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Multiple Connexin Proteins in Single Intercellular Channels: Connexin Compatibility and Functional Consequences

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In vertebrates, the protein subunits of intercellular channels found in gap junctions are encoded by a family of genes called connexins. These channels span two plasma membranes and result from the association of two half channels, or connexons, which are hexameric assemblies of connexins. Physiological analysis of channel formation and gating has revealed unique patterns of connexin–connexin interaction, and uncovered novel functional characteristics of channels containing more than one type of connexin protein. Structure–function studies have further demonstrated that unique domains within connexins participate in the regulation of different functional properties of intercellular channels. Thus, gap junctional channels can contain more than one connexin, and this structural heterogeneity has functional consequences *in vitro*. Moreover, emerging evidence for the existence of intercellular channels containing multiple connexins in native tissues suggests that the functional diversity generated by connexin–connexin interaction could contribute to complex communication patterns that have been observed *in vivo*.

KEY WORDS: Gap junction; intercellular channel; connexin; connexon; compatibility; gating; voltage.

INTRODUCTION

Multicellular organisms evolved a variety of processes whereby cells could communicate with each other. In one such mechanism, neighboring cells exchange ions, second messengers, and small metabolites through intercellular channels present in the morphological structures known as gap junctions. Genes encoding the structural components of these channels have been cloned and comprise a family of highly related proteins, the connexins (reviewed in White *et al.*, 1995a, Bruzzone *et al.*, 1996). Unlike other multimeric transmembrane protein complexes, connexins oligomerize in a post-ER compartment into channels called connexons that span a single cellular membrane. Complete intercellular channels spanning two plasma membranes arise when connexons from adjacent cells align in the extracellular space (reviewed in Musil, 1994).

Intercellular channels allow intracellular signals to directly pass from the cytoplasm of one cell to the cytoplasm of neighboring cells, and are thought to coordinate cellular activities, such as contraction of cardiac and smooth muscle, transmission of neuronal signals at electrotonic synapses, pattern formation during development, and secretion in exocrine and endocrine glands (Bruzzone *et al.*, 1996). These hypotheses have been well supported by a multitude of correlative studies; however, the question of whether one connexin is equivalent to any other connexin in filling these putative roles has not been clarified. Recently, direct experimental evidence from transgenic animals, as well as connexin mutations in human disease, have defined new functions for gap junctions and provided novel insights into the meaning of connexin diversity

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(reviewed in Paul, 1995). The goal of this article is to summarize the different ways that intercellular channels can be constructed from more than one type of connexin protein, and to describe some of the functional consequences of this structural heterogeneity. While these observations are necessarily based on *in vitro* studies, the possibility that mixed connexin channels may exist *in vivo*, and their possible impact on organ physiology will also be discussed.

MOLECULAR DIVERSITY OF CONNEXINS AND INTERCELLULAR CHANNELS

The diversity of the connexin family became clear when amino acid sequencing of gap junction proteins isolated from heart, liver, and lens revealed related but distinct amino termini (Nicholson *et al.*, 1985; 1987; Kistler *et al.*, 1988). The existence of multiple gap junction proteins was rapidly confirmed by the molecular cloning of the connexin genes (Paul, 1986; Beyer *et al.*, 1987). Current nomenclature adds the prefix Cx to the molecular mass (in kD) predicted by DNA sequences to distinguish among different connexins. At present, at least 13 different members of the connexin family have been identified in rodents, and their corresponding genes are expressed in complex, and often overlapping, patterns (Table I). Multiple connexins can be expressed in a single cell type, and an important issue to be resolved is how differences in spatial and temporal distribution of connexins contribute to the function of organs constructing gap junctions from these proteins (Goodenough and Musil, 1993).

The existence of many different connexins suggests that cells expressing more than one gap junction structural protein may form intercellular channels which are heterogeneous in connexin content. This idea is supported by the observation that multiple connexin proteins have been localized within single gap junctional plaques in many cell types, including hepatocytes, lens fibers, cardiac myocytes, and keratinocytes (Nicholson *et al.*, 1987; Traub *et al.*, 1989; Paul *et al.*, 1991; Kanter *et al.*, 1993; Risek *et al.*, 1994). Intercellular channels are structurally more complex than other oligomeric channels, because they require the contribution of two connexons, one from each participating cell. Therefore, there are several possible ways in which different connexins could be incorporated into a single channel. First, a connexon could be homomeric, containing only a single connexin species, or heteromeric containing different connexins (Fig. 1A). As a connexon is composed of six connexins arranged radially around a central pore, many types of heteromeric connexons could be generated, differing in either the number or the spatial organization of

Table I. Expression Patterns of Cloned Rodent Connexins[a]

Connexin	Tissue and cellular distribution	Reference
Cx26	Hepatocytes, pancreatic acinar cells, mammary gland alveolar cells, keratinocytes, kidney, leptomeninges, pinealocytes, intestine	Zhang *et al.*, 1989
Cx30	?	K. Willecke, personal communication
Cx30.3	Skin, preimplantation blastocyst, kidney	Hennemann *et al.*, 1992a
Cx31	Keratinocytes, trophoectoderm, preimplantation blastocyst, kidney	Hoh *et al.*, 1991
Cx31.1	Keratinocytes, preimplantation blastocyst, squamous epithelia	Haefliger *et al.*, 1992
Cx32	Hepatocytes, pancreatic acinar cells, mammary gland alveolar cells, neurons, oligodendrocytes, Schwann cells, thyroid follicular cells	Paul, 1986
Cx33	Testis	Haefliger *et al.*, 1992
Cx37	Endothelium, keratinocytes, heart, stomach, testis	Willecke *et al.*, 1991
Cx40	Endothelium, conductive myocardium (His bundle, Purkinje fibers), preimplantation blastocyst	Haefliger *et al.*, 1992
Cx43	Cardiac myoctes, smooth muscle, endothelial cells, mammary gland myoepithelium, testis, lens and corneal epithelium, pancreatic β-cells, keratinocytes, preimplantation blastocyst, thyroid follicular cells	Beyer *et al.*, 1987
Cx45	Heart, kidney, lung, skin, intestine, preimplantation blastocyst	Hennemann *et al.*, 1992b
Cx46	Lens fibers, kidney, heart, Schwann cells	Paul *et al.*, 1991
Cx50	Lens fibers, corneal epithelium, atrioventricular valves	White *et al.*, 1992

[a] This table is not intended to provide a comprehensive tissue distribution for rodent connexins, rather it illustrates their complex and overlapping patterns of expression. Many cell types express more than one connexin, and multiple connexins have been immunocytochemically localized in single gap junctions. References are provided only for the initial molecular characterization of individual rodent connexins.

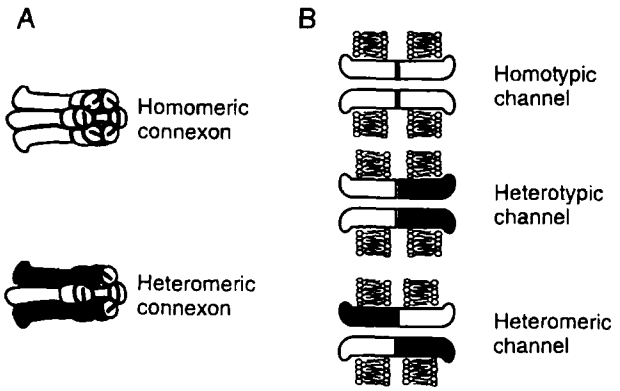

Fig. 1. Schematic representation of the organization of connexins in connexons, and intercellular channels. (A). A connexon is composed of six connexin proteins arranged radially around a central pore. When all six connexins are identical, the resulting connexon is homomeric. When different connexin proteins are present in the same connexon, it is heteromeric. Many types of heteromeric connexons are possible, differing in either the number, or the spatial organization, of different connexin proteins. (B). An intercellular channel is formed from the head-to-head alignment of two connexons in the extracellular space. The association of two homomeric connexons made from the same connexin yields a homotypic channel in which all 12 connexin proteins are identical. A heterotypic channel results from the joining of two homomeric connexons made from different connexins. Heteromeric channels result from the association of a heteromeric connexon with either another heteromeric connexon, or a homomeric connexon. Theoretically, a heteromeric channel could contain up to 12 different connexin proteins.

different connexin proteins. Second, intercellular channels could be generated from different combinations of heteromeric and homomeric connexons (Fig. 1B). Thus, the association of two homomeric connexons made from the same connexin would yield a homotypic channel. Similarly, the alignment of two homomeric connexons made from different connexins would create a heterotypic channel. Finally, a wide variety of heteromeric channel types could be constructed from the interaction of a heteromeric connexon with either another heteromeric connexon or a homomeric connexon. With the 13 known connexin genes, 91 different homotypic and heterotypic channel types can be assembled from homomeric connexons. As heteromeric channels could have unique properties based on both the numbers and spatial arrangement of different connexin proteins incorporated into the paired connexons, the number of physiologically distinct intercellular channel types that could theoretically exist is immense. While it is likely that heteromeric channels do exist *in vivo* (Jiang and Goodenough, 1996), this review will mainly focus on the wealth of information generated by studies of homotypic and heterotypic channels *in vitro*.

CONNEXIN COMPATIBILITY AND THE FORMATION OF HETEROTYPIC CHANNELS *IN VITRO*

The expression of cloned connexin DNAs in cells devoid or depleted of endogenous gap junction proteins has allowed the study of intercellular channels with defined connexin content. The two most popular systems utilized are the *Xenopus* oocyte assay (Dahl *et al.*, 1987), where two cells are paired after the injection of *in vitro* transcribed connexin RNA, and communication-deficient cell lines stably transfected with connexin DNA (Eghbali *et al.*, 1990). Both of these systems have the flexibility to bring two cells expressing different connexins into contact, allowing the properties of heterotypic intercellular channels to be elucidated (Swenson *et al.*, 1989; Bukauskas *et al.*, 1995). Although each technique has drawbacks (for example, both systems express endogenous connexins), they have led to qualitatively similar conclusions (Waltzman and Spray, 1995). Moreover, improvements such as the injection of antisense oligonucleotides to inhibit the endogenous connexin in *Xenopus* oocytes (Barrio *et al.*, 1991) have allowed unambiguous attribution of the recorded properties to the specific connexins being studied. With a few exceptions (Hennemann *et al.*, 1992a; Bruzzone *et al.*, 1994a, 1995), all cloned connexins have been found to form functional intercellular channels in these expression systems. These investigations have led to the elucidation of some of the elementary properties of connexins and to new interpretations of the functioning of intercellular channels.

Formation of a complete intercellular channel requires that two connexons, one contributed by each of the participating cells, become aligned in the extracellular gap. Connexin topological models predict that there are two extracellular domains, whose sequences are well conserved (Fig. 2). Synthetic peptides derived from these domains inhibit intercellular channel formation, confirming that these regions physically interact in the process of connexon–connexon recognition (Dahl *et al.*, 1994; Warner *et al.*, 1995). Sequence conservation in the extracellular domains may play an important role in favoring the head-to-head pairing of connexons each composed of a distinct connexin, thus promoting the formation of heterotypic channels. Studies carried out before the molecular diversity of connexins was apparent documented the frequent occurrence of gap junctional communication between cells derived from different vertebrate organisms and

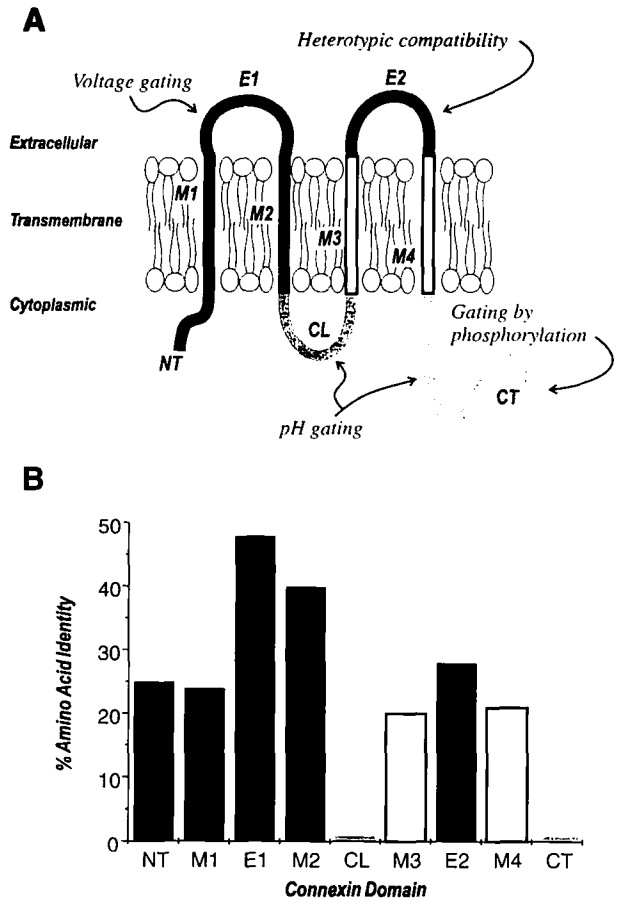

Fig. 2. Membrane topology, structure–function relationships, and sequence identity of connexin proteins. (A). Schematic diagram of a prototypical connexin. Hydropathy plots predict four membrane spanning regions (M1–M4), two extracellular loops (E1 and E2), and three cytoplasmic portions, the amino (NT) and carboxy (CT) terminal domains and the central cytoplasmic loop (CL). Variations in the connexin molecular weight are due to variable lengths of the CL and CT sequences. The putative role of the different domains is inferred from the results of structure–function studies using point mutations and chimeric connexins. Voltage gating properties are influenced by sequences from the NT through M2 (dark gray line, references in text), while pH gating results from interactions between the CL and CT (light gray line, Liu *et al.*, 1993; Ek *et al.*, 1994; Wang *et al.*, 1996; Morley *et al.*, 1996). The E2 domain specifies compatibility in heterotypic channel formation (black line, references in text), and phosphorylation of Tyr and Ser residues in the CT domain results in channel closure, or alteration of unitary conductance and permeability (Swenson *et al.*, 1990; Kwak *et al.*, 1995b). (B). Quantitation of amino acid identity among connexins by domain. Amino acid sequences of twelve rodent connexins were aligned with the PILEUP program (University of Wisconsin Genetics Computer Group). Values represent the percent of amino acid residues that are identical for all twelve connexins in each domain. Domain boundaries reflect the topology presented for Cx32 by Goodenough *et al.* (1988), and are shaded to correspond to the functional domains identified in part A. The first extracellular and second transmembrane domains show the greatest sequence conservation (40–48%). The other transmembrane domains, the second extracellular loop, and the amino terminus are well conserved (20–28%), while the cytoplasmic loop and carboxy terminus are unique.

tissues (Michalke and Loewenstein, 1971; Epstein and Gilula, 1977). Although some restriction patterns of coupling were observed (Kettenmann *et al.*, 1983; Kam *et al.*, 1987), it was generally thought that intercellular communication was a permissive phenomenon occurring each time there were no physical barriers between contacting cells (Hertzberg *et al.*, 1981). The discovery of the multiplicity of connexin proteins, together with the availability of functional expression systems, has allowed the rigorous testing of whether different connexins actually do speak a common language.

While initial reports emphasized that different connexins were able to form heterotypic channels *in vitro* (Swenson *et al.*, 1989; Werner *et al.*, 1989), it has now been established that heterotypic channel formation is a process regulated by a code of compatibility among connexins (Bruzzone *et al.*, 1993; White *et al.*, 1994b). Systematic analyses have demonstrated that adjacent cells programmed to express different connexins have a high probability of not establishing intercellular communication. Thirty-three out of the 60 heterotypic connexin combinations that have been tested failed to generate functional intercellular channels (Table II). While most connexins are able to form channels with approximately half of their tested partners, some extreme examples of compatibility have emerged. Thus, Cx31 is functional only in homotypic configuration, and not in heterotypic combination with the six other connexin partners that have been tested. Connexin 40 is also highly restricted in its ability to make heterotypic channels, functionally interacting with Cx37 and Cx45, but failing to do so when paired with Cx26, Cx31, Cx31.1, Cx32, Cx43, Cx46, and Cx50. In contrast, Cx46 forms heterotypic channels with all connexins tested except Cx40. Intercellular channel formation appears, therefore, to be dominated by rules of compatibility, a characteristic displayed by all members of the connexin family and reproduced in different experimental systems (Elfgang *et al.*, 1995; White *et al.*, 1995b; B. Nicholson, personal communication).

Currently, there are no obvious criteria to predict the pattern of compatibility of any given connexin. On the basis of sequence identity, connexins can be divided into two broad groups, α or β (Gimlich *et al.*, 1990; Paul, 1995). However, available data indicate that connexin compatibility is not simply based on group identity. Although Cx40, Cx46, and Cx50 are all group α connexins, Cx40 does not interact with either Cx46 or Cx50. Furthermore, Cx32 and Cx26,

Table II. Rodent Connexins Exhibit Different Compatibilities in Intercellular Channel Formation[a]

	Cx26	*Cx30*	*Cx30.3*	*Cx31*	*Cx31.1*	*Cx32*	*Cx33*	*Cx37*	*Cx40*	*Cx43*	*Cx45*	*Cx46*	*Cx50*
Cx50	+					+			-	-		+	+
Cx46	+					+			-	+		+	
Cx45	-		-	-	-	-		+	+	+	+		
Cx43	-		+	-	-	-	-	+	-	+			
Cx40	-			-	-	-		+	+				
Cx37	-		+	-	-	-	-	+					
Cx33							-						
Cx32	+	+	-	-	-	+							
Cx31.1	-		-		-								
Cx31	-			+									
Cx30.3	-	-	+										
Cx30	+	+											
Cx26	+												

[a] A (+) indicates the development of coupling, while a (−) signifies that functional channels failed to form. Thus, Cx32 (shaded data) displays a typical pattern of compatibility, forming functional channels in 5 out of the 12 tested combinations. These data were obtained in paired *Xenopus* oocytes and connexin transfected HeLa cells, and are summarized from Elfgang *et al.* (1995), White *et al.* (1995b), and unpublished results from the laboratory of Dr. Bruce Nicholson (SUNY, Buffalo).

which are group β, both readily form channels with group α Cx46 and Cx50 (Table II). To determine the molecular mechanisms allowing connexins to select a compatible partner, several chimeras, whose extracellular sequences were derived from two connexins with different patterns of compatibility, have been tested. These experiments suggest that the second extracellular domain (E2, Fig. 2) plays an active role in the determination of compatibility between some members of the connexin family. For example, Cx43, but not Cx32, has the ability to form heterotypic intercellular channels with *Xenopus* Cx38 (Swenson *et al.*, 1989; Werner *et al.*, 1989). A chimera which contained the amino terminal half of Cx32 (including E1) and the carboxy terminal half of Cx43 (including E2), formed channels with *Xenopus* Cx38 (Bruzzone *et al.*, 1994a). This same chimera was also able to form heterotypic channels with Cx46, but not Cx50 (White *et al.*, 1995b). As Cx32 is compatible with both connexins, and Cx43 functionally interacts with Cx46 but not Cx50 (Table II), the inhibitory effect of a noncompatible E2 outweighed the presence of a compatible E1.

These observations have been confirmed with other chimeric connexins in which only the extracellular domains were exchanged. Replacing the E2 domain of Cx50 with the E2 of Cx46 conferred upon this chimera the ability to form heterotypic channels with Cx43. Similarly, when the E2 domain in Cx46 was substituted with the E2 of Cx50, the resulting chimera no longer functionally interacted with Cx43, but did make heterotypic channels with connexins that were compatible with Cx50 (White *et al.*, 1994b). Thus, the ability to form heterotypic intercellular channels consistently followed the pattern of compatibility specified by the identity of the second extracellular domain. These experiments confirm that the formation of a complete cell-to-cell channel results from the interplay of intrinsic determinants present in different connexins. Other factors in addition to the expression of compatible connexins may also influence the ability of adjacent cells to communicate. For example, dye transfer mediated by Cx43 has been shown to depend on the proper expression and interaction of cell adhesion molecules (Mège *et al.*, 1988; Meyer *et al.*, 1992). Consequently, the expression of compatible connexins may be a necessary, though not sufficient, step for adjacent cells to establish intercellular communication. It also remains unclear how the extracellular loops of connexins in opposing connexons interact (Peracchia *et al.*, 1994). More detailed studies mapping the minimum number of residues that specify selective recognition may provide a more precise definition of the molecular basis for selective interaction between connexins.

FUNCTIONAL CHANGES IN INTERCELLULAR CHANNELS FORMED FROM MULTIPLE CONNEXINS

In addition to exhibiting different compatibilities in heterotypic channel formation, intercellular channels constructed from different connexin proteins also display distinct functional properties with regards to unitary conductance, permeability, and gating (Paul, 1995; Bruzzone *et al.*, 1996). Both the *Xenopus* oocyte assay and communication-deficient cells transfected with connexins have been utilized to study changes in the functional state of intercellular channels under different experimental conditions. The transfected cell system has the resolution to detect single-channel events, which has illuminated unexpected relationships between unitary conductance, permeability, and gating. Thus, it has been systematically shown that specific connexin channels have very different permeability to tracer molecules in the same size range as signaling molecules like cyclic nucleotides and inositol phosphates (Elfgang *et al.*, 1995). Surprisingly, this selectivity is not directly correlated with the unitary conductance of intercellular channels, which can vary by more than an order of magnitude, but is most likely due to direct electrostatic interactions between the channel pore and the permeant molecules (Veenstra *et al.*, 1995). However, treatment with different pharmacological agents that activate protein kinases results in parallel shifts in the permeability and the unitary conductance of gap junction channels (Moreno *et al.*, 1994; Kwak *et al.*, 1995a,b). All of the above cited studies demonstrate different functional properties/regulation of homotypic intercellular channels made from different connexins.

Because intercellular channels can be homotypic or heterotypic, it is of interest to determine whether connexons composed of a particular connexin have intrinsic functional properties regardless of which connexin is present in the opposing connexon. While a few studies have examined the single-channel properties of heterologous cell pairs (Bukauskas *et al.*, 1995; Moreno *et al.*, 1995), the question of whether connexon–connexon interactions modulate functional properties of intercellular channels has not yet been thoroughly addressed in the transfected cell expression systems. One advantage of the paired oocyte assay is that cells injected with different connexin RNAs can be paired to study heterotypic channels. Large numbers of heterotypic combinations can be rapidly assembled and functionally assayed. While connexin permeability and regulation by protein kinases can also be studied in *Xenopus* oocytes (Swenson *et al.*, 1990; Cao *et al.*, 1994), the greatest amount of functional information to date has been generated by studies of the voltage gating behavior of heterotypic and homotypic intercellular channels (Nicholson *et al.*, 1993; White *et al.*, 1995a).

Like conventional ion channels, intercellular channels are voltage sensitive, responding to voltage differences between the two coupled cells (Bennett and Verselis, 1992). Gap junctional conductance can be experimentally modulated by holding one cell in a pair at a constant potential while hyperpolarizing or depolarizing the adjacent cell (Spray *et al.*, 1981). Generally, voltage-dependent changes in conductance happen on a slow time scale (seconds), although significant conductance changes can occur much faster (milliseconds). Equilibrium conductance values can be

plotted over a range of transjunctional potentials to give a characteristic conductance–voltage curve for different connexins. Figure 3 demonstrates how changes in voltage gating properties can occur in intercellular channels assembled from different combinations of homomeric connexons. The conductance–voltage curves of Cx32 and Cx46 homotypic channels (Figs. 3A, 3B) are quite similar, showing equivalent reductions in conductance at large transjunctional potentials of either polarity. Cx50 homotypic channels

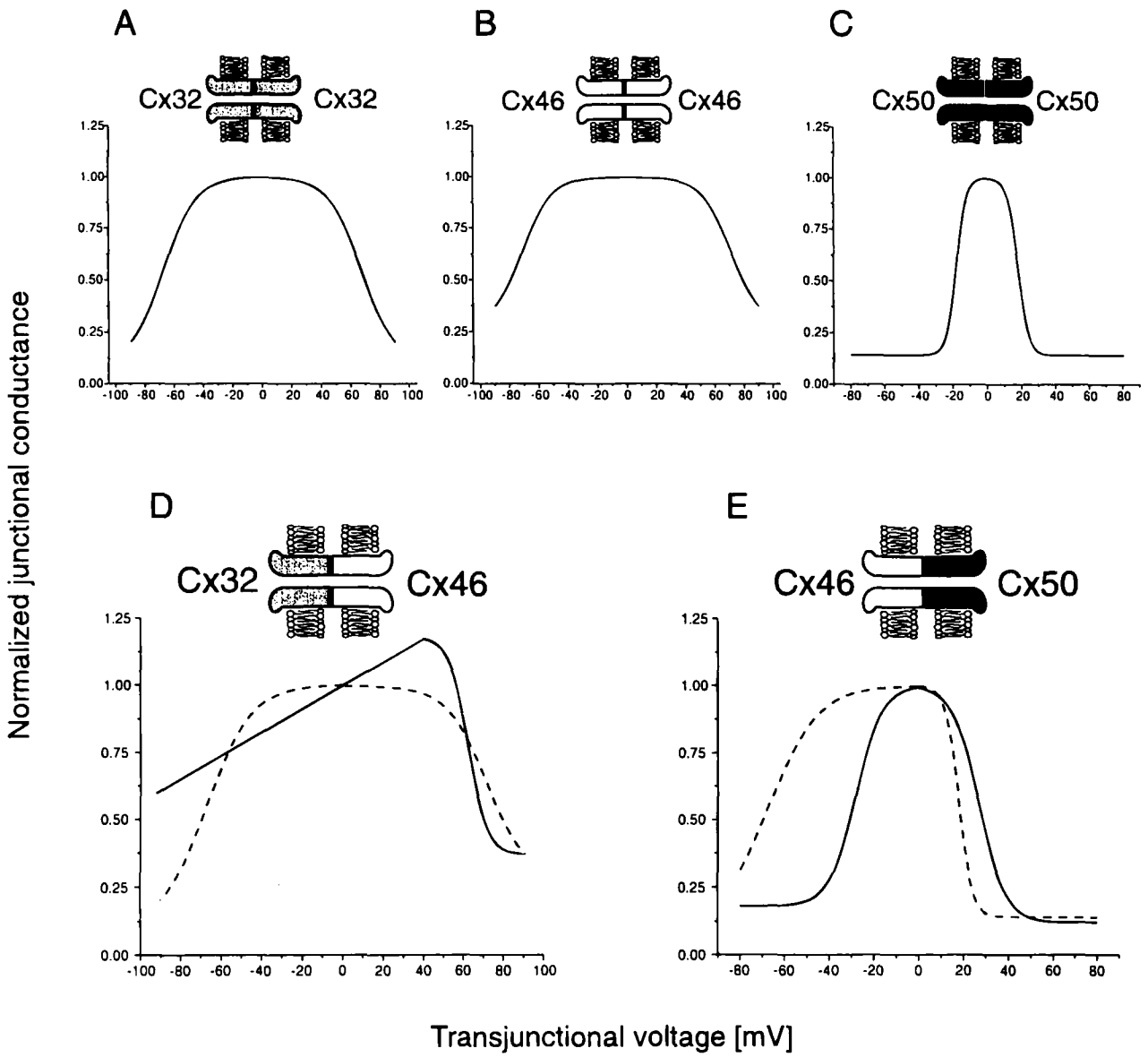

Fig. 3. Comparison of the voltage gating of homotypic and heterotypic channels in paired *Xenopus* oocytes. Solid lines depict the equilibrium conductance values (normalized to the predicted conductance when the transjunctional potential equals zero). (A, B) Homotypic Cx32 and Cx46 channels have similar voltage gating profiles, showing symmetrical reductions of conductance, but only at transjunctional potentials greater than 40 mV. (C) In contrast, Cx50 homotypic channels show large symmetrical reductions in conductance at transjunctional potentials below 30 mV. (D, E) Dashed lines represent the predicted conductance of heterotypic channels based on the properties of homotypic channels. In sharp contrast to the expected curves, the actual equilibrium conductance of heterotypic Cx32/Cx46 channels is highly asymmetric, while that of heterotypic Cx46/Cx50 channels is nearly symmetrical. Thus, the voltage dependence of an intercellular channel is not only due to intrinsic properties of connexins, but can also reflect novel properties arising from connexon–connexon interaction. (Adapted from data presented in Bruzzone *et al.*, 1994a; White *et al.*, 1994a,b, 1995b).

(Fig. 3C) are also symmetrically voltage sensitive, but show greater reductions in conductance at much lower transjunctional potentials than either Cx32 or Cx46 channels. Because these three connexins are compatible with each other, one can test whether the voltage gating of heterotypic channels can be predicted by the homotypic properties. On the basis of the characteristics of homotypic channels, one might expect that heterotypic Cx32/Cx46 channels would still exhibit symmetrical voltage gating while heterotypic Cx46/Cx50 channels would show an asymmetrical conductance–voltage relationship (Figs. 3D and 3E, dashed lines). In reality, heterotypic Cx32/Cx46 channels (Fig. 3D, solid line) show a highly asymmetric voltage dependence, while heterotypic Cx46/Cx50 channels (Fig. 3E, solid line) are symmetrically voltage dependent. Analysis of other heterotypic channels has demonstrated that novel gating properties often result from interactions between different connexins (Barrio *et al.*, 1991; Hennemann *et al.*, 1992c; Bruzzone *et al.*, 1994a; White *et al.*, 1994a,b, 1995b; Chen and DeHaan, 1996). Thus, connexons do not maintain their electrical "fingerprints" and novel functional properties may arise from connexon–connexon interactions in heterotypic channels.

Attempts to understand the molecular basis of the unpredicted voltage gating behavior of heterotypic channels have proved to be extremely difficult. Many efforts have focused on trying to explain the dramatic differences in gating properties observed in homotypic and heterotypic combinations of Cx26 and Cx32. Heterotypic Cx26/Cx32 channels exhibit rectifying behavior, so that over a defined range of transjunctional potentials conductance increases for one polarity and decreases for the opposite polarity (Barrio *et al.*, 1991). The development of simple methods for exchanging or mutating precise domains within connexins (Bruzzone *et al.*, 1991; Rubin *et al.*, 1992a; White *et al.*, 1994b, 1995c), and the ease of expressing these constructs directly from *in vitro* transcribed mRNA in the *Xenopus* oocyte system, have provided a powerful tool for examining this and other aspects of intercellular communication that result from sequence differences between connexins. In one such mutational analysis, Rubin *et al.* (1992a,b) prepared chimeras by substituting defined amino acid segments of Cx32 with the corresponding ones of Cx26, seeking to identify those sequences that could interconvert their voltage dependence. While exchange of the first extracellular domain had the greatest effect, none of the chimeras displayed gating properties that could explain all of the differences between the parent molecules. Taking a different approach, Verselis *et al.* (1994) have postulated that rectification in Cx26/Cx32 heterotypic channels occurs because connexons composed of Cx26 and Cx32 close in response to voltages of opposite polarities. Their analysis identified charge differences in the amino termini which cause connexons composed of Cx26 and Cx32 to close in response to voltages of opposite polarities in intercellular channels, although this assumption has not been verified experimentally with single connexons (see below). Indeed, other residues have also been shown to affect the polarity of voltage gating of Cx26 connexons in intercellular channels. Alteration of charges in all four membrane-spanning domains of Cx26 reversed the polarity of their gating with respect to wild type Cx26 (Yox *et al.*, 1993). Moreover, mutation of a proline residue in the second transmembrane domain of Cx26 resulted in connexons which had a dominant rectifying phenotype when paired with either wild type Cx26 or Cx32, which should exhibit opposite polarities of voltage gating (Suchyna *et al.*, 1993). These authors concluded that this proline, which is conserved among all members of the connexin family, may act as a transduction element between the voltage sensor and the voltage gate of intercellular channels. Together, these studies do not identify a single domain within connexins that dictates all of the voltage properties of gap junction channels. It is likely that complex interactions between different domains, most likely including residues from the amino terminus through the second transmembrane domain, are involved in the specification of a given intercellular channels voltage gating (Fig. 2). In support of this view, the gating behavior of a chimeric connexin consisting of Cx32 from the N-terminus through the second transmembrane domain, fused to Cx43 from the middle cytoplasmic loop to the C-terminus, behaved much more like Cx32 than Cx43 with regard to voltage dependence (Bruzzone *et al.*, 1994a; White *et al.*, 1995b).

Compelling evidence for an explanation for some of the properties of Cx26/Cx32 channels, not based on voltage-gating polarity, has been deduced from single-channel data obtained in the transfected cell expression system. These experiments indicate that unitary conductance in a single Cx26/Cx32 heterotypic channel is differentially modulated by transjunctional potential and shows voltage-dependent rectification (Bukauskas *et al.*, 1995). This apparent violation of the paradigm of unitary conductance may in turn be explained by differences in the relative ionic permeability of Cx26 and Cx32. Cx26 homotypic channels favor cations,

while Cx32 homotypic channels favor anions. In heterotypic Cx26/Cx32 channels, transjunctional potentials of one polarity are favorable to increasing the ionic concentration within the channel pore, thus leading to increasing conductance. Potentials of the opposite polarity result in a lower ionic concentration within the channel, leading to decreasing conductance (Suchyna *et al.*, 1994). As many important signaling molecules are ions at physiological pH, this type of chemical rectification could have profound consequences on intercellular signal transmission between cells coupled by heterotypic channels.

One of the difficulties in trying to unravel the molecular basis of connexin voltage gating has been the inability to dissect differences in the gating of individual connexons out of the more complex behavior of the complete intercellular channel. Recently, it has become possible to directly analyze the gating behavior of single connexons derived from the expression of connexins in solitary *Xenopus* oocytes, or the reconstitution of purified connexons in lipid bilayers (Ebihara and Steiner, 1993; Gupta *et al.*, 1994; Buehler *et al.*, 1995; Ebihara *et al.*, 1995). Therefore, one can directly test whether the voltage gating of connexons is affected by the identity of the connexon in the opposing membrane by comparing the behavior of Cx46 single connexons to Cx46 containing intercellular channels. In single oocytes, Cx46 connexons are opened by cell depolarization, and closed by hyperpolarization (Paul *et al.*, 1991; Ebihara and Steiner, 1993). The voltage-gating properties of homotypic intercellular channels composed of Cx46 are in excellent agreement with those of Cx46 connexons (White *et al.*, 1994b; Ebihara *et al.*, 1995). Surprisingly, the polarity of voltage gating for Cx46 connexons appears to be reversed when they become incorporated into heterotypic intercellular channels with either Cx26 or Cx32 (White *et al.*, 1994a). Application of the types of mutational analysis described above to connexins which can be studied in both single connexons and intercellular channels is likely to clarify the question of whether connexons have intrinsic characteristics with regard to many of their functional properties.

INTERCELLULAR CHANNELS MADE FROM MULTIPLE CONNEXINS *IN VIVO*

While it is clear that intercellular channels can contain multiple connexin proteins *in vitro*, the molecular composition of intercellular channels *in vivo* has been more difficult to ascertain. Single gap junctions often contain multiple connexin proteins; however, the immunohistochemical techniques that have provided this data lacked the resolution needed to look at single intercellular channels. Recently, structural, physiological, and biochemical data have been obtained which indicate that living organisms do indeed assemble intercellular channels containing multiple connexins. In one study, images of rodent liver gap junctions containing Cx26 and Cx32 were obtained with the scanning transmission electron microscope, and subjected to image analysis methods which visualized the individual connexons and determined the mass of each connexon in an intact intercellular channel. The mass distributions were consistent with liver gap junctions containing both homotypic and heterotypic channels, but not heteromeric channels (Sosinsky, 1995). However, Cx26 and Cx32 were able to co-assemble into heteromeric connexons biochemically purified from Sf9 cells infected with recombinant baculoviruses encoding both connexins (Stauffer, 1995). Thus, there could be cell type specific control of the complex processes involved in the assembly of connexins into channels (Musil, 1994).

Other studies have utilized cells isolated from the vertebrate lens, which are coupled by extensive networks of gap junctions (Goodenough, 1992), as model system for the biochemical and physiological study of connexins. Donaldson *et al.* (1995) analyzed the voltage-gating behavior of intercellular channels in freshly isolated lens fiber cells expressing Cx46 and Cx50. Their results suggested a balanced contribution of Cx46 and Cx50, and closely matched the physiological properties of heterotypic Cx46/Cx50 channels reconstituted in paired *Xenopus* oocytes (White *et al.*, 1994b). The view that lens fiber cells may preferentially assemble heterotypic channels was subsequently supported by analysis of intercellular channels solubilized from calf lenses. Single intercellular channel fractions that had been subjected to multiple purification steps still contained equal ratios of the two lens connexins, suggesting that heterotypic or heteromeric channels may be the preferred assembled state in this system (Konig and Zampighi, 1995). Biochemical studies have further shown that heteromeric connexons do exist in the fiber cells. Jiang and Goodenough (1996) solubilized gap junctional plaques from lenses to produce single connexon-rich fractions that were further purified by sucrose gradient centrifugation. Biochemical studies of these fractions have shown that connexins 46 and 50 can be co-immunoprecipitated

from the single connexon pool, demonstrating the presence of heteromeric connexons *in vivo*. Therefore, the lens may express several connexin proteins to take advantage of the novel functional properties that can be generated by the assembly of multiple connexins into the same intercellular channel.

Functional diversity generated by the interaction of multiple connexins in single intercellular channels could contribute to complex communication phenomena that have been observed *in vivo*. As we have discussed, interactions between opposing connexons can generate novel electrical properties. For example, heterotypic channels containing Cx32 often exhibit electrical rectification (Barrio *et al.*, 1991; White *et al.*, 1995b). Thus, heterotypic channels could explain the behavior of rectifying electrical synapses where only orthodromic impulse propagation is allowed (Giaume *et al.*, 1987), although the connexins present in this type of electrotonic synapse have not been identified. While a great deal of effort has focused on voltage gating, other properties, including permeability to ions, second messengers, and metabolites, may be modified by heterotypic or heteromeric interaction.

We speculate that patterns of communication observed in intact tissues could result from intercellular channels containing different combinations of connexins on the two sides of a gap junction. For example, passage of microinjected dyes was readily detected from astrocytes to oligodendrocytes but rarely in the opposite direction (Robinson *et al.*, 1993). Similarly, in the arteriolar wall, microinjected dyes passed freely from endothelial cells to underlying smooth muscle cells; however, transfer in the reverse direction was not observed (Little *et al.*, 1995). The idea that connexin–connexin interaction can alter the permeability to injected dyes is supported by recent studies of transfected cells expressing both Cx43 and Cx45. In ROS cells, Cx43 homotypic channels were permeable to Lucifer yellow, calcein, and hydroxycoumarin carboxylic acid, three different sized fluorescent dyes. When Cx45 and Cx43 were co-expressed in ROS cells, intercellular transfer of hydroxycoumarin carboxylic acid remained the same, while transfer of the other two dyes was markedly reduced (Koval *et al.*, 1995). These effects were not correlated with changes in electrical coupling, and most likely resulted from direct interaction of Cx43 and Cx45 in mixed gap junctional channels. Similar results were obtained in SKHep cells expressing Cx43 and Cx45. In these cells homotypic Cx43 channels were permeable to Lucifer Yellow, while heterotypic Cx43/Cx45 channels were not. Both types of channel were permeable to neurobiotin, a smaller tracer molecule (Moreno *et al.*, 1995). Whether the unidirectional type of dye transfer observed in intact tissues can be duplicated by heterotypic or heteromeric channels reconstituted *in vitro* awaits further testing.

Another example of the potential impact of heteromeric interactions is the demonstration of the dominant negative effect of some mutant connexins (Bruzzone *et al.*, 1994b; Paul *et al.*, 1995). The deleterious consequences of the incorporation of nonfunctional connexins into intercellular channels may be physiologically relevant. Mutations in the Cx32 gene are associated with X-linked Charcot–Marie–Tooth disease, a genetic demyelinating disorder of the peripheral nervous system (Paul, 1995). Some of these mutations are not only devoid of functional activity, but also act as selective dominant negative inhibitors, when coexpressed in paired *Xenopus* oocytes with compatible connexins (Bruzzone *et al.*, 1994b). If this situation occurred *in vivo*, the ability of Cx32-expressing cells to use alternative connexins for intercellular communication would be compromised. The considerable progress made in our understanding of the molecular architecture of intercellular channels with their rules of connexin compatibility, selectivity, and unique gating mechanisms has raised more issues than it has answered. The time is now ripe to leave the protected environment of expression systems to test current working models *in vivo*.

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