

Emerging issues of connexin channels: biophysics fills the gap

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I. Introduction

Connexins are the proteins that form the intercellular channels that compose gap junctions in vertebrates. Connexin channels mediate electrotonic coupling between cells and serve important functions as mediators of intercellular molecular signaling. Convincing demonstration of the latter function has been elusive, as have the experimental tools required for detailed functional study of the channels. Recently, substantial progress has been made on both fronts. Connexin channels are now known to be dynamic, multifunctional channels intimately involved in development, physiology and pathology, and amenable to study by state-of-the-art approaches. A host of developmental and physiological defects are caused by defects in connexin channels, and therefore in the intercellular molecular movement they mediate. The channel structure has been determined to 7.5 Å resolution within the plane of the membrane. Experimental paradigms have been developed that enable application of the tools of modern channel biophysics to study connexin channel structure–function. As a result, the biophysical mechanisms and biological functions of connexin channels now enjoy a vigorous and expanding experimental interest. This article focuses on the former, but with attention to issues likely to have biological consequences.

Connexin channels have historically been regarded by channel physiologists as poor relations of ‘real’ channels. In part, this view developed because the chemical signaling function of connexin channels (its function in most contexts) is less easily defined and less likely to depend crucially on the intrinsically compelling properties of rapid channel gating and selectivity among atomic ions that tend to motivate channel biophysicists. Moreover, with the exception of electrotonic coupling, the study of connexin channels emerged from cell biology and anatomy rather than physiology.

On the pragmatic side, the intercellular structure of gap junction channels makes them difficult to study and requires analytical paradigms different from those applied to other channels. Single-channel recordings of connexin channels were unavailable for many years (and remain technically challenging), as were specific blockers and affinity reagents, which have been immensely beneficial in the study of other channels. In addition, much of the physiological data seemed contradictory or ambiguous, something we now know largely results from diverse connexin channel physiologies, arising from approximately 20 connexin isoforms and their ability to form heteromeric channels.

Much has changed in the last several years. Homomeric channels formed by several connexin isoforms have been characterized in detail using variants of commonly applied molecular and physiological approaches. In addition, new approaches have been devised. The information coming from these studies provides a growing, credible and largely self-consistent framework for understanding connexin channel structure–function. An intriguing set of biophysical issues and questions are emerging from the recent data.

It is hoped that this review will address the (usually) unspoken questions that arise in the minds of most channel physiologists when they hear about connexin channels: Are they real channels? Do they do anything important? Do they do anything interesting from a biophysical perspective? Is there an understanding of their structure–function analogous to that for other channels? What does the terminology mean? What are the key issues? Why is it so difficult to get answers to these questions?

1.1 What? Terminology and general properties

Gap junctions are ubiquitous aggregates of intercellular channels found between most cells across many phyla, from metozoa to chordates (Bennett *et al.* 1994; Becker *et al.* 1998). They (*a*) span closely apposed plasma membranes, (*b*) provide pathways for direct current flow between cytoplasm of coupled cells, and (*c*) can be permeable to molecules the size of cytoplasmic second messengers. Each gap junction channel is formed by two end-to-end hexameric structures called *hemichannels* or *connexons*, each of which spans a single plasma membrane and originates in the cell whose plasma membrane it spans (Goodenough, 1975; Makowski *et al.* 1977; Unwin & Zampighi, 1980; Werner *et al.* 1989). The intercellular, double-membrane-spanning structures will be referred to in this article as *junctional channels*.

In chordates, junctional channels are composed of *connexins* (Beyer *et al.* 1990; Becker *et al.* 1998), currently believed to be a protein family of approximately 20 isoforms (Bennett *et al.* 1994; White *et al.* 1995a; Beyer & Willecke, 2000). The isoforms have approximately 40% overall sequence identity, with much greater identity in the transmembrane and extracellular domains. Many isoforms have species-specific orthologs, bringing the number of naturally occurring connexin variants to over 40 (see Beyer & Willecke, 2000). Some isoforms are restricted to specific species, and some to specific tissues. Most cells express more than one

isoform. The different isoforms compose channels that have widely diverse unitary conductances, permeabilities, subconductance characteristics, voltage sensitivities, and sensitivities to modulatory agents. There is no significant sequence homology with other membrane proteins.

Connexins have not been identified in any invertebrate species, despite many attempts, and despite occasional reports of staining with connexin antisera (Fraser *et al.* 1987; Meiners *et al.* 1991; Janssen *et al.* 1994; Mire *et al.* 2000), nor have connexin genes been found in the completely sequenced genomes of *C. elegans* or *Drosophila*.

Gap junction channels in arthropods and nematodes are *not* formed by connexins, but by a distinct family of proteins called *innexins* (Phelan *et al.* 1998a, b; Phelan, 2000; Phelan & Starich, 2001). It had been thought that innexins might be restricted to Ecdysozoa (the molting clade), but innexin DNA sequences have now been identified in platyhelminthes and molluscs (Alexopoulos *et al.* 2000; Panchin *et al.* 2000). Their localization and function in those organisms are not yet determined. Innexins have no sequence homology with connexins, but do share several features with them, including four transmembrane domains and intracellular N- and C-termini. A single proline at the extracellular end of a transmembrane domain is conserved in all connexins and innexins. Connexins have three nearly invariant cysteines in each extracellular loop; innexins have two (Phelan, 2000). In contrast to these similarities in the proteins, the gene structure of innexins is quite different from that of connexins (Curtin *et al.* 1999). Because the discovery of innexins is recent there is a paucity of structure–function information of identified forms.

Remarkably, channels formed by connexins and innexins have very similar properties in terms of regulation by voltage, pH and lipophiles, and in terms of permeability (Bukauskas & Weingart, 1994; Gho, 1994; Bukauskas *et al.* 1997; Landesman *et al.* 1999), though the pores of innexin channels are likely wider, as a group (Schwarzmann *et al.* 1981). In spite of their convergent functional and structural properties, this article will deal only with channels formed by connexins; it will largely omit information from invertebrate systems.

The first evidence for a direct intercellular pathway permeable to molecules came in the 1920s from studies of dye spread in cardiac cells (Schmidtman, 1925), and was confirmed 40 years later (Loewenstein & Kanno, 1964; Loewenstein, 1965). Evidence for direct intercellular current flow was obtained in the early 1950s (Curtis & Travis, 1951; Weidmann, 1952), though it had been proposed in the 1870s on the basis of physiological and histological studies (Engelmann, 1877). The electrophysiological property called ‘electrotonic coupling’ was first characterized in the late 1950s (Sjostrand *et al.* 1958; Watanabe, 1958; Furshpan & Potter, 1959; Karrer, 1960). Soon thereafter, the morphological structure later called the ‘gap junction’ was described (Dewey & Barr, 1962; Robertson, 1963; Revel & Karnovsky, 1967; Brightman & Reese, 1969). The morphology and physiology were correlated in the next several years (Dewey & Barr, 1962; Bennett *et al.* 1963; Barr *et al.* 1965; Pappas & Bennett, 1966; Payton *et al.* 1969). For most of the next two decades, the functional characterization of gap junction channels was almost exclusively the province of neurophysiologists, who described and analyzed intercellular current flow (Bennett, 1966) and speculated about the cell biological and developmental consequences of an intercellular pathway for diffusion of small cytoplasmic molecules (Bennett, 1966; Bennett & Trinkaus, 1970; Warner, 1973; Pitts, 1978; Loewenstein, 1981). Connexin channels were recognized as dynamically regulated with the demonstration of modulation of intercellular coupling in vertebrates by junctional voltage (Spray *et al.* 1979) and by cytoplasmic pH (Turin & Warner, 1977; Rose & Rick, 1978; Spray

et al. 1981b). The former led to the application of voltage-clamp techniques to connexin channels (Harris *et al.* 1981; Spray *et al.* 1981a). The first cloning of a connexin in 1986 (Kumar & Gilula, 1986; Paul, 1986) led to an explosion of knowledge about connexins and the identification of many connexin isoforms, which enabled application of the techniques of genetic manipulation and heterologous expression. In conjunction with patch-clamp electrophysiology, a wide diversity of connexin channel physiologies was discovered (see Bennett & Verselis, 1992; Bennett *et al.* 1993; White *et al.* 1995a; Peracchia, 2000; Verselis & Veenstra, 2000).

Several recent monographs and special journal issues review various aspects of the physiology and function of connexin channels, as well as their genetics, biochemistry and cell biology (Hertzberg, 2000b; Peracchia, 2000; Lo, 2000; Rozental & Campos de Carvalho, 2000; Rozental *et al.* 2000). The following articles are particularly informative regarding functional properties (Bargiello *et al.* 2000; Barrio *et al.* 2000; Bukauskas & Peracchia, 2000; Delmar *et al.* 2000; Ebihara & Pal, 2000; Peracchia *et al.* 2000c; Skerrett *et al.* 2000; Veenstra, 2000; Verselis & Veenstra, 2000). Much of the material cited as 'submitted' or 'personal communication' in this review will appear in the proceedings of the recent international biannual meeting of connexin researchers to be published in *Cell Communication & Adhesion*, **8** (5/6), 2001.

1.2 Why? Reasons for biophysical study

Defects in molecular movement through connexin channels cause developmental defects, physiological defects, and disease (Peracchia, 2000). Connexins are known or suspected to be involved in a wide variety of biological processes including hematopoiesis, regeneration, cardiac development, lens transparency, fertility, immune system function, protection from oxidative stress and, in some quarters, acupuncture (Mashanskii *et al.* 1983; Shang, 2001) and telepathy (Gollub, 1997). The importance of connexins is underscored by that fact that connexin genes contain IRES (internal ribosome entry site) elements, which are typically found in genes of transcription factors, growth factors, and other genes that need to be specifically and rapidly regulated independently of cell cycle (van der Velden & Thomas, 1999; Werner, 2000).

Connexin channels present for biophysicists the usual kinds of biophysically interesting issues, but each with a twist. They present unique issues as well.

Voltage gating. Hemichannels and junctional channels are voltage gated. The sensitivity to voltage takes several forms, and varies among connexins. There is no S4 domain, so the mechanism for sensing voltage must be different for connexin channels than for other non- β -barrel channels. Junctional channels are typically sensitive to transjunctional voltage, and some to membrane potential as well. Where/how is the voltage sensed?

Permeant selectivity. Many connexin channels exhibit substantial charge selectivity among small permeants (e.g. atomic ions). How is this achieved? They also show remarkable selectivity among larger permeants, including second messengers, that cannot be accounted for solely on the basis of size and overall charge of the permeant. How is the molecular selectivity achieved? Are there specific molecular binding sites in the lumen of the pore?

Channel diversity. Each connexin forms channels with distinct properties, such as selectivity and voltage dependence. Hemichannels and junctional channels can be heteromeric – composed of more than one isoform of connexin. What are the functional consequences?

Substates. Hemichannels and junctional channels typically show substantial gating to substates. What is the structural basis? How do the charge and molecular selectivities differ for different conducting states?

Rectification. Connexin channels can show rectification of unitary conductance when in symmetric solutions that contain no components large enough to block the pores. How does this occur?

pH-dependent gating. Connexin channels have widely varied sensitivities to acidification. Is this due to protonation of the connexin or interaction with a protonated cytoplasmic modulator?

Hemichannels and junctional channels. Hemichannels can operate both as independent, single-membrane channels in plasma membrane and as subunits of junctional channels. What structural features allow this? How do the hemichannels in two cells find each other and interact? What is the specificity of that interaction and what are the structures of the domains that mediate the interaction? How are hemichannel properties modulated by end-to-end (and side-to-side) interactions?

1.3 How? Special issues for study of connexin channels

Connexin channels pose challenges for experimentation and data interpretation beyond those posed by other channels. These challenges have shaped the character and availability of information about connexin function.

The most substantial experimental impediment is that junctional channels are junctional—each channel spans two plasma membranes and both ends of the pore are cytoplasmic. This configuration eliminates the most straightforward electrophysiological approaches (on-cell or excised patch recording) and forces a reliance on various configurations of *dual* whole-cell voltage clamp, which at least squares the probability of success for any given experiment. Single junctional channel recordings can be obtained under some conditions and in some preparations (not paired *Xenopus* oocytes, however due to the high nonjunctional conductance, which is effectively a ‘seal’ conductance in this configuration (see Section 3.1.1)). Control of the ionic environment of the pore is possible only via single or dual whole-cell dialysis. Thus for whole-cell voltage clamp and on-cell patch techniques, the primary domain of modulatory action, the cytoplasmic surface of the channel, is not directly accessible.

The other major impediment is the absence of specific or high-affinity toxins or blockers for connexin channels. Compounds such as TTX and the *connus* toxins have been of inestimable value in structure–function studies of other channels.

Standard patch-clamp techniques can be applied in those few cases where wild-type and genetically altered connexins form hemichannels that are conductive and well-behaved in plasma membrane. In most cases, however, the behavior of hemichannels, and the effects of the end-to-end interactions between hemichannels in junctional channels, must be inferred rather than directly observed. This problem is substantial, and unique to connexin channels. It has led to conflicting conclusions regarding the character and basis for voltage and chemical sensitivity of connexin channels and hemichannels, and to difficulty in elucidating their molecular mechanisms.

The complexity becomes daunting when one considers that each type of hemichannel has the potential to be modulated differently by its end-to-end interaction with every other type

of hemichannel. In fortunate instances, the behavior of a hemichannel inferred from junctional channel behavior compares well with its behavior when it is paired with other types of hemichannels, and/or with its behavior as a single hemichannel in plasma membrane. However the data are typically not so clear and the inferences about 'intrinsic' hemichannel properties in the absence of hemichannel data can become quite model-dependent. Even so, in some cases the properties of hemichannels have been accurately inferred from junctional channel behavior, and vice versa.

2. Molecular and structural context

2.1 Biochemical features

The connexins are identified as a family of proteins by sequence similarity and inferred transmembrane topology (Beyer *et al.* 1990). Despite significant variation in length, primarily in the cytoplasmic domains, several features are common to all. Each connexin has four predominantly hydrophobic, membrane-spanning regions (M1–M4) (Fig. 1). For Cx43, these domains have been shown to be α -helical, on the basis of a 7.5 Å structure (Unger *et al.* 1997). By analogy, the corresponding domains in the other connexins are presumed to be α -helical as well. The carboxy- and amino-domains (CT and NT, respectively) are accessible from the cytoplasm, as is the hydrophilic cytoplasmic loop (CL) domain between M2 and M3. The hydrophilic domains between M1 and M2 and between M3 and M4 are accessible from extracellular space and form extracellular loops E1 and E2 (Zimmer *et al.* 1987; Goodenough *et al.* 1988; Hertzberg *et al.* 1988; Milks *et al.* 1988; Yancey *et al.* 1989; Laird & Revel, 1990; Yeager & Gilula, 1992). The length of the CT varies among connexins and is responsible for most of the differences in molecular mass.

The transmembrane domains and extracellular loops are highly similar across connexins (Fig. 2). E1 and E2 are each characterized by 3 cysteine residues with invariant spacing of CX₆CX₃C and CX₄CX₅C, respectively (except for Cx31, for which the spacing in E2 is CX₅CX₅C, and which does not form channels with any other connexin; Elfgang *et al.* 1995). E2 contains a conserved PCP motif. In spite of a high degree of amino-acid identity and homology in M1–M4 and E1–E2, several key differences in functional properties can be attributed to the few sequence differences in these domains (cf. Verselis *et al.* 1994). The NTs are somewhat homologous, but the CTs and CLs show little homology across connexins. The CT and CL domains contain phosphorylation sites and other sites involved in chemical gating (for reviews, see Delmar *et al.* 2000; Jongasma *et al.* 2000; Lau *et al.* 2000; Peracchia *et al.* 2000; Yeager & Nicholson, 2000), as well as sites that may interact with other proteins including v-src and ZO-1 (Giepmans *et al.* 2001) via SH3/SH2 or PDZ-binding domains (Swenson *et al.* 1990; Kanemitsu *et al.* 1997; Giepmans & Moolenaar, 1998; Toyofuku *et al.* 1998b, 2001). In addition, certain connexins may interact with signaling systems as part of complexes containing β -catenin (Ai *et al.* 2000) and p120ctn, both of which have been co-immunoprecipitated with connexin43 (Ai *et al.* 2000; C. Lo, personal communication) or N-cadherin (Xu *et al.* 2000).

It is intriguing to note that the transmembrane topology of connexin (four transmembrane domains and cytoplasmic carboxy- and amino-termini) is shared by other proteins that mediate close approach or adhesion of plasma membranes, in spite of an absence of amino-acid sequence homology. These include the claudin and occludin proteins of tight junctions

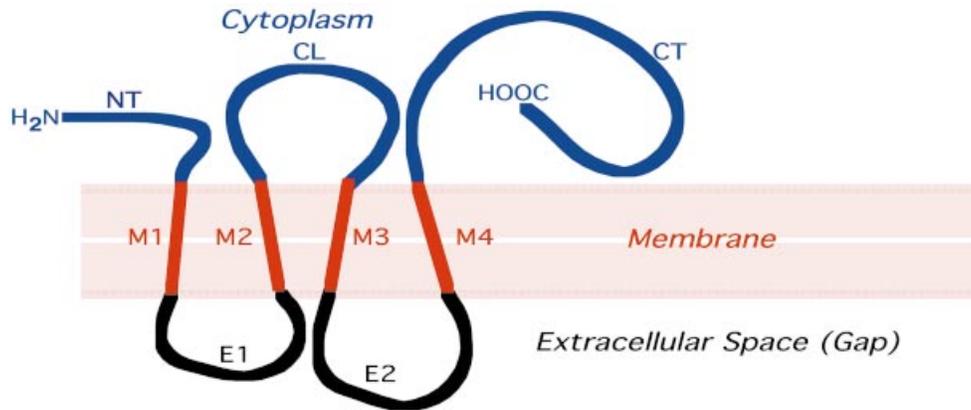


Fig. 1. Membrane topology of a connexin monomer. M1–M4 are transmembrane domains, E1 and E2 are extracellular loops, NT and CT are N-terminal and C-terminal domains, respectively, and CL is the cytoplasmic loop. (Figure from Sosinsky, 2000.)

(Goodenough, 1999; Tsukita *et al.* 1999), and the myelin proteins PLP (Gow *et al.* 1997) and PMP-22 (D’Urso & Müller, 1997), in addition to innexins. Connexins are not members of the tetraspanin family of molecular mediators (Maecker *et al.* 1997).

The most widely used nomenclature for connexins is based on predicted molecular mass – Cx32 for a predicted mass of ~ 32 kDa, Cx43 for a predicted mass of ~ 43 kDa and so on (Beyer *et al.* 1988). Where it is important to designate species-specific orthologs, a lower-case prefix is added, as in cCx42 for chick Cx42, hCx46 for human Cx46.6, mCx32 for murine Cx32, rCx26 for rat Cx26 or zCx45 for zebrafish Cx45. Despite the ease and descriptive utility of this system, it may require modification as additional connexins and species orthologs with similar predicted masses are identified.

Another system (Gimlich *et al.* 1990) distinguishes 3 families of connexin genes and assigns subscripts based on order of discovery: $\alpha 1$ for Cx43, $\beta 1$ for Cx32, $\beta 2$ for Cx26 and so on. The α , β and γ families are distinguished by a combination of gene structure, overall sequence homology, and matching of specific sequence motifs. The length of the CL of the α connexins is approximately double that of the β connexins. Distance matrix analysis of the sequences confirms and extends this grouping, generally identifying the β connexins as Type I and α connexins as Type II (Bennett *et al.* 1994). In the matrix analysis, the large difference between the II/ α and I/ β families compared with the differences between mammalian and amphibian orthologs suggests that the families diverged early in vertebrate speciation. Table 1 identifies known connexins using both nomenclatures.

In this article, the CxXX nomenclature will be used because of its ease and wider use in the literature. Species prefixes will be used when necessary; designations without prefixes generally refer to rat or mouse orthologs.

There are functional differences between some of the species orthologs, in spite of their high sequence identity. The minor sequence differences underlying the functional differences may prove to be quite informative. Among the more dramatic examples are:

- (1) mCx50 and skate Cx35 form hemichannels in plasma membrane, but their species orthologs hCx50 and mCx36, respectively, do not (Zampighi *et al.* 1999; Valiunas & Weingart, 2000; Al-Ubaidi *et al.* 2000; Ebihara, personal communication)

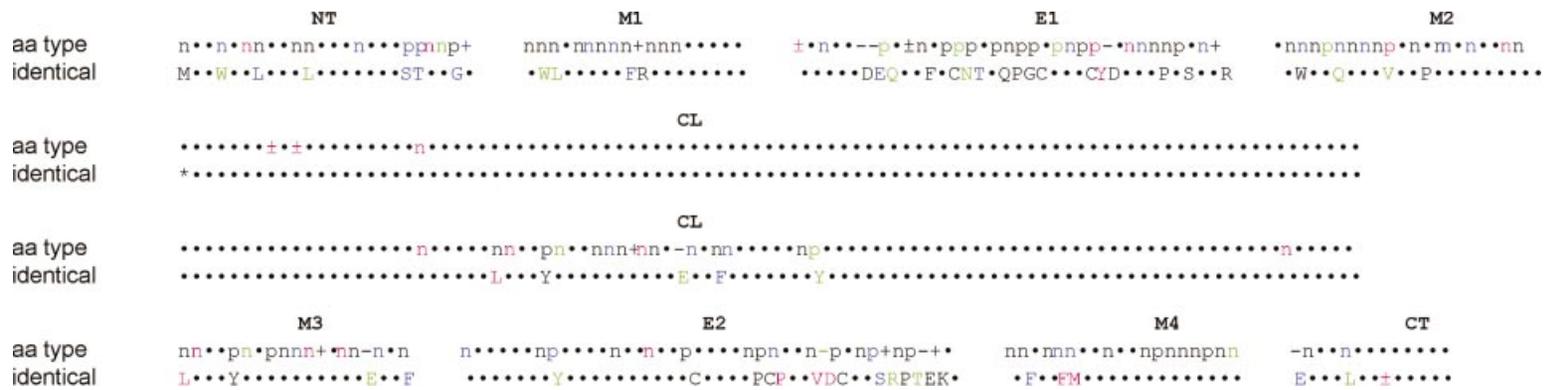


Fig. 2. Connexin consensus sequence. This consensus sequence is based on alignment of the predicted amino-acid sequences of the 20 human connexin genes. Row labeled 'identical' indicates amino-acid identity across all sequences. Row labeled 'aa type' indicates whether the amino acids at this position are polar (p), non-polar (n), negatively charged (-), positively charged (+), or either positively or negatively charged (\pm). Colors indicate number of exceptions to this identity among the 20 sequences: blue for 1, green for 2, magenta for 3 or 4. If there were more than 4 exceptions, no consensus is indicated (\bullet). The position indicated by * is occupied by H and Y in approximately equal numbers of the aligned sequences. The boundaries used for the domains are from Bennett *et al.* (1994) and correspond to the following positions for Cx32/Cx43:

NT: M1-R22/M1-K23

M1: V23-A40/V24-V41

E1: E41-R75/E42-R76

M2: L76-A96/F77-F97

CL: H97-T130/Y98-G150

M3: L131-F149/L151-F169

E2: M150-T188/L170-T207

M4: V189-A207/I208-I226

CT: E208-283/E227-I382.

Table 1. *Connexin nomenclature*

Group	Connexin	Species specificity*	Ortholog(s)	Tissue*
	Cx25	(Human only, to date)		
$\beta 2$	Cx26		Chick Cx31	
β	Cx27.5	Zebrafish	(Mammal Cx26?)	Retina, CNS
γ	Cx29		Human Cx30.2	Glia
$\beta 6$	Cx30			
β	Cx30.2		Human Cx31.9	
$\beta 5$	Cx30.3			
$\beta 3$	Cx31			
$\beta 4$	Cx31.1			
$\beta 1$	Cx32		<i>Xenopus</i> Cx30	
α	Cx32.2	Atlantic croaker		Ovary
α	Cx32.7	Atlantic croaker	Sea bream Cx32.3	Ovary
$\alpha 6$	Cx33			Testis
γ	Cx34.7	Perch		Retina
$\alpha 9$ or γ	Cx36		Skate/perch Cx35	Retina, CNS
$\alpha 4$	Cx37			
$\alpha 2$	Cx38	<i>Xenopus</i>		
	Cx39		Human Cx40.1	
$\alpha 5$	Cx40		Chick Cx42	
α	Cx41	<i>Xenopus</i>		
$\alpha 1$	Cx43			
$\alpha 7$ or γ	Cx45		Zebrafish Cx43.4, Chick Cx56,	Lens fibers
$\alpha 3$	Cx46		Cow, Sheep Cx44	
γ	Cx47		Human Cx46.6,	Retina, CNS, PNS
$\alpha 8$	Cx50		Chick Cx45.6, Sheep Cx49, Zebrafish Cx44.1	Lens
	Cx55.5	Zebrafish		Retina
$\alpha 10$	Cx57		Sheep, pig Cx60, Human Cx62	Ovary
	Cx58	(Human only, to date)		

Most connexins are widely distributed among vertebrate species, and in characteristic sets of tissues. Those with highly restricted species/tissue distribution are noted. In some cases orthologs have different names due to slightly different molecular mass. Some connexins have not been assigned to families or not numbered. A comprehensive review of connexin cloning is found in Beyer & Willecke (2000).

* If highly restricted.

- (2) rCx43 is regulated by PKG, but hCx43 is not, lacking the key serine (Takens-Kwak & Jongsma, 1992; Moreno *et al.* 1994b; Kwak *et al.* 1995b);
- (3) rCx43 has main unitary conductance of 80 pS, and is slightly anion selective, but cCx43 has a conductance of 198 pS and is reasonably cation selective (Veenstra *et al.* 1995);
- (4) the sensitivity of Cx45 to membrane potential (not transjunctional voltage) decreases dramatically in the order of: mCx45 > hCx45 > cCx45 > zCx45 (Barrio *et al.* 1997);
- (5) rCx46, like most connexins, is inhibited by octanol and low pH, but the chick ortholog cCx56 is not sensitive to either (Bukauskas *et al.* 1997).

2.2 Structures

Structurally, connexin channels come in several flavors. The most commonly studied are the

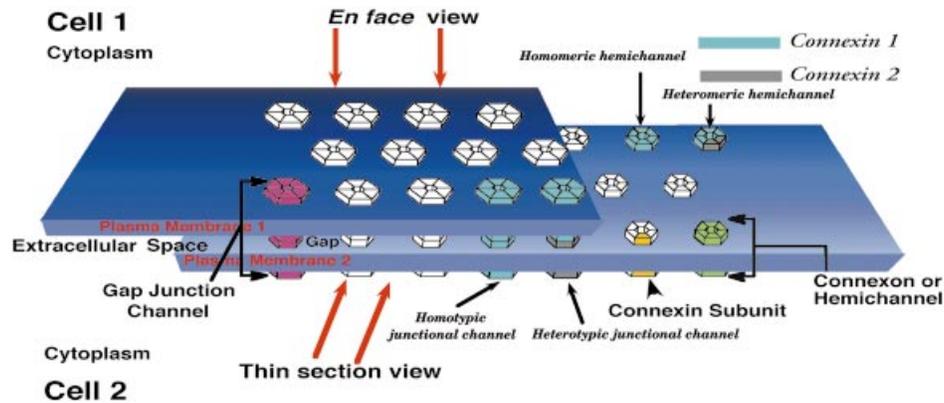


Fig. 3. Connexin structures. *Hemichannels* span each plasma membrane, and interact across extracellular space to form *junctional channels*. The hemichannels may be *homomeric* or *heteromeric*. Junctional channels formed by two hemichannels that are homomeric for different connexins are *heterotypic* channels. (Modified from Sosinsky, 2000.)

simplest – *homomeric junctional channels*, which are two end-to-end hemichannels spanning two membranes, entirely composed of a single connexin isoform. *Heterotypic junctional channels* are end-to-end hemichannels in which each hemichannel is composed wholly of a single connexin isoform, but different isoforms compose the two hemichannels. *Heteromeric hemichannels* are hemichannels composed of more than one connexin isoform (Fig. 3). Junctional channels can also be formed by heteromeric hemichannels. The structural variants provide cells with opportunities for heterogeneous and highly controllable connexin channel physiologies – allowing permeabilities and gating sensitivities specific to a given cell type, developmental stage or physiological condition. Heterotypic and heteromeric channels have been used extensively to investigate structure–function of connexin channels. The closely packed arrays of junctional channels are commonly called *plaques*.

A standard set of abbreviations will be used in this review to denote various structural forms of connexin:

Category	Example	Abbreviation
Connexin isoform	Connexin 32	Cx32
Connexin domain	N-terminal domain of Cx32	Cx32NT
Point mutation	S to D change at position 11 of Cx32	Cx32S11D
Chimeric connexin	M1 domain of Cx26 replaced by that of Cx32	Cx32*Cx26M1
Heteromeric hemichannel	Cx32 and Cx26 in the same hemichannel	Cx26:Cx32
Heterotypic pairing	Cx32 hemichannel paired with Cx26 hemichannel	Cx26/Cx32

2.2.1 Junctional channels

The structure of connexin channels has been investigated by X-ray diffraction and image processing of electron microscopic images of 2D crystals of junctional channels. The highest resolution 3D map (7.5 Å in the plane of the membrane) was derived from tilted low-dose images of frozen-hydrated 2D crystals formed by a heterologously expressed CT-truncated mutant of Cx43 (Unger *et al.* 1997, 1999a, b; Yeager, 1998; Yeager *et al.* 1998). This work is the culmination of many years of structural work on 2D crystals of connexin. Previous

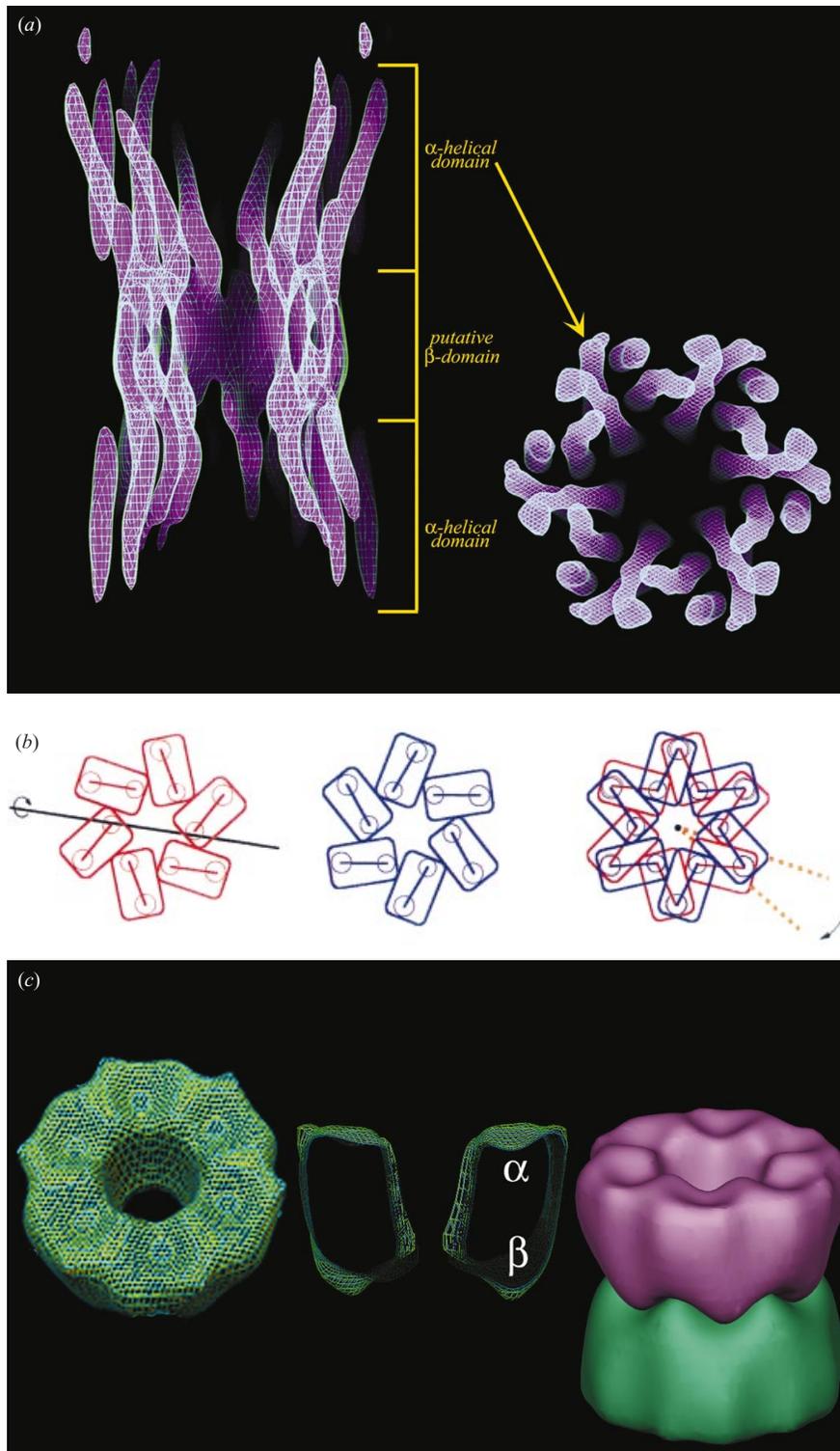


Fig. 4. Connexin channel structure. (a) Reconstruction of junctional channel structure from 2D crystals of junctional channels formed by CT-truncated Cx43. *Left*: cross-sectional view at 7.5 Å resolution

studies were limited to ~ 16 Å resolution due to heterogeneity of the imaged channels and their packing (likely due, in part, to the cytoplasmic CT domain) (Caspar *et al.* 1977; Makowski *et al.* 1977, 1982, 1984a, b; Unwin & Zampighi, 1980; Baker *et al.* 1983; Unwin & Ennis, 1983, 1984; Wrigley *et al.* 1984; Baker *et al.* 1985; Gogol & Unwin, 1988; Sikerwar & Unwin, 1988; Sosinsky *et al.* 1988, 1990; Tibbitts *et al.* 1990; Yeager & Gilula, 1992; Yeager, 1994, 1995). The most recent advances were enabled by the use of heterologously expressed connexin, along with advances in purification of regular lattices of channels from cultured cells (Stauffer *et al.* 1991; Stauffer, 1995).

Homomeric junctional channels have sixfold symmetry around a central pore, indicating that each hemichannel is a hexamer. Hemichannels are approximately 70 Å across and 85–125 Å long, depending on the isoform (i.e. length of the CT domain). It is estimated that 15–20 Å protrudes from the cytoplasmic face of the membrane for Cx32, and 50–60 Å for Cx43. Approximately 15–20 Å extends from the extracellular face into the intercellular gap. Neglecting the contribution of side chains, the hemichannel lumen is ~ 40 Å at the cytoplasmic end, narrows to ~ 15 Å, and then widens to ~ 25 Å within the extracellular vestibule (Yeager & Nicholson, 1996). On the basis of ~ 16 Å resolution reconstructions, it was proposed that hemichannel closure corresponded to rigid connexin monomers undergoing a torsional tilt, sliding against each other and twisting to occlude the lumen of the pore at the cytoplasmic end (Unwin & Zampighi, 1980; Unwin & Ennis, 1983, 1984).

From several lines of structural evidence, the transmembrane domains appear to be α -helical. The published structure shows that the hemichannel is formed by 24 closely-packed rods, corresponding to four α -helical transmembrane domains contributed by each monomer. The α -helices have mixed right- and left-handed packing (Fig. 4*a*). It is not clear whether the imaged channels are in an open or a closed state. As of this writing, the correspondence between the α -helices and the known transmembrane sequences comprising M1–M4 is a matter of active speculation. The 3D map indicates that within the membrane the pore is lined by parts of two α -helices from each connexin monomer. One tilted helix lines most of the length of the pore and has a distinct kink near the extracellular end of the hemichannel, and another lines the pore at the cytoplasmic end. A detailed analysis of connexin sequences and the density within each subunit supports a model in which these helices correspond to M3 and M1, respectively (Unger & Yeager, personal communication). This subject is more fully addressed in Section 4.1.

Structural data show that in junctional channels the two hemichannels are *not* aligned so that each connexin monomer is positioned directly above its positional homologue in the apposing hemichannel. Instead, there is a 30° rotational stagger that brings each monomer into direct apposition with parts of *two* monomers in the apposing hemichannel (Unger *et al.*

within the plane of the membrane. *Right*: end-on view indicating axial tilts of the resolved α -helices. (From Sosinsky, 2000 as adapted from Unger *et al.* 1999b.) *(b)* Schematic of alignment of connexin monomers in junctional channels. Connexin monomers in each of two apposed hemichannels (red and blue) are represented as rectangles, and two α -helices within each as circles. The figure on the right illustrates the proposed rotational stagger between the two hemichannels, causing the α -helices of each monomer to align with α -helices of two connexin monomers in the apposed hemichannel. (Figure from Unger *et al.* 1997.) *(c)* Reconstruction of the structures of hemichannels from 2D crystals of split junctional channels of Cx32. From left to right: cytoplasmic surface, cross-section, and modeled docked structure of junctional channel. (From Sosinsky, 2000.)

1997; Perkins *et al.* 1998a). Two of the transmembrane α -helices in each monomer are nearly in axial alignment with two α -helices in the apposing hemichannel – but the helices are contributed by two different monomers. Schematics of this arrangement (Fig. 4*b*) suggest a tight interlocking arrangement that could account for stability of the junctional channel structure. However it is not likely that the ends of the imaged α -helices make the interhemichannel contacts, but rather the extracellular loops E1 and E2. The density map within the ‘extracellular’ region is not indicative of α -helical structure (Unger *et al.* 1999b), and mutagenesis studies suggest antiparallel β -sheet structures in this domain (Foote *et al.* 1998) (see Section 4.2).

The end-to-end interactions of hemichannels are further informed by atomic force microscopy (AFM) (Hoh *et al.* 1993; Lal & Lin, 2001; Hand *et al.*, In Press) and by 3D electron crystallographic studies (Perkins *et al.* 1997a, 1998a) of ‘split’ junctions of non-truncated connexins – in which the junctional double-membrane structure is separated at the hemichannel–hemichannel interface, allowing examination of the extracellular and cytoplasmic surfaces of the hemichannels. These studies reveal that the extracellular surface topology consists of six alternating peaks and valleys (Fig. 4*c*). It is notable that essentially the same topography was observed in the ‘mechanically-dissected’ AFM images and the more carefully controlled chemical splitting technique used in the crystallographic work. On the basis of this structure, it was proposed that the peaks of one hemichannel fit into the valleys of the other, which requires a 30° rotational stagger between the hemichannels, predicting that seen in the higher-resolution frozen-hydrated images of junctional channels. Animations of 3D reconstructions from electron crystallographic data of single hemichannels and computer-modeled docked structures are available at ncmir.ucsd.edu/~gina/images.html.

2.2.2 Hemichannels

A connexin hemichannel has the structure of a conducting membrane channel, being an oligomeric, integral membrane protein with an aqueous pore in the center. It was formerly believed that hemichannels could not or should not function as channels, that hemichannel opening would be lethal, or would have to be artifactual, without biological relevance or experimental utility. These beliefs are not supported by data. Many studies provide strong evidence of single hemichannel function in native and heterologously expressing cells. For the most part, junctional channels appear to behave as two hemichannels in series. This makes hemichannels important in their own right, and studies of them directly relevant to junctional channels.

The latter point is key – hemichannels can be studied by the techniques commonly applied to other membrane channels, whereas there are powerful limitations on the direct physiological study of junctional channels (see Sections 3.1.1 and 3.2.1). Understanding of junctional channel physiology is facilitated by the ability to infer it from that of the component hemichannels studied individually: In most cases, junctional channels gate as if each hemichannel operates independently (e.g., Bennett *et al.* 1988; Swenson *et al.* 1989; Werner *et al.* 1989; Ebihara *et al.* 1995; Barrio *et al.* 1997; Verselis *et al.* 2000) though there can be some modification of voltage dependence, kinetics and modulatory sensitivity (e.g. Hennemann *et al.* 1992; Bruzzone *et al.* 1994a; White *et al.* 1994;

Ressot *et al.* 1998; Hopperstad *et al.* 2000; Elenes *et al.* 2001; Srinivas *et al.* 2001). Some of these differences may not reflect allosteric effects as much as the superposition of the two forms of junctional voltage dependence that exist in connexin channels. Also, some differences in macroscopic voltage sensitivity in heterotypic channels that had been attributed to structural interactions between hemichannels (Barrio *et al.* 1991) are now understood to arise from rectification of single channel currents due to two dissimilar conductance pathways in series [see Section 5.1; (Bukauskas *et al.* 1995; Oh *et al.* 1999; Suchyna *et al.* 1999)]. There are contradictory data on whether molecular permeabilities of hemichannels and the corresponding junctional channels are different, but this may be due to differences in experimental approaches (Bevans *et al.* 1998; Cao *et al.* 1998).

In native cells. To some, it is counter-intuitive for a hemichannel to open in a native cell under non-pathological conditions. One could expect an open hemichannel to rapidly kill a cell by destruction of the selective permeability of the plasma membrane. However, there are many cases in which cells become permeable to molecules such as Lucifer Yellow (LY) for minutes without lethal effect (Steinberg *et al.* 1987; Buisman *et al.* 1988; Steinberg & Silverstein, 1989). Also, several channels with pores at least as wide as connexin channels are found in plasma membrane, e.g. MscL (Sukharev *et al.* 1997); VDAC (Buettner *et al.* 2000); P2Z/P2X purinergic receptors (Surprenant *et al.* 1997). Under normal conditions, such channels are not toxic; many plasma membrane channels would damage their host cells if they were inappropriately regulated (e.g. the nicotinic acetylcholine receptor), apparently including hemichannels.

The search for open hemichannels began with studies in which two coupled, current-clamped cells were literally pulled apart while voltages and currents were measured (Bennett & Spira, 1968). Within the time resolution of the recordings (~ 0.1 s) there was no discernible electrical leakage to the extracellular medium at the time of physical junction separation, suggesting that junctions ripped apart this way do not yield conducting hemichannels. In this study it was not established whether the hemichannels separated or the junctional channels were pulled out of one of the plasma membranes. More recent studies provide strong evidence that hemichannels originating under more favorable conditions can conduct and gate normally in plasma membranes of single cells.

Compelling evidence for conducting hemichannels in the plasma membrane of native cells was first obtained from the horizontal cells of catfish retina (DeVries & Schwartz, 1992). Solitary horizontal cells, when in a low calcium medium, exhibit a plasma membrane conductance that is permeable to LY. It is regulated by voltage, cAMP, cGMP, dopamine, and intracellular pH in a manner similar to the regulation of gap junction channels in pairs of the same cells. The voltage dependence, pH dependence and pharmacology of the junctional current of paired horizontal cells are fully accounted for by the parameters describing the plasma membrane current, assuming that the junctional channels are formed by two end-to-end plasma membrane channels. Horizontal cells of the skate retina have a similar LY permeable conductance that is consistent, by a variety of pharmacological data, with being mediated by connexin hemichannels (Malchow *et al.* 1993, 1994). As in the catfish horizontal cells, the plasma membrane current in skate was enhanced by low calcium. The hemichannel currents in both species are enhanced by quinine (Malchow *et al.* 1994; Dixon *et al.* 1996). Likely candidates for the connexin(s) that form these hemichannels are Cx35 and Cx34.7, which have been cloned from skate and perch, and localize to the retina (O'Brien *et al.* 1996, 1998). When expressed heterologously, Cx35

produces hemichannel currents that also respond to quinine (White *et al.* 1999). Activation with low external calcium ion and long depolarizing voltages is a consistent feature of plasma membrane hemichannels.

An endogenous membrane conductance in *Xenopus* oocytes that is inhibited by extracellular calcium is now known to be due to hemichannels of Cx38 (Arellano *et al.* 1995; Ebihara, 1996; Zhang *et al.* 1998), which is normally expressed in these cells (Ebihara *et al.* 1989). This was shown by several physiological criteria, as well as complementary anti-sense and sense RNA injections into oocytes. In rat lens fiber cells a plasma membrane current has properties that correspond to the hemichannel properties of Cx46, or rather, more closely, to heteromeric channels of Cx46 and Cx50, which are endogenously expressed in these cells (Eckert *et al.* 1998; Jiang & Goodenough, 1996). Homomeric Cx46 and heteromeric Cx46:Cx50 can form conducting hemichannels when heterologously expressed (Paul *et al.* 1991; Ebihara & Steiner, 1993; Ebihara *et al.* 1999). There is also evidence from dye-uptake and antisense studies that Novikoff hepatoma cells express Cx43 hemichannels in the plasma membrane (Li *et al.* 1996). Lastly, in astrocytes Cx43 hemichannels were visualized with antibodies against E2, which also blocked dye uptake from the extracellular medium (Hofer & Dermietzel, 1998).

In several cell types, connexins have been proposed to be responsible for plasma membrane conductances and single channel activities with properties considered consistent with hemichannels (large conductance, weak charge selectivity, permeability to dyes such as LY, inhibition by octanol or low intracellular pH). Because other high conductance channels are known to exist, one must be cautious in attributing such conductances or permeabilities to connexins (this issue is addressed in detail in Section 3.2.2). Nevertheless, it is probable that some of these currents are indeed mediated by hemichannels. Among the more suggestive studies is work in epithelial urinary bladder cells of *Necturus* (Vanoye *et al.* 1999) rabbit ventricular myocytes (John *et al.* 1999; Kondo *et al.* 2000) and perhaps horizontal cell dendrites in fish (Kamermans *et al.* 2001).

The extracellular release of ATP and other nucleotides during ATP signaling and 'calcium waves' in astrocytes, epithelia and other cells has been attributed to plasma membrane hemichannels (Schlosser *et al.* 1996; Cao *et al.* 1997; Frame & Defejter, 1997; Cotrina *et al.* 1998, 2000; Evans & Sanderson, 1999; Guthrie *et al.* 1999; Graff *et al.* 2000; Homolya *et al.* 2000; Jorgensen *et al.* 2000; Sauer *et al.* 2000; Scemes *et al.* 2000; Suadicani *et al.* 2000; Romanello & D'Andrea, 2001). It is clear that ATP is released and the magnitude of release correlates with connexin expression (Cotrina *et al.* 1998, 2000). However, it is not clear whether the ATP is released through plasma membrane hemichannels or whether modulation of connexin expression affects a downstream, non-connexin ATP-release mechanism. A similar correlation with connexin expression has been found for calcium-dependent modulation of cell volume in a variety of cells (Ngezahayo & Kolb, 1990; Quist *et al.* 2000).

In such cases it is difficult to make a definitive case for functioning hemichannels due to the necessity of distinguishing primary from secondary effects of alteration of connexin expression and function. Induced expression of connexin can have dramatic downstream effects that activate genes and cellular processes not directly mediated by connexin channels (e.g. Lecanda *et al.* 1998). For example, relevant to the case of ATP permeability, expression of connexin genes leads to the induction of several non-connexin genes (Naus *et al.* 2000) and to a profound reorganization of the actin and myosin cytoskeleton that is required for the ATP release (Cotrina *et al.* 2000). These possible effects of hemichannels are intriguing and

Table 2. *Connexins that form hemichannels in plasma membrane*

Connexin	Reference
Cx26	Barrio, personal communication*
Cx30	Valiunas & Weingart, 2000*
Cx32	Castro <i>et al.</i> 1999*
Cx32*Cx43E1	Pfahnl <i>et al.</i> 1997; Hu & Dahl, 1999*
Cx35	White <i>et al.</i> 1999*
Cx35 or 34.7	DeVries & Schwartz, 1992*
Cx37	Puljung <i>et al.</i> 2001
Cx38	Ebihara, 1996
Cx43	Li <i>et al.</i> 1996; John <i>et al.</i> 1999
Cx44	Gupta <i>et al.</i> 1994
Cx46	Paul <i>et al.</i> 1991; Ebihara & Steiner, 1993; Trexler <i>et al.</i> 1996*; Pfahnl & Dahl, 1998*; Hu & Dahl, 1999*; Valiunas & Weingart, 2000*
mCx50	Ebihara <i>et al.</i> 1999; Zampighi <i>et al.</i> 1999; Eskandari & Zampighi, 2000*; Valiunas & Weingart, 2000*
Cx56	Ebihara <i>et al.</i> 1995; Ebihara <i>et al.</i> 1999*

* Single hemichannel recordings.

potentially important, but may not be directly related to hemichannel function in plasma membrane.

In heterologous expression and bilayers. In heterologous expression systems, many connexins form conductive plasma membrane channels, which has been a boon to their study. As for hemichannels in native cells, their currents are typically activated by low external calcium ion and long depolarizing voltages. In most cases the currents are identified by correlation with levels of connexin expression and by modulation by one or more agents known to affect junctional channels. The specifics and caveats of identifying hemichannel currents are discussed in detail in Section 3.2.2. In some cases, single hemichannels have been studied by on-cell and excised patch approaches, as summarized in Table 2.

To date, several connexins have been reconstituted into planar bilayers or liposomes with enough reliability for their functional properties to be investigated: Cx26 (Buehler *et al.* 1995; Falk *et al.* 1997; Bevans *et al.* 1998; Bevans & Harris, 1999a, b), Cx32 (Young *et al.* 1987; Harris *et al.* 1992; Rhee *et al.* 1996; Bevans *et al.* 1998; Bevans & Harris, 1999a, b) and Cx43 (Falk *et al.* 1997; Kam *et al.* 1998; Kim *et al.* 1999). There have been many other reports of bilayer and/or liposome conductances due to connexin channels. Some of them may indeed be hemichannels, but in most cases it is difficult to argue definitively that the channels are formed by connexin, and the data have been difficult to reproduce. Section 3.2.2 summarizes the work and the problem.

The determinants of what allows some connexins to form open hemichannels are not clear. One expects the extracellular loop domains to play a prominent role. However, inspection of the extracellular loop sequences is not informative. In fact, the E1 and E2 sequences of mCx50 and hCx50 are identical, but the former readily forms open hemichannels and the latter does not. This suggests that the factors controlling hemichannel opening are not exclusively localized to the loop domains, and may involve conformational changes in the transmembrane and/or cytoplasmic domains. Supporting this idea is work showing that mutation of H161 in mCx50 eliminates the ability to form open hemichannels, but does not affect the ability to form junctional channels (Beahm & Hall, In Press). Also, it is not clear that the 'gate' that

Table 3. *Heterotypic functional compatibilities among mammalian connexins*

Cx26	Cx30	Cx30.3	Cx31	Cx31.1	Cx32	Cx33	Cx36	Cx37	Cx40	Cx43	Cx45	Cx46	Cx50	Cx57	
+	+	-	-	-	+		-	-	-	-	-	+	+	-	Cx26
	+	+			+		-	+	+	+	+			-	Cx30
		+	-	-	-		-	+	+	+	-			+	Cx30.3
			+	-	-			-	-	-	-			-	Cx31
				-	-			-	-	-	-			-	Cx31.1
					+							+	+	-	Cx32
							-								Cx33
								+	-	-	-				Cx36
									+	+	+			+	Cx37
										+	+			-	Cx40
											+	+	-	+	Cx43
												+		-	Cx45
													+	+	Cx46
														+	Cx50
														+	Cx57

+, Forms heterotypic channels; -, does not form heterotypic channels, blank = not reported.

keeps hemichannels closed under normal conditions is physically located at the extracellular end or composed of E1 or E2.

2.2.3 Heteromeric channels

Like other oligomeric channels, connexin channels can be heteromeric (composed of more than one connexin type). Heteromericity occurs in both native connexin channels and in heterologous expression systems. It can take the familiar form of multiple isoforms in a single hemichannel [a structure analogous to the well-established heteromultimeric potassium channel (Isacoff *et al.* 1990; Ruppertsberg *et al.* 1990)]. Also similar to potassium channels, study of heteromeric connexin channels provides insights into the determinants of channel assembly, the structural determinants of channel function, and the allosteric and cooperative intersubunit interactions that affect modulation of channel physiology.

For junctional channels, there are additional types of heteromeric structures in which the subunit compositions of the two component hemichannels are different, called *heterotypic* channels (see Fig. 4). In typical usage, this term refers to the case where each hemichannel is composed of a single, different isoform. The more complex case, where the hemichannels are themselves heteromeric, are called *heteromeric heterotypic* channels. Heterotypic channels are useful for investigation of *interhemichannel* allosteric and cooperative effects.

Heterotypic junctional channels. Heterotypic junctional channels can form in heterologous expression systems (cf. Swenson *et al.* 1989; Werner *et al.* 1989; Barrio *et al.* 1991; Bukauskas *et al.* 1995). The study of heterotypic channels has been particularly useful for understanding the structural basis of voltage sensitivity and other properties. Homomeric hemichannels will form heterotypic functional junctional channels with hemichannels of some other isoforms and not others (see Yeager & Nicholson, 2000). Investigation of this selectivity is informative as to the structural basis for inter-hemichannel docking (i.e. formation of junctional channels), and may provide insight into functionally or evolutionarily related groups of connexins. Table 3 summarizes data on functional heterotypic formation and non-formation for the mammalian

Table 4. *Heterotypic functional compatibility groups*

Group A	Group B
Cx30.3	Cx26
Cx37	Cx32
Cx40	Cx46
Cx43	Cx50
Cx45	
Cx57	

connexins expressed in *Xenopus* oocytes or mammalian cell lines. Where there are contradictory data, results from mammalian systems are given.

Table 3 makes clear that the α/β grouping only roughly corresponds to functional heterotypic compatibility. From inspection of the table, a more useful grouping can be made in this regard. One feature that emerges is that the pattern of heterotypic *formation* better defines internally consistent groups than does the pattern of *non-formation* of such channels. On this basis, two groups can be defined whose members more consistently form functional heterotypic channels only within each group (Table 4). The exceptions are that Cx43 forms channels with Cx46, Cx30.3 does not form channels with Cx45, and Cx57 does not form channels with Cx40. Cx31, Cx31.1, Cx33 and Cx36 cannot be categorized by these criteria since they apparently do not form heterotypic channels. Connexin 30 appears to be compatible with both groups. There is insufficient information for the remaining connexins.

A major caveat is that this grouping does not distinguish the ability to physically form junctional channels from the ability of such channels to open under the normal conditions for screening. That is, some of the pairs reported not to form heterotypic channels may, in fact, form the heterotypic structure but not open. This may contribute to why the pattern of non-formation is not as consistent as the pattern of formation, as noted above. The data to identify the determinants of junctional channel formation as distinct from the determinants of junctional channel opening do not yet exist.

Do heterotypic junctional channels occur *in vivo*? The more interesting biological question is whether native junctional channels can be structurally (and therefore physiologically) asymmetric due to dissimilar composition of paired hemichannels, either homomeric or heteromeric. The answer is unequivocally positive, since native cells that express non-overlapping connexins can be coupled to one another (e.g., Rash *et al.* 2001). Even where coupled cells express overlapping connexins, structural and physiological asymmetry could arise from a heteromeric hemichannel joined to a homomeric channel, or two heteromeric hemichannels with different stoichiometries or arrangements of isoforms, or even identical heteromeric hemichannels with a rotational displacement. Of course asymmetric physiology (Robinson *et al.* 1993; Little *et al.* 1995b; Venance *et al.* 1995a; Chen & DeHaan, 1996; Zhao, 2000; Zhao & Santos-Sacchi, 2000) may also arise from other sources, such as differences in post-translational modification and interaction with signaling systems in the two cells.

A well characterized case of coupling mediated by non-overlapping connexins occurs between astrocytes and oligodendrocytes. Astrocytes express Cx43 and Cx30 (Dermietzel *et al.* 1989; Nagy *et al.* 1999, Rash *et al.* 2000) whereas oligodendrocytes express Cx45 and Cx32

(Dermietzel *et al.* 1997; Kunzelmann *et al.* 1997; Li *et al.* 1997). Cx32 does not form junctions with Cx43 (Elfgang *et al.* 1995; White & Bruzzone, 1996), but can with Cx30 (Dahl *et al.* 1996). Cx45 can form junctions with Cx43 (Elfgang *et al.* 1995) and with Cx30 (Manthey *et al.* 2001). It is unlikely that Cx32 and Cx45 form heteromeric hemichannels (the specificities for heteromeric compatibility seem to match those for heterotypic compatibility; see below), so it is probable that the hemichannels contributed from the oligodendrocytes are homomeric, and that they interact with either homomeric or heteromeric hemichannels from the astrocytes.

Since cells typically express more than one connexin isoform, it is more difficult to establish in native cells the existence of the more simple case of heterotypic junctional channels composed of homotypic hemichannels. Junctional plaques often contain more than one connexin in the same plaque (Nicholson *et al.* 1987; Kuraoka *et al.* 1993; Risek *et al.* 1994, 1995; Little *et al.* 1995a; Yeh *et al.* 1998; Dahm *et al.* 1999), but optical resolution does not permit direct identification of heterotypic channels as distinct from heteromeric hemichannels.

The first indication of heterotypic channels in native cells came from mass distributions obtained by scanning transmission electron microscopy (STEM) of intact and split liver junctions (Sosinsky, 1995). More recently, double immunogold electron microscopy showed that heterotypic Cx26/Cx32 gap junctions develop by day 7 after birth in the periportal area of rat liver (Iwai *et al.* 2000). The doubly labeled images clearly show Cx32 only on one side of a junction, and Cx26 only on the other. It is particularly interesting that the heterotypic junctions develop in a spatially and developmentally controlled manner. In the ciliary body of the eye, the cells of the outer pigmented cell layer (which express Cx43) and the inner, non-pigmented cell layer form heterotypic channels (Wolosin *et al.* 1997; Vaney & Weiler, 2000), but the other isoform has not been identified.

Single-channel data show that a subpopulation of acutely dissociated pairs of mouse hepatocytes has Cx26/Cx32 heterotypic channels (Valiunas *et al.* 1999b). Interestingly, the heterotypic pairs were more common in neonatal than in adult hepatocytes. To date, this is the only definitive physiological data for heterotypic channels from a native tissue.

Heteromeric hemichannels. Biochemical and physiological experiments have provided substantial direct and indirect evidence for the formation of heteromeric hemichannels in native cells and heterologous expression systems. The biochemical evidence typically involves a combination of co-immunoprecipitation and size/mass characterization of the purified material by velocity sedimentation and/or gel-filtration chromatography. Physiological characterization is typically based on the finding of single-channel conductances and/or voltage dependence that differ from those of the relevant homomeric channels. These approaches have occasionally been supplemented by immunogold electron microscopy. It is interesting to note that the known heteromeric forms are consistent with the known heterotypic specificities of Table 3, suggesting that both helix-helix interactions and extracellular loop interactions can segregate the isoforms.

The existence of heteromeric hemichannels in the cellular repertoire allows considerable fine-tuning of junctional channel physiology (with the reasonable assumption, supported by studies mentioned below in Section 6.2.1, that each connexin monomer within a junctional channel contributes to some degree to the channel physiology). For two connexins, there are 5 possible heteromeric stoichiometries and 12 possible arrangements within a hemichannel. Adding the 2 homomeric possibilities, a total of 14 structural hemichannel forms are possible.

For a junctional channel, a lower limit for the number of possibilities is therefore 196 (14^2), assuming there are no rotary or structural preferences. Fortunately, inspection of the data from heteromeric channels suggests that the number of actual different structures/physiologies is much less.

Cx26 and Cx32. Hemichannels that contain both Cx26 and Cx32 were co-purified by immunoaffinity chromatography from tissues that express both connexins (Harris & Bevens, 1997; Bevens *et al.* 1998; Diez *et al.* 1999; Locke *et al.* 2000). Artfactual monomer exchange between solubilized hemichannels was controlled by cross-linking of connexin structures in native membranes (Kordel *et al.* 1993; Bevens *et al.* 1998; Diez *et al.* 1999). Gel filtration was able to separate by mass the heteromeric structures from homomeric Cx32 and Cx26 hemichannels (Locke *et al.* 2000). The heteromericity had functional effects, altering the selectivity among tracers and second messengers, as well as altering the modulation of channel activity (Bevens *et al.* 1998; Bevens & Harris, 1999a).

Other data in support of heteromeric Cx26:Cx32 hemichannels come from a heterologous expression system (Stauffer, 1995). Connexin structures were purified from singly- and co-expressing Sf9 cells and then co-incubated in detergent. The mixed homomeric structures segregated independently on a gel-filtration column, but the co-expressed connexin structures tended to segregate together. A concern about this study is that the size of the homomeric structures is very large and the distribution very broad (Cx32: 500–1000 kDa with peak at 800 kDa; Cx26: broad peak centered at 550 kDa). These sizes are unreasonably large for single hemichannels even if they bind substantial detergent. For Cx32, with a hemichannel mass of 192 kDa, the measured 800 kDa peak, corresponds to the structure binding 320% of its weight in detergent, whereas it is known that Cx43 hemichannels bind ~17% of their weight of LDAO. This raises a question about whether the structures are in fact aggregates larger than hemichannels. Also, the apparent size of the Cx32-containing structures did not change when heteromeric hemichannels were formed. One expects that as the Cx26:Cx32 ratio increased, the apparent size would decrease and approach that of Cx26 homomeric structures, but this is not the case. The data can be accounted for if the connexin/hemichannel structures are significantly aggregated, and each connexin aggregates primarily with its own kind, but is also capable of some degree of aggregation with the other. The evidence counter to this interpretation is the observation of single, unaggregated structures in the EM, but it is possible that the conditions for EM sample preparation cause the aggregates to disassemble.

Two studies in which Cx26 and Cx32 were synthesized by *in vitro* translation also show the formation of Cx26:Cx32 hemichannels. Size analysis and immunoprecipitation experiments demonstrated that Cx26:Cx32 hemichannels can form when co-translationally inserted into membranes (Falk *et al.* 1997; Ahmad *et al.* 1999) and that Cx32:Cx43 hemichannels do not form (Falk *et al.* 1997). One of these studies (Falk *et al.* 1997) explicitly tested for monomer exchange in detergent solution, and found no evidence for it.

STEM measurements on purified rodent hemichannels, while supporting homomeric and heterotypic Cx26/Cx32 structures, failed to reveal evidence of hemichannel masses corresponding to heteromeric structures (Sosinsky, 1995). They may have been present to some degree and masked by the statistical variations in the measurements or within slightly asymmetric Gaussians of the mass distributions.

Cx46 and Cx50. Co-immunopurification studies (without cross-linking) in combination with velocity sedimentation demonstrated that the lens connexins Cx46 and Cx50 (and their

orthologs Cx56 and Cx45.6) form heteromeric channels (Jiang & Goodenough, 1996). When these connexins are co-expressed heterologously, the resulting hemichannels display macroscopic and single-channel behaviors that differ from those of the homomeric channels. Specifically, expression of Cx46 with Cx50 shifts the voltage activation to more negative potentials, and increases the unitary conductance and open probability (Ebihara *et al.* 1999). The physiological evidence from junctional channels between HeLa cells co-expressing these connexins also supports the presence of Cx46:Cx50 heteromers (Hopperstad *et al.* 2000).

Cx37 and Cx43. The first and most compelling demonstration of heteromeric channels by electrophysiological means was obtained for Cx37 and Cx43 in transfected N2A cells (Brink *et al.* 1997). In this system it was possible to directly compare homotypic and heterotypic single-channel properties with those obtained when both connexins were co-expressed. The resulting channels had conductance and gating properties that could not be predicted from the properties of the two connexins expressed separately. There was a multiplicity of conductances that could not be explained solely by the formation of heterotypic or homotypic channels. This work was followed up by similar studies using Cx40 in addition to Cx37 and Cx43 in transfected NRK cells. The data confirmed Cx37:Cx43 heteromers (as well as Cx40:Cx43 heteromers) (Beyer *et al.* 2000).

Cx40 and Cx43. There is substantial evidence that Cx40 and Cx43 form heteromeric channels in cardiovascular tissues. This was established in a vascular smooth muscle cell line (A7r5) by a combination of co-immunopurification and assessment of single-channel properties (He *et al.* 1999). There were effects on unitary conductance, substate activity, the parameters of voltage dependence and permeability to dye molecules. Not all of these effects were readily predictable from the properties of the parent channels. Similar physiological results were obtained from pairs of canine atrial cells and from pairs of transfected N2A cells (Elenes *et al.* 1999). A study in rat basilar artery smooth muscle cells was less definitive, but nonetheless strongly suggestive of heteromeric Cx40:Cx43 channels (Li & Simard, 1999). In the *Xenopus* oocyte expression system, a functional interaction between Cx40 and Cx43 that modulates the pH sensitivity of junctional channels is almost certainly explained by their presence in the same hemichannels (Gu *et al.* 2000).

There are cases in native cells where some kind of heteromericity occurs, but it is not possible to tell with confidence whether it takes the form of heterotypic junctional channels or heteromeric hemichannels, or both: Cx40, Cx37 and Cx43: (Yeh *et al.* 1998); Cx50 and Cx43 (Dahm *et al.* 1999); Cx43 and Cx45 (Koval *et al.* 1995); Cx43 and unknown connexin(s) (Ochalski *et al.* 1997).

Heteromeric interaction within hemichannels is a likely mechanism in cases where one connexin exerts a negative-dominant effect on another expressed in the same cell. A Cx32 mutant interferes with the channel-forming ability of Cx26 but not Cx40, supporting Cx26:Cx32 hemichannel heteromericity, and suggesting that Cx32 and Cx40 are not heteromerically compatible (Bruzzzone *et al.* 1994b). A fusion protein of Cx43 and an endoplasmic reticulum retention/retrieval signal exerts a dominant negative effect on Cx46 (Sarma *et al.* 2000). These results are consistent with the heterotypic specificities in Table 3. However, wild-type Cx33 inhibits channel-forming ability of Cx37, but not of Cx32 or Cx43 (Chang *et al.* 1996), suggesting an interaction between Cx33 and Cx37 not consistent with the heterotypic specificities. A chimera of the compatible connexins Cx43 and Cx40 interferes with junctional channel formation by Cx43, Cx38 and Cx40, but not Cx32 (Goliger *et al.* 1996) consistent with heterotypic compatibilities. A chimera between the incompatible connexins

Cx32 and Cx43 interferes with Cx38, Cx32 and Cx43, but not Cx37 or Cx46 (Goliger *et al.* 1996). Since the two component connexins are heterotypically incompatible, and the chimera interacts with the wild-type connexins of which it is composed, one might expect it to be incompatible with all connexins. The fact that it is not may reflect the influence of the different domains contributed by each connexin.

An interesting case occurs for several disease-causing mutations of Cx26. They appear to exert a trans-dominant negative inhibition of Cx43 (Rouan *et al.* 2001). Such an effect implies that the mutations (which are in the E1 domain) permit Cx26 to interact directly with Cx43, with which it normally does not.

2.2.4 Junctional plaques

Junctional channels are nearly always found in 2D arrays that can contain from just a few channels to many thousands. The classical electron microscopic images of plaques – large junctional areas in which the plasma membranes of two cells are drawn into close apposition apparently linked by thousands of semi-crystalline intercellular proteins – give the impression of static, stable, and immobile structures. Contrary to this impression, the half-life for turnover of junctional connexin is only a few hours (Musil *et al.* 1990; Fallon & Goodenough, 1981; Traub *et al.* 1987; Laird *et al.* 1991; Laing & Beyer, 1995; Beardslee *et al.* 1998). Recently, time-lapse imaging of GFP-labeled connexin in living cells has not only confirmed that turnover is a highly active process, but that junctional plaques themselves can be highly mobile, moving about within the plasma membranes of coupled cells, budding off and fusing with each other (Holm *et al.* 1999; Jordan *et al.* 1999; Windoffer *et al.* 2000). This may account for the suggestion from a statistical analysis of plaque sizes in published images that plaques grow or shrink by the accretion or removal of 200–300 channel aggregates (Ryerse *et al.* 1984). The mobility of plaques raises questions about why/how the arrays form, what keeps them (transiently) intact, and what causes them to move, bud off, and fuse. This is all the more remarkable given that the plaques are extended regions of contact between two cells.

Rotating 3D volume reconstructions of gap junction plaques formed by GFP tagged connexin imaged by deconvolution microscopy from Falk (2000) are available at www.biologists.org/cgi/content/full/113/22/4109/dc1. Videos of the dynamics of junctional plaques formed by GFP- and EFGP-labeled connexins in live cells are available at www.molbiolcell.org/cgi/content/full/10/6/2033 (from Jordan *et al.* 1999) and www.uni-mainz.de/FB/Medizin/Anatomie/Leube/connexin-movies.html (from Windoffer *et al.* 2000). Videos of transport intermediates, endocytosis and degradation of the fluorescently labeled connexin are also available at these sites.

Other membrane proteins that form 2D arrays typically do so only with the involvement of accessory proteins, often linked to cytoskeletal elements (Kornau *et al.* 1997). Intense scrutiny of junctional plaques has not revealed the obligatory association with cytoskeletal structures seen for other proteins (e.g. Hirokawa & Heuser, 1982). An association of Cx43 with ZO-1 via a PDZ-binding domain on the CT has been demonstrated in several cell types and cell lines (Giepmans & Moolenaar, 1998; Toyofuku *et al.* 1998b), and linked to stabilization of Cx43 at intercalated disks and in plasma membrane, possibly via α -actin (Toyofuku *et al.* 1998b). Actin is also associated with gap junctions in the inner layers of primate lens fiber cells (Lo *et al.* 1996). However, plaque formation cannot require such

interaction because truncation of the Cx43 CT does not inhibit plaque formation (Unger *et al.* 1997) and not all connexins have PDZ-binding domains. Therefore it seems that junctional channels have an intrinsic tendency to aggregate into plaques in plasma membranes, and that extrinsic factors may contribute to plaque structure or stability.

From a biophysical perspective, this indicates that the forces that cause and/or maintain the lateral aggregation of connexin channels are intrinsic to the protein and its immediate environment. The propensity of connexin channels to assemble into lateral aggregates is well documented by self-assembly from solubilized hemichannels and junctional channels (Mazet & Mazet, 1990; Lampe *et al.* 1991; Kistler *et al.* 1993, 1994). In cellular membranes, the packing within plaques has been suggested to arise from repulsive forces between the channels that are opposed by lateral pressure transmitted within the plane of the membrane. This lateral pressure could arise from the effects of intermembrane repulsion (electrostatic and/or steric) where junctional channels are absent, directly (Braun *et al.* 1984; Abney *et al.* 1987) and perhaps mediated by the bending rigidity of the membranes (Bruinsma *et al.* 1994). The membrane movement and undulation of living cells may facilitate the aggregation of particles of similar, smaller diffusion constants that are not constrained by cytoskeletal links.

This view assumes that the individual channels are freely diffusing within the membrane. The movement of channels within plaques has been explored recently by imaging the junctions formed between cells transfected with YFP- and GFP-labeled connexins (Falk, 2000). These data and others (Risek *et al.* 1994; Sosinsky, 1995) show that connexins known to associate (e.g. heterotypically or heteromerically) can co-mingle homogeneously within a plaque, and connexins that do not so associate can remain in separate domains within a junctional plaque. This supports the idea of freely diffusing particles, but also suggests that the inter-channel forces (whether they are attractive or repulsive) are a function of the particular connexins involved. The basis for this is not known – possibilities include isoform-specific organization of lipids around the channels and direct protein–protein interactions. There is no evidence, yet, for the former. The only indication of direct contact comes from supercooled freeze-fracture and rotary-shadowed freeze-etch studies, which yielded images consistent with spoke-like extracellular bridges that connect one particle to another (Peracchia & Peracchia, 1985; Rash & Yasumura, 1992). However, both reports admit the possibility of artifact. Such features have not been seen by any of the other imaging approaches.

What are the functional consequences of the fact that junctional channels are found in plaques? From a molecular signaling point of view, one may imagine that plaques would facilitate the delivery of intercellular signaling molecules to specific sites on a cell's periphery. However the lack of association with cytoplasmic or organellar structure, and their mobility, argue against this.

Aggregation might be thought to enhance the efficiency of chemical or electrical signaling. However, the opposite seems to be the case. Due to overlapping access-resistance domains, tightly packed junctional channels become less efficient mediators of charge and chemical transfer (Hall & Gourdie, 1995). For molecular signaling, calculations show that scattered junctional channels can be an order of magnitude greater in communication efficiency than compact junctional plaques of an equal total number of channels (Chen & Meng, 1995).

The one advantage that plaques can confer is for the case where one cell undergoes a relatively rapid, perhaps regenerative, change in the concentration of a signal, either electrical or chemical. In this case, the density of the molecular signal (or charge) at the points of coupling to the other cells will be greater than if the intercellular channels were dispersed.

Thus, the presence of plaques enhances the ability to transmit transient signals for which there are thresholds or that are regenerative. This has long been obvious for electrical signaling, and is becoming more obvious for chemical signaling (e.g. intercellular calcium waves). Such considerations could apply whether the signal is either an increase or decrease of a chemical signal.

The packing and center-to-center spacing of channels within junctional plaques can be quite variable (Raviola *et al.* 1980; Sosinsky, 1992), but there is no obvious or reliable correlation with conductance state (Miller & Goodenough, 1985). There is evidence for functional junctional channels that are present as dispersed particles (Hülser *et al.* 1997). Some have suggested that plaques facilitate cooperative gating interactions between adjacent junctional channels (Chen & DeHaan, 1992; Manivannan *et al.* 1992; Veenstra *et al.* 1994). This idea is informed by recent work quantitatively correlating GFP-connexin fluorescence with plaque size and junctional conductance (Bukauskas *et al.* 2000). The data indicate that (a) aggregates of junctional channels below a certain size do not contain functioning channels (a minimum size of $\sim 3 \times 10^4 \text{ nm}^2$, or 300 channels, is required) and (b) only a small fraction of the channels in a plaque are functional (5–15%), and the fraction increases with plaque size. Curiously, the observed minimal size for function is the same as that proposed for the aggregates from which plaques are assembled (see above; Ryerse *et al.* 1984). The mechanism for this functional dependence on plaque size is unknown.

Recent work reports positive cooperativity of voltage-dependent gating between Cx30 junctional channels in plaques, based on comparison of steady state and kinetic data from single and multichannel records (Valiunas *et al.* 1999; Valiunas & Weingart, 2001). Multichannel records exhibited greater voltage-sensitivity and more rapid voltage-dependent kinetics than did single channels. Positive cooperativity cannot be accounted for by the considerations of series and access resistance outlined above, which would tend to produce a negative cooperativity. The basis for this interaction between connexin channels is unknown, but it may not be unique (Yeramian *et al.* 1986; Keleshian *et al.* 2000).

3. Experimental approaches and issues specific to the study of connexin channel physiology

This section reviews experimental approaches unique to the study of connexin channels. They are variants on standard techniques, but the differences shape the kinds of data that are obtained and how they can be used. A comprehensive summary of experimental approaches to the study of connexins is found in (Bruzzone & Giaume, 2001).

3.1 Macroscopic currents

3.1.1 Junctional channels

Recording of junctional current requires simultaneous voltage-clamp of two coupled cells. The voltage of each cell of a coupled pair is initially clamped to a common potential so that junctional voltage (V_j) equals 0 mV (Fig. 5). Typically, voltage is imposed across the junctional membrane by stepping the potential in one cell (V_1) while holding that of the other cell (V_2) constant. The current that flows through junctional channels (I_j) as a result of the non-zero V_j (which equals $V_2 - V_1$) is measured as the current supplied to the cell whose

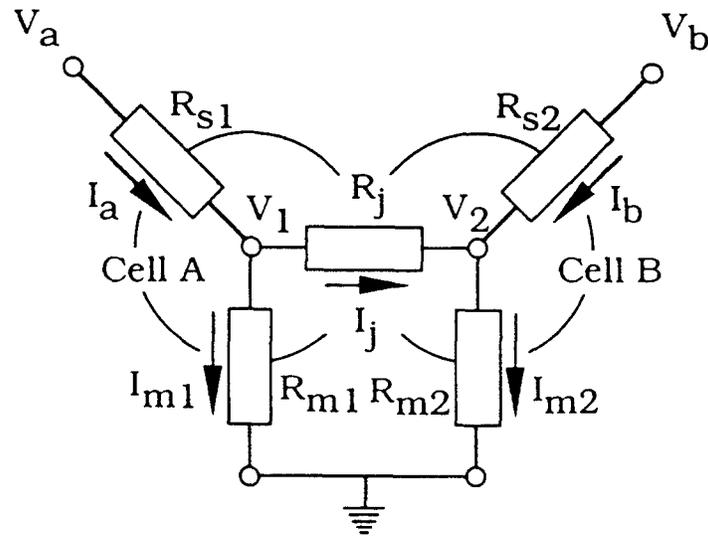


Fig. 5. Equivalent circuit of dual whole-cell patch-clamp for recording junctional currents of two spherical cells. V = voltage; I = current; I_m = current through non-junctional membrane; R_s = series resistance including electrode resistance; R_m = resistances of non-junctional membranes. R_j and I_j are junctional resistance and junctional current, respectively. When $V_a = V_b$, $I_j = 0$. If V_a is then stepped to a more positive potential and V_b held constant, $-I_j$ is recorded as the change in I_a . (from van Rijen *et al.* 1998.)

potential was unchanged (I_2). Even though the currents provided by each circuit are equivalent to the sum of the non-junctional membrane current of that cell plus the junctional current (I_1 or I_2 plus I_j), because the non-junctional potential (V_2) is unchanged, $\Delta I_2 = 0$ and the current supplied by that clamp equals $|I_j|$.

For patch clamp of a single cell the primary source of voltage error is the ratio of the uncompensated electrode series resistance to the membrane resistance. The dual cell configuration has an additional resistance equivalent to a leak resistance in a conventional circuit that is, in fact, the one of interest: the junctional resistance. The error in voltage control due to uncompensated series resistance can therefore be a function of the junctional resistance – the larger the ratio of series resistance to junctional resistance, the greater the error. This error causes underestimation of junctional conductance and its voltage sensitivity.

Another way to appreciate the problem is to recognize that accurate measurement of a membrane conductance requires that the pipette seal conductance be much smaller than the conductance of interest. This is usually not a problem for single-cell patch clamp. However, for coupled cells the substantial nonjunctional membrane conductance contributes the same kind of error for measurement of junctional conductance as the pipette seal conductance does for the measurement of whole cell currents of single cells – it is in parallel with the conductance of interest, and can be equal to it or greater (Moreno *et al.* 1991; Veenstra & Brink, 1992; Wilders & Jongsma, 1992). The lower the membrane resistance the greater the error, and the greater the junctional conductance, the greater the error.

The best way to avoid these errors is to use high resistance, poorly coupled cells and low-resistance electrodes. Failing this, the errors must be estimated, and if necessary, computationally compensated for after the experiments. Equations to do so have been

worked out, based on voltage clamp analysis of a dual whole-cell resistor circuit (Veenstra & Brink, 1992) and on modeled whole-cell currents derived from Kirchoff's Law (van Rijen *et al.* 1998). Both methods require accurate estimation or measurement of the series resistance and membrane resistance (Veenstra & Brink, 1992; van Rijen *et al.* 1998). In general, the measurement is the most accurate when junctional resistance is roughly equal to the input resistance, which must be at least 20-fold greater than the uncompensated series resistance (Verselis & Veenstra, 2000). Since the errors are functions of the junctional conductance, any measurement in which junctional (or membrane) conductances change during an experiment or voltage protocol may have variable accuracy. When determining the accuracy of a measurement one must consider values for junctional conductance over the range anticipated or recorded.

Both methods of off-line compensation assume that the electrical, ionic and structural conditions across the junctions are identical. This is not the case when the cells have different intrinsic resting potentials, the junctional channels are heterotypic, the solutions on either side of the channels are different, and the characteristics of voltage clamp are different. A recent comprehensive analysis shows that these factors can affect the accuracy of measurement of junctional voltage dependence and provides an experimental protocol and equations that correct and compensate for such asymmetries (Veenstra, 2001). Because these asymmetries can affect the initial I_j transient when V_j is stepped, and thereby the time constant of subsequent changes in g_j , a ramp-based protocol is proposed as a reliable alternative to the standard step-based method of assessing voltage-dependent properties. These kinds of considerations may contribute to the variability in published kinetic and steady state parameters of voltage dependence.

An alternative to dual whole-cell patch clamp is dual single-electrode voltage clamp, in which each voltage clamp is achieved with a single microelectrode that is rapidly switched between voltage-measuring and current-applying modes (Brennecke & Lindemann, 1974; Wilson & Goldner, 1975; Finkel & Redman, 1984). This eliminates the direct error due to electrode resistance and has the profound advantage of requiring only one impalement per cell. Such clamps have the disadvantages of greater noise, and require careful adjustment to ensure that the switching frequency is fast enough to record the conductance kinetics faithfully, while slow enough to allow a steady-state potential to develop during the current-injection phase (time constant of the cell) and be measured during the voltage phase (time constant of the electrode). The electrode resistance can constrain the latter. Nevertheless, this approach is useful in cells where the membrane resistance is low and junctional resistance high, or vice versa (Müller *et al.* 1999), or where high temporal resolution of junctional currents is not important.

An additional source of error arises from the fact that junctional channels are closely packed. Calculations show overlap between the cytoplasmic access resistance domains of channels that are as closely packed as junctional channels [as are endplate acetylcholine receptor channels (Matthews-Bellinger & Salpeter, 1983), which ought to be subject to the same considerations]. This effectively leads to an additional access resistance due to flux interaction at the pore openings, even when electrode resistance is fully compensated (Wilders & Jongsma, 1992; Ramanan *et al.* 1994). This can cause underestimation of macroscopic conductance and of voltage dependence. The effect increases with the increased size of junctional plaques. In fact, this effect has been suggested to be responsible for reports of decreased voltage-sensitivity of junctional channels with growth of plaque size (Rook *et al.* 1988, 1990). Of course this does

not apply if the fraction of functioning channels in a plaque is small (Lin & Faber, 1988; Bukauskas *et al.* 2000).

For large cells, voltage-clamp is most reliably achieved by a two-microelectrode voltage clamp for each cell, which avoids the error due to uncompensated pipette resistance (the other sources of error remain, of course). Because it is simple and eliminates electrode series resistance problems, this is the preferred configuration whenever possible. It is well-suited to studies of macroscopic junctional currents in *Xenopus* oocytes and other large cells, such as the blastomeres of early cleavage-stage embryos of amphibians and teleosts (Harris *et al.* 1981; Spray *et al.* 1981a; Dahl *et al.* 1987). Due to the large area of membrane surface being clamped, junctional currents are not typically resolved within 5–10 ms after the onset of a voltage pulse.

For the *Xenopus* system, pairs of oocytes expressing the connexin(s) under study are devitellinized and manipulated into contact. Junctional channels form in several hours or overnight (Ebihara, 1992). The oocyte system does not permit recording of single junctional channel currents due to the low input impedance of the cells, but it is useful for characterization of macroscopic junctional conductance and voltage sensitivity. The primary technical liability is that the junctional conductance can be quite large relative to the membrane conductance, causing errors in voltage control as outlined above.

Another potential problem, not specific to connexins, is that the properties of channels may differ in unexpected ways when expressed in different systems. For example, some aspects of voltage sensitivity differ when the same connexin is heterologously expressed in *Xenopus* oocytes and in mammalian cells (see Section 6 and Anumonwo *et al.* 2000).

A variant of the paired oocyte system has been developed that permits perfusion of the cytoplasm of one of the coupled oocytes (Skerrett *et al.* 2001a). The oocytes are paired in chambers separated by a coverslip in which there is a small hole. After coupling develops through the hole, and the plasma membranes seal adequately to the coverslip, one oocyte is cut open and exposed to the bulk solution on that side, allowing dialysis of the diffusible components. The ability to resolve junctional current is limited by the stability of the system and tightness of the seals, but this approach enables investigation of chemical modulation of junctional conductance and assessment of molecular permeability that would otherwise be impossible. A similar approach was applied to *Fundulus* blastomeres to obtain early data on pH dependence of junctional conductance (Spray *et al.* 1982).

For smaller cells, the technical difficulty of initiating and maintaining four simultaneous microelectrode impalements requires use of either dual whole-cell patch clamp or dual single-electrode voltage clamp. The latter does not permit one to alter the cytoplasmic ionic environment. Currently, the dual patch method is the one of choice for mammalian cells, including primary cultures and cell lines expressing exogenous connexins, having the well-known advantages of whole-cell patch clamp (e.g. fewer electrodes, more stable recordings, some ability to alter cytoplasmic constituents).

As mentioned above, the primary caution is that accuracy of junctional current measurement is affected by the relative values of uncompensated electrode resistances, non-junctional resistances, junctional resistance and seal resistances. For example, if the uncompensated electrode resistance is significant relative to the junctional resistance (R_j ; which can be low), a significant portion of the clamp potential develops across the electrode rather than the junctions, artifactually decreasing the apparent junctional conductance and its voltage sensitivity. Therefore, if R_j (or non-junctional resistance) changes significantly during

an experiment or experimental treatment, the fitness of the voltage control may be compromised. For this reason, careful attention must be paid to these parameters, and it is common to report the relative values of uncompensated electrode resistance, minimal junctional resistance and input resistances. Detailed analyses of the circuitry, sources of error and methods of compensating for them are found in (Rook *et al.* 1988; Giaume, 1991; Veenstra & Brink, 1992; Wilders & Jongsma, 1992; van Rijen *et al.* 1998, 2001; Veenstra, 2001).

The whole-cell patch approach is particularly well-suited for dye-passage experiments due to the low diffusional barrier of the patch pipette. In cases where the concentrations of ions or other cytoplasmic factors must be accurately controlled, the whole-cell patch method is subject to the usual limitations. In particular, differences between pipette concentration of an ion and its steady-state concentration in the cell are functions of the ion's membrane permeability, its diffusion constant, and the electrode resistance (Pusch & Neher, 1988; Mathias *et al.* 1990), as well as the effects of charged components of the cytoplasm that remain.

Primary mammalian cells can be studied as pairs isolated from tissue, or as pairs formed by manipulation into contact following dissociation into single cells. Transfected cells are grown in culture, and pairs identified for physiological study. In these systems, single junctional channels can be recorded using the techniques outlined in Section 3.2.1 below.

Under certain circumstances, intercellular currents do not require intercellular channels – single-membrane channels in closely apposed plasma membranes could give rise to ‘intercellular’ currents. Whether this occurs depends on the relative values of the conductances of the channels, the plasma membrane, the intercellular space and the access to the appositional region via the extracellular space. Ephaptic current flow between neurons is well-known (Arvanitaki, 1942; Faber & Korn, 1989; Jefferys, 1995). Calculations for paired apposed cells show that significant intercellular coupling occurs for the case in which the appositional membrane is significantly more conductive than the non-appositional membrane (Barr, 1963; Bennett & Auerbach, 1969; Heppner & Plonsey, 1970). When this is not the case, low levels of coupling (1–3%) can be attained with interdigitation, and this may be sufficient for entrainment of oscillatory firing of neurons (Vigmond & Bardakjian, 1995). However, the levels of electrical coupling typically generated by this mechanism are quite low.

A key descriptor of junctional conductance is its dependence on junctional voltage. The steady-state parameters of the voltage dependence provide a standard way to compare junctional channels. Essentially, the response of each hemichannel to transjunctional voltage is modeled as if it is a first-order, two-state process in which the energies of the states are exponential functions of voltage (Müller & Rudin, 1963; Ehrenstein *et al.* 1970). This enables fitting to a form of the Boltzmann relation, which yields parameters of voltage sensitivity (n ; number of charges needed to move through V_j to account for the voltage-dependent change in energy) and for the difference in conformational energy at zero voltage (ΔE). While this simplistic view is almost certainly inaccurate, for steady state modeling it provides adequate descriptive fit to the data (discussed in detail in Section 6.1). The steady state data for each polarity of voltage (i.e. closing of each hemichannel) can be modeled independently or as the product of two Boltzmann relations (Spray *et al.* 1981; Revilla *et al.* 1999). A recent implementation incorporates nonlinearities of channel conductance into macroscopic modeling (Vogel & Weingart, 1998).

The conformational energy term ΔE is commonly, and confusingly, expressed as $\Delta E/nq$,

the voltage at which the voltage-sensitive component of the conductance is half-maximal, V_o , where q is the electronic charge. V_o and n are often treated inappropriately as independent descriptors of the voltage sensitivity.

Two often overlooked factors can significantly affect the calculation of these descriptive parameters. For the calculation to be correct, the voltage-dependent component of the junctional conductance must be accurately determined. This component is the maximal junctional conductance (G_{\max}) minus the voltage-insensitive component of the conductance (G_{\min}). G_{\max} is often not a measurable parameter since at zero voltage some channels can be closed, depending on the values of ΔE and n . Also, G_{\min} can be measured only when the data extend beyond voltages where the steady state conductance no longer decreases, which is often not the case.

In the first implementation of this analysis, the system was sufficiently voltage-sensitive that the G_{\min} could be obtained directly (Harris *et al.* 1981; Spray *et al.* 1981a). Also the kinetics of the conductance changes were sufficiently close to first-order that it was justified to determine G_{\max} by explicitly testing for linearity of the $\log[(G_{\max} - G(V))]/[(G(V) - G_{\min})]$ versus V relation for different values of G_{\max} . Fitting to a line rather than a curve allows simple and accurate estimation of V_o and n as long as the linear regression incorporates a correction for the greater uncertainty of the larger values of $\log[(G_{\max} - G(V))]/[(G(V) - G_{\min})]$, that occur near G_{\max} (Bevington, 1969; Harris, 1994). In some cases G_{\min} is estimated from the available data, and G_{\max} fixed at the highest recorded conductance. This can lead to significant error. In other cases, G_{\max} and G_{\min} are treated as free variables to be determined along with n and V_o (Revilla *et al.* 1999). This is more justified, but has the potential for substantial inaccuracy, especially where the character of the data does not sufficiently constrain the parameters (e.g. large G_{\min} , small n).

3.1.2 Hemichannels

Macroscopic hemichannel currents can be studied by conventional whole-cell voltage-clamp techniques. Under normal conditions, hemichannels do not open except at sustained positive membrane voltages. The development of the outward current is slow, developing over many seconds. Reduction of extracellular calcium ion to 1 mM or less shifts the activation to more negative potentials, and dramatically increases the magnitude and rate of rise of the current (Paul *et al.* 1991; DeVries & Schwartz, 1992; Ebihara & Steiner, 1993; Zampighi *et al.* 1999; Ebihara & Pal, 2000). These macroscopic currents are inhibited by alkanols (Li *et al.* 1996; Zampighi *et al.* 1999; Valiunas & Weingart, 2000). The primary challenge is to identify the currents as arising specifically from connexin hemichannels and not from other plasma membrane channels. This caution applies to all studies of hemichannels, whether macroscopic, single channel, or dye permeability studies.

The problem hinges on the fact that there is little to positively identify, by physiological means, a particular membrane current as arising from hemichannels. What were previously thought to be immutable, if not unique, properties of connexin channels are not necessarily reliable identifiers (e.g. permeability to LY, pH sensitivity, calcium sensitivity, charge non-selectivity, sensitivity to octanol). [An early seminal review of gap junction physiology states: ‘A defining characteristic of the electrotonic junction is that junctional resistance is constant’ (Bennett, 1977).] There is no factor that *uniquely* characterizes, activates or inhibits all

connexin channels, other than the ability to form channels through two membranes, which is irrelevant to the problem of identifying hemichannels.

Pharmacological identification is problematic since agents that commonly affect junctional conductance between cells (e.g. pH, calcium, octanol, halothane) affect other channels as well (cf. Hirche, 1985; Quastel & Saint, 1986; Karon *et al.* 1994), do not act on all connexin channels (Rup *et al.* 1993), and, where they do affect connexin channels in cells, may not act on the connexin channel itself but rather via cytoplasmic or membrane components that are dependent on the specific cellular milieu. It was formerly assumed that connexin channels would be non-selective for charge (due to wide pore diameter), but data from heterologous expression systems indicate dramatic differences among connexins in size and charge selectivity as well as unitary conductance (cf. Beblo *et al.* 1995). As mentioned previously, non-connexin protein can also form channels that are permeable to large molecules (Sukharev *et al.* 1997; Surprenant *et al.* 1997; Buettner *et al.* 2000) and that have high conductance and low selectivity (Blatz & Magleby, 1983; Chesnoy-Marchais & Evans, 1986; Young *et al.* 1986; Fox *et al.* 1988).

For these reasons, identification of hemichannel currents in native cells is difficult, and each attempt must be evaluated on its own merits. Fortunately, most studies of macroscopic hemichannel currents are done in cells in which connexin is heterologously overexpressed. Hemichannel currents can then be identified by dependence on transfection with cDNA encoding for connexin, and changes in the currents with mutations in or suppression of the transfected gene. The only caveat is one that applies to all such studies of transfected cells – the possibility that expression of exogenous connexin (indirectly) affects expression of other proteins, including membrane channels, that alter endogenous currents. It is known that transfection with connexin cDNA can induce expression of several unrelated genes (Naus *et al.* 2000) and can affect cellular processes apparently unrelated to connexin expression (Cotrina *et al.* 1998, 2000; Sahenk & Chen, 1998; Scemes *et al.* 2000). The reverse has also been shown – that induced expression of non-connexin genes can affect regulation of connexin channels (Chanson *et al.* 1999, 2001). The problem of identifying single hemichannel currents is even more complex (see Section 3.2.2).

3.2 Single-channel currents

3.2.1 Junctional channels

Currents through single junctional channels between mammalian cells can be recorded by dual voltage-clamp in cases where the number of functioning channels is sufficiently small (Neyton & Trautmann, 1985; Veenstra & DeHaan, 1986; Weingart, 1986; Chow & Young, 1987; Rook *et al.* 1988). Because the current being measured is through only a few channels, the problems due to series resistance mentioned above are not an issue. This can be achieved by selection of highly resistive cells with very low junctional conductance (Veenstra *et al.* 1992), study of newly forming junctional channels (Nishimura *et al.* 1981; Chow & Young, 1987; Rook *et al.* 1988; Bukauskas & Weingart, 1993), or by treatment of coupled cells with agents that close junctional channels. For the latter case, the conditions most often used to reversibly reduce the number of active junctional channels to only a few are cytoplasmic acidification or exposure to any of several lipophilic agents known to reduce open probability of most junctional channels, usually heptanol, octanol or halothane (Turin & Warner, 1977,

1980; Spray *et al.* 1981b; Bernardini *et al.* 1984; White *et al.* 1985; Burt & Spray, 1988, 1989; Rudisuli & Weingart, 1989; Rozental *et al.* 2001). Where examined, acidification has little effect on unitary conductance (Hermans *et al.* 1995). However cytoplasmic acidification has effects on many cytoplasmic constituents and processes, so the lipophilic agents are preferred. To the extent these agents have been characterized, they seem to affect open probability of connexin channels without obvious effect on permeability or conductance (Veenstra & DeHaan, 1988; Burt & Spray, 1989; He & Burt, 2000). Their disadvantages include effects on other membrane proteins (cf. Hirche, 1985; Quastel & Saint, 1986; Karon *et al.* 1994), likely mediated by alteration of lipid bilayer properties and some toxicity. Also, the single-channel activity remaining may not be representative of the entire population of the junctional channels. For example, halothane, has dose-dependent specificities among homomeric channels, and is preferentially less effective on heteromeric channels (He & Burt, 2000). Potential mechanisms of action of these agents are discussed in Section 7.4. Where possible, it is preferable to study cells that are stably but poorly coupled (Veenstra *et al.* 1992).

Under dual whole-cell clamp, junctional channel current is reflected in the clamp current for each cell, but with opposite sign; current entering one cell through a junctional channel perforce leaves the other (Fig. 6). When single junctional channel transitions occur, the clamp current for each cell changes the same magnitude but in opposite directions. This will not occur for conductance transitions of channels in the non-junctional membranes (these transitions will be seen in the clamp current of one cell only). Therefore, even though both clamps record single-channel transitions in non-junctional membranes, junctional channel events can be readily and unambiguously identified. This is aided by the fact that the unitary conductance of connexin channels is typically much larger than that of other membrane channels. This approach has been successfully applied to a variety of native and transfected cells.

Reconstitution of junctional channels in a double bilayer configuration would be advantageous. However it is technically challenging. An attempt to do so was reported 10 years ago, but the absence of follow-up indicates it was either impractical or artifactual (Brewer, 1991). To achieve a double bilayer reconstitution it may be possible to take advantage of the experimental configurations used to study bilayer–bilayer interactions and membrane fusion, in which bilayers are formed across apposed apertures and bulged toward each other by hydrostatic pressure (Neher, 1974; Fisher & Parker, 1984; Fisher *et al.* 1986; Lucy & Ahkong, 1986, 1995). Other technologies may be useful, such as nanofabrication, supported bilayers (Butt *et al.* 1993; Seifert *et al.* 1993; Lu *et al.* 1996) or the use of polymerizable lipids (cf. Büschl *et al.* 1982; Benz *et al.* 1986; Higashi *et al.* 1987).

Formally, analysis of single junctional channel data does not differ from that of other single-channel data. The main limitation is the substantially greater noise in the records (due to the large amount of non-junctional membrane being clamped), instability of the preparation due to two simultaneous patch electrodes, and, where pharmacological treatments are used to reduce the number of channels, instability in the number of active channels. The fact that junctional channels tend to have noisy open-channel currents and prominent gating to substates makes the analysis more difficult and complex.

The dual whole-cell patch clamp method for recording single-channel currents is useful for determination of junctional channel ionic permeability and selectivity. Manipulation of ions must be achieved by dialysis of both cytoplasm via the patch pipettes, and not be toxic to the cells. This imposes substantial technical challenges, which have been admirably met

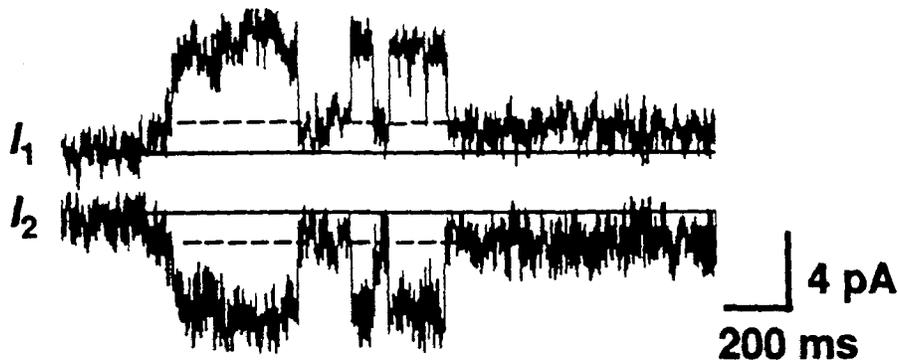


Fig. 6. Single junctional channel currents recorded by dual whole-cell voltage-clamp. I_1 and I_2 are clamp currents for two coupled cells, where V_j is maintained at 40 mV. With the opening of a junctional channel, the current enters one cell and leaves the other, resulting in changes in clamp currents of equal magnitudes in opposite directions. The initial slow opening corresponds to the opening of a newly formed junctional channel. This opening is followed by rapid transitions between a high conductance state and a subconductance state, typical of ' V_j gating' as described in Section 6. (From Valiunas *et al.* 1999a.)

(Veenstra, 2001). Some of the special challenges for selectivity measurements in this system are:

- (1) The ionic replacements required for true bi-ionic experiments (A^+B^- versus C^+B^-) cannot be done in living cells, so it is necessary to assess the contribution to the reversal potential of the asymmetrically distributed ions against a background of symmetrically distributed, but differentially permeable, ions. Therefore one must determine the permeability of each ion in the pipette solutions relative to an anion present in all experiments. Because connexin channels are typically permeable to both anions and cations, the reversal potential due to a salt gradient must also be assessed, and the system osmotically balanced with an uncharged junction-impermeant molecule, such as raffinose.
- (2) Each circuit clamps a cell, not the junction. Therefore a junctional reversal potential is not directly referred to ground or the extracellular medium but to both clamp circuits. Both (voltage-recording) patch electrodes are independently offset from ground by the input resistances and membrane potentials of each cell, which are being dialyzed with non-identical solutions.
- (3) For a junctional channel reversal potential to be measured accurately, the ratios of series resistance to input resistance must be equal for the two cells. Special care must be taken regarding junction potentials at the electrode and solution interfaces of the two (different) internal solutions and the bath solution.

It is easy to see that unless one is careful and confirms before and after each measurement that all the required conditions are being met, errors can be made. This is a primary reason why few groups attempt this kind of measurement, and why ionic selectivity data for connexin channels are not more common.

Because the methods for controlling the number of active junctional channels are indirect, and not as straightforward as simply trying another patch, it is often the case that several junctional channels are recorded from, resulting in ambiguity of gating behavior of the individual channels. To address this, a model-independent method for extracting single-

channel mean, open and closed times from heterogeneous multichannel records has been developed (Manivannan *et al.* 1992; Ramanan *et al.* 1992, 1995; Ramanan & Brink, 1993). The method allows the derivation of information about the number of types of channels present, the number of channels present, and cooperativity between channels (Veenstra *et al.* 1994a; Brink *et al.* 1996). Substate activity and mode-shifting can also be teased out. These statistical methods require recording at least 1000 gating transitions per channel type, and more if there are subtle processes such as mode shifting. This approach makes it possible to characterize a set of channels from long records, which is particularly useful when recordings of single channels are difficult to obtain.

3.2.2 Hemichannels

With the notable exception of 3D structures from crystallography or NMR spectroscopy, most advances in understanding channel structure-function in the last 15 years have derived from the ability to record single-channel currents from genetically manipulated proteins in an accessible configuration (i.e. one that allows application of reagents to one or both sides of the channel). The approaches outlined above for obtaining recordings of single junctional channels are technically demanding and afford limited accessibility to the channel. Fundamental advances in understanding connexin channel biophysics are likely to rely on the ability to record single hemichannel currents.

The electrophysiological techniques for recording single hemichannel currents are the same as those for recording other single channel currents – on-cell patch, excised patch and reconstitution in liposomes or bilayers. The ability to apply these techniques to hemichannels has had two major limitations, both of which are now being overcome. One is that only a subset of wild-type connexins form well-behaved conducting hemichannels (see Table 3). The other is that identifying the channels as formed by connexin protein can be difficult in native cells and in reconstituted systems.

The first problem has been addressed by the use of connexins genetically modified to form conductive hemichannels. The most popular is the chimeric connexin Cx32*Cx43E1 (Cx32 in which the E1 extracellular loop is replaced by that of Cx43) (Pfahnl *et al.* 1997; Oh *et al.* 2000). It forms well-behaved hemichannels that can be studied by on-cell and excised-patch approaches. The liability of such a structural alteration is that it may alter function in ways other than increasing the open probability of the hemichannel – there could be effects on ionic selectivity, molecular selectivity, voltage sensitivity, ligand sensitivity, and kinetics. Comparison of the macroscopic voltage sensitivity of homotypic Cx32 channels and Cx32*Cx43E1 channels indeed shows that the voltage dependence is substantially altered (Oh *et al.* 2000; Purnick *et al.* 2000b).

If the goal is to understand function of a wild-type connexin that does not form well-behaved hemichannels, care must be taken to ascertain the extent and effect of such changes. This can be difficult, since by definition the individual wild-type hemichannel cannot be characterized. The most appropriate comparison is between the single junctional channel behavior of the wild-type and of the mutant connexin being studied. This requires that the mutant form junctional channels as well as hemichannels, and leaves unaddressed whether differences are due to altered hemichannel properties or altered allosteric interactions between the hemichannels. Formally, these effects cannot be deconvoluted, so it is hoped that the two channel behaviors match well.

For studies in native cells, the problem of identifying the channels as hemichannels persists unimproved. The difficulties outlined in Section 3.1.2 for identification of macroscopic hemichannel currents apply to identification of single hemichannels as well. Identification is difficult, and most commonly relies on detailed comparison of the behavior of the single channels with that of putative macroscopic hemichannel currents and with the properties of junctional channels in the same cells. Such correlation is difficult to establish, and fortuitous when it occurs (e.g. DeVries & Schwartz, 1992).

For heterologous expression of connexins in *Xenopus* oocytes or mammalian cells, connexins that form conducting hemichannels can be identified by correlation with the genetic manipulations mentioned above in Section 3.1.2. If expression is low, there may be a statistical sampling problem in showing that expression of a particular single channel correlates sufficiently with sense or antisense effects to identify it as a connexin channel. On-cell and excised patch recordings of single hemichannel currents have been obtained from several wild-type and altered connexins expressed in *Xenopus* oocytes and cultured cells (see Table 3).

Reconstitution of hemichannels into planar bilayers or liposomes offers additional accessibility and potentially enables application of experimental paradigms not easily carried out in cells or patches. Prior to the advent of reliable methods for highly specific purification of arbitrary membrane proteins (e.g. tagging with antibody epitopes or polyhistidine), it was extremely difficult to identify a channel in a bilayer as formed by connexin – substantially more difficult than for most other channels, due to the absence of uniquely defining connexin channel physiology. Pharmacological agents that affect connexin channels in cells may not act on the connexin channel itself but rather on cytoplasmic or membrane components not present in the reconstituted system.

In addition, the sensitivity of the bilayer approach makes it particularly prone to reporting the activity of contaminating channels. In bilayer studies, the ratio of channels added to the bilayer chamber to channels observed is often greater than 10^{10} (i.e. 1 μg of protein corresponds to over 10^{12} channels with the mass of a Cx32 hemichannel). Thus extremely minor, but readily reconstituted, contaminant channels can produce significant and misleading channel activity (Lektin & Osmol, 1986). This concern is particularly valid for reconstitution from preparations of junctional membrane obtained by subcellular fractionation, whose connexin purity may be only one part in 10–100. These numbers also place extremely stringent requirements on the use of polyclonal antisera to identify connexin channels, since a small number of antibody molecules with errant specificity could misidentify the small number of channel molecules active in a bilayer.

Reconstitution into liposomes offers an advantage because there is a discrete number of active units (liposomes) – to present a problem, there must be enough of a minor contaminant to account for the number of liposomes that are permeable. However, excluding this possibility is difficult, and rarely attempted (but see Zampighi *et al.* 1985; Harris *et al.* 1992). Identification of connection channels in liposomes typically relies on liposome permeability to large molecules. Several innovative approaches have been applied (Claassen & Spooner, 1988; Scaglione & Rintoul, 1989; Ghosh *et al.* 1994; Diez & Villalobo, 1996) but compromised by false positives (Jarvis & Louis, 1992) or lack of adequate controls.

Most published reconstitutions of connexin are subject to the concerns described above. One historical class of studies utilizes membrane preparations from native cells enriched for junctional structures. This starting material is morphologically pure (i.e. composed of

junctional membranes as viewed by electron microscopy) but not biochemically pure (i.e. containing only connexin). Such material is reconstituted and the properties of the resulting channels (in bilayers) or permeability (in liposomes) are compared with those of *in situ* junctional channels. On the basis of correspondence of properties, to the extent that it occurs, and in most cases the predominance of connexin in the starting material, the channels are asserted to be formed by connexin. Because of the presence of non-connexin protein, these studies are compromised by the sensitivity of reconstituted systems to channel-forming agents. Furthermore, any deviation from expected properties could be interpreted to indicate either (a) an intrinsic connexin property unmasked by removal from the cellular milieu, or (b) a property of contaminating protein. In this way, almost any channel behavior seen can be justified as connexin-based. In addition, the certainty with which connexin channel properties can be inferred from cellular coupling studies is often questionable (e.g. does pH act via a cellular intermediate or by a direct titration of the connexin?). In some cases, effects of anti-connexin immunoglobulins have been used to aid the identification, but the specificity and purity of the antibody must conform to the strict standards mentioned above, which is rare, even for affinity-purified antisera. It is therefore difficult to be confident that an observed channel activity originates from connexin protein *solely* on the basis of enrichment for junctional membrane by sub-cellular fractionation techniques, or on the basis of the physiological criteria mentioned above.

The early work on reconstitution of channels from lens gap junctions is a case in point (Girsch & Peracchia, 1985; Nikaïdo & Rosenberg, 1985). MIP26 [lens major intrinsic polypeptide (Broekhuyse *et al.* 1976; Goodenough, 1979)] was believed to form gap junction channels in the lens. Its properties in bilayers were characterized as such, using morphologically pure lens junctional membranes as the starting material. MIP26 was suspected not to be a component of gap junctions (Paul & Goodenough, 1983) and was later identified as an aquaporin (AQP0) (Mulders *et al.* 1995; Zampighi *et al.* 1995). The MIP26 bilayer literature prior to Zampighi *et al.* (1989) illustrates the ease with which bilayer and cell fractionation results can be misleading.

Studies in this class reporting connexin channel activity in reconstituted systems have appeared over the last 15 years, using starting material from liver (Lynch *et al.* 1984; Spray *et al.* 1986; Walter *et al.* 1986; O'Terminus & Benefit, 1987; Young *et al.* 1987; Campos de Carvalho *et al.* 1991, 1992; Mazet *et al.* 1992; Diez & Villalobo, 1994, 1996; Ghosh *et al.* 1994), lens (Donaldson & Kistler, 1992; Jarvis & Louis, 1992; Kistler *et al.* 1995) and heart (Claassen & Spooner, 1988, 1989). These studies vary in the rigor of identification of the channel-forming protein.

This review will include data only from reconstitutions in which connexin was positively identified as the channel-forming protein. This means either an appropriate functional assay correlated with antibody identification, or connexin overexpressed in a heterologous system with exclusion of endogenous contaminants.

In general, the channels reported in such bilayer studies are of high conductance and weakly charge-selective, with various degrees of sensitivity to voltage, pH and calcium. From comparison of channel properties with hemichannel records from cells, and the reconstitutions mentioned below, it is likely that some of the studies listed above do indeed report the activity of connexin channels.

Cx26 expressed in insect cells has been reconstituted (Buehler *et al.* 1995). Hemichannel structures were biochemically purified from the cells, solubilized and then incorporated

into bilayers. Control protein from non-infected cells did not show the channel activity. Though it is formally possible that the connexin mRNA induced synthesis of a distinct, highly active channel-forming protein isolated by the conditions used to purify the hemichannels, it is unlikely. It has also been reported that several connexins produced by cell-free translation produced channel activity in bilayers (Falk *et al.* 1997).

One set of studies utilized a novel combination of physiological and immunological criteria to show that a permeability to large molecules induced in liposomes is due to Cx32 (Harris, 1991; Harris *et al.* 1992; see Section 3.4). Protein from junctional membrane was reconstituted into liposomes, which were then fractionated on the basis of permeability to sucrose. The proteins in the two liposome populations were analyzed by Western blotting and stained with a monoclonal antibody. Cx32 in the liposomes was specifically correlated with the permeability to sucrose, and was anti-correlated with impermeability. In addition, no other protein was present in sufficient amounts to account for the number of permeable liposomes. The liposomes were then fused with planar bilayers for direct examination of single-channel properties.

Cx32 and heteromeric Cx26:Cx32 hemichannels have also been immunopurified from native tissue and functionally incorporated into liposomes (Harris, 1991, 1994; Hong *et al.* 1996; Rhee *et al.* 1996; Harris & Bevens, 1997; Bevens *et al.* 1998). Permeability to labeled di- and tri-saccharides was demonstrated. More importantly, it was shown that the permeability of the channels to large molecules could be altered by altering the isoforms that composed the channels. This is compelling evidence that the channel activity is due to connexin. A similar approach has been applied to Cx43 (Kam *et al.* 1998; Kim *et al.* 1999).

None of these studies explicitly establishes that the conducting structures are single-membrane channels, though there is evidence that the connexin is in a hexameric single-membrane-spanning form prior to reconstitution. Though possible, it is unlikely that a double-membrane channel structure would functionally reconstitute in a single bilayer; it is more likely that the structures that were reconstituted were hemichannels rather than junctional channels.

3.3 Molecular permeability

A key characteristic of connexin channels is their permeability to molecules that cannot pass through most other channels (nominal pore diameter 12–14 Å). Intercellular diffusion of fluorescent tracers such as LY is commonly used as an indicator of the presence of functional junctional channels. Tracers are also used to characterize the molecular selectivity of connexin channels for size, charge and molecular species. In some cases the intercellular movement of radiolabeled cytoplasmic molecules is used to characterize the channels. These studies show that the channels formed by the different connexin isoforms have distinct limiting pore diameters, charge preferences and molecular selectivities (Elfgang *et al.* 1995; Veenstra, 1996; Bevens *et al.* 1998; Cao *et al.* 1998; Goldberg *et al.* 1999). Detailed analysis of these differences will undoubtedly lead to clues about their unique structural features.

3.3.1 A selection of tracers

A wide variety of molecular tracer molecules have been used to establish junction channel function. The most common are LY (Stewart, 1978), calcein, neurobiotin, and fluorescein and its derivatives. For systematic exploration of pore properties, one would like a set

of probes for which the relevant dimensions vary in a known fashion, and whose charge and chemistry are similar. Several families of such tracers have been used for this purpose.

The first such family of tracers was a series of fluorescently labeled amino acids and linear peptides that vary in length and charge (Simpson *et al.* 1977; Flagg-Newton *et al.* 1979). Microinjected into cells, these probes provided the fundamental characterization of connexin and innexin channels. They provided the initial estimates of pore diameter, and indications of differences in diameter between connexin and innexin channels, and among connexins (though they were not identified as such). These tracers were later supplemented with linear oligosaccharides and branched glycopeptides (Schwarzmann *et al.* 1981). Because of the variation in structure, chemistry and flexibility of the probes, their utility for more detailed probing of pore properties is limited.

More recently, another set of uncharged probes was developed (Bevans *et al.* 1998). A set of oligomaltoses were labeled at the reducing end with an aminopyridyl group (PA), which is fluorescent, uncharged, and not wider than the diameter of the sugar. The $\alpha 1 \rightarrow 4$ linkages between the saccharide units result in a rigid helical structure for which a full turn requires six saccharide units. Because of this, the axial cross-sectional areas of the oligosaccharides increase with oligomeric number, up to 6 (nomenclature: n -PA, where n is the number of saccharide units). These probes are thus chemically identical, relatively rigid, uncharged, fluorescent, and of a range of sizes. They have been used in reconstituted systems, but may not be suitable for injection into cells since endogenous maltases (α -glucoside hydrolases) may break down the saccharide linkages. This may be prevented by the simultaneous injection of the pseudo-tetrasaccharide acarbose (glycoamylase), which is a high-affinity inhibitor of the α -glucoside hydrolases (Hata *et al.* 1992; Sigurskjold *et al.* 1994). Acarbose is unlikely to permeate connexin channels.

A set of commercially available fluorescent probes, the Alexa family (Molecular Probes, Inc., Eugene, OR), has been successfully employed to compare permeability properties of several connexins (Nicholson *et al.* 2000). This series of dyes was designed to fluoresce at different wavelengths. To achieve this with the same fluorochrome moiety, different carrier structures were generated using conjugated aromatic rings. This resulted in a series of fluorescent structures of different molecular dimensions. However, their structures do not vary systematically and are chemically distinct. Also, although the overall charge is -1 , they carry different numbers of discrete charges at different positions. In spite of this chemical variability, their use as probes of pore size has given systematic and interpretable results.

A set of biotinylated probes has been used recently to distinguish several sizes of connexin channel pores (Mills & Massey, 2000). These are derivatives of biotin ethylenediamine (Neurobiotin; Vector Labs, Burlingame, CA) with different spacer linkages. The structure of the linked ethylenediamine groups is unknown, but their utility as empirical probes of pore size is clear. These molecules are not fluorescent, but are imaged after fixation with a fluorescent streptavidin derivative. These probes have the advantages of being of the same chemistry and charge. At present, only a few members of the series are available commercially.

3.3.2 Junctional channels

A direct and obvious approach to assess molecular permeability of junctions is to inject

fluorescent tracer into a cell through a microelectrode or patch pipette and note whether it spreads to adjacent cells (Loewenstein & Kanno, 1964; Pappas & Bennett, 1966; Furshpan & Potter, 1968). A video of fluorescent dye being injected into a cell and spreading to neighboring cells via junctional channels is available at www.uni-stuttgart.de/bio/biophysik/avi_gap.htm.

This approach has been widely used due to its relative ease, ready application to monolayers of cells, and rapid results. A recent variation is to load a monolayer of cells with Fura-2 and inject Mn^{2+} into a single cell (Niessen *et al.* 2000). Mn^{2+} passing through junctional channels quenches the resting Fura-2 fluorescence in them. The extent of the quenching is an index of junctional permeability to Mn^{2+} .

Cells can also be loaded with tracer by a technique known as 'scrape-loading' (el-Fouly *et al.* 1987) in which a confluent layer of cultured cells is mechanically scored with a rubber policeman in the presence of tracer, which is taken up by injured cells along the score and spreads through junctional channels away from it. Intercellular diffusion of the tracer indicates non-zero junctional permeability. The virtues of scrape-loading are its ease and speed. However, it is difficult to determine accurate absolute or relative permeabilities in this manner. A refinement is to note the number of cells to which the dye spreads during a given time after injection, or to compare the number of 'dye-coupled' cells when different dyes are used. A useful improvement is to load two dyes simultaneously so that differences in the extent of dye-spread through the same junctions can be assessed. A review of these types of approaches is found in (Meda, 2001).

Flow cytometry has been used to determine the extent of dye coupling in populations of cultured cells (cf. Rabito *et al.* 1987; Tomasetto *et al.* 1993; Fick *et al.* 1995; Koval *et al.* 1995; Martin *et al.* 1998a; Czyz *et al.* 2000; Oviedo-Orta *et al.* 2000). The basic paradigm is to label one population of cells with a fluorescent membrane marker (such as Di-I) and another population with a fluorescent cytoplasmic tracer, such as calcein-AM (Tsien *et al.* 1982). After co-culture, the cells are analyzed by fluorescence-activated cell sorting (FACS) to identify cells that contain both fluorescent markers. Coincidence of both markers in the same cell is taken to indicate that the cytoplasmic tracer passed through the junctional channels while the cells were co-cultured. Controls are required to rule out cell fusion, and cell disruption and uptake of tracer by neighboring cells. An advantage of this approach is that analysis is done on large numbers of cells. A disadvantage is that kinetic channel permeability data cannot be obtained, and the extent of transfer cannot be directly normalized to take into account extent of coupling, number of junctional channels, etc.

An assay that is rapid and can give some temporal information is the 'parachute' assay, in which a suspension of cells labeled with a dye such as calcein-AM is pipetted directly onto a monolayer of target cells (Ziambaras *et al.* 1998; Durig *et al.* 2000). Dye spread can then be monitored starting at a roughly synchronous starting point.

These approaches can establish that coupling occurs, and that a dye is permeable, but cannot accurately determine relative permeabilities. This requires examining rates of intercellular diffusion, which is most accurately done in an isolated, two-cell system (Verselis *et al.* 1986b; Imanaga *et al.* 1987). However, even in this case, quantitation can be difficult. The rate of transfer reflects the 'permeability' of the coupled cell system, not exclusively that of the channels. The rate of dye appearance in a coupled cell reflects the number of junctional channels and their open probability, and can be strongly affected by cytoplasmic or nuclear binding as well as cell volume. Unless these factors are controlled for, relative permeabilities

cannot be meaningfully assessed in terms of understanding mechanisms of molecular selectivity. For these reasons, kinetic data are generally more sensitive, trustworthy and informative than steady-state or single time-point measurements.

The channel number and open time variables can be somewhat controlled for by simultaneously co-injecting two tracers into the same cell and monitoring the relative spread of the two tracers. Relative junctional permeabilities to two tracers can be determined by this means, but only if the movement through junctional channels is independent (i.e. the movement of one molecular tracer through the pore does not interfere with the movement of the other), which is not likely for large tracers.

A better approach is to determine the ratio between the measured junctional molecular permeability (P_j) and electrical conductance (g_j ; i.e. permeability to K^+ , Cl^- , Na^+) (Verselis *et al.* 1986b). The P_j/g_j ratio normalizes for junctional area, number of channels, and proportion of open channels. Also, because the atomic ions are small, the condition of independence is more likely to be met. The two parameters can be measured simultaneously for a single tracer, and the results compared between tracers. This approach is limited to measurements between two isolated, coupled cells, but with some modification and approximations it has been applied to monolayers of HeLa cells with surprisingly reasonable agreement with cell pair data (Cao *et al.* 1998). When g_j is very low, it can be difficult to determine whether the permeability per channel is also low – the question is how long to wait for a detectable signal in the coupled cell. This problem has been analytically treated for the two-cell system in Perez-Armendariz *et al.* (1991).

Molecular permeability is difficult to assess even qualitatively in multicellular systems (such as pieces of tissue or sheets of cultured cells). Dye spread under ideal circumstances – a 2D isotropic sheet of identical and equally coupled cells – is a complex process shaped by parameters such as the dye delivery function, cytoplasmic mobility of the dye, junctional permeability of the dye, leakage of the dye across plasma membrane, and binding of the dye in cytoplasm or nucleus. Dye spread in realistic circumstances involves additional parameters such as differences in cell volume and shape, differences in extent and location of coupling to other cells, and heterogeneity of cells regarding metabolism, health and connexin expression. Dye spread in a sheet of cells has been treated analytically in several contexts (Brink & Ramanan, 1985; Safranyos *et al.* 1987; Ramanan & Brink, 1990; Christ *et al.* 1994).

Analysis of dye coupling in 3D tissues is more complex. Models of dye spread in the 3D tissues have been used to estimate intercellular dye diffusion coefficients from confocal and non-confocal images (Rae *et al.* 1996; Eckert *et al.* 1999). To facilitate the targeting of specific cells for dye injection in 3D tissues, an automated system based on image analysis of vitally stained tissue and confocal reconstruction has been described (Kurata *et al.* 1997).

An example of the complexity of interpreting dye-coupling studies in whole tissues was provided by reports that gap junctions between bipolar and amacrine II cells (Vaney, 1997) and between oligodendrocytes and astrocytes (Robinson *et al.* 1993; Zahs & Newman, 1997) were permeable to tracers only in one direction – that there was unidirectional passage of dye through the junctional channels. Indeed the raw data give that impression, but Maxwell may object (Finkelstein *et al.* 1994), and in the former case closer study has shown that dye passage is indeed bidirectional (Trexler *et al.* 2001).

The ease of artifactual findings in whole tissues was recently demonstrated in a study that

compared two methods of assessing junctional dye coupling in early whole *Xenopus* embryos (Landesman *et al.* 2000). Following injection of LY, fluorescence microscopy of whole-mount embryos routinely and erroneously indicated dye coupling. Inspection of fixed and sectioned embryos showed this to be artifactual. The reason for the false positive in the whole mounts is the optical artifacts due to reflected and scattered light, and non-homogeneous pigmentation of the cells. In this case, the more accurate data required a re-thinking of the role of gap junctions in dorso-ventral patterning.

A technique with high potential for accuracy and sensitivity is a variant of the fluorescence recovery after photobleaching (FRAP) method of determining lateral mobility in cell membranes (Peters *et al.* 1974; Axelrod *et al.* 1976; Edidin *et al.* 1976; Koppel, 1979; Délèze *et al.* 2001). The basic paradigm is to load cultured cells with dye, typically by use of acetoxymethyl (AM) ester derivatives of fluorescent tracers (Tsien *et al.* 1982) or 5,6-carboxyfluorescein diacetate (Rotman & Papermaster, 1966; Goodall & Johnson, 1982), which are then removed from the extracellular medium. The fluorescence signal in a cell is rapidly photobleached with a laser. The rate at which fluorescence returns to the bleached cell (i.e. the rate that dye diffuses into that cell from its neighbors through junctional channels) is measured. An effective transfer constant can be determined from this rate. This technique is called 'Gap-FRAP' (Wade *et al.* 1986; Suter *et al.* 1987; Miller, A., 1995; Délèze *et al.* 2001), and has been applied with various degrees of care. It has been adapted to 3D structures (lens) by application of confocal microscopy (Miller, A., 1995; Miller & Hall, 1996). The Gap-FRAP method is essentially a way to perform a dye-spread experiment in a tissue or sheet of cells, in which the time of initiation of dye flux and dye concentration in the source(s) can be measured. Quantitative analysis of rates of recovery usually assume that all the neighbors of the bleached cell have the same number of junctional channels and the same volume. Sources of error include dye leakage to the bath, bleaching during the measurement of fluorescence recovery, and the possibility of photodynamic damage to the cell, all of which can be controlled for.

An imaginative and biologically useful method of assessing junctional channel molecular permeability is to label endogenous cytoplasmic molecules and then trap and identify the ones that pass to other cells (Kolodny, 1971; Goldberg & Lampe, 2001). This unlikely approach has been successfully applied to show transfer of molecules such as ATP and glutathione through junctional channels (Goldberg *et al.* 1998, 1999; Nicholson *et al.* 2000). In brief, the approach is to metabolically label cells (e.g. with [¹⁴C]glucose), identify them by membrane staining with Di-I, and culture them with unlabeled cells. After culture, the unlabeled cells are separated out by FACS, and the labeled molecules analyzed and identified (e.g. by HPLC and TLC). To confirm the validity of the results, the conditions for intercellular diffusion can be tested using a fluorescent dye such as calcein, and coupling can be inhibited by the use of inhibitors of junctional channels. The 'captured' metabolites may be analyzed on any of several specialized HPLC columns with appropriate standards, and, if necessary, further separated by TLC and stained for specific compounds.

Another approach has been developed recently that uses endogenous or exogenous channels to report the intercellular flux of second messengers (Qu & Dahl, In Press). Junctional cAMP flux can be assessed by microinjecting it into one oocyte of a pair that is expressing the connexin of interest. The other oocyte is additionally transfected to express the CFTR protein, which produces a chloride current in response to a rise in cAMP. Thus the appearance of a chloride current in the second cell following cAMP injection into the first can

indicate flux of cAMP through the junctional channels. A similar protocol can be used to detect intercellular IP₃ flux, using the calcium-activated chloride current that is endogenous in *Xenopus* oocytes.

3.3.3 Hemichannels

The tracer permeability of hemichannels in plasma membrane is usually assessed by the ability of cells to become loaded with tracer when it is present in the extracellular medium. This method has been applied to hemichannel studies in *Xenopus* oocytes (Paul *et al.* 1991) and cultured cells (Li *et al.* 1996). The primary concern is the potential of other plasma membrane channels to mediate the tracer permeability. Until a few years ago, it was nearly axiomatic that only connexin channels would be permeable to tracers such as LY or 6-carboxyfluorescein. Now it is known that several types of membrane channels can be permeable to relatively large tracers and amino acids: several varieties of anion or 'chloride' channels (Bosma, 1989), channels activated by extracellular ATP (Steinberg *et al.* 1987; Nuttle & Dubyak, 1994; Surprenant *et al.* 1997), plasma membrane VDAC (Dermietzel *et al.* 1994), and complement channels (Sauer *et al.* 1991). Therefore determination that dye permeability is due to hemichannels must rely on connexin-specific modulation of the permeability, specific inhibition by antibodies, or tight correlation with the properties of junctional channels in the same cells. The caveat mentioned above (Section 3.1.2) regarding the possibility of connexin expression altering membrane permeability by indirect means also applies here.

The potential for misleading results regarding hemichannels is demonstrated by the story of Cx43 hemichannels in a macrophage cell line. It was found that J774 cells that expressed Cx43 responded to extracellular ATP by activation of a plasma membrane permeability to LY and other large molecules. J774 cells that did not express Cx43 did not show this effect. It was reasonably proposed that plasma membrane permeability to LY was due to the opening of Cx43 hemichannels (Beyer & Steinberg, 1991). It has now been shown that ATP-activated LY-permeable channels are not hemichannels (Alves *et al.* 1996), and that, at least in other cells, it is a purinergic receptor (P2Z/P2X; Surprenant *et al.* 1997). In the meantime, it was noted that HeLa cells have a similar response to ATP, and that it correlates with the level of junctional coupling between the cells. This led to the suggestion that cells that express junctional channels at high levels also express an enhanced level of connexin hemichannels in their plasma membrane, which are activated directly or indirectly by extracellular ATP. This was taken as supportive of other data indicating that hemichannels were active in these cells (Liu *et al.* 1995). In fact, it looks like they are (Li *et al.* 1996), but that the correlation between coupling and the ATP-induced conductance has nothing to do with it, since the ATP flux occurs through purinergic receptors. Curiously, it has been shown by another group that the ability of cells to *release* ATP correlates with connexin expression, but the release is not necessarily through hemichannels (Cotrina *et al.* 1998, 2000). These studies point out the need to characterize plasma membrane permeabilities thoroughly before attributing them to connexin.

The FACS-based approach outlined in the previous section can also be used to assess for the presence of hemichannels. A suspension of single cells whose plasma membrane are stained with Di-I, for example, can be loaded with a cytoplasmic tracer, and treated in ways that should allow hemichannels to open, and the ratio of the two tracers in single cells

determined by flow cytometry in the experimental and control populations. The approach using endogenous and exogenous reporter currents outlined above can also be applied to the study of hemichannel permeability to second messengers.

3.4 Other

A technique called transport-specific liposome fractionation (TSF) that can assess aspects of both channel activity and molecular permeability was developed for the study of connexin channels, and can also be applied to other channels (Harris *et al.* 1989, 1992; Sugawara & Nikaido, 1994; Rhee *et al.* 1996; Harris & Bevans, 2001).

Purified connexin is incorporated into unilamellar liposomes by gel-filtration of a mixture of octylglucoside-solubilized connexin and lipid. The liposomes are fractionated on the basis of permeation of osmolytes (urea and sucrose) through the reconstituted channels. Specifically, TSF employs buoyant density sedimentation of liposomes by ultracentrifugation through an iso-osmolar density gradient formed of urea- and sucrose-containing buffers. This procedure separates, into distinct bands, liposomes with open channels permeable to urea, and sucrose from liposomes that are without such channels. Liposomes that do not contain open connexin channels (i.e. are not permeable to urea and sucrose) migrate to an equilibrium position in the upper part of the gradient. For liposomes that contain open connexin channels, the osmolytes exchange through the channels and the liposomes migrate to a lower position determined only by lipid density (Fig. 7). Any significant channel open probability (P_o) results in sufficient osmolyte exchange to cause the required change in density. TSF is therefore an all-or-none assay of per-liposome channel activity.

Effects of test compounds on channel activity are assessed by exposing connexin or connexin-containing liposomes to the compounds prior to or during a TSF centrifugation. Effects on channel activity are quantified as changes in the fraction of liposomes in the lower band relative to that for connexin or liposomes not exposed to the test compound. The fractional change in distribution of liposomes between the two bands is a quantitative measure of the fractional change in activity of the population of the channels, after statistical correction for liposomes containing more than one channel. The change in liposome density can result from brief channel openings, so only when P_o changes above or below a low threshold value are changes in channel activity detected (typically P_o between 0.01 and 0.001).

TSF is also used for studies of molecular permeability by loading tracers into the liposomes. After TSF, the tracer:liposome ratio in each population (i.e. upper and lower bands) is determined. Tracers retained in the lower band are impermeable through the reconstituted, functional channels that caused the change in liposome density. The tracer:liposome ratio in the upper band serves as a positive control for tracer loading and non-specific tracer leakage.

This technique can be applied in a variety of ways to obtain information about reconstituted channels. Its limitations are that it does not allow kinetic measurements of gating or assessment of relative (non-zero) permeabilities. It is an equilibrium measurement that reflects essentially all-or-none phenomena.

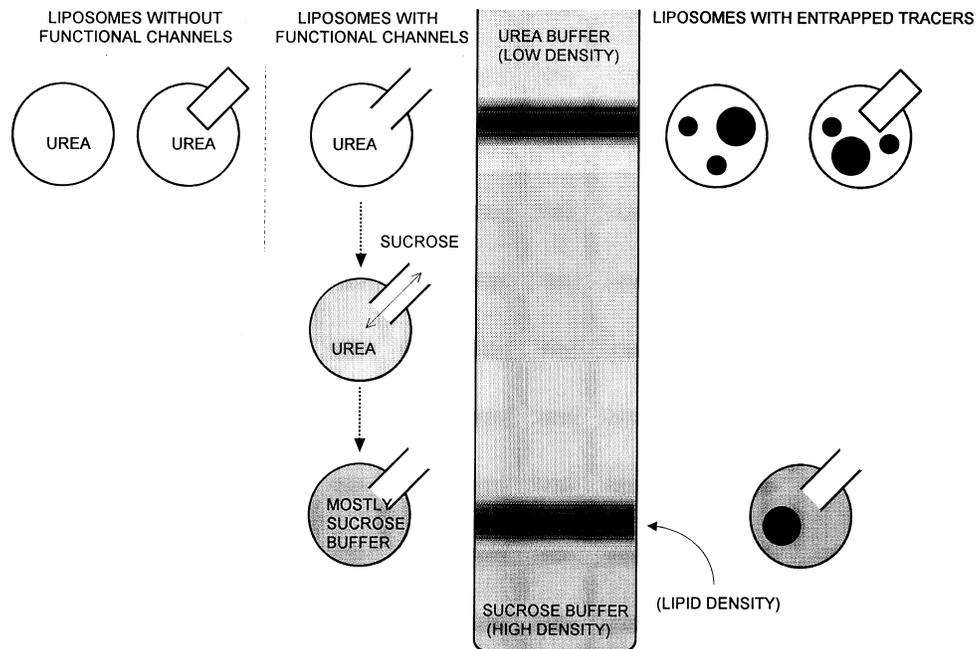


Fig. 7. Schematic of transport specific fractionation (TSF). Urea and sucrose exchange through reconstituted open hemichannels, causing the liposomes that contain the hemichannels to become more dense and move to a lower position in the density gradient. Liposomes in the lower band lose entrapped tracers that permeate the hemichannels.

4. Structural issues

4.1 What lines the pore?

Among channels with the same ionic selectivity, the key pore-lining amino acids – those that determine selectivity – can be identified as highly conserved across isoforms (e.g. as for potassium channels). However, since different connexins form channels with different molecular and charge selectivities, this approach is not available. In fact, it is likely that the amino acids responsible for selectivity are those that are anti-conserved across connexins. This does not aid in identifying pore-lining regions. One hopes that the structural scaffold of the pore is conserved, but this does not require a strict amino-acid identity.

The third transmembrane domain of connexins, M3, contains polar or charged residues at every fourth position, similar to the amphipathic helical motif thought to form the pore-lining domain of the nicotinic acetylcholine receptor (nAChR) and other channels (Karlin & Akabas, 1995). In further analogy with the nAChR, the pattern is of small polar residues flanked by larger, non-polar residues. Over the displacement of nearly 6 regular turns, the hydrophilic residues form a stripe that sweeps through an angular displacement of $\sim 250^\circ$. Given these features and the conservation of this pattern in all connexins, M3 was proposed to be the primary structural determinant of the pore, prior to the availability of experimental data (Milks *et al.* 1988).

A peptide corresponding to the N-terminal segment of the E2 domain of Cx32 that could not

be externally labeled was found to permeabilize *Xenopus* oocytes and induce channel activity in bilayers, leading to the suggestion that it forms part of the channel pore (Dahl *et al.* 1994). This suggestion draws on analogy with the pore-forming P-region of voltage-gated ion channels. In this view, all or part of the sequence LYPGYAMVRLVK (positions 156–167) forms an extended β structure within the membrane, perhaps in concert with M3. While an intriguing suggestion, that a peptide containing $\sim 60\%$ non-polar residues forms channels does not compellingly argue that it forms the pore of a native connexin, only raises the possibility. There is no homology with the much more hydrophobic *Shaker* channel P-region, and the pattern of polar, charged and non-polar residues is not similar. Nevertheless, it is notable that of the several published antibodies against E2 that bind to extracellular locations, none significantly overlap the region defined by this peptide. The one report of an antibody raised against this domain gave binding too weak for investigative purposes (Milks *et al.* 1988).

The first experimental, though indirect, evidence for channel-lining positions was obtained from studies exploring the determinants of the polarity of voltage sensitivity (Verselis *et al.* 1994; Oh *et al.* 2000). These studies showed that residues at the NT (N2 for Cx32; D2 for Cx26) and at the M1/E1 border (E41 and S42 for Cx32; K42 and E43 for Cx26) interact to determine the voltage polarity to which the hemichannel responds (in bold type in the sequences below).

Cx32NT:	1	M NWTGLYTLLSGVNRHSTAIGR	22
Cx32M1:	23	VWLSVIFIFRIMVLVVA	40
Cx32E1:	41	E SVWGDEKSSFICNTLQPGCNSVCYDHFFPISHVR	75
Cx26NT:	1	M DWGTLQSI LGGV NKHSTSIGK	22
Cx26M1:	23	IWLTVLFIFRIMILVVA	40
Cx26E1:	41	K EVWGDEQADFVCNTLQPGCKNV CYDHHFPISHIR	75
Cx46E1:	42	E EVWGDEQSDFTCNTQQPGCENVCYDRAFPISHIR	76

These positions are known to be localized at least some of the time on opposite sides of the membrane, and mutations at them act as intragenic ‘suppressors’ of changes in polarity of voltage sensitivity (di Rago *et al.* 1990; Papazian *et al.* 1995). This functional linkage suggests either that they interact directly or experience the same voltage field. Either possibility can be accommodated if they both lie close to or in the aqueous pore, consistent with the early suggestion based on kinetic analysis of macroscopic junctional currents that charge-sensing residues lie within the aqueous pore (Harris *et al.* 1981). This placement implicates M1 as a pore-lining domain in Cx32 hemichannels.

More direct evidence for M1 involvement came from SCAM (scanning cysteine accessibility mutagenesis; Akabas *et al.* 1994) applied to hemichannels formed by Cx46 and a mutant of Cx32 (Cx32*Cx43E1) expressed in oocytes (Zhou *et al.* 1997). Plasma membrane conductance induced by these hemichannels was substantially and specifically reduced by reaction with maleimido-butyryl-biotin (MBB; 537 Da) at two positions in M1 (I33 and M34) in Cx32 and the corresponding positions (I34 and L35) in Cx46. Three positions in M3 and at the M3/E2 border showed significant, but smaller, effects (S138, E146 and M150) in Cx32*Cx43E1. This was the first direct evidence for the involvement of M1 in the pore lining.

Cx32M1:	23	VWLSVIFIFR IM VLVVAA	40
Cx46M1:	24	VWLTVLFIFR IL VLGAAA	41
Cx32M3:	131	LWWTYV IS VVFRL LE AVF	149
Cx32E2:	150	MY VFYLLYPGYAMVRLVKCEAFPCPNTVDCFVSRPTEKT	188

This study looked only at effects on macroscopic plasma membrane conductance, not single hemichannels, and MBB was applied only from the outside. Thus the interpretation is complicated by the possibility that modification at the accessible positions modified properties other than permeation, and that some of these effects are due to modification at externally accessible sites that are not part of the pore. Negative findings could result from the large size of MBB restricting complete entry into the pore.

A different kind of evidence for M1 involvement in the pore was obtained from studies of a CMTX-inducing mutation at position 26 of Cx32. Access resistance measurements demonstrated that a S26L mutation had little effect on channel function except to narrow the pore near the cytoplasmic opening (Oh *et al.* 1997). This could suggest that M1 contributes to the pore lining of Cx32, but could also mean that the S → L change altered the tilt of another helix to narrow the pore.

Cx32M1: 23 VWLSVIFIFR**IM**VLVVAA 40

Pursuing the potential involvement of M1, the Cx32 M1 was replaced by the Cx46 M1 in Cx32*Cx43E1 chimeric hemichannels. This chimera and Cx46 hemichannels have different unitary conductances and gating properties (Hu & Dahl, 1999). While there was not a clean and complete exchange of permeability properties, the trends were in the correct direction. Specifically, the unitary conductance of the Cx32*Cx46M1*Cx43E1 channels was identical to that of wild-type Cx46 channels. More recent work showed that the reciprocal swap (M1 of Cx46 replaced by that of Cx32) produced channels with Cx32-like conductance. Furthermore, swap of only the C-terminal half of M1 produced the effect (positions 33–41 of Cx46), whereas swap of only the N-terminal half of M1 had no effect on conductance (Dahl, personal communication). These are compelling data that M1 lines at least part of the pore. The absence of effect of the N-terminal half of M1 of Cx32 is inconsistent with the suggestion from the earlier work that S26 is physically in the pore.

Single channel data also suggest that part of E1 lines the pore. A series of domain swaps demonstrate that the E1 domain of Cx46 hemichannels defines in large measure the charge selectivity and single-channel $I-V$ relations of the pore [Trexler *et al.* 2000; see Section 5.1.3(a)]. Mutagenesis of the NT of Cx32 suggests that up to 10 residues from the N-terminus may affect polarity of V_j -gating (see Section 6) (Purnick *et al.* 2000b). An NMR solution structure of the NT domain suggests that the NT could bend into the vestibule of the channel (Purnick *et al.* 2000a). In addition, recent SCAM studies show that modification of several positions in the N-terminal end of E1 of Cx46 (E43, G46, E48, D51; in bold above) with MTSET or MTSES alters the $I-V$ relations in a way consistent with the charge difference in these reagents (Kronengold *et al.* 2001; Kronengold, Trexler, Bukauskas, Bargiello & Verselis, personal communication). Furthermore, D51 was accessible for this modification from either end of the pore. These data make a compelling case for the N-terminal portion of E1 being exposed to the pore in Cx46.

The data described thus far, all derived from hemichannels with the exception of the S26L

study, suggest that M1 and E1 contribute to the pore, presumably in the transmembrane and extracellular regions, respectively. In particular, replacing either the M1 or the E1 domain of Cx46 with that of Cx32 yields hemichannels with pore properties resembling that of Cx32. This implies that either the results are coincidental, that the pore properties are determined by M1–E1 interactions (with either Cx32 domain able to induce functionally equivalent changes to the other domain of Cx46) or that either Cx32 domain has a dominant effect on the Cx46 pathway.

Unpublished data from point mutations in these domains also support the involvement of M1 and E1. An M1 L35G mutation in Cx46 hemichannels both increases the unitary conductance 35% and induces a wider pore (Dahl, personal communication), and an E1 E48K mutation in Cx43 junctional channels increases the unitary conductance 2.6-fold without apparent effects on other properties (Brink, Kumari & Valiunas, personal communication).

Another SCAM study suggests otherwise (Skerrett, Ahmed, Shin, Kasparek & Nicholson, submitted). In this work, cysteine substitutions were made at a substantial fraction of the positions in the transmembrane domains of Cx32. MBB was applied to the cytoplasmic face of oocyte junctions via the perfused oocyte technique. Modifiable cysteines were considered to be in the pore if MBB was effective from the cytoplasmic side but not from the outside of the oocytes, and also if the character of the voltage sensitivity was not dramatically altered (taken to indicate a change not specific to the pore). Using these criteria, 2 sites in the cytoplasmic half of M2 were identified, 1 site in M4, and 6 sites in M3, 3–4 positions apart that form a stripe on one face of the helix. On the basis of these findings, it was proposed that M3 forms most of the transmembrane pore. M1 sites identified by previous SCAM studies were accessible for modification from the outside and therefore discounted. In the previous work, the N2 position in the NT, and E41 and S42 positions in E1 were suspected to be in the pore precisely because of dramatic effects on voltage gating, so it is likely that this study discarded changes at those sites. Given this, and the fact that the size of MBB could limit its accessibility, only the positive results of this study are meaningful, and argue strongly for the substantial contribution of M3 to the pore lining, and possibly a contribution from M2. The primary caveat to this work is the difficulty in controlling for MBB accessibility at non-pore sites from the cytoplasmic face of the channels.

Several studies report effects on unitary conductance of modification of the cytoplasmic CT and/or CL domains (cf. Fishman *et al.* 1991; Spray *et al.* 1992; Kumari *et al.* 2000; Manthey *et al.* 2001). These changes could be effected by allosteric effects on other transmembrane domains or by affecting entry into the pore, but are unlikely to line the pore in the usual sense.

The published 7.5 Å resolution structure of a CT-truncated Cx43 channel shows that 2 of the 4 transmembrane helices line the pore (Unger *et al.* 1997, 1999; Yeager, 1998). One of these helices ('helix B') is virtually perpendicular to the plane of the membrane. The other ('helix C') is tilted through the plane of the membrane $\sim 24^\circ$, narrowing the pore at the extracellular domain. Near the boundary with the extracellular gap, helix C bends to become nearly perpendicular with the plane of the membrane. The trajectory and positioning of the four helices suggest that there is no direct connectivity between helix A and helix B at the cytoplasmic end of the channel, whereas the cytoplasmic ends of helices B, C and D appear to cluster.

Taking this structural information from a truncated Cx43 with the suggestions from the

data summarized above for Cx32 and Cx46 as a basis for speculation, several possible assignments for the identification of the helices may be made.

If the kink in helix C is produced by the highly conserved proline at position 86, the only proline in a membrane-spanning region, then helix C corresponds to M2 (also suggested in Ri *et al.* 1999; Purnick *et al.* 2000a). If the suggestion from the data indicating involvement of M1 is correct, then helix B must correspond to M1. The inferred absence of direct connectivity between the cytoplasmic ends of helices A and B, and the clustering of cytoplasmic ends of B, C and D permits the suggestion that helix D corresponds to M3 and helix A to M4. In this view, moving from the cytoplasmic end of a hemichannel toward the extracellular end, the pore is first lined primarily by M1 (possibly up to residue M34) then by M2 (R75 to T86).

This configuration is not supported by the data from the Cx32*Cx46 chimeras indicating a strong influence of the extracellular half of M1, or the MBB accessibility of M3 in the perfused system. Also, the proline in M2 is two-thirds of the way toward the cytoplasm, inferred from its sequence position, yet the bend in helix C is at the extracellular end of the hemichannel.

Alternatively, if helix C is M2, but the MBB data implicating M3 are considered, then helix B would correspond to M3. This is inconsistent with any of the data implicating M1, and is contradicted by first-order calculations of energies based on pair potential functions between residues for different helix-packing arrangements (Nunn *et al.* 2001).

If, however, the kink in helix C does not correspond to the proline in M2, the constraints are relaxed. The MTSET and MTSES data on Cx46 hemichannels would then favor the pore being lined at the cytoplasmic end by M1 (helix B) and the MBB data on Cx32 junctional channels would suggest M3 (helix C) lining the pore for most of the length of the membrane region, moving from the cytoplasm outward. This is the conclusion based on inferences from an unpublished 6 Å resolution density map (Unger & Yeager, personal communication). That work relies primarily on the above data to define M1 and M3 as composing helices B and C, and then identifies helix D as M4 on the basis having the greatest exposure to lipid and by far the least conserved residues exposed to it. This forces the assignment of helix A as M2. Given this, the trajectories of the cytoplasmic densities extending from helices B and C (not sufficiently resolved in the 7.5 Å structure), specifically the appearance of a lack of direct connectivity between helices A and B at the cytoplasmic end, suggests that they correspond to the separate structures M1/NT and M2/CL rather than M2 and M3, which are joined by the CL.

Another possibility is that helix B, contributing to the cytoplasmic end of the lumen, is M2. This is based on a bend in helix B near the extracellular end that is not clear in the 7.5 Å structure, but is resolved in the 6 Å structure. This would make helix C correspond to M3. This incorporates the positive SCAM results at two positions in M2, but ignores the data implicating M1.

There are several loose ends with the last two assignments. The criterion used to identify helix D as M4 would also identify M2 as not exposed substantially to lipid, since its sequence is the most highly conserved of all the transmembrane domains. Also, the proline kink in M2 is ignored, in spite of the necessity of proline generating a kink and the compelling argument made for its role in gating, based on mutagenesis and Monte Carlo structural simulations (Ri *et al.* 1999), discussed in Section 6.2.2. There is heavy reliance on SCAM data using MBB as a probe, but one must consider that MBB is large enough that its accessibility may be

restricted (though there is evidence it can permeate some connexin channels; Skerrett, Ahmed, Shin, Kasparek & Nicholson, submitted), and that reactivity in crevices accessible from the cytoplasm are not excluded. If this pillar supporting M3 is removed, the assignments mentioned first above become possible.

These considerations are based on structural data largely confined to the protein domains within the membrane. They are not informative regarding the pore-lining domains that lie in the extracellular gap, where the two hemichannels are joined, and are exposed to extracellular space rather than membrane lipid. The actual intercellular structure is presumably formed by the 24 E1 and E2 domains of the two hemichannels, and as mentioned above and in Section 5.1.3, E1 likely lines the pore in this region.

Clearly, no single assignment is consistent with all the data. In part, this no doubt arises because the data is from hemichannels and junctional channels, and from several connexins. Aside from the cautions about interpretation of SCAM data mentioned above, other factors may play a role, including fluctuations between channel conductance states, whether the 7.5 Å map is of an open or closed channel, the effects of truncation on that structure, and the fact that the data come from different connexins. At the present time, one way to resolve the conflicts is to consider that the amino acids exposed to the pore differ substantially in the various conductance states, possibly due to a combination of helical/connexin rotations and tilts. Such movements could translate a particular residue from the pore to a crevice or an inaccessible or differently accessible site. It is possible that this can be accounted for by the rotation and tilt model of gating originally proposed by Unwin (Unwin & Ennis, 1984).

4.2 Docking between hemichannels

The mechanism by which two hemichannels dock end-to-end to form junctional channels is not understood. Hemichannels protrude only about 20 Å from the plasma membrane (Yeager, 1998). Connexins are not glycosylated, eliminating the possibility that apposed hemichannels initially interact via extended glycosides. In cells, other membrane associated molecules presumably interact and bring the membranes into close apposition. Altering intercellular adhesion, either up or down, has profound effects on the efficacy of gap junction formation (Mege *et al.* 1988; Musil *et al.* 1990; Meyer *et al.* 1992). To date, there is no evidence for the direct involvement of a facilitating extracellular factor (a 'dockase') in the docking and binding process.

The binding reaction must involve several elements. It must be initiated by contact between connexin domains exposed to the extracellular aqueous environment. This contact must lead to a stable protein-protein seal that excludes ions, and must enable or allow the opening of the pore. This process could proceed in any of several ways.

One possibility is that the domains that seal off the extracellular end of the hemichannel are the same domains that interact across the junction. One could envision domains that seal against each other to close the pore and then rotate outward to seal against the corresponding domains of the apposed hemichannel, simultaneously de-occluding the lumen. A potential triggering step is that the close approach of the extracellular domains of the hemichannels displaces a postulated calcium ion(s) that keeps the extracellular end sealed. In this scenario, docking and opening of the gate that normally keeps hemichannels closed would be part of the same process.

Another possibility is that the binding and the opening are two distinct processes, mediated

by distinct but allosterically related structural elements. This scenario permits docking without opening of the pore; docking would enable opening, but not require it. There is evidence for a dissociation between docking and opening (Hülser *et al.* 2001; Zhu, Weber, Ciutaru, Kasperek & Nicholson, personal communication). In this view, the physical gate need not be at the extracellular end of the hemichannel, and could even be identical with the gates that respond to voltage or other modulators. If the ‘docking gate’ is deep in the pore, one expects some pore-lining residues to be accessible from the outside even when the hemichannels are closed. At the present time, there are no direct data on this point, but it is known that the gate that keeps hemichannels closed is extracellular to position 35 in Cx46 hemichannels (Pfahnl & Dahl, 1998).

There are fragmentary data on the postulated docking-enabled hemichannel gate. Replacing the E1 of Cx32 with that of Cx43 allows hemichannels to open (Pfahnl *et al.* 1997), suggesting E1 involvement. Also, heterotypic docking of wild-type Cx45 with CT-truncated Cx45 (which does not form homotypic functional junctional channels) seems to enable the truncated Cx45 hemichannel to open (Hülser *et al.* 2001), a ‘dominant positive’ effect. Cx35 forms functional hemichannels but its rodent ortholog does not, with essentially no difference in E1, but minor differences in E2 (Al-Ubaidi *et al.* 2000). On the other hand, mCx50 and hCx50 have identical E1 and E2 sequences, but the former forms open hemichannels and the latter does not (Zampighi *et al.* 1999; Valiunas & Weingart, 2000; Ebihara, personal communication).

4.2.1 Structural and molecular basis

What is the structural basis for end-to-end interactions between hemichannels – recognition, binding and connexin specificity? These interactions must involve the extracellular domains of hemichannels, composed of the two extracellular loops (E1 and E2) of each connexin monomer. At the highest available resolution, there is little structure to the extracellular domains of hemichannels. The limitation on resolution in these studies is disorder in the arrays and the necessity of reconstructing the interacting extracellular domains of two end-to-end hemichannels. Studies of chemically split junctions permit imaging of the extracellular face by deep-etch freeze fracture, but no significant structural features were revealed. The extracellular surface of hemichannels has been explored by atomic force microscopy following ‘force dissection’ of the gap junctions, in which the AFM probe literally strips off one layer of hemichannels from an isolated junctional membrane (Hoh *et al.* 1991, 1993; Lal *et al.* 1995; Hand *et al.*, In Press). Direct imaging by this technique reveals a central pore formed by a hexagonal protein structure. There is also an indication of modulation in the height of the protein at the periphery, which could contribute to a ‘locking together’ of the hemichannels in a junctional structure (Hoh *et al.* 1993).

In several reports, purified junctional structures show a remarkable ability to spontaneously form end-to-end structures. Solubilized connexin incorporated into liposome membranes show particles apparently causing aggregation of the liposomes (Mazet & Mazet, 1990). When dialyzed in the absence of lipid, double-layered connexin structures formed (Lampe *et al.* 1991). In the most dramatic illustration, purified recombinant hemichannels formed long filaments composed of end-to-end hemichannels, which under other conditions coalesced into 2D sheets of filaments (Stauffer *et al.* 1991). Filament formation was promoted by oxidizing

agents. The end-to-end interactions can occur in the absence (Stauffer *et al.* 1991) or presence (Kistler *et al.* 1993) of lipids.

What is the nature of the chemical interaction between hemichannels? Studies that investigate the formation or splitting of channels between cells to address this question are necessarily indirect and limited by the inability to distinguish modulation of chemical interactions between the hemichannels from the host of biological processes that could influence their interaction. For example, perfusion of liver with hypertonic solutions causes splitting of junctions, but this is most likely a cellular or tissue response to hypertonicity rather than a specific and direct disruption of the interhemichannel contact; exposure to the same solutions following homogenization of the tissue does not result in splitting (Goodenough & Gilula, 1974). Perfusion with calcium-free solutions enhances splitting (Peracchia, 1977); simple incubation of liver pieces in calcium-free medium also splits junctions (Hirokawa & Heuser, 1982). On the other hand, studies that examine interactions between purified hemichannels may be criticized for (a) using material subjected to harsh conditions during purification, (b) using a non-representative (i.e. damaged, immature, about to be internalized) population of channels, and (c) studying a process that in cells may involve factors other than connexin (e.g. adhesion molecules).

For the formation studies, extracted lens junctional membranes were solubilized with octylpolyoxyethylene (8-POE) into a population of single hemichannels. Some assembly of single hemichannels into end-to-end pairs was induced by exchange of the 8-POE with octylglucoside or decylmaltoside (Kistler *et al.* 1994). This process was unaffected by presence of 5 mM DTT or 500 mM NaCl, suggesting that ionic interactions and disulfide exchange are not involved under these conditions. Studies that characterize the conditions of splitting of junctional channels have typically used as starting material the small fraction of a tissue's junctional membranes that are resistant to solubilization by ionic detergent (Fallon & Goodenough, 1981; Baker *et al.* 1983), high pH (pH 12) (Hertzberg, 1984), or lower pH (9.5) with 4 M urea (Kistler *et al.* 1994). Conditions used to split isolated junctions include exposure to 8 M urea at pH 10 (Manjunath *et al.* 1984; Goodenough *et al.* 1988), 8 M urea at pH 12 (Goodenough & Gilula, 1974; Milks *et al.* 1988; Zhang & Nicholson, 1994), and pH 2 (Goodenough & Gilula, 1974). The harshness of these conditions limits their value as probes of molecular interactions, but the data are consistent with hydrophobic interactions stabilizing the end-to-end hemichannel interactions.

A parametric study investigated minimal conditions required for substantial splitting of junctions without membrane disruption (Ghoshroy *et al.* 1995). Exposure to 4 M urea at pH 8 in the presence of EGTA reliably split junctions without destruction of the hemichannels or the membrane, perhaps suggesting the contributions of hydrogen bonding to the stability of the junctional structure. Less concentrated urea, lower pH or omission of EGTA substantially reduced the splitting; 5 mM DTT was without effect. Since a chaotropic agent is required for disruption, these data strongly indicate that hydrophobic forces play a major role in stabilization of end-to-end interactions. Splitting under these conditions is not reversible; removal of urea does not cause reassembly. This may mean that the hydrophobic domains involved in the interaction become inaccessible to the aqueous environment, perhaps forming a 'gate' on the extracellular end of the pore (suggested in Peracchia *et al.* 1995). Potential hydrophobic domains involved in such a process are reviewed in Peracchia *et al.* (1995) and Ghoshroy *et al.* (1995).

The enhancing effect of EGTA on splitting supports suggestions from cellular work that

calcium stabilizes the end-to-end interactions (Peracchia, 1977). If so, it is important to explore by site-directed mutagenesis the roles of conserved potential calcium-binding sites in E1 and E2 (e.g. the three conserved aspartate and glutamate residues). It should be noted that low pH, which would neutralize such sites, is effective in breaking junctional channels apart, even in the absence of chaotropic agents. On the other hand, high pH is also effective, and EGTA alone has no effect (Zimmer *et al.* 1987; Ghoshroy *et al.* 1995).

A scenario emerges from these studies in which calcium ions coordinate the structure of interhemichannel hydrophobic domains. These interactions can be disrupted by removal of the calcium or by presence of chaotropic agents. Once disrupted, the hydrophobic domains interact within a hemichannel and become inaccessible from the aqueous environment, and are therefore unable to readily reassemble into junctional channels. Hydrophobic interactions are known to play key roles in dimerization reactions of proteins (Divita *et al.* 1995; Larsen *et al.* 1998; Koltzsch & Gerke, 2000). Intriguingly, there may also be a role for calcium in stabilizing the 'closed' structure, since removal of extracellular calcium causes hemichannels of some connexins to open in plasma membrane, as previously noted. Thus calcium, normally present at millimolar concentrations outside of cells, would also assist in stabilizing conformations favorable to intrahemichannel hydrophobic interactions.

Strictly speaking, these considerations apply only to disruption of interactions between hemichannels that have survived the conditions involved in purifying junctional membranes from cells. In fact, connexin structures the size of hemichannels can be immunopurified from plasma membrane solubilized by non-ionic detergents such as octylglucoside; these channels are undoubtedly solubilized by the ionic detergent and the alkali extraction methods, and discarded (Harris, 1994; Rhee *et al.* 1996). The yield from such studies is up to 40% of the total connexin in a tissue. Therefore conclusions drawn about the nature of the hemichannel interactions from studies using purified junctions may only apply to the fraction of junctional channels that survive the junction isolation procedures. That a substantial fraction of junctional channels can be broken apart by mild, non-ionic detergents implies that a substantial fraction of junctional channels are held together by more moderate forces.

On the other hand, freeze-fracture and thin section studies of dissociated cardiac cells showed junctions that remained attached to one of the separated cells (Mazet *et al.* 1985), and it has been reported recently that junctional channels can be internalized intact into one cell of a coupled pair (Jordan *et al.* 2001), suggesting remarkable stability under normal conditions.

Another approach to analyzing the docking interactions is to examine the effects of extracellular application of synthetic peptides whose sequences correspond to the extracellular loops E1 and E2 on the formation and stability of functional junctional channels (reviewed in Berthoud *et al.* 2000). Several such peptides have been shown to have precisely this effect, as if they mimic the homophilic-binding domains, whereas peptides corresponding to cytoplasmic regions did not (Dahl *et al.* 1994; Eugenin *et al.* 1998; Kwak & Jongsma, 1999). A detailed study found that for both loops, the peptides most effective in blocking formation were those toward the C-terminal ends of the loops (Warner *et al.* 1995). The proposed mechanism is that the peptides interact with the loop domains of the hemichannels that can potentially form junctional channels, blocking their interaction with the loop domains of an apposed hemichannel. It is tempting to think that in doing so the peptides assume the conformation they would have if part of an apposed hemichannel. How-

ever, none of the peptides appeared to be able to effect hemichannel opening, though this was not tested explicitly in some of the studies. There was no synergy between the effects of peptides directed against each loop, suggesting that each loop interacts with domains of the other loop (e.g. E1 with E2 and vice versa) in the apposing hemichannel. The sequences were: CNTLQPGC for Cx32 E1 (53–60), SHVR for both Cx32 (72–75) and Cx43 E1, and SRPTEK for Cx32 (182–187) and Cx43 (204–209) E2.

In an intriguing set of studies it was shown that peptides containing these sequence motifs were also effective in disrupting electrical coupling between cells already joined by gap junctions (Chaytor *et al.* 1997, 1998; Boitano & Evans, 2000) with a rate at least 4-fold greater than the most rapid reported turnover of plasma membrane connexin (time constant of 20–30 min as opposed to 120 min) (Laird *et al.* 1991; Beardslee *et al.* 1998; Berthoud *et al.* 1999). This raises the intriguing possibility that the homophilic binding between hemichannels is dynamic, and that the peptides interfere with ongoing binding and unbinding reactions. Such postulated reactions could occur in ‘stable’ junctional channels, or be part of the rapid turnover of junctional channels (i.e. the docking of new pairs of hemichannels, undocking of hemichannels fated for internalization, and possible re-docking). It is also possible that the peptides interact with exposed regions of the patent junctional channels and disrupt the junctional structures. These possibilities suggest that junctional channels are more plastic than previously thought.

What are the structures of the extracellular domains of hemichannels? As mentioned previously, each extracellular loop contains 3 cysteine residues with identical spacing across all connexins except one. It is well-established that disulfide linkages are not made between end-to-end hemichannels or adjacent hemichannels (Dupont *et al.* 1989), or between connexin monomers within hemichannels (John & Revel, 1991), despite an earlier report to the contrary (Manjunath & Page, 1986). There is positive evidence from studies using very different approaches that there are disulfide linkages between the two extracellular loops of a connexin monomer (John & Revel, 1991; Rahman & Evans, 1991), probably initially formed in the ER (Rahman *et al.* 1993). There is also evidence for disulfide-exchange reactions taking place during junctional channel formation (John & Revel, 1991). It has been suggested that these linkages stabilize the structure of these domains so that the inter-hemichannel adhesion can be achieved. Substitution of any of the cysteines with serine inhibits formation of junctional channels in the paired *Xenopus* oocyte system (Dahl *et al.* 1991, 1992), though in these studies the possibility of a disruption of connexin processing, assembly, or insertion prior to hemichannel docking remains.

The most informative data about the structure of the extracellular loops in junctional channels comes from innovative work in which the invariant cysteines in E1 and E2 were not eliminated, but displaced (Foote *et al.* 1998). The first and third cysteines within each loop were displaced sequentially in the same direction and then in opposite directions, and the ability to form functional junctional channels was assessed. Displacements of one position eliminated functional activity, as expected. However the surprising result was that function was rescued only when the first cysteine of one loop was displaced in one direction *and* the third cysteine in the other loop was displaced in the *other* direction. Furthermore, junctional channel activity occurred only when the cysteines were displaced in steps of two (e.g. the first cysteine of E1 moved back two positions and the third cysteine of E2 moved forward two positions). Function was not seen when the displacements were in the same direction. The periodicity of the mutations with recovered function suggests a β structure, and the

restoration of function with opposite displacements suggest interactions between anti-parallel strands. These findings and others are best explained if the two loops are in stacked β structures stabilized by inter-loop disulfide bonds between the first and third cysteines in each loop. Presumably there is a third inter-loop disulfide between the second cysteine of each loop (Fig. 8a). The anti-parallel β loops contributed by apposing and adjacent connexin monomers would interact to form β -barrel structures. CD spectra support the presence of some β structures in isolated junctional membranes, but not in the transmembrane domains (Cascio *et al.* 1990). Furthermore, 6 Å maps show vertical 'threads' of density on the perimeter of the channel, consistent with β structure (Unger & Yeager, personal communication).

These data suggest that the two hemichannels interact to form a junctional structure via formation of inner and outer anti-parallel β barrels composed of interdigitating loops from each hemichannel (Fig. 8b). Each barrel would be formed by 24 strands of either E1 or E2 loops. The disulfide bonds would link the two barrels, connecting the E1 and E2 contributed by the same connexin monomer. This model of the junctional channel, when taken with the evidence that, at least for some connexins, E1 plays a large role in defining pore selectivity (Sections 4.1, 5.1.3), suggests that the β barrel formed by the E1 loops would be innermost. The pattern of disulfide linkages is most readily accommodated if the E1 and E2 loops are stacked in an anti-parallel configuration, as shown in Fig. 8a. It is less likely that the disulfide linkages between the first and third cysteines in each loop would have to cross one another as shown in one of two possible arrangements in (Perkins *et al.* 1998a), forming a cysteine-knot structure often found in growth factors (Sun & Davies, 1995).

A concentric double β -barrel structure has not been observed in other proteins to date. A β barrel formed from strands entering the barrel from opposite ends of the barrel has not been reported either, however the efficacy and strength of this kind of interaction is demonstrated in the lateral interaction between β strands that mediate cadherin homophilic binding (Leckband & Sivasankar, 2000). The channels of bacterial porins are formed by single β barrels (Jap *et al.* 1991; Weiss *et al.* 1991); in that case the barrel is monomeric (all strands are part of the same peptide). A closer analogy is with the β barrel of α -hemolysins in which each pair of strands of the barrel is contributed to by each of seven subunits (Song *et al.* 1996; Gouaux, 1998). Stacked β sheets are common in several structural proteins (cf. Parkhe *et al.* 1997) and are seen in dimerization interfaces as well; the homodimerization of the DNA-binding domain of HIV-1 integrase is mediated by two stacked β structures stabilized by hydrophobic interactions and hydrogen bonding (Lodi *et al.* 1995; Eijkelenboom *et al.* 1999), as is the interaction between oppositely oriented β sheets in TGF- β 2 dimers (Sun & Davies, 1995). Dimerization by the double, edge-to-edge extension of anti-parallel β sheets is seen in the gelation factor of *Dictyostelium* (McCoy *et al.* 1999) and in the dimerization of transthyretin (Blake *et al.* 1974, 1978).

In other β -barrel channels, channel selectivity is determined by strands that dip into the lumen (MacKinnon, 1995; Schirmer *et al.* 1995; Phale *et al.* 1997; Wang *et al.* 1997), some as short as 9–16 amino acids (Schirmer *et al.* 1995; Phale *et al.* 1997). If the analogy is carried further in connexins, one may look for the involvement of strands of similar length, perhaps contributed by the regions N-terminal to the first cysteines of E1 or perhaps E2. Inspection of the sequences shows that there are 12 and 18 residues, respectively, between the extracellular ends of M1 and M3 and the first cysteine of E1 and E2, respectively. The fantasy is that a component of residues 41–52 of the E1 of Cx32 dip into the pore to affect charge selectivity and also interact with N2 to mediate voltage gating. This could include

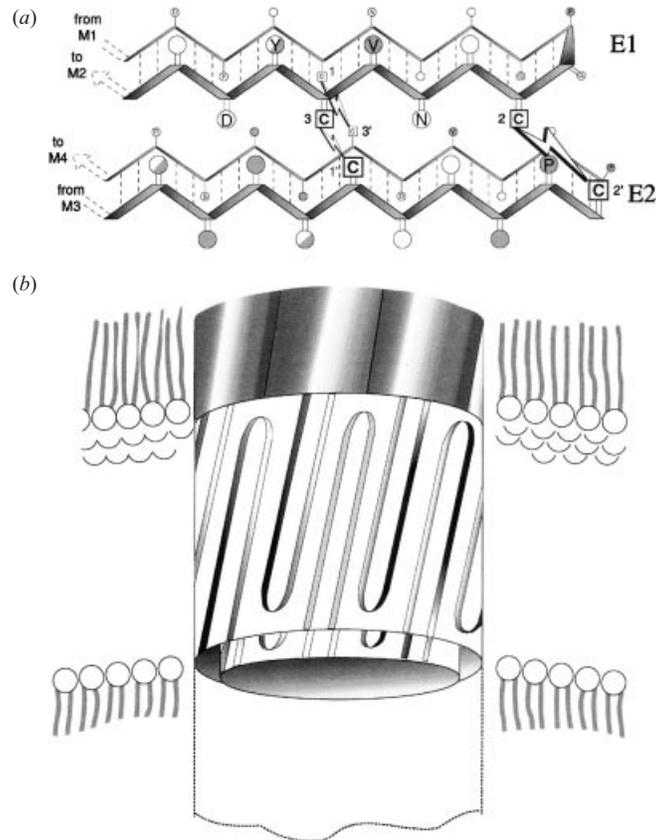


Fig. 8. Proposed docking domain structures. (a) Schematic of proposed relations between β structures formed by the extracellular loops (E1 and E2) of a single connexin, based on the work of Foote *et al.* (1998). Each loop forms a pair of anti-parallel β strands internally stabilized by hydrogen bonds. The two pairs of strands (E1 and E2) are stabilized relative to each other by disulfide bonds. In this model, immediately in front of (and behind) these two pairs of strands are two more pairs contributed by the apposing hemichannel (originating from the right in this diagram), with the E1s adjacent to each other and the E2s adjacent to each other. This interdigitated structure is extended by alternating contributions from each of the apposed hemichannels to form two concentric β structures, as illustrated in (b). The data from Foote *et al.* (1998) do not address which ring of β structures is innermost (that formed by E1 or E2), but other data (Trexler *et al.* 2000) suggest that it is E1. (From Foote *et al.* 1998.)

D51 and the three other positions in the E1 of Cx46, shown to influence charge selectivity (Kronengold *et al.* 2001; Kronengold, Trexler, Bukauskas, Bargiello & Verselis, personal communication), and E41 and S42 of Cx32, shown to affect voltage sensitivity (Verselis *et al.* 1994), and possibly E48 of Cx43, which strongly affects unitary conductance (Brink, Kumari & Valiunas, personal communication); more C-terminal regions of E1 have not been directly implicated in these properties.

A cautionary note is that the data upon which this interpretation is based do not distinguish between the formation of junctional channels *per se*, and the formation of junctional channels that are open. For this distinction to be made, biochemical or structural information must be obtained in addition to functional information.

Table 5. *Determinants of heterotypic specificity*

	K167 equiv.	Charge	N175 equiv.	Charge
Group A				
Cx30.3	A	Non-polar	H*	10% +
Cx37	V	Non-polar	H	10% +
Cx40	V	Non-polar	H	10% +
Cx43	T	Polar	H	10% +
Cx45	C	Polar	H	10% +
Cx57	K	+	N	Polar
Group B				
Cx26	K	+	N	Polar
Cx32	K	+	N	Polar
Cx46	R	+	N	Polar
Cx50	R	+	N	Polar

* Histidine is approximately 10% protonated at physiological pH.

4.2.2 Determinants of specificity of interaction

What determines the specificity of interhemichannel interactions – whether a hemichannel composed entirely of one connexin can form a heterotypic junctional channel with a hemichannel composed of a different connexin? Specificity of heterotypic channel formation is readily determined by pairing *Xenopus* oocytes expressing different connexins (although it is probable that non-connexin factors (e.g. adhesion molecules) help to define coupling specificity between cells expressing endogenous connexins). Table 3 summarizes the known specificities. The E2 extracellular loop is thought to be the primary structural component responsible for the specificity. This is based on studies in which chimeric connexins with substituted E2 domains were constructed. In every case, the origin of the E2 sequence determined the selective affinity of the connexin (Bruzzone *et al.* 1994a; White *et al.* 1994a). Sequence alignments for E2 of groups A and B reveal that the groups can be distinguished by the charge character at positions corresponding to K167 and N175 of Cx32 (Table 5).

Recent unpublished studies take advantage of this correlation and show by mutagenesis that, at least for Group B, the specificity is determined by these two positions (Zhu, Weber, Ciubotaru, Kasperek & Nicholson, personal communication). By changing the character of these residues to be more hydrophobic, Cx32 can be made to couple heterotypically like a Group A connexin (Cx32A). If the loop domains of apposing hemichannels are anti-parallel as suspected, one can postulate that ionic interactions between apposing 167 and 175 positions (K and N for Cx32) favor interaction, and that the more hydrophobic interactions between the corresponding positions in Group A (V and H for Cx40) favor their interaction. Thus changing the residues in Cx32 to be more hydrophobic would allow interactions with Group A, and disfavor interactions with Group B.

Interestingly, hemichannels of the altered Cx32 (Cx32A) and wild-type Cx32 can form heterotypic structures but do not function. This suggests either that the specificities above are really about the ability to open, not to dock *per se*, or that the Cx32A/Cx32 docking occurs via an abnormal structure that does not permit opening.

The compatibility determinants described above do not tell the whole story; other positions or structural factors may play a role. By sequence, Cx57 should be in Group B, but its compatibility is that of Group A. Cx30, which by sequence is also in

Group B, is unique in forming heterotypic channels with both compatibility groups. Furthermore, peptides corresponding to regions of E2 of Cx40 seem to inhibit the formation of Cx40 junctional channels but not Cx43 channels, and vice versa (Kwak & Jongsma, 1999), even though the E2 sequences should be compatible by the scheme described above.

Sequence comparison also offers an explanation for one of the exceptions noted in the compatibility group in Table 4, the absence of coupling between Cx30.3 and Cx45. Cx45 has a positive charge immediately adjacent to its position 175 equivalent (K217) that is unique to this position in either group. Cx30.3, three positions before the position 167 equivalent, has a positive charge unique within Group A (R160). If the strands from the apposed hemichannels have an anti-parallel orientation, the two unique positive charges could interact to inhibit binding.

In Cx31 the spacing between the first and second cysteine in E2 is greater by one than for all other known connexins. This may contribute to its apparent inability to form channels with any other connexin. Variant cysteine spacing cannot, however, account for the other cases where heterotypic channels do not function (Cx31.1 and Cx36).

In addition to the sequence determinants, the structure of the contacting domains is expected to have an influence. The displacement of a single cysteine in the E2 of Cx32 not only disrupts the ability to form functional channels, as expected, but enables formation of channels with a connexin with which it normally does not (Foote *et al.* 1998). Further evidence for the role of structure comes from studies of chimeric proteins showing that cytoplasmic domains can affect heterotypic coupling specificity (Haubrich *et al.* 1996).

As for the previous section, these compatibilities and incompatibilities do not distinguish junctional channel formation from junctional channel opening.

5. Permeability and selectivity

More than other properties, permeability and selectivity are at the core of connexin channel function. A large literature documents a wide variation of conductance, ionic selectivity and molecular permeability of channels formed by the various connexin isoforms. These properties are investigated in two major ways.

One is via electrical measurements, which reveal what can be inferred about the permeation pathway and process from unitary conductances, reversal potentials and single-channel $I-V$ relations. This approach draws on the highly evolved analytical framework applied to other channels.

Another approach is via measurements of molecular flux, which reveal what can be inferred about the permeation pathway and process from knowing which molecules move through the pore, and how well. This approach is less rich analytically, but draws in interesting ways on classical flux studies. It addresses directly the property of connexin channels most central to their biological function in inexcitable cells.

Given the latter, one may ask if it is worthwhile to understand the determinants of electrical conductance – the selectivity and permeation of atomic ions – of a channel whose function in most tissues is the selective permeation of much larger cytoplasmic molecules. Rephrased, why seek to understand the permeability to K^+ or Cl^- if the permeation pathway is designed to select for or against specific molecules, and not atomic ions? Will not the electrical measurements be informative only of an epiphenomenon – the largely irrelevant ion flux through a pore designed for another function?

This view is short-sighted for several reasons. One is that the role of connexin channels in nerve and muscle is sufficient justification; the character of electrical signaling in excitable tissues may depend crucially on the details of how atomic anions and cations permeate the pore and interact with each other while doing so. For example, as described below, the $I-V$ relations for several junctional channels and hemichannels rectify in symmetric salt. Understanding how this occurs addresses a fundamental mechanism of electrical signaling in nerve and muscle.

Another reason is that the mechanisms of ionic selectivity and interactions in a large pore are likely to be reprised in other macromolecules. Fundamental mechanisms of electrostatic interaction in this pore will be relevant to mechanisms in other constrained, yet minimally dehydrated environments such as access pathways to active sites of enzymes and the catalytic and binding clefts of enzymes and nucleic acids, as well as the inner and outer vestibules of ion-specific ion channels, and other channels with wide pores. Most ion channels are charge-selective, being highly permeable to either cations or anions. Understanding how anions and cations move through the same pathway, perhaps in opposite directions, requires a different analytic formalism. Such an understanding will also be relevant to the operation of other wide pores, such as porins, VDAC, and several channels that mediate toxicity (e.g. colicins, tetanus toxin, diphtheria toxin, botulinum toxin). In a reductionist view, ionic and intermolecular interactions in a wide pore, as in a connexin channel, are intermediate between those that occur on planar, fully hydrated surfaces and those that occur in narrow, 'dehydrated' pores – which describes interactions on the surfaces of most macromolecules.

Several features of connexin channels warrant special attention in this regard. Permeation of small atomic ions through them is fundamentally different from that through narrow pores such as *Shaker* or KcsA, in which the selectivity region is as long as connexin pores are wide (12 Å). To permeate those pores, an ion must interact at a specific site (e.g. the GYG region of KcsA). If its interactions at that site are not sufficiently favorable, the ion will not permeate. The same ion permeating a connexin channel does not need to bind to a specific site in the pore to pass through. It will experience a spectrum of forces as it permeates, but binding to a specific site is not obligatory for the ion to permeate. The mechanism that underlies much of our thinking about ionic selectivity is precisely the requirement to bind to an unavoidable site in a pore. This means that while the conductance and permeability of atomic ions through connexin channels will be informative, they do not arise from the kind of interactions that occur in narrower pores.

A second feature of connexin pores that requires some conceptual adjustment is that they are substantially permeable to both cations and anions. This adds a complex set of intra-pore electrostatic interactions that affect flux – attractive interactions between the mobile ions.

Both features are shared with several other channels, for which the classical GHK formulations of permeability do not hold (Borisova *et al.* 1986; Zambrowicz & Colombini, 1993; Franciolini & Nonner, 1994b). For these channels, as for connexin channels, conductance measurements are not faithful predictors of permeability.

Connexin pores have an additional feature not shared with other channels. In extracellular solutions the anions and cations are roughly the same size, and therefore are able to either enter or not a plasma membrane channel to roughly equal degrees. In cytoplasm, the concentration of anions small enough to enter a connexin pore is much less than the concentration of cations able to do so – $\sim 3.5 \text{ mM Cl}^-$ versus $\sim 140 \text{ mM K}^+$ (Adrian, 1961; McDonald & DeHaan, 1973; Maughan & Godt, 1989). This means that the normal charge-screening that

Table 6. *Approximate unitary conductances of junctional channels*

	pS (in 120–150 mM salt)
Cx26	140
Cx30	180
Cx31	100
Cx32	60
Cx36	15
Cx37	310
Cx40	175
Cx43	100
Cx45	30
Cx46	140
Cx50	220
Cx57	27

is a function of ionic strength is strongly skewed within connexin pores, which are accessible only by cytoplasmic ions; potassium ions and chloride ions can enter freely, but the concentration of K^+ is more than 10-fold that of Cl^- . Though this has not been treated analytically, a consequence must be to amplify the relative influence of positive charges inside the pore, if they are not part of charge-pairs. This implies that ionic replacement experiments during whole-cell dialysis (where small anions and cations are present in equal concentrations) may produce an electrostatic environment in and around the pore that does not reflect the native situation. The same situation may apply to sites of restricted access (e.g. crevices) of any protein (or nucleic acid) exclusively exposed to cytoplasm.

5.1 Among the usual ions

5.1.1 Unitary conductance

Recordings of single junctional channels in native and heterologous systems reveal a wide range of maximal single-channel conductances and subconductance states (for a recent compilation, see Verselis & Veenstra, 2000). The maximal conductances are referred to as ‘main-state’ conductances, whether or not they are the most occupied. In native tissues, main-state conductances of junctional channels range between ~ 20 and ~ 300 pS. Most junctional channels have several subconductance states, but several have none. The subconductance states can be as small as one-tenth of the main-state conductance. The magnitude of the unitary conductance may be influenced by surface charge, even where the channel is not charge-selective (Banach *et al.* 2000). A selective summary of the approximate conductances of main states of homomeric wild-type connexin channels determined in heterologous expression systems under roughly comparable conditions is given in Table 6.

In any given experiment, the recorded unitary conductances of homomeric channels typically have a broader variance about the mean than do most other channels. The reasons for this are unclear, but may reflect the inherent noisiness of such recordings (due to voltage clamp of two whole cells as a requirement for obtaining single-channel records; see Section 3.2.1). Most of the differences in reported mean values can be accounted for by differences in the species orthologs used, or by investigator experimental technique.

The unitary conductances of some hemichannels are somewhat rectifying, decreasing in a curvilinear fashion with positive potentials. Though first documented in excised patches in symmetric salt for Cx46 (Trexler *et al.* 1996; Pfahnl & Dahl, 1998, 1999; Trexler *et al.* 2000), a similar rectification is seen for Cx30, Cx46 and Cx50 in on-cell recordings (Valiunas & Weingart, 2000). Not surprisingly, the rectification is greater in the asymmetric conditions of the on-cell recordings. Accordingly, for Cx30 the on-cell data predicted a somewhat greater rectification than was seen in the (symmetric conditions of) junctional channel recordings.

Where there are single-channel data for hemichannels and for homotypic junctional channels formed by the same connexin, the conductances of the hemichannels predict the conductances of the corresponding junctional channels quite well (for Cx30, Cx46 and Cx50) with one exception (Cx32). There are fewer data for heterotypic unitary conductances, and they are complicated by the fact that heterotypic channels rectify strongly, due to the two hemichannels having different ionic selectivities (see Section 5.1.3 below). Nevertheless, conductances near $V = 0$ of heterotypic channels calculated from conductances of homotypic channels (where the conductivity of a homomeric hemichannel is presumed to be twice that of the homomeric junctional channel) correspond roughly for most cases for which there are data (predicted \rightarrow actual in pS: 84 \rightarrow 48 and 100 for Cx26/Cx32; 127 \rightarrow 100–150 for Cx40/Cx43; 85 \rightarrow 56 for Cx32/Cx46; 75 \rightarrow 120 for Cx43/Cx46; 51 \rightarrow 52 for Cx43/Cx44).

Serine phosphorylation can affect the distribution between main state and the substate occupancies of certain connexins. This is a gating effect, and is covered in Section 7.3.

5.1.2 Selectivity

On the basis of permeability to large tracers such as LY, it was believed for many years that connexin channels were so wide that they would be essentially non-selective among ions and molecules whose size allowed them to enter the pore. This idea persisted despite the fact that an informed consideration of the effect of charge on the walls of a ~ 15 Å pore predicts at least moderate charge selectivity. Experimental data on ionic selectivity for junctional channels similar to that routinely obtained for other channels was difficult to obtain before the era of dialysis through patch electrodes.

The presence of negative charge in the pores of connexin channels was inferred from dye-coupling studies (Flagg-Newton *et al.* 1979), as it had been for innexin channels (Brink & Dewey, 1980). Differential staining by anionic and cationic negative stains in electron microscopy of rat liver junctions (primarily Cx32) also suggested anionic charge in the pore (Baker *et al.* 1985). Measurement of charge selectivity by more conventional means (dual dialysis through patch electrodes) was first achieved in mammalian cells in 1985, when reversal potential experiments suggested a P_{K^+}/P_{Cl^-} ratio of 1.45 (Neyton & Trautmann, 1985) for junctional channels likely formed either by Cx26 or heteromers of Cx26:Cx32 (Meda *et al.* 1993) based on unitary conductance, also implying negative charge. However, an early reconstitution study showed an anionic selectivity for Cx32 (Harris *et al.* 1992) implying positive charge in the pore. It has since become clear that Cx32 is indeed a mildly anion-selective channel (Suchyna *et al.* 1999). Nevertheless, most junctional channels have a preference for cations, consistent with slight fixed negative charge in the pore. The first systematic examination of selectivity of an identified connexin expressed in mammalian cells (Cx37) showed a cation selectivity of 2.3 [(Veenstra *et al.* 1994b); later revised to 3.5

(Veenstra, 1996b)]. This was particularly notable since the unitary conductance was large, even for a junctional channel (300 pS).

Charge selectivity varies substantially with connexin isoform. Most data are conductance-mobility ratios derived from experiments in which symmetric ion substitutions were made via patch pipettes. Selectivity measured in this way varies over at least an order of magnitude, from nearly 10:1 for chick Cx45 to $\sim 1:1$ for rat Cx32 (Veenstra *et al.* 1995; Veenstra, 1996). The magnitude of the selectivity is *not* inversely correlated with unitary conductance – that is, selectivity is not greater in channels with smaller conductance as would be expected for a simple narrowing of a pore with charged walls. In the two cases where selectivity has been determined from reversal potentials in asymmetric salt solutions, P_{K^+}/P_{Cl^-} is much greater than that calculated from conductance-mobility ratios (7.7 instead of 2.3 for cCx43, and 6.9 rather than 3.4 for rCx40) (Beblo & Veenstra, 1997; Wang & Veenstra, 1997). It has been proposed that this difference arises due to the presence of Donnan potentials generated by fixed charges in the pore, and that they dominate the conductance ratios (Veenstra, 2000). The $P_{K^+}:P_{Cl^-}$ for Cx46 was recently determined to be 7:1 (Trexler *et al.* 2000).

The information on selectivity among cations or among anions is sparse, but intriguing. Overall, the selectivities determined from reversal potentials in asymmetric solutions are not dramatic. For rCx43, and for rCx46 hemichannels (Trexler *et al.* 1996), relative selectivities among cations correlated with aqueous mobility except for a preference for Rb^+ over Cs^+ , which suggests a weak anionic site (Wang & Veenstra, 1997). The selectivity sequence among anions roughly follows their aqueous mobilities. However, the experimentally measured reversal potentials for anions could not be described by the GHK equation using the P_{K^+}/P_{Cl^-} ratio determined from a single asymmetric salt reversal potential. This implies an interaction between anions and cations that influences selectivity (Wang & Veenstra, 1997).

A more dramatic example of this occurs for rCx40, where the cation sequence follows the aqueous mobilities, but the anion sequence does not match either the aqueous mobilities or an Eisenman sequence (Beblo & Veenstra, 1997). The permeabilities are equal for all the non-atomic anions tested, and that of the atomic anions vary *inversely* with their aqueous mobilities. That is, the electrical conductance of the pore is *reduced* by anion permeation. This is a complex form of the anomalous mole fraction effect – one that effectively involves different affinities for anions and cations. One explanation for this behavior is that complexes of permeant anion–cation pairs form in the pore and interact with fixed anionic sites (Beblo & Veenstra, 1997). In this case, the selectivity among anions and the component of the current carried by anions would be functions of salt concentration and the specific cation present. Similar mechanisms have been proposed to account for interactions between permeant anions and cations in certain other channels (Borisova *et al.* 1986; Franciolini & Nonner, 1994a, b). This kind of interaction can only occur in wide pores, and can be viewed as a localized, intra-pore charge screening effect.

The selectivity of subconductance states has not been extensively explored. Where it has, the data are consistent with a narrowing of the pore (i.e. reduced permeability to molecules that permeate the main state) and/or an enhanced charge selectivity (see Veenstra *et al.* 1994b; Trexler *et al.* 1996; Valiunas *et al.* 1997; Oh *et al.* 1999; Qu & Dahl, In Press). Future studies may address whether unexpected ionic or molecular selectivities appear in the substates.

5.1.3 Nonlinear single-channel I - V relations and their molecular determinants

As expected for channels composed of two oppositely oriented subunits in symmetric solutions, homotypic junctional channels show single-channel I - V relations that are symmetric around $V = 0$ (e.g. Veenstra *et al.* 1994b; Beblo & Veenstra, 1997; Wang & Veenstra, 1997; Suchyna *et al.* 1999; Valiunas *et al.* 1999b; Trexler *et al.* 2000). They also retain the charge selectivity of the component hemichannels (cf. Trexler *et al.* 2000). However, rectifying single-channel I - V relations are observed in currents through single hemichannels as noted above (Trexler *et al.* 1996; Pfahnl & Dahl, 1998, 1999; Trexler *et al.* 2000; Valiunas & Weingart, 2000) and through some heterotypic junctional channels in symmetric solutions (Bukauskas *et al.* 1995; Brink *et al.* 1997; Oh *et al.* 1999; Suchyna *et al.* 1999; Valiunas *et al.* 1999b, 2000), but not others (Elenes *et al.* 2001). Exploration of the origins of these rectifications have been informative as to their thermodynamic basis, the molecular determinants of selectivity, and identification of pore-lining amino acids. The two most extensively studied instances are described below.

In one case, a homomeric junctional channel (Cx30) has been reported to have a slightly rectifying unitary conductance (Valiunas *et al.* 1999a). The basis for this is not clear, though it is suggested to follow from detailed modeling of unitary conductances (Vogel & Weingart, 1998).

(a) *Cx46 and its E1*. The main state of rCx46 hemichannels is largely cation selective ($P_{K^+}:P_{Cl^-}:10:1$), high conductance (~ 300 pS) and inwardly rectifying in symmetric physiological saline (Trexler *et al.* 1996). The rectification takes the form of sublinear conductance at positive voltages and supralinear conductance at negative voltages. Cation selectivity and inward rectification can be accounted for by fixed negative charges near the extracellular end of the pore (impeding anion flux in both directions, and depressing entry of anions into the pore from outside). High salt on the outside reduces the cation selectivity to a greater degree than high salt on the inside, as if it screens fixed negative charges near the extracellular end of the pore (Green & Andersen, 1991). It also tends to linearize the single-channel I - V relation (Trexler *et al.* 2000), as expected.

These data were modeled using a form of Poisson–Nernst–Planck (PNP) theory (Chen *et al.* 1997b, 1999; Nonner *et al.* 1998; Nonner & Eisenberg, 1998). PNP theory is a continuum model of the distribution of ions within a right cylindrical pore for a given charge profile of the pore wall under a given set of ionic and voltage conditions. When calculations are made at different voltages, the results can be transformed into a predicted I - V relation. As it is a continuum model, it ignores the physical properties of the permeating ions except for charge and mobility. Because of this, it may be somewhat more applicable to wide pores than narrow ones [in which there is specific ion coordination as in the *Shaker* channel selectivity region (Roux & MacKinnon, 1999)]. Even so, PNP theory has reasonably accounted for the shapes of I - V relations of several ion channels, including a heterotypic junctional channel, discussed below (Chen *et al.* 1997a, b; Oh *et al.* 1999; Suchyna *et al.* 1999).

The version used to model the Cx46 hemichannel data was the 1D form, and did not include the modified Poisson equation that incorporates induced charge effects (Trexler *et al.* 2000). In this context, the modeling is more a test of the PNP theory than of the hypothesis of negative charge near the external mouth of the pore. Nevertheless, PNP calculations for a single, fixed negative charge near the extracellular end of the pore (0.25 pore length wide, centered 0.25 of the pore from the extracellular end) match the I - V relations and selectivity of the rCx46 hemichannels under symmetric and asymmetric salt conditions.

Studies of Cx46 junctional channels showed that the cationic selectivity was preserved when the hemichannels were joined, indicating that the end-to-end interactions between hemichannels in the dimerization reaction did not alter the charge selectivity. PNP calculations for two end-to-end hemichannel profiles accounted reasonably for the $I-V$ observed under asymmetric conditions.

Studies of chimeric hemichannels in which portions of Cx46 (140 pS; $P_{K^+}:P_{Cl^-}::7:1$) were replaced by the corresponding portions of Cx32 (50 pS; $P_{K^+}:P_{Cl^-}::1:1.2$) argue strongly that the E1 domain is a major determinant of the charge selectivity and thus is exposed to the permeation pathway (Trexler *et al.* 2000). Specifically, the Cx46*32E1 hemichannel has an outward rectification, a lower conductance and a slight anion selectivity. Higher salt on the inside tends to linearize the $I-V$ relation (there are no data on the effect on selectivity). On this basis it was proposed that in Cx46 the E1 domain forms a functionally significant portion of the pore lining, that it contains the negative charges that define selectivity, and that the E1 of Cx32 does not replace them.

Changing the sign and reducing the magnitude of the charge of the PNP model accounted for the anionic selectivity, but no amount of manipulation of the position or magnitude of the single charge could account for the $I-V$ relations. It is concluded that other factors, presumably also structurally contributed by the E1 of Cx32, play a role.

The effects of predicted asymmetric charge distributions in heterotypic channels were examined as well. Cx32/Cx46 channels have a dramatic outward rectification, as would be expected for hemichannels with pore charges of opposite sign (see Section 5.3.2 below for a detailed consideration). Rat Cx43 homotypic channels are essentially non-selective regarding charge. Cx43/Cx46 channels have an outward rectification as well as that expected for an asymmetric charge distribution in the pore (negative on the Cx46 side, zero on the Cx43 side), but of smaller magnitude. The results of PNP calculations for these heterotypic channels mimic the experimental findings.

What features of the E1 domains of Cx46 and Cx32 might be responsible for the observed differences in $I-V$ relations? The relevant sequences are compared below:

```

CX32E1:  41  ESVWGDEKSSFICNTLQPGCNSVCYDHFFPISHVR    75
          p   + p n  n   pp   ?n   n
          *   * * * * * * * * * * * * * * * *
          -   p - p  p   -p   +n   n
CX46E1:  42  EEVWGDEQSDFTCNTQQPGCENVCYDRAFPISHIR    76
    
```

*: identical; n: non-polar; p: polar; +, -: charge differences as noted; underlined: partially charged at pH 7.0.

Comparison of the amino-acid sequences shows that the Cx46E1 could present a more negative charge to the pore than Cx32E1; it has 3 more negative charges and 1 less partially ionized group. Both E1 domains contain 4 other residues that are partially charged at neutral pH, and whose protonation near physiological pH is therefore most readily modifiable by the local environment (Perutz *et al.* 1969, 1971). The positioning of the polar groups offers additional opportunities for effecting differences in charge selectivity, in addition to those due to the additional negative charges in Cx46E1 and the modifiable protonation of cysteine and histidine residues.

Of the amino acids that change when the Cx46E1 is replaced by Cx32E1, all but two

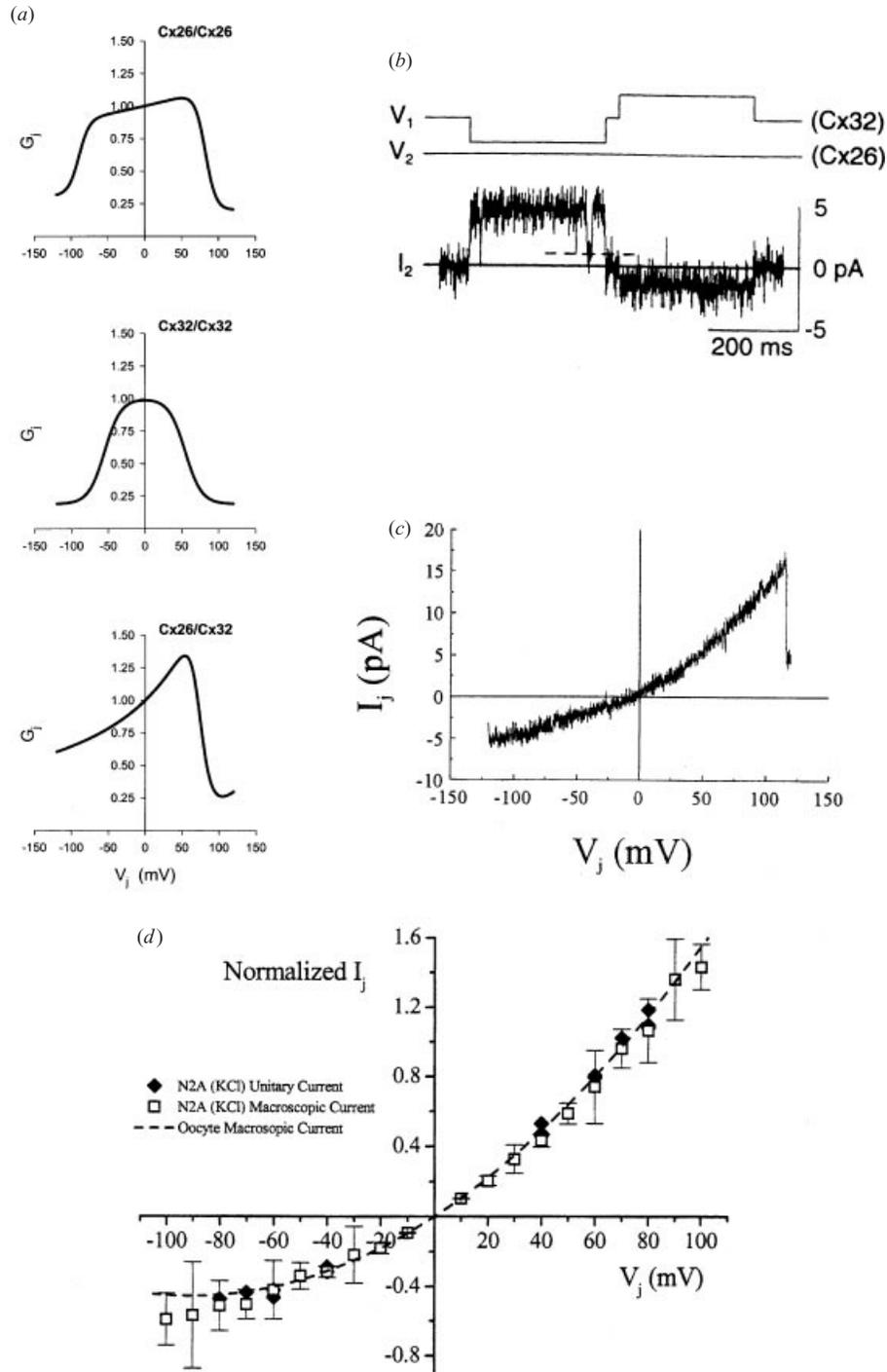


Fig. 9. Single-channel basis of macroscopic rectifying junctional currents. (a) Normalized macroscopic steady-state $G-V$ relations for homomeric Cx26, homomeric Cx32 and heterotypic Cx26/Cx32 junctional channels expressed in oocytes. Lines are calculated from equations in Barrio *et al.* (1991) that closely fit the data. The asymmetry of the Cx26/Cx26 $G-V$ relation is due to voltage dependence of initial conductance commonly seen for Cx26 in oocytes. (b) Single-channel conductances for Cx26/Cx32 junctional channels. The single channel $I-V$ relations for homomeric Cx32 and Cx26 channels are linear.

change to smaller or similar-sized R groups, and those that are larger (T → I; A → F) are uncharged and non-polar. In every case where there is a charge change from negatively charged to uncharged but polar, the polar residue is smaller (E → S; D → S; E → N). In the one case of a polar residue changing to one positively charged (Q → K), the positively charged residue is larger. These changes are consistent with the changes in selectivity. The sole exception to this trend of potentially decreased negativity in the pore is the R → H change (H is ~ 10% protonated at pH 7.0, and smaller).

Recent work shows that the Cx46 conductance phenotype can be largely restored to the Cx46*32E1 chimera by making the point mutations K49Q and S51D – that is, by changing only the residues in those two positions back to what they are in Cx46 (Kronengold *et al.* 2001; Kronengold, Trexler, Bukauskas, Bargiello & Verselis, personal communication). These changes replace a positive charge with a polar residue (K → Q), and a polar residue with a larger, negatively charged residue (S → D). It was shown that an S51C change largely restored the phenotype as well. This cysteine was accessible by MSTET and MTSEA from either the extracellular or cytoplasmic side of the channel, and alter the single-channel $I-V$ relations in a manner consistent with their respective charges. Thus the mutagenesis and the modeling converge to a common mechanism and structural element.

(b) *Cx32 and Cx26*. The Cx32 homotypic junctional channel has a main state conductance of approximately 60 pS in physiological salt, and is somewhat anion selective. The Cx26 homomeric junctional channel has a main state conductance of ~ 140 pS and is somewhat cation selective. Both homotypic junctional channels have linear $I-V$ relations. However, the heterotypic Cx26/Cx32 junctional channel has a strongly rectifying main-state conductance in symmetric solutions (Bukauskas *et al.* 1995). The conductance increases with positive potentials on the Cx32 side of the junctional channel (1.7-fold between -50 and +50 mV; 2.3-fold between -100 and +100 mV) (Fig. 9). The basis for this rectification has been explored theoretically by two groups, and experimentally by one of them. It is likely that single-channel currents through Cx30/Cx32 channels show similar rectification, by extrapolation from macroscopic data (Dahl *et al.* 1996).

In one case, a variant of the PNP theory was applied in which the charge selectivity of the component hemichannels was modeled either as due to difference in bulk-pore partition coefficient and intra-pore diffusion constant, or as a difference in Donnan-induced surface potentials at the mouths of the pore (Suchyna *et al.* 1999). In either case, when a potential is applied, there is an initial mismatch between the anion and cation fluxes, due to the different selectivities of each half of the pore. The requirement that the ionic fluxes in each half of the junctional channel be equal induces both a distortion of the potential within the pore and an accumulation or depletion of anions and cations within the pore, depending on the direction of current flow. These flux-induced field distortions are required to equalize the currents in the two halves of the channel. For the case where the selectivity is due to intra-pore diffusion constants, not surface charge at the entrance, the rectification arises because one voltage polarity causes an accumulation of ions (both cations and anions) in the pore, and the other polarity a depletion of them.

(From Bukauskas *et al.* 1995.) (c) Single-channel $I-V$ relations for Cx26/Cx32 junctional channels obtained with a voltage ramp. (From Oh *et al.* 1999.) (d) Comparison of macroscopic and single-channel $I-V$ relations of Cx26/Cx32 junctional channels. The macroscopic behavior is accounted for by the rectification of the single-channel conductance. The oocyte macroscopic data are from Barrio *et al.* (1991). (From Suchyna *et al.* 1999.)

When the experimentally measured values for selectivity are incorporated, both implementations predict a rectification of the same shape as that observed experimentally, but of a somewhat smaller magnitude. Interestingly, when the $I-V$ relation is calculated imposing a constant field (an unrealistic constraint in this system), the fit is better. The deviations from the data may be explained by the simplifications of the modeling, which include assuming a physically and electrically featureless, cylindrical pore, the mobile charges as points occupying no physical space, and an absence of hemichannel–chemical interactions.

This implementation of PNP theory did not permit determination of the relative roles of the differences in selectivity and conductance in producing the rectification, nor did it address whether the rectification *requires* that the selectivities of the two halves of the pore be different for anions and cations. Nevertheless, the fundamental character of the rectification is accounted for by the properties of the component hemichannels, as inferred from their properties in homotypic channels.

The other application of PNP theory to Cx26/Cx32 channel rectification is less analytic but takes advantage of a series of mutants to establish its utility in accounting for the character of a variety of shapes of $I-V$ relations, as well as identifying potentially influential amino-acid positions (Oh *et al.* 1999). For Cx32 and Cx26 homotypic channels, symmetric charge profiles were generated for which the PNP predictions for $I-V$ relations, unitary conductance and charge selectivity matched those of the experimental data. The simple series combination of these charge profiles for a Cx32 hemichannel and a Cx26 hemichannel produced $I-V$ relations, conductance and selectivity that closely matched those of Cx26/Cx32 heterotypic single channels.

Studies on determinants of the polarity of V_j gating of Cx32 and Cx26 had suggested that charges at positions 1 and 2 (including the positive charge of the N-terminal methionine) in both connexins, and position 42 in Cx26, and possibly position 41 in Cx32 (both in E1), sense the junctional voltage (summarized in Section 4.1). Given that these positions are not in the explicit transmembrane domains, it was proposed that they sense the field by virtue of being in the aqueous pore (Verselis *et al.* 1994). Charges at these positions were therefore good candidates for determinants of charge selectivity as well. Studies of single-channel $I-V$ relations of chimerae and point mutations support this idea. A series of chimeric channels demonstrated (a) symmetric $I-V$ relations for all homotypic channels, (b) symmetrically rectifying relations for homotypic channels where the charges were changed from wild type, (c) slight asymmetric rectification when a charge-changed connexin is heterotypically paired with the wild type of the other, (d) strong rectification when such a connexin is (now, heterotypically) paired with its own wild type.

On the basis of these studies, the charge profiles used initially in the PNP calculations incorporated charges of the appropriate polarities near the ends of the junction channels, and a positive charge deeper in the pore for Cx26 (corresponding to E42). In addition, the profile for Cx32 included a small negative baseline charge, corresponding to a smeared charge along the length of the pore (possibly due to backbone carbonyls). The profile of Cx26 required a much smaller baseline charge of opposite polarity. These charge distributions accurately reproduced the rectification of wild-type Cx26/Cx32 channels. However, they could not account for the shape of the $I-V$ relations for one set of chimerae. To simultaneously match the shapes of the $I-V$ relations of the mutants and the degree of rectification of the Cx26/Cx32 channel, two additions were made to the charge profiles – a negative charge at W3 of both connexins, possibly provided by the quadrupole moment of the aromatic ring,

and a negative charge in Cx32 corresponding to E41. With these changes, reasonable fits to all the $I-V$ relations were obtained. To summarize, the $I-V$ relations of the Cx26/Cx32 channels could be accounted for by incorporation of charges corresponding to those at positions 1, 2, 3, 41 and 42, plus smeared charge.

As for the implementation of PNP to the Cx46 hemichannels in Section 5.1.3(a), one must keep in mind the well-known limitations of the PNP theory (Miller, 1999). In addition, while each of the elements of the charge profiles in this study has a basis in structure, the relative magnitudes, widths, and to some extent, positions, of the fixed charges, as well as the magnitude of the baseline charge, are all adjustable parameters, as are the ionic mobilities within the pore. On the other hand, the criteria for an acceptable fit to the data include shape of $I-V$ over ± 150 mV, correct charge selectivity in 1:10 salt gradient, and correct channel conductance. Given these criteria and the variety of structural mutants the model was required to fit, and given the simplifications of the PNP approach, the explanation appears robust.

5.2 Among large permeants

The studies described above are not very informative as to the internal structure of the connexin pore or its interactions with large molecules, yet these properties are the primary determinants of the intercellular molecular signaling function of connexin channels. Connexin channels vary greatly in their permeability to molecular tracers, but the mechanisms of discrimination are unexplored. Permeant molecules can be considered in three categories.

For uncharged permeants (i.e. cytoplasmic molecules such as sugars), in addition to diffusion constant, the primary determinants of absolute and relative permeability are the minimum diameter of the pore and how long it is, respectively. For these permeants, the absolute selectivity is based on entry into the pore, and relative permeability roughly determined by size (as reflected in intra-pore diffusion constant) with larger permeants diffusing somewhat more slowly within the pore due to ‘frictional’ forces (see Renkin, 1954; Verselis & Veenstra, 2000).

At the other extreme are molecules for which the determining factor is a specific molecular interaction with the pore (e.g., site-specific, thermodynamic binding). This could produce selectivity among second messengers of similar size. Such effects could take the form of an absolute selectivity [in which a permeant must interact with the channel to permeate, analogous to the requirement that an ion must interact strongly at the ‘selectivity filter’ of an ion-selective channel in order to permeate (Hille, 1975; Doyle *et al.* 1998)], or a relative selectivity [in which the permeation of a particular cytoplasmic molecule is substantially enhanced over that of others of similar size, analogous to the facilitated but not exclusive permeability of maltose through maltoporin (Freundlieb *et al.* 1988; Klebba *et al.* 1994; Schirmer *et al.* 1995)].

The intermediate class of permeants (large and charged but interacting with the pore only via non-specific means, such as local charge field effects) likely includes most potential cytoplasmic permeants. The primary determinant of selectivity would be interactions between the permeant and charge on the wall or mouth of the pore, to which it would come into closer approach than would an atomic ion.

It is therefore important to know three kinds of information about connexin pores: their limiting diameter, their charge selectivity among large molecules, and their ability to select

or facilitate the passage of specific cytoplasmic molecules. The data on this subject have not generally been gathered in a way that readily distinguishes among these classes of potential molecular selectivity. The discussion that follows attempts to do so.

5.2.1 Uncharged molecules

For the most part, estimates of limiting pore diameter have been inferred from studies using charged, heterogeneous tracers. Uncharged probes have been utilized for only a few identified connexins. They offer the most direct way to separate the influence of size from other parameters affecting permeability; they can define the limiting diameter of a connexin channel, independent of charge. They have been successfully employed in two ways. One is to use uncharged molecules whose flux through the pore can be monitored by chemical detection, fluorescence or radioactivity. The other is to use uncharged molecules to affect the flux of atomic ions through the pores.

A set of oligomeric, uncharged, fluorescent sugars (described in Section 3.3.1) was used with the TSF system described in Section 3.4 to characterize the pore size of homomeric Cx32 and heteromeric Cx26:Cx32 hemichannels (Bevans *et al.* 1998). The tracers were loaded into a population of liposomes, of which a large fraction contained immunopurified hemichannels. The TSF fractionated the liposomes into two populations, one composed of liposomes with functional hemichannels, and one without. By HPLC analysis of the tracers retained in the liposomes in each population, it was determined which tracers were permeable; permeable tracers diffuse out through the hemichannels soon after loading, and the tracers retained in the liposomes without functional channels serve as internal controls for trapping and leakage. The data showed that tracers 3-PA and smaller (i.e. derived from trisaccharide, or smaller) were permeable through Cx32 channels (and larger ones were impermeable), whereas only tracers 2-PA and smaller were permeable through heteromeric Cx26:Cx32 channels. The presence of Cx26 clearly reduced the diameter of the pore. Since the population of Cx26:Cx32 channels used was heterogeneous and uncharacterized (i.e. the stoichiometries of the isoforms composing the channels in the population were variable and unknown), it was not possible to say how many Cx26 monomers were required in a hemichannel to exclude the 3-PA tracer. The difference between the minimum axial van der Waals cross-sectional dimensions of 2-PA and 3-PA in projection is about 0.5 Å in the smaller axis (4.4×3.4 Å *versus* 4.4×3.8 Å). Hydration, molecular flexibility, and other factors prevent a precise determination of the cross-sectional pore dimensions, but it is significant that such a small difference in size could be distinguished. It is also significant that the difference in size is in the minor axis, which suggests that the outline of the pore of the Cx26:Cx32 heteromeric channels at the narrowest point is not circular, consistent with the different (heteromeric) monomers protruding differently into the pore rather than a symmetrical, cooperative narrowing with heteromericity.

A more elegant approach examined the effects of non-electrolytes of several sizes [including a series of polyethylene glycols (PEGs)] on the unitary conductance of junctional channels formed by wild-type Cx32 and a S26L Cx32 mutant (Oh *et al.* 1997). With 50 mM of a PEG too large to enter the pore (PEG 900), the unitary conductance of wild-type Cx32 was essentially unchanged from that in normal saline. With successively smaller PEGs, the unitary conductance remained constant until the change from PEG 400 (hydrodynamic radius 7 Å) and PEG 300 (hydrodynamic radius of 5.8 Å), with which the conductance dropped to

nearly one-half its former value. It stayed at this level with smaller non-electrolytes down to the size of glycerol. The change in unitary conductance occurs when the non-electrolyte is small enough to enter the pore, where it impedes ion flux (Krasilnikov *et al.* 1992; Bezrukov & Vodyanoy, 1993). These data place the limiting pore diameter roughly between 6 and 7 Å, if the hydrodynamic radius is the relevant size. The S26L mutant, which causes peripheral demyelination in humans (see Section 8), did not show a corresponding drop in conductance even with nonelectrolytes down to the size of glycerol (hydrodynamic radius of 3 Å). This suggests that the S → L substitution narrows the pore substantially so that it excludes even glycerol (on the basis of these studies it was proposed that the S26L mutation would cause cyclic nucleotides to be excluded, as a molecular basis for the pathology).

Large, uncharged molecules are used to maintain osmotic balance in asymmetric dual internal dialysis experiments. From these studies, it is clear that mannitol and stachyose (radius ~ 6.4 Å) are not permeable through Cx40 channels (Beblo & Veenstra, 1997), but that mannitol is permeable through rCx43 channels, which are impermeable to raffinose (radius ~ 5.6 Å) and stachyose (Wang & Veenstra, 1997). This indicates that the limiting diameter of rCx43 channels is greater than that of rCx40 channels. Recently it has been shown that sucrose can reduce the unitary conductance of Cx46 hemichannels (Dahl, personal communication).

Unpublished work with PEGs and mannitol shows that Cx26 and Cx37 are not permeable even to PEG 200, but are permeable to mannitol (Gong & Nicholson, In Press).

These studies suggest the following rankings in terms of limiting diameter: Cx32 > Cx26 ~ = Cx37 and rCx43 > rCx40.

5.2.2 Charged molecules

Large charged probes of connexin channels have also been utilized in two ways. One is to electrically measure the charge carried through the pore by large organic ions such as tetra-alkylammonium derivatives. The other is to examine the flux of charged fluorescent tracers such as LY.

The tetra-alkylammonium derivatives have a long and respected history as probes of ion channels (Hille, 1975; Dwyer *et al.* 1980; Hess *et al.* 1986). TMA, TEA and TPA, but not TBA have been shown to be permeable through gap junctions composed of unidentified connexins in several systems (Weingart, 1974; Verselis *et al.* 1986a, b; Kurjiaka *et al.* 1998). Tetra-alkylammonium ions have been applied to three identified connexins to date. Junctional channels of rCx40 were permeable to TMA (radius 3.47 Å) and TEA (radius 4.00 Å), but increasingly blocked in a concentration and voltage dependent manner by TBA (radius 4.94 Å) (Beblo & Veenstra, 1997), TPcA (radius 5.25 Å) and THxA (radius 5.75 Å) (Musa & Veenstra, 1999). Channels formed by rCx43 were permeable to TMA and TEA (TBA experiments were unsuccessful; Wang & Veenstra, 1997). Hemichannels formed by Cx46 were permeable to both TMA and TEA (Trexler *et al.* 1996).

Data from studies of fluorescent tracers are difficult to interpret in terms of pore properties because, with some obvious exceptions, the molecules differ in shape, flexibility, charge, charge distribution, and chemical interactions. Thus, when differences in permeability are seen, inferences about which property of the tracer made the difference must be made with care. Ideally, to examine charge selectivity one would like a set of probes of equal size,

Table 7. *Tracers mentioned*

Tracer	MW (Da)	Size (Å)	Charge
Alexa350	349	14.1 × 13.7 × 8.1	-1
Alexa488	570	14.1 × 13.7 × 11.5	-1 (-2, +1)
Alexa594	759	17.4 × 17 × 14.8	-1 (-2, +1)
Calcein	623		-4 (-6, +2)
Calcein blue	321		
6-carboxyfluorescein (6-CF)	376	12.6 × 12.7 × 8.5 (Brink & Ramanan, 1985)	-2
4,6-diamidino-2-phenyl-indole dihydrochloride (DAPI)	279	15.4 × 6.0 (Elfgang <i>et al.</i> 1995)	+2
Dichlorofluorescein (DCF)	401	12.3 × 12.7 × 5.5 (Brink & Ramanan, 1985)	-1
Ethidium broimide (EB)	314	11.6 × 9.3 (Elfgang <i>et al.</i> 1995)	+1
Hydroxycoumarin carboxylic acid (HCCA)	206		-6
Lucifer yellow (LY)	443	10.6 × 9.5 (Elfgang <i>et al.</i> 1995), 12.6 × 14 × 5.5 (Brink & Ramanan, 1985)	-2
Neurobiotin (NB)	287	12.7 × 5.4 (Elfgang <i>et al.</i> 1995)	+1
Propidium iodide (PI)	441	12.9 × 9.3 (Elfgang <i>et al.</i> 1995)	+1

structure and comparable chemistry, yet differing in the magnitude of evenly distributed charge.

The initial characterization of connexin pore diameter was carried out with a set of fluorescently labeled amino acids, linear peptides and sugars (Flagg-Newton *et al.* 1979; Schwarzmann *et al.* 1981). Several mammalian cell types were studied. Two points were noted: (1) that the different cells had different permeability profiles to the tracers, and (2) that the cut-off was between 16 and 20 Å diameter, based on Corey–Pauling models of the probes. The data also suggested that the more negatively charged the probe, the less the permeability.

Since that early work, a vast literature has reported the use of charged, usually fluorescent, molecular tracers to establish and characterize junctional coupling. The discussion here is limited to those cases where a systematic effort was made either to characterize the pore of an identified connexin with several tracers, or to compare the permeability properties of several connexins to one or more tracers. These criteria exclude a large body of data in which single tracers were tested in single connexins in a variety of systems, assessed idiosyncratically. They also avoid the confusions that would result from comparing data from different systems gathered in different ways. The properties of the dyes used in the discussed studies are summarized in Table 7

The first study to systematically and definitively show connexin-specific molecular permeabilities used four fluorescent tracers of different charges and sizes compared side by side (Elfgang *et al.* 1995). The tracers LY, propidium iodide (PI), ethidium bromide (EB) and 4,6-diamidino-2-phenyl-indole dihydrochloride (DAPI) were microinjected into single cells of HeLa cell lines individually transfected to express each of seven connexins. A rough electrophysiological estimate of junctional conductance showed that it was within a factor of 2 across the cell lines. The degree of permeability was taken as the number of cells to which the dye spread under standard conditions. Each connexin had a characteristic ‘fingerprint’ of relative permeabilities to the tracers, establishing clear distinctions among the permeation pathways. However, given the differences in the probes and the possible interactions between

size and charge selectivity, it was difficult to conclude much about the specific nature of the pores from these data. However it was clear that the permeability profile of each connexin was distinct.

A more recent study extended this analysis to a comparison of wild-type and chimeric Cx26 and Cx30 permeabilities to LY and neurobiotin (NB). In HeLa cells, Cx26 is permeable to LY while Cx30 is not. A chimeric Cx26 containing the CL and CT of Cx30 (Cx26*Cx30CLCT) was not permeable to LY, while the reciprocal exchange (Cx30*Cx26CLCT) was permeable. The data indicate that whatever else may play a role in determining permeability to large anions, the CL and CT domains can have a defining effect, whether direct (as a filter at the pore opening) or indirect (by affecting the structure of transmembrane domains). These domain swaps affected unitary conductance in a similar manner (voltage dependence was also altered, but not in a readily explainable manner).

In another study, the permeability to several dyes of cells expressing Cx43 and both Cx43 and Cx45 were compared (Koval *et al.* 1995). When normalized to conductance, compared to the permeability of Cx43 channels, the co-expression of Cx45 had no effect on the permeability of hydroxycoumarin carboxylic acid (HCCA) and calcein blue, but substantially reduced the permeability to calcein and LY. All the dyes were negatively charged. This indicates that the Cx45 contributes to channels that are narrower and/or more cation selective.

The first attempt to control for probe size while changing charge compared the permeabilities to two structurally similar molecules of different charge, 6-CF (6-carboxyfluorescein) and DCF (dichlorofluorescein) (Veenstra *et al.* 1995). Molecular modeling shows their van der Waals surfaces to be nearly identical, yet their electrostatic potential contours are very different, with 6-CF carrying an additional exposed negative charge. By comparing the extent of dye coupling in transfected cells, it was possible to establish a ranking of permeability for large, negatively charged permeants: rCx43 > cCx43 > rCx45 \gg Cx37 = Cx40. This sequence does not match the sequence of cation/anion selectivity from the conductance-mobility data above (Section 5.1.2), which would have predicted the sequence rCx43 > cCx43 > hCx37 > Cx40 > Cx45. Greater selectivity than expected from the conductance-mobility data could indicate a narrower pore in a charged region. This would suggest that Cx37 and Cx40 had the narrowest pores.

A more quantitative analysis was carried out in HeLa transfectants. The permeability to LY and DAPI, two oppositely charged tracers, was assessed by the kinetics of dye spread and normalized to an indirect index of the channel number [this approximates a P_i/g_i ratio normalized across connexins (Verselis *et al.* 1986b)]. The calculated LY permeability decreased in the order of Cx32 > Cx26 > Cx45. This would not be informative of itself, but it was also found that the permeability to DAPI increased with the opposite order Cx32 < Cx26 < Cx45 (Cao *et al.* 1998; Nicholson *et al.* 2000). This strongly suggests that these sequences are dominated by charge, not size. The greater LY permeability for Cx32 over Cx26 was confirmed by kinetic analysis of LY spread in paired *Xenopus* oocytes.

The same system was used to assess the sizes of the pores for Cx32, Cx26 and Cx43 (Nicholson *et al.* 2000) using three Alexa probes. The probes differ from each other by the number of conjugated aromatic rings and the number of fixed charges, but have the same overall charge of -1 ; they are not ideal probes, but better than many. The permeability of each probe was assessed for each connexin by the same analysis and normalization as previously. Size of probe did not matter for permeability through Cx43 or Cx32, with

somewhat increasing permeability to the smaller probes. Cx26 showed the same permeability as Cx32 to the smallest probe, and drastically reduced permeability to the larger ones. The simplest explanation is that the limiting diameter of Cx43 is wider than that of Cx32, which is wider than that of Cx26. This inference is bolstered by the data from uncharged tracers that Cx26 hemichannels are narrower than Cx32 hemichannels (Bevans *et al.* 1998). Unpublished work has extended this study to several other connexins (Gong & Nicholson, In Press). In terms of limiting diameter, the combined results may be summarized as: Cx43 > Cx32 ≫ Cx26 ~ = Cx40 ~ Cx45 ≫ Cx37.

In another study, the permeability of Cx43 and Cx32 channels to calcein was assessed on a rough per-channel basis. It was found that the permeability through Cx43 channels was slightly greater than that through Cx32 channels, ratio of 1.08:1.0 (Goldberg *et al.* 1995, 1999).

A recent study directly compared the DAPI and LY permeabilities of Cx32, Cx43 and Cx46, normalized for junctional conductance. Cx46 was impermeable to LY and permeable to DAPI, whereas the opposite results were found for Cx32. Cx43 channels were permeable to both dyes (Trexler *et al.* 2000). This is in accord with Cx46 being relative cation selective and Cx32 being relatively anion selective [supported by hemichannel data (Harris *et al.* 1992) and junctional channel data (Oh *et al.* 1997)].

The conclusions from these studies are self-consistent. The fact that the experiments used very different methods and probes lends credence to their summative conclusions. Although it is not much to show for such difficult and tedious work, these studies suggest that:

- (1) for limiting diameter: Cx43 > Cx32 ≫ Cx26 ~ = Cx40 ~ Cx45 ≫ Cx37;
- (2) Cx43 is wider and/or less cation selective than Cx45;
- (3) Cx43 is wider and/or less charge selective than Cx32 and Cx46;
- (4) for permeability to large anionic molecules: Cx32 > Cx26 > Cx45 ≫ Cx37, Cx40
Cx32 ≫ Cx46
Cx43 > ~ Cx32;
- (5) for permeability to large cationic molecules: Cx45 > Cx26 > Cx32 Cx46 ≫ Cx32.

For comparison, these connexins ranked by decreasing unitary conductance are:

$$\text{Cx37} > \text{Cx40} = \text{Cx46} > \text{Cx43} = \text{Cx26} > \text{Cx32} > \text{Cx45}$$

and by decreasing selectivity for cations are:

$$\text{Cx40} > \text{Cx46} = \text{Cx43} > \text{Cx45} = \text{Cx37} = \text{Cx26} > \text{Cx32}.$$

These data also show that the characterization of the permeability of the connexins to large molecules bears little relation to their unitary conductances or to their charge selectivity among atomic ions.

5.2.3 Cytoplasmic/signaling molecules

One of the first functional descriptions of gap junctions was their ability to mediate intercellular movement of metabolites (Subak-Sharpe *et al.* 1966, 1969; Gilula *et al.* 1972). That cytoplasmic molecules diffuse through junctions is a trivial point. That *different* connexin channels allow *different* cytoplasmic molecules to diffuse from cell to cell, or favor the movement of specific cytoplasmic molecules where the discrimination is not simply due to

size and overall charge, is not. The mechanisms of such discrimination are of acute biological and medical interest.

The movement of cAMP and RNA through junctional channels was demonstrated nearly 30 years ago (Kolodny, 1971; Tsien & Weingart, 1974; Lawrence *et al.* 1978). Since then, evidence has been gathered strongly indicating that junctional channels or hemichannels junctions can be permeable to IP₃ (Sáez *et al.* 1989; Charles *et al.* 1992; Christ *et al.* 1992; Kam *et al.* 1998), nucleotide triphosphates and cyclic monophosphates (Tsien & Weingart, 1976; Pitts & Simms, 1977; Yamamoto & Kataoka, 1985; Brissette *et al.* 1994; Bevans *et al.* 1998; Kam *et al.* 1998), amino acids (Alvarez, 1973; Brissette *et al.* 1994; Wang & Veenstra, 1997; Vaney *et al.* 1998), glucose and its metabolites (Tabernero *et al.* 1996; Giaume *et al.* 1997), and calcium ion (Sáez *et al.* 1989; Christ *et al.* 1992; Tabernero *et al.* 1996; Giaume *et al.* 1997). The summary below will focus on some recent data for which the connexin involved was positively identified, or that allow comparisons between connexins.

In a liposome system analyzed by TSF, Cx32 hemichannels purified from native cells were shown to be permeable to both cAMP and cGMP (Bevans *et al.* 1998). Using the same approach, the results for a heterogeneous population of Cx26:Cx32 heteromeric channels also purified from native cells gave unexpected results. In a typical sample, approximately one-fourth of the channels were permeable to cAMP, and the rest were not. This suggested that some of the stoichiometries and/or arrangements of Cx32 and Cx26 in the hemichannels in this population permitted cAMP to permeate, but most did not. Since these were native channels, these data provided evidence that control of connexin stoichiometry/arrangement in channels could control whether cAMP was able to diffuse from cell to cell. A more surprising result was that for the same liposome population, approximately two-thirds of the hemichannels were permeable to cGMP. Clearly some of the stoichiometries/arrangements of isoforms could allow cGMP to permeate, but the more interesting finding was that in the same population of hemichannels, different subsets of the hemichannels were permeable to the two different second messengers. This strongly suggests that at least some of the hemichannels were permeable to cGMP but not to cAMP.

This finding has several implications. One is that control of connexin stoichiometry/arrangement can not only determine whether cyclic nucleotides can pass from cell to cell, but which one. Support for this was gained by demonstration that the hemichannel populations with increased proportions of Cx26 had decreased cGMP permeability. A study in cells has demonstrated that molecular permeability through junctional channels can be quite sensitive to the ratio of expression of two isoforms with different selectivities (Burt *et al.* 2001). Furthermore, the discrimination between cAMP and cGMP is unlikely to be on the basis of size or charge, and more likely to involve chemical recognition (perhaps of hydrogen-bonding ability, in which these two molecules differ). It is congruent that Cx32, which has a wider pore limiting diameter, does not make this discrimination. The exploration of the basis of this important discrimination will be interesting on several levels.

Calcium and IP₃ can pass between rat hepatocytes, for which the predominant connexin is Cx32 (Sáez *et al.* 1989). Such IP₃ flux is thought to be partially responsible for the intercellular propagation of calcium waves seen in several systems. The cell-to-cell signal can have a paracrine component, involving extracellular release of ATP and its subsequent inter-

action with purinergic receptors (Jorgensen *et al.* 2000; Paemeleire *et al.* 2000), in addition to a gap junction-mediated IP₃ pathway (Jorgensen *et al.* 2000; Scemes *et al.* 2000; Newman, 2001; Romanello & D'Andrea, 2001).

It has been demonstrated in transfected cells that Cx43 can support such calcium waves (Charles *et al.* 1992; Toyofuku *et al.* 1998a). In airway cells, intracellularly applied antibodies against Cx32 but not Cx43 domains inhibited the endogenous calcium waves, suggesting that Cx32 is also permeable to IP₃. Both Cx32 and Cx43 can support IP₃-mediated calcium waves in transfected C6 glioma cells (Boitano *et al.* 1998; Fry *et al.* 2001).

The relative ability of several connexins to support IP₃-mediated calcium waves was tested in HeLa transfectants (Niessen *et al.* 2000). The data from the different cell lines was normalized to a novel index of junctional coupling – the extent of Mn²⁺-induced quenching of Fura-2 fluorescence in confluent cells. The data show that Cx32 channels were able to support calcium waves 2.5 times better than did Cx43 channels, and 3–4 times better than did Cx26 channels. Compared to cells that normally express both Cx32 and Cx26, cells that were lacking Cx32 had a 25 times greater IP₃ threshold for initiation of intercellular calcium waves (Niessen & Willecke, 2000).

To directly determine which cytoplasmic molecules have different permeabilities through different connexins, the ‘metabolite capture’ approach was applied to cells transfected to express Cx43 and Cx32 (Goldberg *et al.* 1995, 1999) (see Section 3.3.2). The rates of transfer of ¹⁴C-labeled metabolites were adjusted for the different levels of coupling in the two cell lines (assessed by calcein transfer, shown to be equally permeable through Cx32 and Cx43 channels). The data show that glutathione and glutamate were approximately an order of magnitude more permeable through Cx43 channels than Cx32 channels. Furthermore, ATP and ADP were substantially more permeable than glutathione/glutamate through Cx43 channels, but only minimally permeant through Cx32.

This experiment shows that not only can these compounds pass through connexin channels, but the two connexins discriminate differently among this set of permeants. It should also be noted that the permeability assessed by charged tracer (calcein) bears no relation to the permeabilities to the endogenous permeants.

Recent work using the CFTR-mediated chloride current as a sensor for cAMP (described in Section 3.3.2) indicates that cAMP has a substantial permeability through Cx46 hemichannels and through heterotypic Cx43/Cx46 junctional channels (Qu & Dahl, In Press). The same study shows that increasing the occupancy of the conductance substate induced by V_j -gating (see Section 6) substantially reduces the macroscopic flux of cAMP through both the Cx46 hemichannels and Cx43/Cx46 junctional channels, despite an increased voltage driving force. This is a proof-of-principle that the V_j -gating mechanism can modulate flux of second messengers while retaining electrical coupling. Table 8 summarizes data on cytoplasmic permeability through channels formed by identified connexins.

Regarding the main conductance state, one may question the biological utility of an intercellular pathway that can discriminate among second messengers only poorly (i.e. less than a factor of 10). What difference would it make whether cAMP permeates a junctional channel several times better than IP₃? Such a difference would be important for any signaling with a temporal component. A growing literature supports the idea that oscillatory changes in signaling molecules convey information distinct from changes in steady-state levels (Rothman & Lenard, 1977; Hajnóczky *et al.* 1995; Jafri & Keizer, 1995; Dupont *et al.* 2000) and relative rates of permeation across junctions would have

Table 8. Permeability to cytoplasmic molecules

Connexin	Permeable to	Much less permeable to	Impermeable to
Cx32 hemichannels	cAMP, cGMP, Ca, IP3	Glutamate, glutathione	ADP, ATP
Cx26:Cx32 hemichannels	cAMP*, cGMP*	IP3	cAMP*, cGMP*
Cx32	IP3	Glutamate, glutathione, ADP, ATP	
Cx40	Glutamate		
Cx43	Glutamate, glutathione, ADP, ATP	IP3	
Cx46 hemichannels	cAMP		
Cx46	cAMP		
Cx43/Cx46	cAMP		

* Permeable or not depending on stoichiometry/arrangement of subunits.

a defining effect on how well such signals are transmitted. In addition, second-messenger molecules can have constrained lifetimes and diffusional persistence. The lifetimes of cAMP and IP3 in cytoplasm are ~ 60 s (Bacskai *et al.* 1993) and from 9 to 60 s depending on cell type (Kasai & Petersen, 1994; Wang *et al.* 1995; Sims & Allbritton, 1998), respectively. Regenerative waves of calcium ion, IP3 and cGMP (Honda *et al.* 2001) have been observed. Due to these factors, even modest selectivity at cellular junctions could have a major impact on the strength, character and location of the transmitted signal. The effect of signaling molecule lifetime on transmission of signals in coupled systems has been treated analytically (Klein & Mayer, 1997; Ramanan *et al.* 1998; Dupont *et al.* 2000) and support the importance of relative permeabilities in intercellular molecular signaling.

Some of the compounds that pass through junctional channels can affect junctional conductance directly or indirectly (such as cAMP). The effective intercellular diffusion constant of such a compound can have a key role in the recruiting of coupled cells into a syncytial unit (Christ *et al.* 1994). Thus there are ways that differences in molecular selectivity that are small by biophysical standards can have large consequences in biological contexts.

6. Voltage sensitivity

Connexin channels have several voltage-sensitive processes. The most prominent and well-characterized is the sensitivity of macroscopic junctional conductance to the magnitude of transjunctional voltage (V_j) (Fig. 10). The conductance is typically maximal at $V_j = 0$ and relaxes symmetrically and slowly (over hundreds of milliseconds to seconds) for hyperpolarizations or depolarizations of either cell. Usually a small portion of junctional conductance is V_j -insensitive. Nearly all naturally occurring connexins form channels with some degree of this type of voltage sensitivity, with widely varying parameters.

A single-channel basis of V_j sensitivity has been proposed from the behavior of single junctional channels and single hemichannels. It is called ' V_j -gating' and involves rapid voltage-sensitive transitions between a primary open state and one or more subconductance states, but not to a fully closed state.

In addition to V_j -gating, a second gating process dependent on junctional voltage is seen in single-channel currents (Trexler *et al.* 1996). Unlike V_j -gating, it involves slow transitions

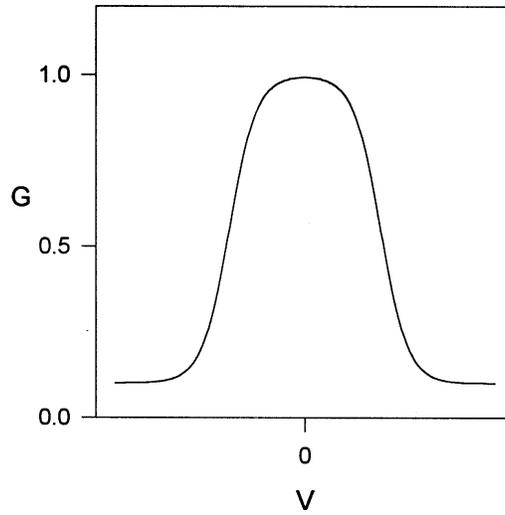


Fig. 10. Typical macroscopic steady-state conductance *versus* junctional voltage relations for junctional channels.

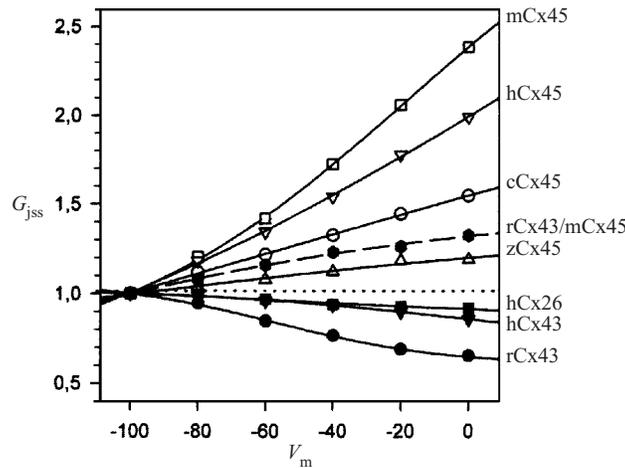


Fig. 11. Membrane potential (V_m) dependence of steady-state junctional conductance. (From Barrio *et al.* 2000.)

into and out of a fully closed state. It is termed ‘loop gating’ because the transitions resemble the slow initial opening transitions seen when insect gap junction channels (composed of innexin) first form (Bukauskas & Weingart, 1994), presumably initiated by contact between the extracellular loops E1 and E2. Loop gating can also contribute to V_j dependence of macroscopic junctional conductance. This is an area of ambiguity and controversy. The loop gating process may be related to what is termed ‘chemical gating’ (gating by ions and ligands), discussed in Section 7.

Several connexins show a sensitivity of the steady-state junctional conductance to the membrane potentials of the cells, as distinct from V_j (Barrio *et al.* 2000) (Fig. 11). This has been called ‘ $V_{i,o}$ sensitivity’ (for ‘inside-outside’) or ‘ V_m -gating’. When the holding potentials of two coupled cells are simultaneously depolarized while maintaining $V_j = 0$, the

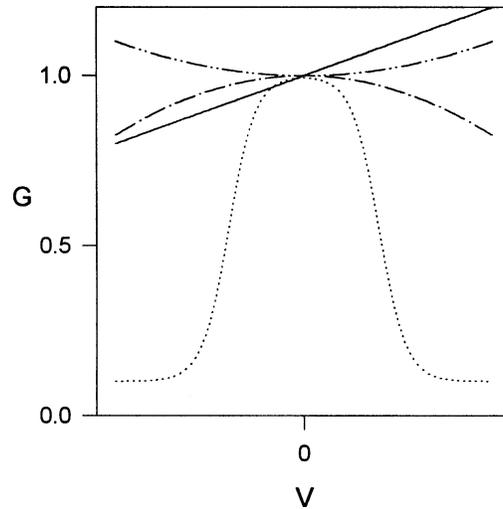


Fig. 12. Voltage sensitivity of instantaneous conductance. Upper curved lines illustrate forms of voltage sensitivity of initial ('instantaneous') junctional conductance. Dotted line is steady-state G - V relation, for comparison.

junctional conductance changes with a time constant of seconds, as opposed to fractions of a second for V_j sensitivity. V_m sensitivity of this kind increases conductance with depolarization for some connexins, and decreases it for others. Recent work has started to elucidate the molecular determinants of V_m -gating, but single-channel correlates have not yet been identified. Macroscopic V_m - and V_j -gating appear to be independent. To date, this type of V_m sensitivity has been reported almost exclusively for connexins expressed heterologously in oocytes, and not for the same connexins expressed in mammalian cells.

In addition to the *bona fide* voltage-dependent channel gating described above, macroscopic junctional currents have been described as having apparent V_j sensitivity of the *initial* or *instantaneous* junctional conductance. Historically this voltage sensitivity was attributed to gating transitions with kinetics too rapid to resolve under dual whole cell clamp. It appears that this is not the case. Initial conductances that increase or decrease symmetrically about $V_j = 0$ (see Fig. 12) occur only when certain connexins are expressed in coupled *Xenopus* oocytes (see Oh *et al.* 1999; Teubner *et al.* 2000) and therefore likely to be either specific to the oocyte system or to the experimental constraints they impose (see Section 3.1.2).

A more interesting case occurs where the initial conductance is monotonically dependent on V_m and has been termed 'fast V_{i-o} ' or 'fast V_m ' voltage dependence. As illustrated in Fig. 12 (solid line), fast V_m sensitivity is an apparent decrease of initial junctional conductance with hyperpolarization of either cell and an increase with depolarization of either cell. A slight V_m sensitivity of this type is seen in homotypic channels formed by Cx26 expressed in *Xenopus* oocytes (but not in N2A cells; Oh *et al.* 1999), where it is superimposed upon a V_j sensitivity (Barrio *et al.* 1991b; Rubin *et al.* 1992b). Similar V_m sensitivity is seen in Cx30 channels expressed in HeLa cells (Valiunas *et al.* 1999a) and in heterotypic Cx43/Cx45 channels expressed in N2A cells (Elenes *et al.* 2001). Its mechanism is unknown.

A more dramatic form of apparent V_m sensitivity of initial conductance occurs in most heterotypic channels containing Cx32 independent of expression system (Rook *et al.*

1988; Barrio *et al.* 1991; Rubin *et al.* 1992a; Suchyna *et al.* 1993, 1999; Bruzzone *et al.* 1994a; White *et al.* 1995b; Dahl *et al.* 1996; Oh *et al.* 1999; Valiunas *et al.* 1999b). Recordings of single junctional channels show that these rectifying macroscopic currents are due to non-linearity (i.e. rectification) of currents through single junctional channels (Bukauskas *et al.* 1995; Oh *et al.* 1999; Suchyna *et al.* 1999), not to voltage-dependent gating. Analysis of this rectification of unitary conductance of Cx26/Cx32 heterotypic channels has led to informative quantitative modeling of permeation (Oh *et al.* 1999; Suchyna *et al.* 1999) discussed in Section 5.1.3, and a possible mechanism for classical rectifying electrical synapses (Furshpan & Potter, 1959; Auerbach & Bennett, 1969).

V_m sensitivity of initial conductance is also seen in many invertebrate coupled systems (Obaid *et al.* 1983; Spray *et al.* 1984; Verselis *et al.* 1991; Bukauskas *et al.* 1992, 1997; Churchill & Caveney, 1993; Bukauskas & Weingart, 1994; Chanson *et al.* 1994b; Ghossein, 1994) where the junctional channels are presumably formed by innexins. Not all innexins have V_m sensitivity, however (see Jaslove & Brink, 1986; Giaume *et al.* 1987; Phelan *et al.* 1998b; Landesman *et al.* 1999).

V_j and V_m sensitivity of *initial* conductance will not be considered further in this review.

As should be clear from the summary above, description and analysis of connexin voltage sensitivity is complex. Each isoform makes channels with distinct voltage sensitivities, the parameters of which can vary with the cell in which it is expressed, several isoforms can co-mingle in the same junctional channel, and end-to-end interactions between hemichannels can modify voltage gating. For these reasons, and because of the additional voltage-dependent phenomena described above, the typical analysis is complex, jargon-intense and seldom understood by those not actively working in the field.

One way to view V_j -sensitive behavior of junctional channels, and to appreciate the unique features and challenges to its study, is to imagine two generic voltage-sensitive channels in adjacent cells with their extracellular ends tightly glued together. Allow their oppositely oriented S4 domains to sense only the field developed across the dimer, not the field between the inside and outside of the cells. Now replace the S4 domains with generic, uninformative sequences, and allow each of the two physical voltage gates to only partially close the channel. Permit each isoform of the channel-forming protein to form channels with different polarities and degrees of voltage sensitivity, and where the gates close to different partial degrees. To make analysis challenging, limit most experimental manipulation to the study of the dimeric channel, not the single-membrane components, but allow a few of the isoforms to form functional single-membrane channels, without knowing how the absence of end-to-end contact affects gating or voltage-sensing. For good measure, add a second voltage-sensitive gate that completely closes the channel at the extracellular end of each single-membrane subunit and eliminate the use of open-channel blockers and specific toxins as investigative tools. This now approximates the situation for study of voltage sensitivity in connexin channels. For a more complete understanding, sensitivity to V_m should be added to the mix, in some cases.

Due to this situation, analysis of connexin voltage-dependence and its structural basis is largely indirect, and therefore prone to error. Much of the information about hemichannel function is inferred from comparison of the properties of homotypic channels with those of various heterotypic channels, yet each different end-to-end interaction (e.g. a Cx32 hemichannel paired with a Cx32 hemichannel *versus* a Cx26 or a Cx46 hemichannel) can differently alter gating properties of each component hemichannel. One cannot assume that

all properties seen in single hemichannels are retained in the corresponding junctional channels, and vice versa.

This complexity presents substantial challenges. Key issues are highly controversial and the direct information to resolve them is lacking. The discussion that follows will not attempt a summary and synthesis of all known facts about the V_j and V_m dependencies of all connexins in all their forms. Instead it will focus on key issues that may help to elucidate how voltage-driven conformational transitions occur in connexins, and thus provide an alternative to the S4 motif for voltage sensitivity in proteins with α -helical transmembrane domains.

As mentioned above, at the single-channel level, in the connexin literature ‘ V_j -gating’ refers to the rapid transitions to and from a small subconductance state and ‘loop gating’ refers to the slower transitions to and from a fully closed state (Trexler *et al.* 1996; Banach & Weingart, 2000; Brink, 2000) (Fig. 13). It appears that each hemichannel possesses these two gating mechanisms. Both can be voltage sensitive. The polarities of gating have been shown to be the same in homomeric junctional channels and in the corresponding hemichannels (Bukauskas *et al.* 2001). For a given hemichannel, loop gating closes with hyperpolarization, but V_j -gating can close with either polarity of voltage. This can result in bipolar gating of single hemichannels (Oh *et al.* 2000). Macroscopic V_j dependence is likely to include contributions from both processes. To minimize confusion this review will continue to use the term ‘ V_j -gating’ as defined above, but the reader is cautioned that this does not imply that V_j -gating is necessarily the basis for, or even a contributor to, any given instance of macroscopic V_j sensitivity. The reader is also cautioned that for nearly every statement regarding voltage sensitivity of connexins there is at least one counter-example.

Because of the existence of two types of V_j sensitivity observable at the single-channel level (V_j - and loop gating), it is often difficult to attribute macroscopic voltage-dependent changes to one or the other; assignment is made with greatest certainty in single-channel recordings. However, most data on the molecular bases of the voltage sensitivities are from the macroscopic junctional currents readily obtainable from the *Xenopus* oocyte preparation. In most cases, changes in macroscopic voltage sensitivity due to mutagenesis and/or heterotypic pairings have been attributed without justification to changes in V_j -gating. This is likely the cause of much of the controversy in this area.

Credible assignment of macroscopic voltage sensitivity to one or the other mechanism requires that the range of transjunctional voltages tested be sufficient to reveal whether the conductance goes to a non-zero value, or if the conductance continues to drop toward zero with larger voltages. The former case (as in the figures above) is indicative of V_j -gating (as it does not involve transitions to a fully closed state), and the latter indicates loop gating. When they superimpose, one expects an inflection at the extremes of the G - V relations. When there is neither an inflection nor a leveling-off of the conductance, one cannot draw conclusions about the dominant mechanism involved. This view requires a re-evaluation of the published data regarding molecular mechanisms.

Recently, modifications of the CT have been shown to apparently eliminate V_j -gating while leaving loop gating intact, permitting the two processes to be dissociated (Bukauskas *et al.* 2001; Moreno, Chanson, Anumonwo, Scerri, Gu & Delmar, submitted).

It has been proposed to refer to the single-channel V_j -sensitive processes as ‘fast V_j -gating’ and ‘slow V_j -gating’ for the processes defined above as ‘ V_j -gating’ and ‘loop gating’, respectively (Bukauskas *et al.* 2001). This makes descriptive sense in the modern age, but may be confusing since in the past these terms have referred to other phenomena.

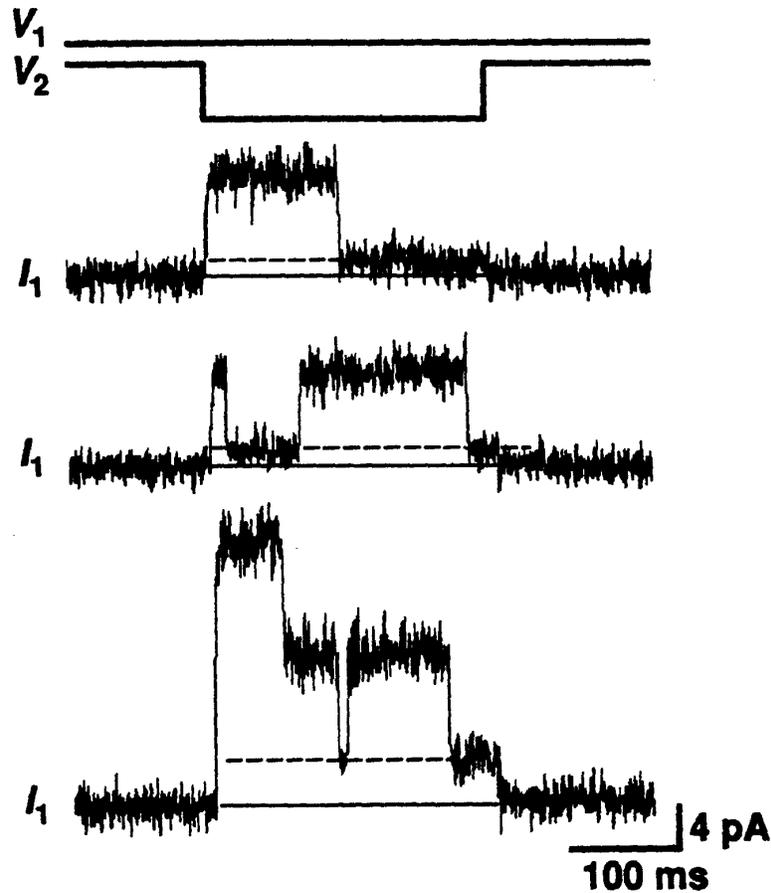


Fig. 13. V_j - and loop gating in a Cx30 junctional channel. Channel is open at onset of each current trace, and the first upward transition in each trace reflects the imposition of a junctional voltage. Following this, in each current trace the transitions are (from left to right): Upper trace: rapid transition to subconductance state (V_j -gating) \rightarrow slow transition to closed state (loop-gating). Middle trace: rapid transition to subconductance state (V_j -gating) \rightarrow rapid transition to fully open state (V_j -gating) \rightarrow rapid transition to subconductance state (V_j -gating) \rightarrow slow transition to fully closed state (loop-gating). Lower trace: rapid transition to subconductance state (V_j -gating) \rightarrow transition to fully open state (V_j -gating) \rightarrow transition to subconductance state (V_j -gating) \rightarrow slow transition to fully closed state (loop-gating). (From Valiunas *et al.* 1999a). Loop-gating opening transitions are seen in Fig. 6, which is from the same cell pair.

6.1 Macroscopic transjunctional voltage sensitivity

Dependence of junctional conductance on V_j has been described for many connexins (cf. Spray & Bennett, 1985; Bennett & Verselis, 1992; White *et al.* 1995a). For homotypic channels, steady-state junctional conductance is typically maximal at $V_j = 0$ and relaxes symmetrically to lower values with junctional polarizations of either polarity (Spray *et al.* 1981a). Even at high voltages, there is usually a voltage-insensitive component of the junctional conductance (G_{\min}). For each voltage polarity, the steady-state G - V relation for the voltage-sensitive component is roughly sigmoid and can be fitted to a first approximation by a model in which the open and closed states of junctional channels are described by a Boltzmann distribution, where the energies of the states are exponential functions of voltage

(Harris *et al.* 1981). The voltage-dependent behavior of the conductance changes is characterized by two parameters: the limiting steepness of the G - V relation (reflected in the n parameter of the Boltzmann formulation; see below) and by the transjunctional voltage required to cause the voltage-sensitive component of the conductance to be reduced to one-half of its maximal value (the V_o term). The voltage-induced conductance relaxations typically occur over hundreds of milliseconds to several seconds. It is recognized that in most cases the use of the Boltzmann distribution to describe the changes is only a convenient descriptor of the phenomenology, and not an appropriate representation of mechanism, since relaxations often cannot be fitted by a single exponential (Willecke *et al.* 1991; Revilla *et al.* 1999) and more than one closed state is required to fit kinetic data for some connexins (Ramanan *et al.* 1999). The parameters are defined below.

Parameters describing junctional voltage-dependence of macroscopic junctional conductance (all voltages are V_j 's)

G_{\max}	maximal junctional conductance
G_{\min}	V -insensitive component of the junctional conductance
$G_{\max} - G_{\min}$	V -sensitive component of the junctional conductance
$G = G(V) + G_{\min}$	Junctional conductance at a given voltage V (sum of V -dependent and V -independent components)

For a single polarity of junctional voltage

$G(V) = (G_{\max} - G_{\min}) / (1 + \exp(-A*(V - V_o)))$	conductance of the V -sensitive component at voltage V
$G = (G_{\max} - G_{\min}) / (1 + \exp(-A*(V - V_o))) + G_{\min}$	total conductance at voltage V (includes V -sensitive and V -insensitive components)
$A = (n*q) / (k*t)$	energy term expressing voltage sensitivity (voltage-dependent component of energy difference between open and closed states)
n	equivalent number of charges q moving through voltage field V
q	unitary charge
k	Boltzmann's constant
t	temperature
V_o	voltage at which voltage-induced change in conductance is half-maximal (i.e. where energies of open and closed states are equal). = $\Delta E / (n*q)$ where ΔE is the energy difference between open and closed states at $V=0$

For all junctional voltages (assuming a homotypic junctional channel)

$$G = [(G_{\max} - G_{\min}) / (1 + \exp(-A*(V - V_o))) + G_{\min}] * [(G_{\max} - G_{\min}) / (1 + \exp(A*(V + V_o))) + G_{\min}]$$

Every parameter of V_j sensitivity (n , V_o , G_{\min}) is different for channels formed by each connexin isoform (e.g. White *et al.* 1995a). For some connexins, the n is as great as that of neuronal sodium channels, and V_o sufficiently small that under some conditions coupling and uncoupling among cells can be regenerative, have thresholds and can be bistable (i.e. cells can be stably coupled or uncoupled, depending on the prior voltage history) (Harris *et al.* 1983; Baigent *et al.* 1997). G_{\max} often cannot be experimentally observed, since there may be some voltage-sensitive closure of channels even at $V = 0$; it is calculated as a free variable, as are the other parameters, in fitting the above equations to data (see Section 3.1).

For homotypic channels the symmetry of the G - V relations around $V_j = 0$ reflects the action of identical V_j -sensitive gating mechanisms in each of the oppositely oriented

Table 9. Polarity of V_j gating

Closes with inside	
Positivity/depolarization	Negativity/hyperpolarization
Cx26	Cx30
Cx37	Cx32
Cx38	Cx43
Cx40	Cx45
Cx46	
Cx50	

hemichannels that compose each junctional channel (Harris *et al.* 1981). From such relations one can infer apparent voltage-gating parameters for each hemichannel, at least as exhibited when the hemichannels are in junctional channels.

To a first approximation, V_j sensitivity of junctional channels is wholly a property of the component hemichannels. That is, in a junctional channel the hemichannels function largely independently in series; the V_j -sensitive changes in macroscopic junctional conductance reflect the combination of V_j -sensitive properties inferred for each hemichannel from their behavior in homotypic channels (Ebihara *et al.* 1995; Barrio *et al.* 1997). Most data supporting this are indirect, coming from studies in which connexin isoforms that form homotypic channels with different V_j properties are studied in heterotypic configurations. The typical experimental protocol is to measure the V_j properties in junctions formed between two *Xenopus* oocytes, each heterologously expressing a different connexin isoform.

For example, homomeric Cx40 channels have a symmetric, moderate voltage sensitivity and homomeric Cx37 channels a steeper and faster sensitivity. When a heterotypic channel is formed, one polarity of junctional voltage induces a response like that of Cx40 and the other like that of Cx37 (Hennemann *et al.* 1992; Bruzzone *et al.* 1993). This kind of result led to the view that V_j sensitivity of junctional channels is mediated by a sensor/transduction mechanism integral to each hemichannel, not one formed by the interaction of two hemichannels. There is evidence for modulatory end-to-end interactions in some cases (Hennemann *et al.* 1992; Bruzzone *et al.* 1994a; White *et al.* 1994; Hopperstad *et al.* 2000; Elenes *et al.* 2001).

As indicated above, each hemichannel of a junctional channel appears to close in response to a single polarity of voltage. However the polarity to which it closes is different for different connexins – some close with depolarization (referred to as ‘positive gaters’) and some close with hyperpolarization (called ‘negative gaters’) (Table 9). These polarities have been established by non-systematic study of the macroscopic V_j behavior of homotypic and heterotypic channels, initially based on a mutational analysis of Cx32 and Cx26 channels showing that wild-type Cx32 closes with hyperpolarization and Cx26 with depolarization (Verselis *et al.* 1994). There is consensus on these polarity assignments, with the exception of Cx30, for which the data are ambiguous (Dahl *et al.* 1996; Valiunas & Weingart, 2000), but more consistent with closing with negativity in the view of the author. On the other hand, loop gating seems to close with hyperpolarization for all connexins.

As alluded to above, the character of V_j dependence can be complex. A fundamental complexity is the relationship between the voltage-sensing abilities of the two hemichannels. Where the V_j sensitivity appears to be primarily first-order for each polarity, there is

evidence that the operation of the V_j -gating mechanism in one hemichannel can be affected by the position of the gate in the other (Harris *et al.* 1981). It appeared that one V_j mechanism did not respond substantially to V_j unless the V_j -gate of the other hemichannel was open. This was termed ‘contingent gating’, and has since been observed for several other connexins (Chanson *et al.* 1993; Steiner & Ebihara, 1996), but not others (Wang *et al.* 1992). It was originally proposed that the mechanism for contingency was that the voltage being sensed by the V_j sensors was that within the lumen of the pore, so that when one gate was closed, most of the V_j field within the pore dropped across that gate, dramatically reducing the fraction of the field sensed by the other. This mechanism is unproven, but the involvement of NT and proximal E1 domains in V_j -gating, outlined below, supports the idea that V_j is sensed within the pore.

6.2 Microscopic voltage sensitivity – V_j -gating

At the level of the single junctional channel, V_j -gating mediates transitions between a fully open maximally conducting ‘main’ state and a low-conductance substate (Veenstra *et al.* 1994b; Bukauskas & Peracchia, 1997; Oh *et al.* 1997; Valiunas *et al.* 1999b). These transitions are rapid, and there can be intermediate substates. The lowest conductance substate gives rise to the voltage-insensitive G_{\min} component of the macroscopic conductance (Bukauskas & Weingart, 1994; Moreno *et al.* 1994; Perez-Armendariz *et al.* 1994; Oh *et al.* 1999; Valiunas *et al.* 1999a). These properties are carried through to the level of single hemichannels (Trexler *et al.* 1996). In the discussion below, closing due to V_j -gating refers to transition to the lowest substate, not to the fully closed state.

6.2.1 Molecular basis – voltage sensor

The amino-acid sequences of connexins have no homology with the S4 domain of other non- β -barrel voltage-sensitive channels, even though gating of connexin channels can be quite voltage-sensitive. The V_j dependencies of Cx32 and Cx26 have been examined in detail by combined molecular and physiological approaches. Study has focused on this pair of connexins because they readily form heterotypic and heteromeric channels, and, more importantly, because they form homomeric hemichannels that gate with opposite V_j polarity (Barrio *et al.* 1991; Verselis *et al.* 1994). That is, Cx32 hemichannels close when their cytoplasmic side is negative and Cx26 hemichannels close when their cytoplasmic side is positive. This was shown in an elegant series of studies of heterotypic channels formed by wild-type Cx32 and Cx26 and mutants of them with altered voltage-sensitivity (Verselis *et al.* 1994).

By exploiting the fact that these polarities of response are retained in junctional channels, mutational analysis showed that the polarity of the voltage sensitivity can be determined by the polarity of the second amino acid of the NT (N2 in Cx32; D2 in Cx26) (Verselis *et al.* 1994). When the amino acid at this position is negatively charged (e.g. N2E or N2D for Cx32; D2 for Cx26) the hemichannel closes with inside positivity. When the amino acid is uncharged (e.g. N2, N2Q, A, R or K for Cx32; D2N, Q, R or K for Cx26) the hemichannel closes with inside negativity, whether the rest of the molecule is Cx32 or Cx26. These data are consistent in every instance with this position (*a*) sensing the field and therefore residing within the membrane, and (*b*) moving toward the cytoplasm with channel closing. This is remarkable in suggesting that a portion of the NT domain is inside the pore when the channel is open,

Table 10. Determinants of polarity of V_j -gating

	Charge at positions			Net charge	Closes with voltage inside
	2	41	42		
Wild-type Cx32	0	–	0	–1	Positive
	–	–	0	–2	Negative
	0	+	–	0	Enhanced positive
	–	+	–	–1	Positive
Wild-type Cx26	0	–	–	–2	Negative
	–	+	–	–2	Negative

and is driven in the direction of the cytoplasm by voltage to cause channel closure. This is reminiscent of the proposed movement in VDAC of the voltage-sensing N-terminal domain that occupies a pore-lining position in the open state, and whose movement out of the pore leads to a closed state (Mannella, 1998).

How much of the NT senses the field has not been well-defined. Charge changes at positions up to position 11 have effects on the voltage dependence, indicating an upper bound of 10 residues (Oh *et al.* 2000; Purnick *et al.* 2000a, b). However, double mutant studies showed that position 2 has the greatest single effect (Oh *et al.* 2001). In addition, these charge changes have effects, as yet uncharacterized, on conductance and selectivity, consistent with exposure to the pore lumen.

Cx32NT: 1 **M**NWTGLYTLLSGVNRHSTAIGR 22
Cx26NT: 1 **M**DWGTLLQSILGGVNVKHSTSIGK 22

While the charge at position 2 can determine polarity of response, the reversed-polarity mutants were not as steeply voltage-sensitive as the wild-type channels. This suggests that other charged residues are involved in sensing voltage. It has been proposed that charged residues at the M1/E1 border (positions 41 and 42) are directly involved, since making them more negative in Cx26 (K41E and E42S) enhances the voltage sensitivity. Making them less negative in Cx32 (E41K and S42E) enhances the voltage sensitivity of Cx32, consistent with the polarity of the changes at position 2. This suggests that residues at both the NT and the M1/E1 border sense voltage. Presumably the positive charge required to establish the normal gating polarity (closure for cytoplasm positive) is contributed by the N-terminal methionine. Furthermore, making the M1/E1 residues of Cx32 less negative (E41K and K41E) suppresses the ability of the N2D mutation to change polarity of gating. Though not arising from a quantitative analysis, this result is similar to the intragenic suppression paradigm applied to the *Shaker* channel to identify direct interaction of residues that are not adjacent in the sequence (Papazian *et al.* 1995). Even more compelling, retaining the wild-type negative charge E41 and making an S42E mutation (adding a negative charge) reverses the gating polarity, as did the N2D change. These data are summarized in Table 10.

These findings make a straightforward story in which polarity and steepness of voltage sensitivity are determined by additivity of charges at these three positions. This seems to hold for Cx26 and Cx32 as well as Cx37, Cx40, Cx46, Cx50 and Cx45, but cannot be generalized to all connexins. For example, Cx38 has the same charges as Cx32 at these positions, yet seems to gate with opposite polarity (Bradshaw *et al.* 1993; Dahl *et al.* 1996), and Cx43 has two negative charges at these positions and gates with the same polarity as Cx32 (Werner *et al.*

1989). This scheme is consistent with the uncertain assignment of Cx30 as closing with negative voltages.

To observe V_j -gating of Cx32 at the single hemichannel level, a chimeric connexin was generated in which the E1 of Cx43 replaced that of Cx32 (Cx32*Cx43E1) (Oh *et al.* 2000). This chimera forms functional hemichannels amenable to patch-clamp in oocyte plasma membrane (Pfahnl *et al.* 1997). While the gating was not identical to that of wild-type Cx32, it was possible to identify the V_j -gating transitions. On this background, an N2E mutant (which reverses V_j -gating polarity) was made. Different ratios of RNA coding for Cx32*Cx43E1 and Cx32N2E*Cx43E1 were co-injected into oocytes and the V_j -gating observed at the single hemichannel level. The formation of heteromeric hemichannels (hemichannels containing monomers with and without the mutation) was clear from the observation that the channels now displayed V_j closure to substates at *both* polarities. Furthermore, the results from the injection of different ratios of the RNAs demonstrated that *each* connexin monomer was capable of causing V_j -gating independent of the other subunits forming the hemichannel. The extent of reversed polarity gating of the channels was roughly accounted for by a binomial distribution of reversed-gating connexin monomers – V_j -gating is not concerted within a hemichannel, but can be initiated individually by each monomer. These are remarkable findings.

It was asserted that these findings argue against voltage-sensing charges being anywhere except at the NT (i.e. that the M1/E1 charges are not involved). This assertion would be supported by (a) an analysis of the steepness of the voltage dependence of the individually gating monomers, and (b) recordings from single hemichannels in which the M1/E1 charges were altered (though this may not be possible since the hemichannels open only if the native E1 is replaced by that of Cx43, and E1 begins at position 41). It would be satisfying (and fortuitous) if the voltage sensitivities for the monomers were as predicted – establishing that no other charges were directly involved.

6.2.2 Molecular basis – transduction and/or state stability

Amino acids at other positions can also affect voltage gating, but cannot be considered ‘sensors’ since they are not charged. For example, in Cx26, substitution of a P at position 87 with G, L, or A produces a dramatic change in voltage response such that the altered hemichannel is closed at $V = 0$ and open at the same high voltages at which it normally closes (Suchyna *et al.* 1993). On first inspection it appears as if the polarity to which the hemichannel responds is changed. However this would produce a junctional channel that is open at $V = 0$ and closes only with one polarity (both hemichannels having opposite orientations and opposite polarities of voltage sensitivity). It is as if the voltage sensor was unaltered but the effect of its motion in the field reversed so that the movement that formerly closed the channel now opens it, and vice versa. On this basis it was proposed that P87 in Cx26 is involved in the transduction between voltage sensing and the gate. The other possibility is that the mutation dramatically stabilizes the closed state so that the $G-V$ relation is shifted along the voltage axis. In either case there must be interference with the mechanism in the other hemichannel that would normally close at the voltages at which the mutated hemichannel opens.

P87 is located in M2, conserved in all known connexins, and the only proline predicted to lie in a transmembrane domain. The presence of proline in transmembrane α -helices induces

‘proline kinks’ consisting of a bend and a twist in the helical backbone (Barlow & Thornton, 1988; Ballesteros & Weinstein, 1995). This kink is a locus of flexibility in the α -helix – a proline kink disrupts the stabilizing hydrogen bonds internal to the helix, and allows a portion of the helix to interact with and move among other nearby regions of the protein, with which it may interact (Pastore *et al.* 1989; Williams & Deber, 1991; Ballesteros & Weinstein, 1992; Konvicka *et al.* 1998). This property makes proline-containing transmembrane α -helices useful elements for the transduction of molecular movement induced by signal reception (e.g. voltage, ligand, light) into movements that alter the properties of the protein.

However, in Cx32 a P87G substitution gates normally (Verselis *et al.* 1994), suggesting that the mechanism of transduction is different. But if this change is accompanied by two other charge-neutral changes in M2 that do not alter polarity of gating by themselves (S78A and L83M; which convert the M2 sequence of Cx32 into that of Cx26), the voltage gating is altered in the same way as for the P87G or P87L change alone in Cx26 (Ri *et al.* 1999). These changes suggest that P87, S78 and L83 are either involved in the transduction of a voltage-driven change into the movement of a gate, or in the stabilization of the open state.

```
Cx32M2:  76 LWSLQLILVSTPALLVAM    93
Cx26M2:  76 LWALQLIMVSTPALLVAM    93
```

Is there a fundamental difference in the V_j -gating transduction between Cx32 and Cx26? To answer this question, the detailed role of P87 and its neighbor T86 in voltage transduction was explored in Cx32 (Ri *et al.* 1999; Bargiello *et al.* 2000). P87A results in a slight shift in the conductance-voltage relation to favor the closed state, and P87V does not express functional channels. From Monte Carlo simulations of the structure of M2 containing these mutations it was clear that the magnitude of the functional changes did not correlate with the proline-induced bend *per se* (since all three mutations eliminate it) but rather with increased flexibility in bending and solvent accessibility of the adjacent carbonyls.

Modeling of the M2 of wild-type Cx32 revealed two features of interest: an average bend angle of 37° , in contrast to the 26° bend seen in proline kinks of other known structures, and a hydrogen bond between the hydroxyl side chain of T86 and the backbone carbonyl of I82. Computationally, it was shown that substitutions of A, N, V or L at position 86 would disrupt this link and S and C would partially compensate. Actual mutations of T86 showed that the voltage dependence of the conductance and kinetics shift progressively toward lower potentials in a manner roughly correlated with the degree to which hydrogen bonding potential at this position was disrupted. This shift corresponds to a relative destabilization of the open state. Remarkably, the mutation that produced the greatest shift (T86L) produced a G - V relation that closely resembled that of the P87G mutation in Cx26. This suggests a common mechanism for both connexins – that in Cx26, the bend in M2 required to keep the channel open is provided by P87, and without substantial contribution from the T86–I82 hydrogen bond. Therefore elimination of P87 would shift the G - V relations. For Cx32, the T86–I82 hydrogen bond serves to enhance the bend caused by P87, and that greater bend angle correlates with relative stabilization of the open state.

```
Cx32M2:  76 LWSLQLILVSTPALLVAM    93
                |←→|
                H-bond
```

Assuming that the closed state(s) induced by the mutations at T86 correspond to the closed states induced by V_j , the following mechanism was proposed for Cx32 V_j -gating: V_j causes

a voltage sensor at the NT (and possibly at the M1/E1 interface) to move toward the cytoplasmic end of the channel. This movement disrupts the H-bond between T86 and I82 in M2. Without this bond, the proline kink reverts from 37° to something substantially less ($\sim 20^\circ$). This reduction in bend angle results in the movement of the C-terminal part of M2 (the part toward the cytoplasm) to narrow the pore at the cytoplasmic end. This type of molecular switch based on proline-induced kinks has been seen in several membrane proteins (Sansom & Weinstein, 2000). For Cx26, which closes with opposite V_j polarity, the sensor may move in the other direction to decrease the bend in M2 by other means. Recent unpublished work shows that elimination of the corresponding proline in Cx43 (P88S) destabilizes both the open and closed states, corresponding to a decrease in the energy of the transition state separating them (Brink, Kumari & Valiunas, personal communication).

This view is consistent with one interpretation of the 7.5 Å structure (Unger *et al.* 1999b). If the kinked helix C corresponds to M2, its extracellular end is perpendicular to the plane of the membrane, but bent (presumably at P87) so that it passes through most of the membrane at an angle oriented away from the pore's central axis. If the extracellular end of M2 remains in place, a decrease in the bend angle will move the region on the other side of the bend at P87 (toward the cytoplasm) to close in toward the central axis of the pore. A narrowing at the cytoplasmic end of the pore by V_j -gating is also suggested by data showing that the V_j -induced substate of Cx32 has an enhanced rectification, which can be most readily explained by increased charge density near the cytoplasmic mouth (Oh *et al.* 2000). In this view the mutations at P87 exert their effect by enabling the relaxation of the bend, favoring the closed state. In the absence of the proline the bend is presumably partially maintained by other interactions.

Given that M2 may not correspond to helix C, it is important to note that the fact that the C-terminal part of M2 moves to close the pore is not to say that it lines the pore itself – as noted above, there is strong evidence that M1 lines the pore in this region (see above and Krasilnikov *et al.* 1995; Zimmermann & Walz, 1997). The proposed mechanism is that the centripetal movement of the cytoplasmic part of M2 displaces other domains, perhaps M1, that physically occlude the pore.

A phenotype similar to that of the Cx26 P87G mutation is produced by homotypic channels in which E1 and E2 of Cx40 are replaced by those of Cx43 (Cx40*Cx43E1,E2) (Haubrich *et al.* 1996). The macroscopic conductance is minimal at $V_j = 0$ and increases with voltages of either polarity. There is no ready explanation for this behavior, particularly since heterotypic channels formed with wild-type Cx40 or Cx43 show no indication of altered V_j -gating.

Mutagenesis and SCAM studies have identified positions in several transmembrane domains that affect voltage sensitivity whose accessibility differs with the conductance state of the channel (Skerrett *et al.* 2000, 2001b; Skerrett, Ahmed, Shin, Kasperek & Nicholson, personal communication). The ways in which voltage sensitivity is affected varies, but taken as a whole the data suggest that voltage-induced gating involves rotation of M1, and a host of changes in interactions between the transmembrane α -helices. As for the data on pore-lining positions summarized in Section 4.1, these data also support the 'tilt and rotate' model of gating proposed by Unwin and colleagues many years ago (Unwin & Ennis, 1984).

The voltage dependence of a Cx26*32NT-M2 chimera is identical to that of native Cx32, suggesting that for these connexins the V_j -sensing/transduction mechanism is wholly contained within the NT-M2 domain and does not involve the CL, E2 or CT domains (Verselis *et al.* 1994). However, noting that Cx26 does not have much of a CT, other data

suggest that the CT of Cx32 can affect some aspects of V_j -gating (Martin *et al.* 1998b; Revilla *et al.* 1999).

Recent data support involvement of the CT domain in the V_j -gating mechanism of Cx43. Addition of EGFP to the CT of Cx43 completely eliminates V_j -gating, as seen at both macroscopic and single-channel levels, leaving loop gating and chemical gating intact (Bukauskas *et al.* 2001). This suggests that the CT of Cx43 participates in V_j sensing or transduction, or forms part of the physical gate. Truncation of the CT of Cx43 or of Cx40 eliminates the residual state that is characteristic of V_j -gating (Anumonwo *et al.* 2001; Moreno, Chanson, Anumonwo, Scerri, Gu & Delmar, submitted). It is not clear whether the effect is due to an elimination of V_j -gating or an elimination of a structural element that normally keeps the V_j -gate from fully occluding the pore, though inspection of the Cx40 single channel records appears to favor the latter. The most surprising element of this study is that adding back the truncated domains as free peptides restores the residual conductance state, and the CT of Cx43 did so for truncated Cx40 as well (Anumonwo *et al.* 2000, 2001). Furthermore, the CT of Cx43 could restore the normal gating of Cx40 whether added as a free peptide or concatenated to the CT-truncated Cx40 (Anumonwo *et al.* 2001). This recapitulates the effects of this truncation and peptide on pH sensitivity (see Section 7.2). The data are consistent with the peptide associating with a cytoplasmic domain to either enable V_j -gating, or to keep the V_j -gate from occluding the lumen completely. This mechanism cannot account for the presence of a residual conductance state in Cx26 (Valiunas *et al.* 1999b), which has almost no CT domain. Also, several modifications to the CT domain of Cx37 (addition of GFP, and several kinds of truncations) had little effect on V_j -gating (Kumari *et al.* 2000).

In yet another twist on this issue, it was recently reported that heterotypic pairing of Cx45 and Cx43 also eliminates the prominent residual state of Cx43, and alters its voltage sensitivity, in similar fashion to that produced by the CT truncation above (Elenes *et al.* 2001). This suggests an effect on V_j -gating mediated by heterotypic, but not homotypic, hemichannel docking.

6.3 Microscopic voltage sensitivity – loop gating

As mentioned above, several wild-type connexins form functional hemichannels in cell membranes. The typical protocol for revealing them, either by macroscopic currents or dye uptake/leakage, involves reduced extracellular calcium ion and prolonged depolarization. This result seems counterintuitive for hemichannels inferred from junctional channel behavior to open with cytoplasmic negativity, such as Cx46. Detailed examination of the gating of single Cx46 hemichannels in cell-attached and excised patch recordings reveals bipolar gating (Trexler *et al.* 1996). At inside-positive voltages the channels undergo rapid transitions between a fully open and a small subconductance state, typical and consistent with V_j -gating observed in junctional channels. At inside-negative voltages the channels undergo slow (tens of milliseconds) irregular transitions between fully open and fully closed states that can include sojourns in several conducting substates. Thus the channels tend to be closed at negative potentials, open to a substate at high positive potentials, and fully open at intermediate voltages.

The slow, ill-defined transitions to and from the closed state are dubbed ‘loop gating’. Despite the implication, there is no evidence that E1 and/or E2 are directly involved in loop

gating. In all connexins studied, loop gating has the same polarity of voltage sensitivity, closing with hyperpolarization, no matter what the polarity of V_j -gating in the same channels. Slow transitions that may correspond to loop gating were first observed in cardiac cells (DeHaan, 1988; Veenstra & DeHaan, 1988).

Detailed studies of V_j -gating and loop gating in single channels of wild-type Cx43 and channels in which V_j -gating is eliminated by addition of EGFP to the CT indicate that the operation of the two gates is contingent (Bukauskas *et al.* 2001). The data suggest that the two sensors are in series in the lumen of the pore, and that the sensitivity of each to V_j changes with the position of the other gate. This is reminiscent of the mechanism proposed for contingency of macroscopic V_j -gates in each hemichannel (Harris *et al.* 1981).

There is some reason to think that the loop gate is physically located toward the extracellular end of the hemichannel. It had been previously shown by cysteine scanning mutagenesis followed by reaction with the large thiol reagent MBB that L35 of Cx46 appeared to lie in the pore (Zhou *et al.* 1997). It has been demonstrated that when Cx46L35C hemichannels are closed by inside-negative voltages (the polarity that closes the loop gate, but not the V_j -gate of Cx46) L35C is accessible by MBB from the cytoplasmic but not the extracellular end of the channel (Pfahnl & Dahl, 1998). This is strong evidence that the loop gate is extracellular to the L35 position. It also indicates that the pore occlusion in the loop gate closed state is focal, not along the whole length of the pore.

Cx46M1: 24 VWLTVLFIFRI**L**VLGAAA 41

Recent work from the same laboratory investigated the effect of calcium ion on loop gating (Pfahnl & Dahl, 1999), defined as opening of Cx46 hemichannels in oocytes with depolarization (the opposite polarity from V_j gating in Cx46). As mentioned above, opening of hemichannels in the plasma membrane requires low calcium and prolonged depolarization. The data show that at a depolarization to -10 mV, Cx46 hemichannels are open at 1 mM calcium and largely closed at 2 mM, though complete inhibition requires up to 5 mM calcium. Hyperpolarization (the polarity that closes the loop gate and opens the V_j -gate of Cx46) increases the sensitivity to extracellular calcium, but intracellular calcium has no effect. Depolarization (which opens the loop gate and closes the V_j -gate) reduces the sensitivity to extracellular calcium, but the channels become sensitive to intracellular calcium. The previous work had shown that residue L35C can be modified by intracellular MBB when the channel is closed by depolarization and intracellular calcium. When the channel is closed by calcium at -10 mV, L35 is not accessible from outside by MBB. These data indicate that, like the loop gate, the site of calcium action to close single Cx46 hemichannels is extracellular to position 35 and to the V_j -gate. The kinetics of calcium-induced closure were not examined, so it cannot be established whether calcium induces loop gating closure, or if the calcium gate is distinct from the loop gate.

The relation between the voltage-sensitive loop gate and other forms of gating in connexin channels is unclear. Slow transitions to closed states from subconductance states are often seen in response to exposure of junctional channels and hemichannels to a variety of chemical agents, including CO_2 (Bukauskas & Peracchia, 1997) and several lipophilic compounds (Weingart & Bukauskas, 1998). If the sensor and/or closing mechanism are the same, studies of regulation by these agents could reveal structural information about the loop-gating mechanism. However, at this time the correlation is only on the basis of similar irregular, slow transitions. For example, a detailed study of pH gating in Cx46 hemichannels showed

that it is strongly modulated by a voltage-induced change in accessibility of or affinity for protons (Trexler *et al.* 1999). This raises the possibility that the two gating processes are linked; ‘normal’ loop gating may be mediated or modulated by the proton concentration at ‘normal’ pH.

The precise relationships between microscopically described V_j -gating and loop gating and the voltage sensitivity of macroscopic junctional and hemichannel conductances are ambiguous. On one hand, there seems to be a clear criterion for assigning microscopic mechanisms to macroscopic conductance changes: the presence of a G_{\min} indicates V_j -gating, and absence of a G_{\min} indicates loop gating. On this basis one would assign most macroscopic junctional conductances to V_j -gating. In addition, based on analysis of the gating behavior of single Cx46 hemichannels one would infer that each hemichannel in a homotypic Cx46 junctional channel would close in response to cytoplasmic positivity. However, recordings of macroscopic currents through Cx46 hemichannels show strong activation of the conductance with depolarization, the polarity of response appropriate for loop gating. Furthermore, these depolarization-induced macroscopic currents through hemichannels largely account for the macroscopic voltage dependence seen in the junctional currents, implying that the macroscopic voltage sensitivity for Cx46 junctional channels is in fact mediated by loop gating. The situation is further complicated by the observation that the junctional currents show a G_{\min} (indicating V_j -gating) whereas the plasma membrane currents do not (consistent with loop gating, as is the polarity of response). The size of the Cx46 hemichannel subconductance state predicts a limiting G_{\min} of 0.4 for the junctional channels, not the 0.24 that is observed. Yet, for Cx46 the polarity of hemichannel closure inferred from its behavior in heterotypic channels is the same as the polarity for V_j -gating in the single hemichannel records (Werner *et al.* 1989; Bruzzone *et al.* 1994; White *et al.* 1995a, b). How can these findings be reconciled? It is not easy. Some options are that when hemichannels form junctional channels:

- the loop-gating voltage sensors operate the V_j -gate, and the V_j sensors are disconnected from a gate;
- loop gating shifts to much larger potentials along the voltage axis or is disabled, leaving V_j -gating that just happens to have very similar parameters;
- there is no real G_{\min} in the junctional currents (e.g. with high voltages the conductance continues to drop).

None of these explanations are satisfying, or even consistent with all the existing data. It is interesting to note that a hint of bipolar gating is seen in some records of macroscopic hemichannel currents of other lens connexins (Ebihara *et al.* 1999). This ambiguous situation is likely to persist until experiments are carried out in which gating is quantitatively compared for single hemichannels, macroscopic hemichannel currents and junctional currents formed by the same connexin, under comparable conditions, preferably accompanied by mutational analysis.

6.4 V_m -gating

Though initially described some time ago for innexins (Verselis *et al.* 1991) and connexins (Barrio *et al.* 1991), quantitative and molecular analysis of V_m -gating has been recent. The most well-described cases are for several Cx45 species orthologs, which open upon

depolarization (Barrio *et al.* 1997), as does Cx57 (Manthey *et al.* 1999) (see Fig. 11). Several other connexins close with depolarization [Cx26, Cx43 and Cx30 (White *et al.* 1994; Jarillo *et al.* 1995; Barrio *et al.* 2000)] and several are insensitive to V_m Cx32, Cx40, Cx42, Cx46, Cx56, Cx38 (Barrio *et al.* 2000). The magnitude and V_m sensitivity of the conductance changes vary greatly among connexins, from 10 to 250% over 100 mV, and 0.5–0.8 gating charge, respectively. The V_m and V_j gating processes do not seem to interact, suggesting independent sensors and gating mechanisms. To reveal V_m -gating, a specific voltage protocol must be used in which junctional conductance is assessed at relatively long times (tens of seconds) following displacement of both coupled cells to identical depolarizing and/or hyperpolarizing potentials. Experimentally it is readily distinguished from V_j sensitivity by this protocol and its much longer time-course.

The structural basis of steady-state V_m sensitivity in Cx43 was investigated (Revilla *et al.* 2000), and the findings have implications for the relationship between this form of gating and the others exhibited by connexin channels. Truncation of the CT at position 242 but not 257 eliminated V_m -gating. Furthermore, removal of charges at the two positions in the intervening positions that are charged in the wild-type channel has self-consistent effects on V_m -gating. Specifically, elimination of the positive charge at position 243 (R243Q) reduces the sensitivity, and elimination of the negative charge at 245 (D245Q) enhances the sensitivity. Removal of both charges achieved the same effect. These changes had no effect on V_j -gating. Other studies support the idea that the V_m - and V_j -gates are distinct, but can interact at large V_j . Because V_m could reduce the conductance below the G_{\min} achieved by V_j -gating it was inferred that V_m -gating could mediate transitions into and out of a fully closed state. There is no evident correlation between V_m sensitivity and any other functional property of the channel.

These findings suggest that closure by depolarizing V_m is mediated by outwardly directed movement of a positively-charged voltage sensor that includes positions 243 and 245. This could imply that this part of the CT senses the field in the pore. Because the R243Q mutation did not eliminate V_m -gating, other charges must be involved as well. The fact that this domain is physically close to domains thought to be involved in chemical gating of Cx43 (see Section 7) allows speculation that the influence of V_m on these charges interacts with chemical gating and vice versa. This relationship is as yet unexplored.

7. Direct chemical modulation

Direct gating of connexin channels by ligands is of key importance in intercellular signaling. Except in excitable tissues, the voltage sensitivity of connexin channels in most cases is not of a character readily seen to play a biological role (i.e. it is not significant at voltages near normal transjunctional potentials). The intercellular channel location and the cytoplasmic location of the modulatory sites make it difficult to identify ligands that directly regulate junctional channels. These factors also present problems for applying ligands and assessing their effects – junctional channels cannot be studied in excised patches, and cytoplasmic application of ligands via patch electrodes can cause cellular effects that complicate assessment of direct effects on the channels. Identification of endogenous ligands of connexin channels is required to understand the physiology of intercellular signaling, and is important for biophysical investigations because it can lead to the use of affinity reagents to explore connexin structure–function. Unfortunately, the biophysical study of connexin channels has

been hindered by the absence of specific affinity reagents or toxins, which have been vitally important in elucidating molecular mechanisms of other channels (Strichartz, 1987; Miller, C., 1995).

Junctional coupling is reduced by exposure of cells to various compounds (reviewed in Spray, 1994; Bruzzone *et al.* 1996; Goodenough *et al.* 1996; Kumar & Gilula, 1996; Alves *et al.* 2000; Rozental *et al.* 2001). Data on the actions of ligands on connexin channels come from a spectrum of experimental approaches that differ in the ways ligands are applied and their effects assessed. Accordingly, there are apparent contradictions and the validity of the conclusions vary. Modulation of junctional properties due to extracellular application of membrane-impermeant drugs (e.g. acetylcholine) or to manipulation of second messengers (e.g. cAMP) rarely reflects *direct* action of the drug on connexin channels; an agent applied intracellularly could act directly on the channel or via signaling systems. Agents can be applied to connexin channels by (a) intracellular application via patch electrode or microelectrode, (b) use of membrane-permeant agents, (c) perfusion of the cytoplasmic face of junctions, (d) exposure to hemichannels in an excised patch configuration, or (e) exposure to hemichannels reconstituted into liposomes or bilayers. The perfused cell and excised patch methods preserve the channels in their native membrane environment and presumably with membrane-associated components that may be involved in modulation of channel properties. The reconstituted systems can unambiguously show direct effects, but the channels are separated from cellular elements that may determine the character of modulation in cells.

Methods of assessing drug effects on connexin channel gating are also varied. They include all the approaches outlined in Section 3, and their attendant limitations. For example, single time-point studies of dye coupling cannot distinguish with confidence whether a decrease in dye coupling results from reduced channel open time or reduced permeability to the dye due to a narrowed pore.

Intriguing, indirect evidence of an endogenous modulator of connexin channel has been described recently (Le & Musil, 2001). In the chick lens, up-regulation of ERK (extracellular signal-regulated kinase; part of the MAP kinase cascade) increases dye-coupling between lens cells without affecting junction formation, assembly or turnover, or the levels, synthesis or level of phosphorylation of any of the three connexins expressed in these cells. The apparent dye permeability per channel structure increases, indicating an increase in the fraction of active channels, the open probability or the width of the pore. The effect of ERK activity on dye coupling takes many hours to become evident, suggesting that it does not occur via modulation or modification of pre-existing cellular components, but rather by a change in the level or activity of a direct or indirect modulator of connexin channel function. A possible mode of action is reduction of the level of an inhibitor that tonically interacts with voltage or chemical gating mechanisms to keep low the fraction of active channels in a plaque (as described in Section 2.2.4). Difference between Cx43 species orthologs (their amino acid sequences are 92% identical) and in the cellular milieu may account for the apparently different actions of ERK on the chick lens Cx43 and the rodent Cx43 described above. The modulation of intercellular molecular permeability by ERK in the lens has clear biological relevance; the ERK activity is stimulated by FGF (fibroblast growth factor) in a way that accounts for the characteristic patterns of dye coupling and fluid flow that maintain lens transparency.

The discussion below considers modulators that are either commonly used experimentally, or may act directly on connexin channels.

At the single-channel level, the transitions described in Section 6 as loop gating (slow transitions between the fully closed state and an open state) closely resemble the transitions induced by certain ligands. The similarity of the transitions suggests similar or shared molecular mechanisms for the effects of these ligands. Whether or not this is so, at this time it is convenient to speak of ‘chemical’ gating as a class of transitions (slow transitions that completely close a channel; $\tau \sim 9\text{--}30$ ms) that may be related to the voltage dependent loop gate, in distinction from V_j -gating transitions (rapid transitions that close only to a non-zero conductance state; $\tau < 2$ ms) (Bukauskas & Peracchia, 1997, 2000; Bukauskas *et al.* 2001). It is particularly noteworthy that channel closing due to chemical gating can be reversed by voltage (Peracchia *et al.* 1999).

On the basis of single-channel recordings, chemical gating includes the effects of pHi, *n*-alkyl alcohols, halothane, fatty acids and calcium ion. On the basis of elimination of detectable macroscopic currents, other lipophiles can be tentatively considered to operate by this mechanism as well. Glycyrrhetic acid and its analogs, however, do not completely eliminate coupling and so may operate by a distinct mechanism, as may cyclic nucleotides. Phosphorylation can affect substate occupancy, and may cause full closing as well, but there is no indication that it involves either V_j or chemical gating mechanisms.

7.1 Phosphorylation

Phosphorylation of proteins is a widespread mechanism of functional modulation. Effects of phosphorylation on channel function include stabilization of open or closed states and alteration of kinetics of transitions between states (Levitan, 1999). Most connexins contain likely sites of phosphorylation in the cytoplasmic CT domain, and all connexins examined except for Cx26 have been demonstrated to be phosphoproteins, either *in vivo* or *in vitro* (Jongsma *et al.* 2000; Laird & Sáez, 2000). The most thoroughly studied connexin, Cx43, can be phosphorylated at multiple sites by multiple kinases, and contains three tandem PKA/PKC consensus motifs in the CT (positions 362–373). An extensive literature describes the possible role of phosphorylation in regulation of trafficking of connexin to the plasma membrane, assembly into junctional plaques and degradation (Musil & Goodenough, 1991; Brissette *et al.* 1994; Lampe, 1994; Laird *et al.* 1995). This literature, as well as the more restricted literature dealing with possible direct effects on channel function, were recently reviewed in (Lampe & Lau, 2000; Lau *et al.* 2000).

In many cases, it is difficult to discern whether phosphorylation affects junctional coupling by a direct effect on channel gating or permeation, or by effects on the trafficking, assembly or turnover of junctional channels.

Injection of the free catalytic subunit of PKA can cause a rapid increase in junctional communication mediated by Cx43, possibly indicating a direct effect on either gating or permeability (Nnamani *et al.* 1994). In cells expressing a S364P mutation, coupling was not affected by PKA, in contrast to controls, making S364 a required locus for PKA modulation (Britz-Cunningham *et al.* 1995). Intriguingly, somatic mutations at S364 or at R362, which is part of the same consensus phosphorylation site, are specifically correlated with cardiac developmental defects in humans (Dasgupta *et al.* 2001).

One well-established direct functional effect of connexin phosphorylation is that serine phosphorylation of human or rat Cx43 by PKC but not PKA alters the frequency of entering the dominant unitary conductance state, favoring instead one of substantially

lower conductance and low permeability to LY (Moreno *et al.* 1992, 1994; Takens-Kwak & Jongma, 1992; Kwak *et al.* 1995b; Lampe *et al.* 2000). Specifically, the reduction of unitary conductance and permeability that follows treatment with TPA requires phosphorylation at S368 (Lampe *et al.* 2000). If S368 is changed to another amino acid, TPA has little functional effect. Analysis of single channel records indicate that the effect is to favor transition to a substate rather than to physically alter the conductance pathway. Modulation of substate occupancy by phosphorylation has also been seen in other channels (Lee *et al.* 1995).

Rat Cx43 (rCx43) can also be phosphorylated by a G kinase (PKG), which similarly shifts the occupancy toward lower conductance states. Treatment with phosphatase has the opposite effect. The only PKG consensus phosphorylation site is in the CT. Interestingly, human Cx43 (hCx43) does not show this modulation by G kinase – and differs from rCx43 by the absence of S257 in the PKG consensus phosphorylation site (Kwak *et al.* 1995b).

Thus it appears that phosphorylation at distinct sites by distinct kinases has similar effects on substate occupancy. The molecular mechanism of this modulation is not known.

The bulk of the work on regulation of connexin gating via phosphorylation deals with modulation of Cx43 by the MAP kinase ERK and by the oncogene products v-Src and c-Src. For both kinase systems, the evidence is indirect (there are no single channel records), but suggestive.

In mammalian cells, ERK activation by EGF can reversibly disrupt junctional communication within minutes, without obvious disruption of plaques (Lau *et al.* 1992; Gourdie *et al.* 1993; Warn-Cramer *et al.* 1996, 1998). To have the full effect, it must phosphorylate the CT of Cx43 at S255, S279 and S282. The speed of the response suggests a direct effect on gating. A direct action of purified ERK on Cx43 was supported by liposome studies (Kim *et al.* 1999).

In both oocytes and in mammalian cells it has been known for some time that activation of the v-Src oncogene leads to a rapid reduction of junctional communication (Atkinson *et al.* 1981; Swenson *et al.* 1990). Early work in the oocyte system indicated that v-Src-induced inhibition of coupling required phosphorylation of Y265 (Swenson *et al.* 1990). Later work strongly indicated that the rapid inhibitory effect of v-Src was mediated by a downstream activation of ERK, and that phosphorylation of Y265 was not required (Zhou *et al.* 1999). The inhibition of channel activity required the portion of the CT domain that includes the three ERK serine sites mentioned above, and occurred in the absence of Y265. This suggested that, in oocytes, phosphorylation of Y265 resulted in altered trafficking or assembly rather than direct effects on channel gating.

Truncation of the CT at position 245 eliminated the v-Src effect, but a surprising finding was that adding back the truncated domain as a free peptide (by co-injecting the appropriate cDNA) restored it. This suggests that ERK-mediated inhibition is mechanistically similar to the pH_i inhibition described below in Section 7.2, in which the CT domain (albeit a somewhat different part of it) acts as a blocking particle. In one case the association is promoted by serine phosphorylation, and in the other by protonation of a connexin site and/or soluble factor. Recent studies of the voltage dependence of the effect support the idea that the blocking particle enters the junctional voltage field, suggesting it acts as an open-channel blocker (Moreno & Nicholson, personal communication). Single-channel analysis should be most informative. This mechanism may account for similar effects of truncation and replacement of CT peptide on insulin-induced uncoupling (Homma *et al.* 1998)

In mammalian cells, however, the v-src-induced inhibition appears to operate by a different mechanism – inhibition of Cx43 channel activity requires direct, processive phosphorylation of Y265 and Y247 by v-Src without apparent involvement of the MAP kinase pathway (Lin *et al.* 2001). Similar effects have been reported for the action of c-Src (Giepmans *et al.* 2001; Toyofuku *et al.* 2001), also in mammalian cells. The reason for the different results in the mammalian and amphibian systems is unclear, but may arise from differences in the cell types, related differences in expression mechanisms protocols or differences in the timing of connexin and *src* expression in the various studies.

Effects of phosphorylation of other connexins are less well-characterized. Activation of PKC causes phosphorylation of rat Cx45 and the appearance of a new conductance state (Kwak *et al.* 1995a) but phosphorylation of mouse Cx45 increases the occupancy of existing open states (van Veen *et al.* 2000). Activation of PKA seems to favor the higher conductance states of Cx40 (van Rijen *et al.* 2000), whereas PKC phosphorylation of Cx32 is reported to induce a new, higher conductance state (Sáez *et al.* 1986; Chanson *et al.* 1994a).

7.2 Cytoplasmic pH and aminosulfonates

Changes in intracellular pH (pH_i) can affect junctional conductance (Turin & Warner, 1977; Spray *et al.* 1981b). The sensitivity to changes in pH_i varies with cell type and connexin isoform (Campos de Carvalho, 1988; Liu *et al.* 1993; Spray, 1994; Delmar *et al.* 1995; Hermans *et al.* 1995). Decrease of pH_i from physiological levels typically produces a decrease in junctional conductance (Iwatsuki & Petersen, 1979; Turin & Warner, 1980; Spray *et al.* 1981b) and in permeability to large tracers (Schuetze & Goodenough, 1982; Connors *et al.* 1984). The reduction of junctional conductance can be to zero, and is typically reversible with return of pH_i to normal values. Though it is clear that the CL and CT are involved, the precise molecular mechanisms are not fully understood, and may differ among connexin isoforms. They have been proposed to involve direct protonation of connexin (Spray *et al.* 1981b), pH-facilitated interaction between connexin cytoplasmic domains and/or cytoplasmic factors (Delmar *et al.* 1995; Bevans & Harris, 1999a), indirect effects of undissociated weak acids in the membrane (Rudy, 1995; Dunina-Barkovskaya *et al.* 2000), changes in ionized calcium concentration, and/or activation of calmodulin (Rink *et al.* 1980; Peracchia *et al.* 1996, 2000a; Peracchia & Wang, 1997; Torok *et al.* 1997). At the single-channel level, low pH clearly induces full channel closure by the slow mechanism alluded to above (Bukauskas & Peracchia, 1997).

For Cx43, Cx40 and Cx32, work in the paired *Xenopus* oocyte expression system suggests a molecular mechanism for pH sensitivity. In these studies, pH_i and junctional conductance were monitored continuously while controlled, slow, pseudo-steady-state acidification and re-alkalization of the cytoplasm was carried out. The fundamental finding is that truncation of the CT effectively eliminates the pH sensitivity, and adding back the CT as a free peptide (by co-injection of cDNA) restores it (Morley *et al.* 1996). In fact, the Cx43 CT can induce pH sensitivity in truncated (pH insensitive) Cx40, and vice versa (Stergiopoulos *et al.* 1999). The histidine at position 95 (H95), which is in the CL of nearly all connexins, was proposed to be part of the receptor, since mutation of it eliminates pH sensitivity (Ek *et al.* 1994). The proposed mechanism is that low-pH facilitates interaction of the cytoplasmic Cx43 CT with a receptor domain elsewhere in the connexin molecule, causing the channel to close (Liu *et*

al. 1993; Ek *et al.* 1994; Ek-Vitorin *et al.* 1996; Morley *et al.* 1996, 1997). The mechanism is analogous to the ball-and-chain N-type activation mechanism of voltage-dependent potassium channels (Armstrong & Bezanilla, 1977; Hoshi *et al.* 1990; Zagotta *et al.* 1990). Studies using a perfused double-oocyte preparation indicate that a cytoplasmic component is required for the pH sensitivity of Cx43 (Nicholson *et al.* 1998). On the other hand, Cx46 hemichannels appear to be directly gated by protonation, without a cytoplasmic requirement (Trexler *et al.* 1999). pH sensitivity is intrinsic to hemichannels, since the pH sensitivity of homotypic hemichannels of Cx46 and Cx38 accounts fully for the pH sensitivity of the corresponding homomeric junctional channels (Francis *et al.* 1999). In the same study, heterotypic pairings of connexins with dissimilar pH sensitivities indicated that the pH sensitivity could be affected by hemichannel–hemichannel interaction.

In *Xenopus*, Cx32 channels are weakly pH sensitive (Liu *et al.* 1993; Wang & Peracchia, 1996). From studies of chimerae of Cx32 and Cx38 (which is more pH sensitive than Cx32), a model of pH sensitivity has been proposed (Wang *et al.* 1996; Wang & Peracchia, 1996, 1997; Peracchia & Wang, 1997) that is mechanistically similar to that proposed for Cx43, though it differs in some details. It involves a receptor domain in the N-terminal half of the CL that interacts competitively in a pH-sensitive manner either with the C-terminal half of the CL to close the channel, or with a proximal region of the CT, which does not close the channel. A Cx43 CT peptide can enhance the degree of pH sensitivity of Cx32 channels, suggesting conservation of receptor structure across connexin isoforms (Morley *et al.* 1996). Several CMTX mutations in the CL and CT, but not in E1, alter pH sensitivity as well (Ressot *et al.* 1998).

In both these models from *Xenopus* data, interactions between cytoplasmic domains of connexins are favored by protonation – a region of the CT interacts with a receptor region of the CL in a pH-dependent manner to close the channels.

At the single-channel level, the opening and closing CO₂-induced transitions have similar kinetics. The transitions are often irregular, perhaps indicating the reconfiguring of several structural elements. The channels behave as if each hemichannel contains a V_j -gate and a chemical gate that operate concurrently. Channels in the substate induced by one polarity of V_j could be moved to the fully open state by a rapid change in V_j polarity, but if the channel was in the fully closed state induced by low pH, changing the V_j polarity had no effect (Bukauskas & Peracchia, 2000), suggesting that the gates are in series.

A TSF study using purified and reconstituted homomeric Cx32 and heteromeric Cx26/Cx32 investigated the direct effects of pH on connexin hemichannel activity in liposomes (Bevans & Harris, 1999a). Activity of the heteromeric channels was affected by changes in pH, but not in the manner anticipated from cellular studies. The pH effect was strongly modulated by and *dependent on* the pH buffer present. Investigation showed that pH sensitivity was in fact a sensitivity to the concentration of the protonated form of aminosulfonate pH buffers, including HEPES, MES, TAPS and taurine, and not to pH *per se*. Experiments that used the non-aminosulfonate pH buffers maleate, bicarbonate or Tris instead showed negligible pH sensitivity, as did experiments using derivatives of taurine lacking an aminosulfonate moiety. The sensitivity to aminosulfonate depends on isoform composition of the channels, being essentially absent in homomeric Cx32 hemichannels, and present in hemichannels that also contained Cx26.

These data show a direct modulatory effect of protonated aminosulfonates on connexin channel activity. The significance is twofold: (a) it provides a molecular mechanism by which

changes in pH_i can affect connexin channel activity, and (b) it identifies a class of cytoplasmic compounds that directly and reversibly regulates connexin channel activity. The active compounds share a common structural motif: a protonatable amine moiety separated from an ionized sulfonate moiety by 2 or 3 methylene groups. Of the aminosulfonates tested, TAPS appeared to have the greatest efficacy. TAPS is the only compound tested with three methylene groups between the protonatable amine and the ionized sulfonate moiety. It is possible that this structural difference contributes to the increased efficacy.

The data also suggest that at least some of the effects on connexin channels that have been attributed to direct action of low pH_i are mediated (or modulated) by endogenous protonated aminosulfonates or related compounds, rather than by protonation of connexin. pH may also have effects not revealed in the TSF system, and it could act directly on other connexins, as is the case for Cx46 hemichannels (Trexler *et al.* 1999).

Taurine is a ubiquitous aminosulfonate. It interacts with many proteins, the most prominent of which are the inhibitory glycine receptor (GlyR) (Young & Snyder, 1974; Akagi & Miledi, 1988; Schmieden *et al.* 1992) and several amino-acid transporters (Chesney *et al.* 1990). Though the structure of the taurine-binding site on the GlyR is not known, the most important sequence elements have been defined (Schofield *et al.* 1996). The site is thought to be formed by parts of at least two non-contiguous segments. The segments are: (a) an aromatic-small-aromatic sequence (159–161; ‘loop-2’), and (b) a cationic-X-aromatic-X-small sequence (200–204; ‘loop-3’). Cx32 and Cx26 each have four loop-2-like motifs and three loop-3-like motifs, all in the putative transmembrane and extracellular domains. However Cx26 contains an additional loop-2-like motif, that Cx32 lacks, in its small C-terminal cytoplasmic domain (YLEF, 212–214). Also, one of the loop-3-like motifs of Cx26 partially extends into the cytoplasmic N-terminal (NT) domain (K·W·T, 22–26), unlike that of Cx32.

These similarities are not striking, but suggest the possibility that the CT and/or NT regions of Cx26, but not Cx32, interact with taurine or similar compounds, perhaps in concert with other domains. If so, a taurine-binding site could be composed of the CT domain (providing a loop-2-like motif) and the region of the transition between the NT and M1 (providing a loop-3-like motif). The NT-to-M1 transition is thought to be at the mouth of the pore (Oh *et al.* 1997), possibly providing a direct site of action. Intriguingly, the CT peptide of Cx43 that is required for its pH sensitivity and can confer some pH sensitivity on Cx32 (Morley *et al.* 1996), contains two loop-2-like motifs, one of which is precisely aligned with that of Cx26 (YVF; 230–232), and the other of which has been positively identified as essential for pH modulation (YAY, 265–267) (Ek-Vitorin *et al.* 1996).

It is thus possible that aminosulfonates regulate some connexin channels by occupying a binding site composed of a part of the CT domain and another domain, perhaps at the NT–M1 boundary. The effect on channel activity could be due to occupancy of the site, or the conformational changes caused by coordination of disparate parts of the connexin molecule.

The amino-acid sequences of several high-affinity taurine transporters have been determined, but the residues that interact with taurine have not yet been identified (Liu *et al.* 1992; Smith *et al.* 1992; Uchida *et al.* 1992; Ramamoorthy *et al.* 1994; Vinnakota *et al.* 1997). The affinities of these transporters for taurine range from 4 to 40 μM , and there are several regions of potentially significant homology with connexins. An invertebrate odorant receptor

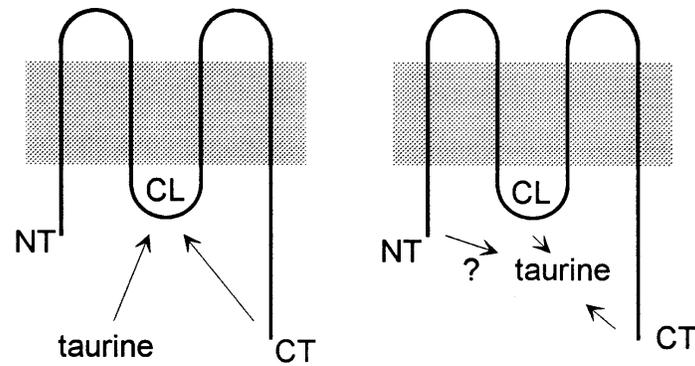


Fig. 14. Two possible mechanisms for aminosulfonate action. *Left*: aminosulfonate and CT domain of connexin independently interact with a receptor domain in the CL to effect channel closure. *Right*: aminosulfonate and the CT domain form a complex that interacts with other connexin domains to effect channel closure. (From Bevans & Harris, 1999a.)

has been shown to have two high-affinity binding sites for taurine (K_d of 18 μM and 6 μM), but the amino-acid sequence has not yet been determined (Olson & Derby, 1995).

The consensus view of pH sensitivity of Cx43 and Cx32*Cx38 chimerae from the *Xenopus* system is that a pH-dependent interaction between segments of the CT domain and the CL inhibits channel activity (Delmar *et al.* 1995). There is now evidence from combined surface plasmon resonance and NMR studies that the Cx43 CT interacts with the C-terminal half of the CL in a pH-dependent manner, albeit in the presence of the aminosulfonate HEPES, and that this involves histidines at positions 126 and 142 (Duffy *et al.* 2001). The CL–CT interaction is not inconsistent with the aminosulfonate interaction with heteromeric Cx26:Cx32 channels described in the TSF system. Two mechanisms can be proposed that incorporate both sets of data.

One mechanism is that *either* protonated aminosulfonate *or* competent CT (i.e. that of Cx43 or Cx38, but not Cx32) can bind to the receptor domain to close the channel. The other is that to close the channel a complex must form between *all three* elements – protonated aminosulfonate, competent CT, and receptor. Each hypothesis is consistent with much of the data and has distinct consequences and predictions (Fig. 14).

In cells, the first mechanism involves a competition between the available CTs and the available cytoplasmic protonated aminosulfonates. Interaction between the CT and receptor would be pH-sensitive and involve protonation of at least one connexin site. An ineffective Cx32 CT would account for the relative pH insensitivity of homomeric Cx32 channels (Liu *et al.* 1993; Wang & Peracchia, 1996), behaving as a competitive blocker in binding to the receptor but not effecting channel closure. The increased pH sensitivity with Cx26 content would be due to the very short CT of Cx26 – with increased Cx26 in Cx26:Cx32 heteromeric hemichannels, the number of bulky Cx32 CTs present decreases and access to the receptor increases, permitting aminosulfonates in the solution or cytoplasm to interact with the receptor and effect closure. In this view, Cx26 subunits would effectively decrease the local Cx32 CT ‘blocker’ concentration, and/or act as ‘spacers’ to alleviate steric crowding by Cx32 CT and prevent access by aminosulfonates.

It is possible that the CT interacts with the connexin pore in a manner analogous to that of N-type inactivation of potassium channels, in which a single bound CT ‘particle’ occludes the pore, interacting with ‘receptor’ regions from several subunits (MacKinnon *et al.* 1994).

For K⁺ channels, one particle–receptor complex is sufficient to block the channel. It is unlikely that a single taurine could serve the same steric function in a connexin pore. Therefore, for the analogy to hold, either it would have to occlude the pore in association with cytoplasmic domains, or several molecules of taurine would have to bind to several subunits and collectively occlude the pore.

For the second mechanism, pH-dependent channel closure requires both aminosulfonate and competent CT. The aminosulfonate could interact directly with both other elements, or could bind to one to enable binding of the resulting two-element complex to the remaining element to form a ternary complex. In this case the pH insensitivity of homomeric Cx32 would arise from an inability of the Cx32 CT to form this complex, rather than from steric crowding or occupancy of the receptor as above. In cells, pH sensitivity of Cx32 would be enhanced by co-expression of the Cx43 CT, providing a competent CT domain that effectively interacts with both the receptor and cytoplasmic aminosulfonate. For this mechanism to account for the pH sensitivity of the Cx26:Cx32 channels one must postulate that the small Cx26 CT participates in this complex formation (perhaps via the loop-2-like motif mentioned above), if the aminosulfonate site is similar to the taurine site of the GlyR.

These hypotheses predict that since Cx26 lacks a bulky CT, channels formed by homomeric Cx26 in cells should be pH-sensitive (i.e. allow free access to the receptor by cytoplasmic modulators). The model for pH sensitivity of C43, which relies exclusively on a full-length CT for pH sensitivity (Ek-Vitorin *et al.* 1996), if applied to Cx32 and Cx26, predicts that homomeric Cx26, lacking most of the CT, would be pH insensitive. Recent work has established that homomeric Cx26 channels are in fact highly pH sensitive (Stergiopoulos *et al.* 1999), as both models proposed here predict. CT-truncated Cx32 is not as pH sensitive as Cx26 (Werner *et al.* 1991). This suggests that for these two isoforms the properties of the receptor domains differ, or the short CT of Cx26 plays a role that the Cx32 CT cannot. Also, junctional channels formed by tandem Cx32–Cx32 dimers, in which three of the CTs are not free but linked to the NT of the adjacent subunit, are more pH sensitive than channels formed by unlinked subunits (Peracchia *et al.* 1999).

These hypotheses also predict that cellular junctions would lose their pH sensitivity if maleate, for example, were perfused or dialyzed into the cytoplasm instead of an aminosulfonate buffer, and that it would be modified by the substitution of TAPS or MES.

Functional interaction between aminosulfonate and CT does not require that they act at the same site – the possibility of homotropic or heterotropic cooperative linkages allows for interaction of ligands' effects without requiring competition for the same binding site. For either mechanism, 'pH regulation' of connexin channels between cells would be modified by application of exogenous aminosulfonates.

That the naturally occurring amino-acid taurine directly modulates connexin channel activity is intriguing. Taurine is found in many different mammalian tissues, where it can be found at relatively high cellular concentrations (Green *et al.* 1991; Huxtable & Michalk, 1994). At physiological pH, essentially all taurine is fully protonated, so changes in overall taurine concentration could affect activity of aminosulfonate-sensitive channels. Even so, there is no evidence that taurine is an endogenous modulator of connexin channels in cells. Other cytoplasmic aminosulfonates, of which there are many, may be more effective, and therefore act over different ranges of pH.

The preceding discussion is based on the idea that the mechanisms of pH sensitivity are common across the connexins, which may not be the case. Truncation of the CT of Cx37,

mCx50, Cx45 (Stergiopoulos *et al.* 1999), Cx32 (Werner *et al.* 1991; Wang & Peracchia, 1997) or Cx46 (hemichannels) (Trexler *et al.* 1999) has little effect on pH sensitivity assessed in oocytes. Curiously, truncated hCx50 is pH insensitive when expressed in N2A cells (Xu *et al.* 2001), which could reflect differences in species orthologs or in expression systems. For these connexins, pH dependence may rely exclusively on soluble factors, such as aminosulfonates or pH-dependent kinase activity (Yahuaca *et al.* 2000), or on direct protonation of other domains, as for Cx46 (Trexler *et al.* 1999).

7.3 Calcium ion

Calcium has a long and contentious history as a modulator of connexin channels. It is clear that increased cytoplasmic calcium can cause junctional channels to close. It is not clear whether the effect is due to calcium ion directly, to concomitant changes in pH, or to calcium action on other cytoplasmic elements, such as calmodulin, as is the case for L-type calcium channels (Zuhlke & Reuter, 1998; Peterson *et al.* 1999; Qin *et al.* 1999) and at least one type of calcium-activated potassium channel (Fanger *et al.* 1999). If raised calcium ion levels are sufficient to close the channel, either directly or via calmodulin, one would expect the effect to be reversible, and reasonably independent of pH. It is likely that the role of calcium ion in gating of connexin channels differs among connexins.

In several cellular systems there is an apparent interaction between the effects of calcium and pH on coupling (De Mello, 1980; Lazrak & Peracchia, 1993; Firek & Weingart, 1995). If pH is maintained near neutrality, near millimolar levels of calcium ion are required to close the channels. It is clear that in some cases low pH can have effects independent of changes in calcium levels (Spray *et al.* 1982; Noma & Tsuboi, 1987). On the other hand, the effects of low pH can be lessened if the calcium buffer present does not release calcium when the pH is lowered (Lazrak & Peracchia, 1993). The one single-channel study of closure by calcium ions provides an explanation for some of these results, indicating that a site sensitive to near-millimolar calcium in the extracellular part of the pore can be accessed from either end of the pore, depending on the voltage (Pfahnl & Dahl, 1999). Since this study was carried out on hemichannels, this site may be the one at which calcium ion acts to keep plasma membrane hemichannels closed (see Sections 2.2.2, 3.1.2, 3.2.2, 4.2), and which may also correspond to the loop/chemical gate. There is no clear demonstration that in the documented absence of a pH change that calcium ion levels below millimolar can reversibly close connexin channels. This is not to say that calcium ion is not involved or an important modulator of effects on connexin channels. For example, manipulation of calmodulin expression in oocytes can dramatically alter the sensitivity of junctions to pH changes (Peracchia *et al.* 1996, 2000a, b), but it is not clear whether this is due to direct interaction of calmodulin with connexin, or downstream affects of the global alteration of calmodulin-mediated processes in the cells.

7.4 Lipophiles

Several classes of hydrophobic compounds reduce connexin channel activity (Bernardini *et al.* 1984; White *et al.* 1985; Davidson *et al.* 1986; Burt & Spray, 1988, 1989; Davidson & Baumgarten, 1988; Rudisuli & Weingart, 1989; Guan *et al.* 1997; Rozental *et al.* 2001). These compounds are commonly used to reduce macroscopic junctional

conductance so that single junctional channels can be recorded. Where their actions have been studied in detail they reduce the open probability of junctional channels without affecting unitary conductance (Veenstra & DeHaan, 1988; Burt & Spray, 1989; He & Burt, 2000). Their specific mechanisms of action may not be identical but they likely act via partitioning into the plasma membrane and affecting the physical properties of the bilayer, such as fluidity, stiffness, and local curvature, and/or of the lipid microenvironment of the connexin channels. Given their diverse chemical structures and similarity of effects, it is not likely that they interact with specific receptor sites. All these agents exert effects on other membrane proteins (cf. Hirche, 1985; Quastel & Saint, 1986; Karon *et al.* 1994), so they cannot be used as sole identifiers of junctional channels. In addition to the lipophiles discussed below, inhibitory effects have been noted for gossypol (Ye *et al.* 1990), ginseng saponin (Hong *et al.* 1996) and arachidonic acid (Giaume *et al.* 1989; Schmilinskyfluri *et al.* 1997) [which can exert its effects by other mechanisms as well (Fluri *et al.* 1990; Massey *et al.* 1992; Polonchuk *et al.* 1997; Martinez & Sáez, 1999)]. Where lipophile effects on membrane proteins have been studied, their effects strongly correlate with mole fraction in the bilayer and with the ability to induce disorder in the lipid acyl chains (Anel *et al.* 1993; Mitchell *et al.* 1996). Both properties can be strongly modulated by the specific lipid composition of the membrane (e.g. saturation, acyl chain length, presence of cholesterol). Furthermore, increasing chain length of *n*-alkyl alcohols or fatty acids can have a biphasic effect on acyl chain disorder, with shorter lengths increasing disorder and longer lengths decreasing it. These membrane composition-dependent effects are superimposed on the inherent chemical and structural differences of each lipophile.

Given this, the specificity of lipophile effects is likely to be mediated not by global changes in membrane properties (though they can occur), but by alteration of the acyl chains of the boundary lipids that are specifically associated with the connexins (Burt, 1989; Burt *et al.* 1991). With this in mind, one should not be surprised to find that different fatty acids, for example, affect different connexins in a differential manner – this likely reflects the ability of the fatty acids to have different efficiencies in acyl chain disruption for different lipids, and the possible preference of different connexins to have different lipids around them. These considerations no doubt contribute to the apparently contradictory claims and reports for the effectiveness or ineffectiveness of specific fatty acids in inhibiting connexin channels. The same logic follows for other lipophiles, offering the possibility, but not requirement, for a degree of specificity among connexin isoforms, and for connexin channels.

It is interesting to think that the same factors account for the *enhancement* of connexin junctional channel activity reported for γ -linolenic acid (Jiang *et al.* 1997) and hemichannel activity reported for quinidine/quinine (Malchow *et al.* 1994). Investigation of the ways in which acyl chains are disrupted by lipophiles that have opposite effects on the same connexin may be informative as to molecular mechanism.

7.4.1 Long chain *n*-alkyl alcohols

The *n*-alkyl alcohols heptanol and octanol produce a well-characterized, rapid, and reversible inhibition of gap junctional conductance (Délèze & Hervé, 1983; Spray & Burt, 1990). At the single-channel level, they reduce open-channel probability with little other effect (Veenstra & DeHaan, 1988; Spray & Burt, 1990; Bastiaanse *et al.* 1997). The potency of the effect of alkanols is inversely related to chain length. Hexanol is inactive at concen-

trations at which alkanols with longer chains are active, and is used as a control (Spray, 1994). These alkanols are effective on almost all known connexins, the one exception being cCx56, which is also unaffected by cytoplasmic acidification (Rup *et al.* 1993). Fluorescence anisotropy experiments showed that the effects were specifically correlated with a decrease in the fluidity of the cholesterol-rich membranes in which junctional channels reside, in spite of an increase in bulk membrane fluidity elsewhere (Bastiaanse *et al.* 1993). Use of an agent that alters fluidity only of non-cholesterol-rich regions had no effect on the junctional channels. These studies, and most applications of the *n*-alkyl alcohols, use concentrations of 1–2 mM. At such concentrations, they inhibit a variety of membrane proteins (Armstrong & Binstock, 1964; Upreti *et al.* 1980; McLarnon & Quastel, 1984; Hirche, 1985; Quastel & Saint, 1986; Llinás *et al.* 1987; Takens-Kwak *et al.* 1992; Garcia-Dorado *et al.* 1997), and likely affect other processes as well. However, recent studies in smooth muscle and cardiac myocytes make a strong case for the selective action of the alkanols on connexin channels when used at concentrations between 20 and 300 μM (Garcia-Dorado *et al.* 1997; Christ *et al.* 1999). In this work, effects on other membrane and cellular processes did not occur below 1 mM. It is possible that this specificity occurs due to the localization of junctional channels in cholesterol-rich membranes.

7.4.2 Fatty acids and fatty acid amides

Oleamide (*cis*-9-octadecenamide) and anandamide (arachidonoyl-ethanolamide) are recently described reversible inhibitors of gap junction communication (Venance *et al.* 1995a; Guan *et al.* 1997; Boger *et al.* 1998a). They are fatty acid amides, members of a growing class of membrane-localized signaling molecules (Devane *et al.* 1992; Di Marzo *et al.* 1994; Collin *et al.* 1995; Cravatt *et al.* 1995; Maurelli *et al.* 1995; Giang & Cravatt, 1997). The *cis*-isomers have more potent effects on coupling and greater membrane solubility than do the *trans*-isomers (Guan *et al.* 1997). These compounds have direct or indirect effects on many other proteins, including 5-HT receptors (Huidobro-Toro & Harris, 1996; Boger *et al.* 1998b), GABA receptors (Yost *et al.* 1998), sodium channels (Verdon *et al.* 2000), calcium channels (Gebremedhin *et al.* 1999) and cannabinoid receptors (Collin *et al.* 1995). However, the effects on coupling require substantially higher concentrations (μM versus 10–100 nM) than for effects on other channels. In addition, the structural requirements for activity are much less specific (Boger *et al.* 1999). Where investigated, the effects on connexins appear to be mediated by G-protein-coupled receptors, rather than cannabinoid receptors (Venance *et al.* 1995b; Sagan *et al.* 1999), and may be dependent on cell type (Venance *et al.* 1995b; Guan *et al.* 1997b). In some systems oleamide has been shown to have more specific effects than 40 μM GA (glycyrrhetic acid; see below) or 3 mM heptanol (Guan *et al.* 1997). At present there are no reported single-channel studies of fatty acid amide action. It was initially reported from a projection map that oleamide induced a substantial change in connexin channel structure. However, a preliminary report from a somewhat lower resolution 3D structure does not reveal significant changes (Cheng *et al.* 2001).

A variety of fatty acids have inhibitory effects on junctional channels (Giaume *et al.* 1989; Fluri *et al.* 1990; Burt *et al.* 1991; Hirschi *et al.* 1993; de Haan *et al.* 1994; Lavado *et al.* 1997). Fatty acids, and particularly *cis*-unsaturated fatty acids, affect a wide variety of cellular functions (cf. Ordway *et al.* 1989; Xiao *et al.* 1995, 1997; Xiao-Xian *et al.* 1998). In general, activity is positively correlated with the degree of *cis*-unsaturation and negatively correlated

with chain length. The ability of fatty acids to affect the function of membrane proteins is a function of their ability to induce disorder in the lipid acyl chains of the membrane (Anel *et al.* 1993). Thus the fact that *trans*-oleamide is less effective than *cis*-oleamide may not reflect specificity of action for connexins, only that the *trans* isomer does not disrupt acyl chains as much.

7.4.3 Halothane

The inhalation anesthetic halothane is widely used to inhibit junctional communication (Palmer & Slack, 1969; Burt & Spray, 1989; Spray & Burt, 1990). It is the most common agent used to reduce junctional conductance so that single-channel currents can be recorded. Its effects are rapid, reversible and nontoxic. The effect at the single-channel level is purely on gating; unitary conductance is not affected (Burt & Spray, 1988; He & Burt, 2000). Where tested, different connexins (Cx40, Cx43) have slightly different sensitivities to halothane (He & Burt, 2000). Surprisingly, heteromeric channels were significantly less sensitive than either of the corresponding homomeric channels. This could indicate that the structural changes induced by halothane are somewhat different for each connexin, and that they can interfere with each other when in a heteromeric configuration. Halothane appears to be effective on all connexins. As expected, its action is not specific for connexin channels; it affects a large number of channels and receptors (reviewed in Rozental *et al.* 2001). There is no reason to think that its mechanism of action on connexin channels is different from its mechanism of action on other membrane proteins, or separate from its actions as an anesthetic. Effects may be mediated by alteration of acyl chain packing, as for the alkanols (Baber *et al.* 1995). On the other hand, recent work with model proteins shows that halothane can bind with high affinity to appropriately sized hydrophobic cavities in proteins to stabilize helix–helix configurations (Johansson *et al.* 2000).

7.5 Glycyrrhetic acid and derivatives

The α and β stereoisomers of 18-glycyrrhetic acid (GA) and a related compound, 18-carbenoxolone (CA; GA-3 β -O-hemisuccinate) are naturally found in licorice, and reversibly inhibit gap junction communication in long-term culture at low concentrations without apparent toxicity at concentrations of 10–75 μM (Davidson *et al.* 1986; Davidson & Baumgarten, 1988; Goldberg *et al.* 1995). These triterpenoid saponins can have toxic effects at higher concentrations in long-term culture (Le & Musil, 1998). The reduction in coupling is never complete; electrical coupling is reduced only about 25% and there is residual dye coupling even at toxic concentrations (Martin *et al.* 1991; Goldberg *et al.* 1996). This could indicate partial pore block or closure. There are no reported direct effects of either compound on other ion channels. However several reports indicate nonspecific effects at concentrations greater than 40 μM (Taylor *et al.* 1998; Santicoli & Maggi, 2000) or even lower (Coleman *et al.* 2001). GA was noted to affect only junctional conductance at 10 μM , but affected connexin phosphorylation patterns at the higher concentrations often used (Le & Musil, 1998). In one study the effects of 10 μM GA on uncoupling were not accompanied by effects on other channels, as was the case with lipophiles (Venance *et al.* 1998). There is some variability in the effectiveness of GA on different connexins. It may therefore have a greater degree of specificity for connexins or among connexins than other uncoupling agents. Aside from their effects on connexin channels, GA and CA inhibit 11 β - and 3 α -hydroxysteroid dehydrogenases,

which metabolize cortisol to its inactive forms (Monder *et al.* 1989; Akao *et al.* 1992), and are responsible for the adverse effects of licorice abuse (Epstein *et al.* 1977; Cugini *et al.* 1983; Benediktsson & Edwards, 1994; Kelly *et al.* 1998). The enzyme inhibitory effects and the connexin inhibitory effects are both sensitive to isomeric structure and occur at similar concentrations. The metabolic precursor, glycyrrhizic acid, can be used as a control. In the absence of general or non-specific effects via the above enzymes, it seems likely that these compounds act directly on connexin channels (Davidson & Baumgarten, 1988). There are no reports of junctional insensitivity to these compounds, nor are there single-channel records of their effects. The uncoupling is not accompanied by a change in connexin expression, but is accompanied by disruption of the physical packing of the channels as seen by electron microscopy and freeze-fracture. There are conflicting data on whether this change is accompanied by phosphorylation in all connexins (Goldberg *et al.* 1996; Guan *et al.* 1996), and it clearly affects Cx26 channels, which lack CT phosphorylation sites (Zhang & Nicholson, 1989; George *et al.* 1998).

7.6 Cyclic nucleotides

An intriguing direct modulatory action of cAMP and cGMP on hemichannels formed by Cx32 and Cx26 has been demonstrated in the TSF system (Bevans & Harris, 1999b). The effect is not mediated by a protein kinase, as in Section 7.1 above, but rather by direct nucleotide interaction with a selective, high-affinity site on connexin itself; the effect occurs in purified and reconstituted connexin, in the absence of cytoplasmic or native membrane constituents. Accessibility to the site is apparently limited when the hemichannels are in liposome membranes, where action requires millimolar concentrations. However, when hemichannels in lipid-detergent micelles are exposed to nanomolar concentrations of the nucleotides, channel activity was inhibited following incorporation into liposomes. This inhibition was not reversible in the liposomes. The possibility of kinase activity is excluded by the absence of ATP, divalent cations or native membrane components. The modulatory sensitivity is specific for purine cyclic monophosphates (cPMPs), not occurring with nanomolar levels of AMP, ADP, ATP, cTMP, or cCMP. It is not likely that the inhibition of channel activity by cPMPs is due to physical occlusion of the pore, since cPMPs can permeate reconstituted Cx32 channels (Bevans *et al.* 1998). The chemical specificity and nanomolar affinity suggest a biological role.

These data are the first evidence of a modulatory high-affinity binding site for a second messenger on connexin channels. Fitting of the dose-response data to the Hill equation indicated subnanomolar K_d and n_H between 1 and 2. The properties of homomeric Cx32 and heteromeric Cx26:Cx32 channels were distinct but similar.

That activity inhibition persists in the absence of free nucleotide after reconstitution suggests that once the cPMP is bound and the channel is incorporated into a membrane, it is unable to dissociate. This 'locking-in' may occur by conformational changes secondary to binding. Examples include the conformational changes that occur in calmodulin (Ikura *et al.* 1992; Meador *et al.* 1992) and the molecular chaperone DnaK (Zhu *et al.* 1996) upon binding of peptide ligands. Binding at an active site can involve conformational changes that stabilize the bound complex (Joseph *et al.* 1990; Jia *et al.* 1995), and render it inaccessible to bulk solvent (Wierenga *et al.* 1992). Alternatively, dissociation of ligand may be blocked by

membrane lipids, where the bound ligand is shielded from the aqueous phase by the phospholipid bilayer following reconstitution.

Cyclic nucleotides can permeate some junctional channels (Tsien & Weingart, 1976; Pitts & Simms, 1977; Lawrence *et al.* 1978; Murray & Fletcher, 1984; Piccolino *et al.* 1984; Fletcher & Greenan, 1985; Qu & Dahl, In Press) as well as hemichannels (Bevans *et al.* 1998), so the inhibition at subnanomolar levels seems paradoxical. However, to inhibit the channels already in membranes, millimolar levels are required, which are well above cytoplasmic concentrations (Rich *et al.* 2000).

The activity of cyclic nucleotide analogs is informative regarding the nature of the binding site and the structural requirements for ligand activity. Two fluorescent analogs, ϵ -cAMP (1, N^6 -etheno-cAMP) and MANT-cGMP (2'-(*N*-methylantraniloyl)-cGMP, and the photoaffinity reagent 8-azido-cAMP, were effective at the same concentrations as the parent compounds. Since ϵ -cAMP and 8-azido-cAMP are derivatized on the purine ring, and MANT-cGMP derivatized on the ribose sugar, and all three are effective at nanomolar levels, it appears that additions to the 2, 8, N^6 and 2'-hydroxyl groups do not preclude efficacy or steric fit into the binding site.

There are several classes of cyclic nucleotide-binding sites. The most well-known is the CAP family, which includes the *E. coli* catabolite activator protein (CAP), cyclic nucleotide-dependent protein kinases and cyclic nucleotide-gated ion channels (Weber *et al.* 1989; Shabb & Corbin, 1992). However, Cx32 and Cx26 do not contain the amino-acid sequences that are strictly conserved at the cyclic nucleotide-binding domains of the CAP family. Neither is there obvious sequence similarity with the postulated cGMP-binding domain of cGMP-specific phosphodiesterases (PDE) (McAllister-Lucas *et al.* 1995).

Unlike the CAP and PDE families of cyclic nucleotide receptors, which have micromolar affinities, the cAR1 and cAR3 chemoattractant receptors of *Dictyostelium* have nanomolar affinities for cyclic nucleotides (Johnson *et al.* 1992), and mediate physiological responses at subnanomolar levels (VanHaastert, 1995). Further analogy with the cAR family is provided by the finding that MANT-cGMP is as effective on connexin as cGMP, suggesting that substitutions at the ribose 2'-OH position do not interfere with binding. An unsubstituted ribose 2'-OH group is *required* for binding of cGMP by the CAP family of receptors (Francis *et al.* 1990), but not for cAR1 receptors (VanHaastert & Kien, 1983).

The amino acids involved in the cyclic nucleotide binding to cAR1 have not yet been identified. However, a region of 26 amino acids (148–173) thought to control access to the binding pocket (Kim & Devreotes, 1994; Kim *et al.* 1997) shows ~ 25% identity and ~ 60% homology with a region of Cx32 and Cx26 that spans the cytoplasmic loop and the third transmembrane domain (M3). The same level of identity and homology extends through the 8 positions N-terminal to this sequence before dropping off. This sequence similarity is only suggestive – further analogy with the cAR family must await positive identification of the relevant amino acids.

		+ *++ *			* ++ *+ *++++
Cx26:	123–130:	TQKVRIEG	131–155:	SLWWTYTTTSIFFRV	–IFEAVFMYVFY
cAR1:	140–147:	KNTVQFVG	148–173:	NWCWIGVSFTGYRFG	LFYGPFLFIWA
Cx32:	122–129:	RHKVHISG	130–154:	TLWWTYVISVVFRL	–LFEAVFMYVFY
		++ *++ *		* * + +++ ** +++++	

M3 domain of connexin

(* , Identity; + , homology; alignment and homologies from Align using BLOSUM50)

That connexin channels have subnanomolar specific affinities for cAMP and cGMP and that binding of these cyclic nucleotides closes connexin channels is likely to be of physiological importance. One possible function is to keep hemichannels closed while they are in Golgi or ER membranes. The binding site may be accessible during initial membrane insertion and/or channel assembly, when cytoplasmic cPMPs could bind and thus ensure that connexin hemichannels remain closed during trafficking to the plasma membrane. Docking of apposed hemichannels to form the full intercellular channel could cause dissociation of these nucleotides from connexin channels.

The apparent low affinity for cPMPs in liposomes could arise from restricted accessibility to a high-affinity site, as would occur if the binding site were partially obscured by membrane lipids, or if the reconstituted channel was only rarely in a conformation that enabled access to the site. Cx32 is co-translationally inserted into ER membrane (Zhang *et al.* 1996; Falk *et al.* 1997). Intriguingly, it has been shown that during co-translational insertion of polytopic membrane proteins, the transmembrane segments inserted through the ER membrane are stabilized in a salt-accessible compartment, apparently not interacting directly with lipid (Borel & Simon, 1996; Hancin *et al.* 1996). Thus regions of a folded membrane protein that will be later blocked from aqueous access by membrane lipid appear to be transiently accessible to cytoplasmic components.

The fact that the cyclic nucleotide sensitivity is enhanced at least six orders of magnitude by detergent leads to the suggestion that the micellar environment renders the cPMP binding site(s) accessible. NMR studies show that non-denaturing detergent such as that used in the TSF studies increases molecular motion of and accessibility to protein domains that are exposed to lipid when in bilayers (Stopar *et al.* 1996; Kálmán *et al.* 1997). There is particularly enhanced accessibility to residues at the membrane-water transition (Spruijt *et al.* 1996; Stopar *et al.* 1997). There is evidence that non-denaturing detergents do not fully coat the hydrophobic surfaces of proteins (unlike SDS), leaving a somewhat open structure (Makino *et al.* 1975; Cavalieri *et al.* 1976) accessible to small, hydrophilic ligands.

Furthermore, the predicted number of amino-acid residues for connexin transmembrane helices suggests that the hydrophobic core of the protein is relatively short, composed of five helical turns (28 Å) (Yeager & Gilula, 1992) rather than the six or more typical of α -helical transmembrane segments of channel proteins (Bowie, 1997). Junctional channels may require cholesterol or specific lipids to accommodate this reduced axial hydrophobic distance, yet not occlude modulatory sites. In other systems, differences in membrane lipid composition are known to affect accessibility to binding sites as well as other aspects of channel function (Matthews, 1982; Reynolds, 1982; Devaux & Seigneuret, 1985; Conforti *et al.* 1990; Ryan *et al.* 1996; Zanello *et al.* 1996; Bastiaanse *et al.* 1997).

7.7 Other candidates

A recent paper describes a potentially direct gating effect of retinoids on junctional channels and hemichannels in bass horizontal cells (Zhang & McMahon, 2000). All-*trans* retinoic acid produced a reversible drop in junctional conductance within minutes at 10 μM concentrations applied to the outside of the cells, but not from the inside. The bleached form had no effect. The inhibition was unaffected by alteration of cytoplasmic ATP or GTP, addition of G-protein inhibitor, non-specific and specific kinase inhibitors or phorbol ester. There was no effect on normal non-junctional currents or glutamate- or kainate-induced currents. Effects

were also seen in macroscopic hemichannel currents and on single hemichannels in excised patches. The pharmacological profile suggests similarity to the retinoic acid receptor of the β or γ subtypes. The action in cells and in patches develops slowly (over minutes), which could indicate an indirect action via membrane-delimited receptor-mediated effects.

Quinine and quinidine have enhancing effects on hemichannel currents of teleost Cx35 (Malchow *et al.* 1994; Dixon *et al.* 1996; White *et al.* 1999; Al-Ubaidi *et al.* 2000). The mammalian (rodent) ortholog, Cx36, does not form functional hemichannels (Al-Ubaidi *et al.* 2000), but recent unpublished work shows that quinine has a reversible *inhibitory* effect on *junctional* channels formed by rat Cx36, teleost Cx35, as well as Cx50 and perhaps Cx45 (Srinivas *et al.* 2001). No effect was seen on junctional channels formed by Cx26, Cx32, Cx40 or Cx43. Effects were seen at macroscopic and single-channel levels, and were consistent with access from the cytoplasm. The EC50 was approximately 50 μM . The mechanism of channel block/closure is unclear, but the rapid onset indicates a direct interaction between quinine and the connexin channel. These data make clear that modulatory sensitivity (to quinine, in this case) can differ for the hemichannel and junctional channel structural forms.

8. Connexinopathies

A rapidly growing literature documents physiological and developmental abnormalities arising from defects in connexin expression and mutations in the connexin coding sequences. These data come from human genetic studies and from studies of transgenic mice in which connexin sequence or expression is altered. This literature has been reviewed and summarized recently (Abrams & Bennett, 2000; Kelsell *et al.* 2001a, b) and compilations of connexin polymorphisms and disease-causing mutations can be found at www.iro.es/deafness.html, www.uia.ac.be/dnalab/hhh/main.html, molgen-www.uia.ac.be/CMTMutations and at NCBI's Online Mendelian Inheritance in Man server (www3.ncbi.nlm.nih.gov/omim/searchomin.html). Syndromes associated with defects in specific connexins are listed in Table 11.

Since most cells express more than one connexin, Table 11 makes clear that in most cases one connexin cannot functionally compensate for the absence of another. This is confirmed in studies showing that replacement of the expression of Cx43 with either Cx32 or Cx40 under the control of the same promoter (a 'knock-in') results in partial rescue of the embryonic lethal 'knockout' phenotype, but that isoform-specific developmental defects remain (Plum *et al.* 2000). This is a clear demonstration of the specificity of the biological function of each isoform. Other studies have shown that expression of Cx26 can exert negative growth control of HeLa cells whereas expression of Cx40 or Cx43 cannot, even though all three successfully mediate electrical coupling (Mesnil *et al.* 1995). Expression of Cx32 in pancreatic β -cells, which endogenously express several other connexins, causes reduced glucose-induced release of insulin (Charollais *et al.* 2000). On the other hand, Cx43 rescues an embryonic lethal phenotype of a Cx31 knockout (Plum *et al.* 2001), and either Cx43 or Cx45 can compensate for the other to permit bone formation *in vitro* (Minkoff *et al.* 1999).

For connexins, as for most membrane proteins, most mutations result in defects in trafficking, folding or assembly (see VanSlyke *et al.* 2000). These defects typically produce a functional knock-out of the affected connexin. They can also inhibit functional expression of other connexins by a 'dominant negative' effect via heteromultimerization with non-mutant connexins. In addition, by analogous mechanism it is possible that mutant connexins could

Table 11. *Connexinopathies*

Connexin	Pathology	System	Possible mechanism
Cx26	Recessive non-syndromic deafness (DFNB1) (Kelsell <i>et al.</i> 1997)	Human	Impaired circulation of K ⁺ to endolymph via sensory hair cells, supporting cells and fibrocytes in cochlea
	Dominant non-syndromic deafness (DFNA3) (Kelsell <i>et al.</i> 1997)	Human	
	Palmoplantar keratoderma (PPK) (mutational overlap with DFNA3; abnormal callusing of palms and soles) (Kelsell <i>et al.</i> 2000)	Human	
	Vohwinkel syndrome (VS) (mutational overlap with DFNA3; deafness and callusing of digits leading to autoamputation) (Maestrini <i>et al.</i> 1999)	Human	
	Embryonic lethal (Gabriel <i>et al.</i> 1998)	Mouse KO	
Cx30	Dominant nonsyndromic deafness (DFNA3) (Grifa <i>et al.</i> 1999)	Human	Impaired transfer of glucose across the trophoblast layers of the placenta
	Clouston's hidrotic ectodermal dysplasia (HED) (palmoplantar hyperkeratosis, hair and nail defects) (Lamartine <i>et al.</i> 2000)	Human	
Cx30.3	Ethrythrokeratoderma variabilis (EKV) (hyperkeratosis and red patches in skin) (Macari <i>et al.</i> 2000)	Human	
Cx31	Progressive high-tone deafness (Xia <i>et al.</i> 1998)	Human	
	Ethrythrokeratoderma variabilis (EKV) (hyperkeratosis and red patches in skin) (Richard <i>et al.</i> 1998)	Human	
	Dominant and recessive nonsyndromic deafness (Coucke <i>et al.</i> 1999; Liu <i>et al.</i> 2000)	Human	
	Peripheral neuropathy (Lopez-Bigas <i>et al.</i> 2001)	Human	
Cx32	60% embryonic lethal due to placental dysmorphogenesis (Plum <i>et al.</i> 2001)	Mouse KO	Reduced labyrinth and spongiotrophoblast size
	X-linked form of Charcot-Marie-Tooth disease (CMTX), peripheral demyelinating neuropathy) (Bergoffen <i>et al.</i> 1993)	Human	Impaired function of reflexive junctions between myelin layers (Scherer <i>et al.</i> 1995)
	Late-onset disorganization of peripheral myelin (Anzini <i>et al.</i> 1997; Scherer <i>et al.</i> 1998)	Mouse KO	Impaired function of reflexive junctions between myelin layers (Scherer <i>et al.</i> 1995)
	Enhanced susceptibility to hepatic tumors (Temme <i>et al.</i> 1997)	Mouse KO	
	Compromised hepatic glucose mobilization (Chanson <i>et al.</i> 1998)	Mouse KO	
	Enhanced susceptibility to chemical hepatocarcinogenesis; delayed liver regeneration (Omori <i>et al.</i> 2001)	Mouse liver KO	
Cx36	Cortical asynchrony; defect in retinal processing (Guldenagel <i>et al.</i> 2001)	Mouse KO	Disrupted electrical coupling

Table 11. (*cont.*)

Connexin	Pathology	System	Possible mechanism
Cx37	Polymorphic marker for atherosclerotic plaque development (Boerma <i>et al.</i> 1999) Infertility (Simon <i>et al.</i> 1997)	Human	
		Mouse KO	Arrested oocyte maturation due to impaired coupling between oocytes and granulosa cells
Cx40	Cardiac conduction defects and impaired regulation of vasodilation (Kirchhoff <i>et al.</i> 1998; Simon <i>et al.</i> 1998)	Mouse KO	Impaired cardiac electrical coupling
Cx43	Visceroatrial heterotaxia (defect in left-right asymmetry leading to cardiac malformations and multiple organ defects) (Britz-Cunningham <i>et al.</i> 1995) and hypoblastic left heart syndrome (Dasgupta <i>et al.</i> 2001) Perinatal lethal: defects of conotruncus and right ventricle leading to obstruction of cardiac outflow (Reaume <i>et al.</i> 1995; Sullivan <i>et al.</i> 1998)	Human	
		Mouse KO	Disruption of neural crest cell migration
	Craniofacial abnormalities and delayed skeletal ossification (Lecanda <i>et al.</i> 2000)	Mouse KO	Osteoblast defect
	Small gonads, paucity of germ cells and immature follicles (Juneja <i>et al.</i> 1999)	Mouse KO	
	Structural defect in lens (Gao & Spray, 1998)	Mouse KO	Altered osmotic balance in the lens
	Diverse congenital abnormalities (spina bifida, anencephaly, myeloschisis, limb malformation, cleft palate, failure of hematopoiesis, cardiovascular deformity) (Becker <i>et al.</i> 1999)	Mouse, embryonic-knockdown	
	Defects in hematopoiesis (Montecino-Rodriguez <i>et al.</i> 2000)	Mouse KO	
	Sudden cardiac death due to ventricular arrhythmia (Gutstein <i>et al.</i> 2001)	Mouse cardiac KO	Slowed ventricular conduction velocity and increased anisotropy
	Hypotension and bradycardia (Liao <i>et al.</i> 2001)	Mouse endothelial KO	Elevation of plasma NO
	Cx45	Embryonic lethal: defective cardiogenesis and vasculogenesis (Kruger <i>et al.</i> 2000; Kumai <i>et al.</i> 2000)	Mouse KO
Cx46	Autosomal dominant zonular pulverulent cataract (Mackay <i>et al.</i> 1999)	Human	
	Nuclear cataracts at 2–4 wk (Gong <i>et al.</i> 1997)	Mouse KO	Altered calcium homeostasis
Cx50	Autosomal dominant zonular pulverulent cataract and microphthalmia (Berry <i>et al.</i> 1999)	Human	
	Autosomal dominant zonular pulverulent cataract and microphthalmia (White <i>et al.</i> 1998)	Mouse KO	
	Nuclear opacity cataract (Steele <i>et al.</i> 1998)	Mouse KO	

disrupt the function of other proteins with which they interact directly. When the mutants form functional channels, the pathology could be caused by altered permeability properties or by altered channel regulation. While the information in Table 11 makes clear the medical importance of appropriately functioning connexin channels, and facilitates funding of connexin research, it is not informative regarding the molecular bases of the pathological conditions – What are the molecules that must permeate junctional channels to produce a normal phenotype? What are the molecular signals that erroneously or abnormally pass through connexin channels to cause problems? How does the biophysics of connexin channels determine the changes in intercellular signaling that produce pathology? What are the downstream molecular events that are disrupted because of defects in connexin channel function?

To answer these questions it may be useful to examine the properties of mutated connexins that form functional channels yet cause physiological or developmental defects. By such examination one can hope to infer the molecular basis of the defect, or at least to identify the functional property of the connexin that is responsible. Unfortunately, for most connexins, at this time the number of identified mutants that form functional channels is small. Naively, one expects the mutations in the CL and CT domains to primarily affect channel regulation, and mutations in the other domains to affect the pore or voltage dependence directly or indirectly, and chemical regulation indirectly.

Cx32 mutants are the most widely studied, due to the extraordinary number of CMTX-inducing human mutations, which are distributed across the entire coding sequence. As will probably be the case for other connexins, there is good evidence that mutations in Cx32 cause other, perhaps subclinical, syndromes in addition to CMTX (Abrams *et al.* 2000). Several CMTX Cx32 mutants form functional channels in oocytes and/or transfected mammalian cells (comprehensively reviewed in Abrams *et al.* 2000). Point mutations are located in M1, E2 and CL. One mutation produces a 5-residue deletion in the CL, and several mutants lead to truncation of the CT from C217 onward. Some of the mutants show a shift in the conductance–voltage relations so that the occupancy of the low-conductance V_j -induced substate is favored (e.g. Oh *et al.* 1997). Presumably this substate permits electrical coupling but inhibits most molecular movement. Other mutants show enhanced sensitivity to cytoplasmic acidification [e.g. E102G and Del111-116 in the CL and R220stop in the CT (Ressot *et al.* 1998)]. However, the behavior of some mutants is only minimally altered, showing no significant alteration of steady-state G – V properties or pH sensitivity, e.g. L56F (Ressot *et al.* 1998). One of these is the S26L mutant described in Section 4.1, which seems to narrow the pore (Oh *et al.* 1997). It is noteworthy that moderate narrowing of Cx32 channels correlates with reduced permeability to cyclic nucleotides (Bevans *et al.* 1998).

Most of the mutations of Cx26, Cx30.3 and Cx31 known to cause deafness and skin disorders are early truncations. The missense mutations that have been functionally characterized do not form channels and can act as dominant negatives (Richard *et al.* 1998, 2000; White, 2000).

The polymorphism of Cx37 reported to be a marker for atherosclerotic plaque development (P319) was studied at the single-channel level expressed in RIN cells. The plasma membrane expression, junctional channel formation, unitary conductance and voltage dependence was unaltered compared with S319, which is not correlated with plaque development (Kumari *et al.* 2000). This suggests that the P319 polymorphism, which is in the CT domain, affects modulation of channel gating by factors other than voltage.

The somatic point mutation in Cx43 that appears to cause the laterality defects in humans (S364P) is also located in the CT domain (Dasgupta *et al.* 2001). Removal of the wild-type serine at this position alters the modulatory effects of phosphorylation at this site.

In Cx46, the point mutation N63S in E1 is sufficient to cause cataracts in humans (Mackay *et al.* 1999). It does not form junctional channels in *Xenopus* oocytes (Pal *et al.* 2000).

The P88S mutation in Cx50 (in M2; see Section 6.2.2) that causes cataracts in humans does not form junctional channels in the *Xenopus* system, and functions as a dominant negative (Pal *et al.* 1999). The D47A mutation in E1 that causes cataracts in mice does not form functional channels in the *Xenopus* system (Xu & Ebihara, 1999).

The use of pathology-producing mutations to explore connexin structure–function is still in its infancy. One hopes that as more of the mutations are screened functionally that promising candidates for study will be identified.

9. Summary

This summary is a proposed synthesis of available information for the non-specialist. It does not incorporate all the published data, is inconsistent with some, and reflects the biases of the author.

Connexin proteins have a common transmembrane topology, with four α -helical transmembrane domains, two extracellular loops, a cytoplasmic loop, and cytoplasmic N- and C-terminal domains. The sequences are most conserved in the transmembrane and extracellular domains, yet many of the key functional differences between connexins are determined by amino-acid differences in these largely conserved domains. Each extracellular loop contains three cysteines with invariant spacing (save one isoform) that are required for channel function.

The junctional channel is composed of two end-to-end hemichannels, each of which is a hexamer of connexin subunits. Hemichannels formed by some connexin isoforms can function as well-behaved, single-membrane-spanning channels in plasma membrane. In junctional channels, the cysteines in the extracellular loops form intra-monomer disulfide bonds between the two loops, not intermonomer or inter-hemichannel bonds. The end-to-end homophilic binding between hemichannels is via non-covalent interactions. Mutagenesis studies suggest that the docking region contains β structures, and may resemble to some degree the β -barrel structure of porin channels. The two hemichannels that compose a junctional channel are rotationally staggered by approximately 30° relative to each other so that the α -helices of each connexin monomer are axially aligned with the α -helices of two adjacent monomers in the apposed hemichannel. At present there is a published 3D map with 7.5 Å resolution in the plane of the membrane, based on electron cryomicroscopy of 2D crystals of junctional channels formed by C-terminal truncated Cx43. The correspondence between the imaged transmembrane α -helices and the known transmembrane amino-acid sequences is a matter of debate.

Each of the approximately 20 connexin isoforms produces channels with distinct unitary conductances, molecular permeabilities, and electrical and chemical gating sensitivities. The channels can be heteromeric, and subfamilies among connexins largely determine heteromeric specificity, similar to the specificities within the voltage-dependent potassium channel superfamily. The second extracellular loop contains the primary determinants of the

specificity of hemichannel–hemichannel docking (analogous to the tetramerization domain of potassium channels).

The 7.5 Å map shows that each monomer exposes only two transmembrane α -helices to the pore lumen. However the conductance state of the imaged structure and the effects of the C-terminal truncation are unknown, so it is possible that other transmembrane domains contribute to the lumen in other functional states of the channel.

In the transmembrane region, SCAM and mutagenesis data suggest that parts of the first three transmembrane α -helices are exposed to the lumen. Some of these data are contradictory, but may reflect conformational or isoform differences. There is reason to think that the first part of the N-terminal cytoplasmic domain can line the pore in some conformations. In the extracellular part of junctional channels, the N-terminal portion of the first extracellular loop is exposed to the lumen.

The unitary conductances through connexin channels vary over an order of magnitude, from 15 pS to over 300 pS. There is a range of charge selectivities among atomic ions, from slightly anion selective to highly cation selective, which does not correlate with unitary conductance. There appear to be substantial ion–ion interactions within the pore, making the GHK model of assessing selectivities of limited value. Pores formed by different connexins have a range of limiting diameters as assessed by uncharged and charged probes, which also does not correlate with unitary conductance (i.e. some have high conductance but have a narrow limiting diameter, and vice versa). Channels formed by different connexins have different permeabilities to various cytoplasmic molecules. Where it has been assessed, the selectivity among cytoplasmic molecules is substantial and does not correlate in an obvious manner with the size selectivity data derived from fluorescent tracer studies, suggesting there are chemical specificities within the pore that enhance or reduce permeability to specific cytoplasmic molecules, functionally analogous to the ability of some porins to facilitate transport of specific substrates. For example, heteromeric channels with different stoichiometries or arrangements of isoforms can distinguish among second messengers. The differences in permeability to cytoplasmic molecules have biological consequences; in most cases one connexin cannot fully substitute for another.

Voltage and chemical gating mechanisms largely operate within each hemichannel, though there is evidence for inter-hemichannel allosteric effects as well. There are at least two distinct gating mechanisms. One (V_j -gating) is a voltage-driven mechanism that governs rapid transitions between conducting states. Its voltage sensor involves charges in the first several positions of the cytoplasmic N-terminal domain and possibly in the N-terminal part of the first extracellular loop, which may both be exposed to the lumen of the pore in some states. The polarity of V_j -gating sensitivity is connexin-specific, closing with depolarization for some connexins and with hyperpolarization for others. The polarity can be reversed by point mutations at the second position. The lower conductance states induced by V_j -gating correspond to physical restrictions of the pore, and thus restricted or eliminated molecular permeation. Since the channels are not fully closed by V_j -gating, it can be seen as a way to eliminate molecular signaling while leaving electrical signaling operational.

A second, independent gating mechanism mediates slow transitions (~ 10 – 30 ms) into and out of non-conducting state(s). These transitions can occur in response to voltage ('loop gating'), chemical factors such as pH and lipophiles ('chemical gating'), and the docking of two hemichannels (sometimes called the 'docking gate'). These slow transitions may reflect a common structural change induced by these several effectors (electrical, chemical and

homodimerization). Alternatively, they could reflect distinct gating processes responding to one or more of these effectors, that are indistinguishable at the single-channel level and have yet to be resolved mechanistically.

The slow or loop gate closes with hyperpolarization. As a result, where V_j -gating closes with depolarization, individual hemichannels can close in response to both polarities of voltage (but only to a subconductance state for the V_j -gating polarity). Because of this, it is difficult to assign a macroscopic voltage sensitivity, or its modification due to mutagenesis, chemical modification or heteromeric interactions, to one or the other of these very distinct voltage-sensitive processes. This distinction can be made reliably only at the single-channel level.

The V_j -gating voltage sensor and the loop-gating voltage sensor appear to be independent structures, since the V_j -gating voltage sensitivity can be modified without effect on loop gating. For some connexins, certain modifications of the C-terminal domain seem to interfere with the operation of the V_j -gate while leaving loop gating unaffected.

In some connexins, but not all, the chemical sensitivity to pH can involve interactions between regions of the C-terminal domain and cytoplasmic loop. Whether these regions exert their effects directly by physically blocking the pore, or by allosteric mechanisms (which may be more consistent with the relatively long time-course of closure) is not clear. For several connexins, truncation of the C-terminal domain eliminates the pH sensitivity, and co-expressing the domain with the truncated connexin restores the pH sensitivity. This has a functional resemblance to the particle–receptor mechanism for N-type inactivation of *Shaker* channels. What is being protonated is not clear, and may involve cytoplasmic factors, such as endogenous aminosulfonates. For other connexins, the action of pH does not involve the C-terminal domain and seems due to direct protonation of connexin.

PKC phosphorylation of serine(s) in the C-terminal domain can affect the substate occupancy of at least one connexin. Phosphorylation of serines in the C-terminal domain by MAP kinase appears to facilitate an interaction between it and an unknown receptor domain to eliminate coupling. This process has yet to be studied at the single-channel level. It also has a functional analogy to the particle–receptor model of channel inactivation. Both MAP kinase phosphorylation-induced and pH-induced inhibition can be mediated in truncated connexins by the corresponding free peptide. However, the relation between these two mechanisms are unexplored, as are specific mechanisms of direct endogenous regulation of connexin channel activity.

Major questions include:

- (1) What are the pore-lining sequences and how do they change in the different conductance states?
- (2) What are the determinants of molecular permeability among cytoplasmic molecules?
- (3) How does heteromericity influence channel function?
- (4) What are the structural gating elements and conformational changes that underlie the rapid transitions of V_j -gating?
- (5) What is the relationship between the loop, chemical and docking gating processes, and what are the conformational changes that accompany each?
- (6) What is the structural basis of hemichannel–hemichannel docking and its relation to opening of hemichannels?
- (7) What are the cytoplasmic ligands that regulate connexin channel function?

- (8) Are there fundamental mechanistic or structural differences in the gating and permeability processes of different connexins?

The stage is set for direct, molecular analysis of these issues. One looks forward to the application of the approaches that reveal structural interactions and dynamics that have been applied recently to other channels, and to higher resolution structural maps. Information from such studies, guided by an awareness of the intimate and fundamental biological function of connexin channels, will help us to understand the link between molecular mechanisms and what cells are able to do if they talk to each other.

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