.E. Morley, S.M. Taffet, M. Delmar, Intramolecular interactions mediate pH regulation of connexin43 channels, Biophys. J. 70 (1996) 1294–1302.
[59] X.G. Wang, C. Peracchia, Chemical gating of heteromeric and heterotypic gap junction channels, J. Membr. Biol. 162 (1998) 169–176.
[60] H. Gu, J.F. Ek-Vitorin, S.M. Taffet, M. Delmar, Coexpression of connexins 40 and 43 enhances the pH sensitivity of gap junctions: a model for synergistic interactions among connexins, Circ. Res. 86 (2000) e98–e103.
[61] K. Stergiopoulos, J.L. Alvarado, M. Mastroianni, J.F. Ek-Vitorin, S.M. Taffet, M. Delmar, Hetero-domain interactions as a mechanism for the regulation of connexin channels, Circ. Res. 84 (1999) 1144–1155.
[62] D.S. He, J.M. Burt, Mechanism and selectivity of the effects of halothane on gap junction channel function, Circ. Res. 86 (2000) E104–E109.
[63] J.M. Burt, A.M. Fletcher, T.D. Steele, Y. Wu, G.T. Cottrell, D.T. Kurjiaka, Alteration of Cx43:Cx40 expression ratio in A7r5 cells, Am. J. Physiol., Cell Physiol. 280 (2001) C500–C508.
[64] J.M. Burt, T.D. Steele, Selective effect of PDGF on connexin43 versus connexin40 comprised gap junction channels gap junction channels, Cell Adhes. Commun. 10 (2003) 287–291.
[65] G.T. Cottrell, R. Lin, B.J. Warn-Cramer, A.F. Lau, J.M. Burt, Mechanism of v-Src- and mitogen-activated protein kinase-induced reduction of gap junction communication 1, Am. J. Physiol., Cell Physiol. 284 (2003) C511–C520.
[66] B.R. Kwak, M.M.P. Hermans, H.R. De Jonge, S.M. Lohmann, H.J. Jongsma, M. Chanson, Differential regulation of distinct types of gap junction channels by similar phosphorylating conditions, Mol. Biol. Cell 6 (1995) 1707–1719.
[67] H.V. Van Rijen, T.A. van Veen, M.M. Hermans, H.J. Jongsma, Human connexin40 gap junction channels are modulated by cAMP, Cardiovasc. Res. 45 (2000) 941–951.
[68] G.S. Goldberg, P.D. Lampe, B.J. Nicholson, Selective transfer of endogenous metabolites through gap junctions composed of different connexins, Nat. Cell Biol. 1 (1999) 457–459.
[69] E.B. Trexler, M.V.L. Bennett, T.A. Bargiello, V.K. Verselis, Voltage gating and permeation in a gap junction hemichannel, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5836–5841.
[70] R.D. Veenstra, H.Z. Wang, D.A. Beblo, M.G. Chilton, A.L. Harris, E.C. Beyer, P.R. Brink, Selectivity of connexin-specific gap junctions does not correlate with channel conductance, Circ. Res. 77 (1995) 1156–1165.
[71] R.D. Veenstra, H.-Z. Wang, D.A. Beblo, E.C. Beyer, P.R. Brink, Ion permeability of connexin-specific channels, variations on a common theme? Biophys. J. 66 (1994) A259.
[72] V. Valiunas, E.C. Beyer, P.R. Brink, Cardiac gap junction channels show quantitative differences in selectivity, Circ. Res. 91 (2002) 104–111.
[73] P.A. Weber, H.C. Chang, K.E. Spaeth, J.M. Nitsche, B.J. Nicholson, The permeability of gap junction channels to probes of different size is dependent on connexin composition and permeant-pore affinities, Biophys. J. 87 (2004) 958–973.
[74] V. Valiunas, Biophysical properties of connexin-45 gap junction hemichannels studied in vertebrate cells, J. Gen. Physiol. 119 (2002) 147–164.
[75] J.L. Beny, C. Pacicca, Bidirectional electrical communication between smooth muscle and endothelial cells in the pig coronary artery, Am. J. Physiol., Heart Circ. Physiol. 266 (1994) H1465–H1472.
[76] T.L. Little, J. Xia, B.R. Duling, Dye tracers define differential endothelial and smooth muscle coupling patterns within the arteriolar wall, Circ. Res. 76 (1995) 498–504.
[77] M. Koval, S.T. Geist, E.M. Westphale, A.E. Kemendy, R. Civitelli, E.C. Beyer, T.H. Steinberg, Transfected connexin45 alters gap junction permeability in cells expressing endogenous connexin43, J. Cell Biol. 130 (1995) 987–995.
[78] I.V. Budunova, S. Carbajal, T.J. Slaga, The expression of gap junctional proteins during different stages of mouse skin carcinogenesis, Carcinogenesis 16 (1995) 2717–2724.

[79] W.R. Loewenstein, Junctional intercellular communication and the control of growth, Biochim. Biophys. Acta 560 (1979) 1–65.
[80] D.T. Kurjiaka, T.D. Steele, M.V. Olsen, J.M. Burt, Gap junction permeability is diminished in proliferating vascular smooth muscle cells, Am. J. Physiol., Cell Physiol. 275 (1998) C1674–C1682.
[81] G.T. Cottrell, Y. Wu, J.M. Burt, Functional characteristics of heteromeric Cx40–Cx43 gap junction channel formation, Cell Adhes. Commun. 8 (2001) 193–197.
[82] N.S. Peters, A.L. Wit, Myocardial architecture and ventricular arrhythmogenesis, Circles 97 (1998) 1746–1754.
[83] P.R. Brink, Gap junctions in vascular smooth muscle, Acta Physiol. Scand. 164 (1998) 349–356.
[84] Y.F. Wang, E.E. Daniel, Gap junctions in gastrointestinal muscle contain multiple connexins, Am. J. Physiol.: Gasterointest. Liver Physiol. 281 (2001) G533–G543.
[85] A.A. Auerbach, M.V. Bennett, A rectifying electrotonic synapse in the central nervous system of a vertebrate, J. Gen. Physiol. 53 (1969) 211–237.
[86] A.T. Smith, N. Santama, S. Dacey, M. Edwards, R.C. Bray, R.N. Thorneley, J.F. Burke, Expression of a synthetic gene for horseradish peroxidase C in *Escherichia coli* and folding and activation of the recombinant enzyme with Ca^{2+} and heme, J. Biol. Chem. 265 (1990) 13335–13343.
[87] X. Lin, R.D. Veenstra, Action potential modulation of connexin40 gap junctional conductance, Am. J. Physiol., Heart Circ. Physiol. 286 (2004) H1726–H1735.
[88] X. Lin, M. Crye, R.D. Veenstra, Regulation of connexin43 gap junctional conductance by ventricular action potentials, Circ. Res. 93 (2003) e63–e73.
[89] D.P. Kelsell, J. Dunlop, H.P. Stevens, N.J. Lench, J.N. Liang, G. Parry, R.F. Mueller, I.M. Leigh, Connexin 26 mutations in hereditary non-syndromic sensorineural deafness, Nature 387 (1997) 80–83.
[90] I. Gottfried, M. Landau, F. Glaser, W.L. Di, J. Ophir, B. Mevorah, N. Ben Tal, D.P. Kelsell, K.B. Avraham, A mutation in GJB3 is associated with recessive erythrokeratodermia variabilis (EKV) and leads to defective trafficking of the connexin 31 protein, Hum. Mol. Genet. 11 (2002) 1311–1316.
[91] F. Rouan, C.W. Lo, A. Fertala, M. Wahl, M. Jost, U. Rodeck, J. Uitto, G. Richard, Divergent effects of two sequence variants of GJB3 (G12D and R32W) on the function of connexin 31 in vitro, Exp. Dermatol. 12 (2003) 191–197.
[92] N.K. Marziano, S.O. Casalotti, A.E. Portelli, D.L. Becker, A. Forge, Mutations in the gene for connexin 26 (GJB2) that cause hearing loss have a dominant negative effect on connexin 30, Hum. Mol. Genet. 12 (2003) 805–812.