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Review

Connexin channel permeability to cytoplasmic molecules

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Abstract

Connexin channels are known to be permeable to a variety of cytoplasmic molecules. The first observation of second messenger junctional permeability, made ~30 years ago, sparked broad interest in gap junction channels as mediators of intercellular molecular signaling. Since then, much has been learned about the diversity of connexin channels with regard to isoform diversity, tissue and developmental distribution, modes of channel regulation, assembly, expression, biochemical modification and permeability, all of which appear to be dynamically regulated. This information has expanded the potential roles of connexin channels in development, physiology and disease, and made their elucidation much more complex—30 years ago such an orchestra of junctional dynamics was unanticipated. Only recently, however, have investigators been able to directly address, in this more complex framework, the key issue: what specific biological molecules, second messengers and others, are able to permeate the various types of connexin channels, and how well? An important related issue, given the ever-growing list of connexin-related pathologies, is how these permeabilities are altered by disease-causing connexin mutations. Together, many studies show that a variety of cytoplasmic molecules can permeate the different types of connexin channels. A few studies reveal differences in permeation by different molecules through a particular type of connexin channel, and differences in permeation by a particular molecule through different types of connexin channels. This article describes and evaluates the various methods used to obtain these data, presents an annotated compilation of the results, and discusses the findings in the context of what can be inferred about mechanism of selectivity and potential relevance to signaling. The data strongly suggest that highly specific interactions take place between connexin pores and specific biological molecular permeants, and that those interactions determine which cytoplasmic molecules can permeate and how well. At this time, the nature of those interactions is unclear. One hopes that with more detailed permeability and structural information, the specific molecular mechanisms of the selectivity can be elucidated.
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Keywords: Connexin; Gap junction; Molecular permeability; Second messengers

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1. Introduction

Aside from the ability to pass electrical current, the signal property of gap junctions is that they mediate intercellular movement of cytoplasmic molecules. This property underlies the widespread interest in the biological functions of gap junctions. While proof-of-principle of functional intercellular molecular signaling through these channels was demonstrated many years ago (Lawrence et al., 1978), elucidation of the specific roles that gap junctions play in development, physiology and pathology has remained elusive. There are many kinds of connexin channels, each with distinct permeability properties, and it is difficult to experimentally determine exactly which cytoplasmic molecules can pass through the channels, how well, and which do not. It has become evident that the simple indices of channel permeability (unitary conductance, charge selectivity, permeability to fluorescent dyes) do not correlate well with each other, or with permeability to cytoplasmic molecules (Veenstra et al., 1995; Veenstra, 1996; Nicholson et al., 2000; Goldberg et al., 2004; Weber et al., 2004; Bedner et al., 2006; Hernandez et al., 2007). This has motivated the recent efforts to investigate the permeation of cytoplasmic molecules through specific types of connexin channels, reviewed in this article.

Generically, the pores of connexin channels are sufficiently wide (nominally ~12 Å) to be permeable to a wide variety of cytoplasmic molecules. There is evidence for permeation through at least some types of connexin channels of virtually all soluble second messengers, amino acids, nucleotides, calcium ions and glucose and its metabolites (Harris, 2001).

Until a few years ago, the expectation was that any cytoplasmic molecule of appropriate size would permeate connexin channels, and that the limiting (i.e., minimum) pore diameter would be the primary determinant of molecular permeation. It had also been widely presumed that, due to the relatively large pore diameter, there would not be significant charge selectivity among permeants. Both expectations were incorrect—pore diameter, while obviously important, does not account for the observed molecular selectivities, and many connexin channels exhibit substantial charge selectivity, even among monovalent atomic ions (Verselis and Veenstra, 2000).

A variety of approaches have been applied to investigation of the permeability properties of connexin channels, well reviewed in Verselis and Veenstra (2000). These studies mostly involve measurement of unitary conductance, charge selectivity among atomic ions (e.g., K, Cl) and/or permeability to charged fluorescent tracer molecules. They reveal a striking heterogeneity of pore properties. The unitary conductances of connexin junctional channels are broadly distributed over the range ~10 pS to over 300 pS, depending on the connexin isoform (Veenstra et al., 1994, Verselis and Veenstra, 2000; Kreuzberg et al., 2005). The charge selectivities range from slightly anion selective (1.1:1 for Cx32; Suchyna et al., 1999) to highly cation selective (10:1 for Cx45; Veenstra et al., 1995). These vastly different unitary conductances and charge selectivities suggest that the pores of different connexin channels have diverse properties, structural and/or electrostatic.

Molecular permeabilities have been assessed using several classes of fluorescent tracer molecules, of various charges, sizes and chemistries. Each type of connexin channel has characteristic permeabilities among these tracers (cf. (Elfgang et al., 1995; Cao et al., 1998)). These studies show that the magnitudes of unitary

Table 1
Relative pore sizing with uncharged tracers

Connexin	Permeable tracers	Impermeable tracers	Reference(s)
26h[a]	G2PA[b] and smaller, sucrose	G3PA and larger	Locke, 1998
26/32h[c]	G2PA and smaller, sucrose	G3PA and larger	Bevans et al., 1998; Locke et al., 2004
26j	PEG200 and smaller	PEG300 and larger	Gong and Nicholson, 2001
32h	G3PA and smaller, sucrose	G4PA and larger	Bevans et al., 1998; Locke et al., 2004
32j	PEG300[d] and smaller	PEG400 and larger	Krasilnikov et al., 1995
	PEG300 and smaller	PEG400	Gong and Nicholson, 2001
37j	Triethylene glycol and smaller	PEG200 and larger	Gong and Nicholson, 2001
40j		Mannitol, raffinose, stachyose	Beblo and Veenstra, 1997
43h	G4PA and smaller, sucrose	G5PA	Locke et al., 2004
43j	Mannitol	Raffinose, stachyose	Wang and Veenstra, 1997
46h	Sorbitol, glucose	Sucrose, stachyose	Qu and Dahl, 2004; Ma and Dahl, 2006

[a]In this and the subsequent tables, for reasons of space the isoform composition of the connexin channels is denoted only by the identifying number, with the prefix "Cx" omitted. The suffix "h" denotes "hemichannel" and the suffix "j" denotes "junctional channel". For example, 43h refers to hemichannels formed by Cx43, and 32j refers to junctional channels formed by Cx32.
[b]G*n*PA refers to maltosaccharides with *n* glucose units, derivatized with an aminopyridyl group (uncharged), as described in Bevans et al. (1998).
[c]Heteromeric channels are denoted by the numbers of the two connexin isoforms separated by "/".
[d]PEG*xxx* refers to polyethylene glycol with molecular weight *xxx* Da.

conductance do not correlate with the sizes of the permeant tracer molecules, with some of the larger tracer molecules permeating channels with low conductances (e.g., Cx32), and unable to permeate channels with higher conductances (e.g., Cx26) (Veenstra et al., 1995; Cao et al., 1998). This suggested that a simplistic view of the lumen of the connexin channel as a right cylinder was incorrect, and that the pore likely had the complex structure of most other eukaryotic membrane channels. The high conductance–small permeant case can be accounted for by a pore that is wide except for a very short constriction that would restrict molecular flux yet have minimal effect on electrical conductance. The low conductance–large permeant case can be accounted for by a pore that is just wide enough over its entire length to allow a given permeant to pass, or more complex properties, such as permeant-induced flexibility of the pore.

The fluorescent tracers have been used successfully and extensively to report the existence and extent of junctional communication and hemichannel function. They are less informative as investigational tools regarding the nature of the permeability pathway itself. Even the most widely used set of dyes, the Alexa series, vary dramatically in shape and spatial distribution of chemical moieties and in charge.

A few studies have used sets of highly homologous uncharged molecules (sugars, polyethylene glycols) to directly assess the limiting pore width of connexin channels. However, even for these, one cannot exclude the possibility that the molecules have interactions with the walls of the pore that affect permeation, as for maltose and the maltoporin channel (Schirmer et al., 1995; Wang et al., 1997). However, with this caveat aside, the use of uncharged permeants has been informative (Table 1; for a summary, see Section 5.2.1 of Harris, 2001).

The studies listed in Table 1 suggest the following ranking of limiting pore diameter:

$Cx43 > Cx32 > Cx26 = Cx26/Cx32 > Cx37 > Cx46$ and $Cx43 > Cx40$. (For comparison, data using the Alexa dyes suggest the following ranking of pore diameter: $Cx32 \geqslant Cx43 \gg Cx26 \geqslant Cx40 = Cx45 \geqslant Cx37$ (Weber et al., 2004)).

More recently, efforts have been made to characterize the permeability of connexin channels to cytoplasmic molecules. In some sense, knowledge of which signaling or regulatory molecules can pass through which types of connexin channels, and how well, is the "holy grail" of connexin channel function. The widespread interest in connexin channels by biologists in many disciplines has been frustrated by the absence of a satisfying answer to the questions: What is/are the important endogenous chemical signal(s) that can pass through gap junction channels? Are they different for different types of connexin channels, and if so, how?

We now know that the second question can be answered in the affirmative. We also know the answers to the other questions will not be simple, for reasons including: (1) the existence of over 20 connexin isoforms, which

have distinct pore properties, as evidenced by different unitary conductances, charge selectivities and permeabilities to tracers, (2) the fact that almost all of the isoforms can form heteromeric channels with substantial numbers of other isoforms, and (3) the possibility that the permeability properties of a given connexin channel can be dynamically modulated (e.g., by voltage, reversible post-translational modification and perhaps ligands). Therefore, there will be many answers, and each will be specific to a particular structural and biochemical channel configuration, defined by the cellular biology of the coupled cells at the specific time an interaction occurs.

Until this is worked out, the challenge is to define the permeabilities to specific cytoplasmic molecules through connexin channels of various compositions. The data thus far suggest that different connexin channels can have highly distinct and differential permeabilities among cytoplasmic molecules, and that these bear little discernible relation to the permeabilities to nonbiological fluorescent tracers that have been used to date.

2. Assessment of connexin channel permeability to cytoplasmic molecules

The data on permeation of cytoplasmic molecules through connexin channels come from a variety of experimental approaches. Some involve direct measurements of molecular flux through unambiguously identified connexin channels. Other studies reply on indirect methods to infer the identity of the transferred compounds or that the transfer is through connexin channels. Previous reviews focused specifically on this topic include Goldberg et al. (2004) and Nicholson et al. (2000). This section discusses the various methods used.

2.1. Junctional channels

2.1.1. Pathway identification

Because connexin channels are likely to be unique in forming direct intercellular pathways for molecular movement in vertebrates, identifying them as the pathway of observed transfer of compounds between cells is relatively straightforward. However, even for this simple case, several potentially complicating factors must be considered and controlled for.

Cytoplasmic bridges can be found between cultured mammalian cells (cf. Bukauskas et al., 1992; Valiunas et al., 1999). Their contribution to intercellular molecular transfer can be assessed by conditions that close gap junction channels, such as low pH or any of the multitude of "gap junction blockers." Demonstration of lack of transfer of gap junction-impermeable compounds, such as dextrans, serves the same purpose.

The most problematic case is where intercellular Ca signaling is taken as evidence of gap junction-mediated molecular communication. Such signaling is now well established to occur by two distinct pathways—gap junctional and paracrine—and both may operate in a given situation (Boitano et al., 1992; Sanderson, 1996; Giaume and Venance, 1998; Homolya et al., 2000; Braet et al., 2001; Jorgensen et al., 2002; Schuster et al., 2002; Bennett et al., 2006; Henriksen et al., 2006; Scemes and Giaume, 2006). The former involves intercellular diffusion through gap junction channels of a molecular signal, usually considered to be IP3 and/or Ca. This signal elicits intracellular Ca release, which regenerates the signal. The paracrine pathway involves extracellular release of ATP via any of several candidate mechanisms (e.g., connexin hemichannels, pannexin channels, P2XY activated channels; see Section 2.2), which interacts with puringeric receptors on a neighboring cell, in turn inducing Ca entry. Therefore, intercellular Ca signaling may be due to gap junctional permeation by IP3 and/or Ca, or to ATP release, with connexin hemichannels being one of the possible pathways. This means that observations of intercellular Ca signaling cannot be designated as junctional unless the paracellular pathway is excluded. This may be done by the use of suramin, oxidized ATP or other agents that either block "postsynaptic" purinergic receptors or that degrade ATP.

There is the possibility that intercellular molecular transfer can be mediated by junctional channels formed by pannexin (Bruzzone et al., 2003; Baranova et al., 2004; Barbe et al., 2006). Pannexin1 (panx1) forms junctional channels when overexpressed in the *Xenopus* oocyte system (Bruzzone et al., 2003) and in at least one cell line (vanden Abeele et al., 2006). Pannexin gene and/or protein expression have been demonstrated in

a wide variety of tissues (Bruzzone et al., 2003; Baranova et al., 2004; Dvoriantchikova et al., 2006; Litvin et al., 2006; Locovei et al., 2006, Ray et al., 2006), but expression of panx1 in most cell types used in permeability studies to date has not been directly assessed. This raises the possibility that junctional permeability mediated by pannexin channels could contribute to permeability that has been attributed to connexin channels, particularly if expression of the two proteins is positively correlated. However, it has been noted that junctional channels formed by panx1 have not been demonstrated in any native system (Dahl and Locovei, 2006), and it was recently demonstrated that panx1 does not form junctional channels when expressed in neurons or glia (Huang et al., 2006).

2.1.2. Compound identification

Identification of specific cytoplasmic compounds that pass through junctional channels is achieved by several means. One is by monitoring the appearance of a specific compound in one cell in response to increase in the concentration of that compound in a coupled cell. This can be via a fluorescent sensor for the compound itself (e.g., FURA-2 for Ca (Sáez et al., 1989), H30 for cAMP (Hernandez et al., 2007; Ponsioen et al., 2007), LIBRA for IP3 (Hernandez et al., 2007), or by the activity of channels that are regulated by a compound (e.g., CNG (Bedner et al., 2006) or CFTR (Qu and Dahl, 2002) channels for cAMP). One must consider that the elevation of a compound may be a secondary consequence of intercellular movement of a distinct, un-"sensed" compound.

As mentioned above, the most ambiguous case is that where Ca signaling is observed to spread between cells. Where established to be junctional, as opposed to paracellular, the signaling is usually considered as evidence that IP3 permeates the junctional channels. However, junctional Ca flux may also be involved. In some cases, it is clear that regenerative Ca signaling is mediated by IP3 flux and that the contribution of junctional Ca flux to this process is minor (Venance et al., 1997; Leybaert et al., 1998). In other cases it appears that junctional Ca flux is a major factor (Sáez et al., 1989; Boitano et al., 1992; Christ et al., 1992). Computational modeling has supported both scenarios, and delineated synergistic roles for both (Sneyd et al., 1994, 1998; Höfer, 1999; Höfer et al. 2001, 2002; Iacobas et al., 2006).

A second approach involves "metabolite capture," in which radiolabeled compounds are harvested specifically from recipient cells (cells not radiolabeled, but co-cultured with metabolically radiolabeled donor cells) and then identified biochemically (Goldberg et al., 1998, 1999, 2002; Goldberg and Lampe, 2001; Alexander and Goldberg, 2003). The caveat here is that the compounds thus identified may not be those that passed through the junctions, but rather their metabolic products.

A "bioassay" approach was used to monitor transfer of siRNA between cells, in which the specific action of the siRNA reports its transfer (Valiunas et al., 2005). In this case, there is little doubt as to the permeable molecular species.

2.2. Hemichannels

2.2.1. Pathway identification

Studies involving hemichannels have much greater potential for artifactual findings, except in the cases where purified connexin protein is used. Most such studies involve assessment of the release of cytoplasmic compounds from cells into the extracellular medium. Proof that connexin hemichannels are the pathway for release are highly problematic because of other plasma membrane channels that can be permeable to large molecules, whose expression or regulation may be affected by the treatments that affect connexin channels. The difficulties and criteria for identifying hemichannel function in cells are comprehensively reviewed in Spray et al. (2006).

The most prominent non-connexin pathway is the large pore activated by purinergic P2X7 receptors, which was shown recently to be formed by panx1 (Pelegrin and Surprenant, 2006; Locovei et al., 2007).

Activation of P2X7 receptors leads to activation of a conductance to small cations, including Ca. It also leads to slower activation of a much larger pore, which is permeable to Lucifer yellow, ATP and glutamate (Alves et al., 1996; Ballerini et al., 1996; Surprenant et al., 1996; Rassendren et al., 1997; Bisaggio et al., 2001; North, 2002; Duan et al., 2003; Parpura et al., 2004; Suadicani et al., 2006). The activity is enhanced by low extracellular Ca (Virginio et al., 1997), which is also a common method of activating connexin hemichannels.

A recent report showed that in glial cells the ATP release that occurs during paracrine intercellular propagation of Ca signaling is through P2X7-activated channels, and not Cx43 hemichannels (Suadicani et al., 2006). It was also shown that the large P2X7-activated (i.e., panx1) channel was blocked by several compounds widely used to block connexin channels.

It is now clear that the larger P2X7-activated channel is in fact a panx1 channel (Pelegrin and Surprenant, 2006; Locovei et al., 2007). In independent work, it was shown that panx1 channels are permeable to ATP and mechanosensitive (as are some connexin channels; Bao et al., 2004b), and that several of the pharmacological agents that inhibit connexin junctional channels also inhibit panx1 channels (Bruzzone et al., 2005; Locovei et al., 2006; Pelegrin and Surprenant, 2006). In addition, they can be indirectly but robustly activated by extracellular ATP stimulation of P2Y receptors, which increases submembrane Ca, which in turn activates the panx1 channels (Bao et al., 2004a; Dahl and Locovei, 2006; Locovei et al., 2006). As pannexin channels are widespread and there are as yet no pharmacological agents that specifically block them (unlike P2X7 receptors), release of ATP and other cytoplasmic molecules through them—either by their direct activation or via activation of P2X7 receptors—must be considered a significant possibility that is not controlled for in most of the studies on this issue; these recent findings call into question many of the previous conclusions regarding connexin channel properties that are based on molecular leakage studies.

Other channels that can be permeable to large molecules include maxi-anion channels (Bosma, 1989; Sabirov and Okada, 2004), the CFTR channel (Reisin et al., 1994), plasma membrane VDAC (Dermietzel et al., 1994; Rostovtseva and Colombini, 1996; Okada et al., 2004), and non-connexin mechanosensitive or volume-sensitive channels (Rutledge et al., 1999; Hisadome et al., 2002; Takano et al., 2005; Bader and Weingart, 2006).

Several methods can be used to eliminate the potential contributions of these channels to observed release of cytoplasmic molecules, however these controls are not applied in many of the published studies. It stands to reason that if connexin hemichannels mediate the flux into the medium of ATP (and/or glutamate, as has been reported), other cytoplasmic molecules ought to be released into the extracellular medium as well, but this has not been systematically investigated. Each study must be evaluated on its own merits, with the following considerations in mind.

Inhibition by blockers: A variety of organic compounds are used to block gap junctions (reviewed in Harris, 2001; Rozental et al., 2001). However, none are specific for connexin channels. This limits their utility as means to determine whether cellular release of a cytoplasmic component is through a connexin channel. As mentioned above, many of the commonly used connexin channel blockers also block panx1 channels, either studied directly (Bruzzone et al., 2005; Locovei et al., 2006; Pelegrin and Surprenant, 2006) or as P2X7-activated channels whose activity is enhanced by low Ca (Suadicani et al., 2006). The compounds so characterized include glycerrhetinic acid, carbenoxolone, mefloquine, octanol and heptanol. These data make a strong case for not relying on these agents as the sole means to identify release via connexin channels.

Correlation with connexin expression: In some studies, release of cytoplasmic compounds is examined as a function of connexin expression. This is accomplished by comparing the release from cells not expressing connexin, due to absence of endogenous expression, or to genetic deletion or siRNA knockdown, with that from cells that express connexin, either endogenously or via transfection. In several systems, there is clear correlation between connexin expression and ATP release (cf. Cotrina et al., 1998, 2000). However, a large literature documents the multifaceted biological effects of induced connexin expression. Altered connexin expression has been shown to have dramatic effects on the expression of literally hundreds of other gene products (Naus et al., 2000; Iacobas et al., 2003, 2004, 2005), which may have downstream effects on non-connexin plasma membrane proteins and other mechanisms of release. Such changes could alter expression or regulation of the other candidate channels. However, in at least in one type of cell, P2X7 receptor expression itself is not affected by connexin expression (Suadicani et al., 2006), though panx1 expression was not assessed. These concerns are difficult to address; while there are good data suggesting that cellular ATP and other molecules can permeate connexin hemichannels, in many studies it is not clear that connexin hemichannels are the channels through which ATP release occurs. In several cases there is a notable lack of correlation between the conditions under which ATP release occurs and the conditions required for significant opening of the relevant connexin hemichannels. This important issue has been discussed in detail in several excellent review

articles (Bennett et al., 2003; Goodenough and Paul, 2003; Sáez et al., 2003; Contreras et al., 2004; Barbe et al., 2006; Dahl and Locovei, 2006; Evans et al., 2006).

Dependence on low Ca: Most connexin hemichannels are kept closed by normal extracellular Ca levels; hemichannel activity is enhanced when the Ca concentration drops below ~0.5 mM (Ebihara and Steiner, 1993; Ebihara, 1996; Beahm and Hall, 2002; Ebihara et al., 2003). Unfortunately, this can also enhance other permeability pathways in the plasma membrane, including P2X7 receptors. Panx1 channels on their own, however, seem to be insensitive to these changes in extracellular Ca (Bruzzone et al., 2005).

Application of peptide inhibitors of connexin channels: Potentially, the most specific means to identify permeability due to plasma membrane hemichannels is the use of connexin mimetic peptides. These are small peptides that correspond to segments of specific connexins. Some of the peptides that correspond to the extracellular regions of connexin protein have been shown to block hemichannels when applied extracellularly (Warner et al., 1995; Boitano et al., 1998; Chaytor et al., 1998; Evans et al., 2006). The mechanism of action of these peptides is unclear; presumably they interact with the extracellular domains of the hemichannels, as they are likely to during homophilic binding end-to-end docking of hemichannels, but in such a way that the channels are unable to open. This is somewhat counterintuitive, since such interaction between correctly folded extracellular loop domains would *promote* conductive channels. As yet, there are no published reports regarding the specificity of these peptides for connexin channels (i.e., testing against other plasma membrane channels that may mediate release of cytoplasmic components). In one case, a peptide mimetic for a cytoplasmic connexin segment inhibited ATP release when applied extracellularly (De Vuyst et al., 2006), which is difficult to explain; extracellular application of peptides corresponding to cytoplasmic segments ought to be a good *negative* control for specificity. Perhaps most distressing in this context is unpublished work indicating that connexin mimetic peptides substantially reduce currents though panx1 channels at the same concentrations that block connexin channels (G. Dahl, pers. commun.).

2.2.2. Compound identification

Identification of compounds released by cultured cells is fairly straightforward, since such compounds can be directly recovered and analyzed biochemically.

2.3. Relative permeabilities

To understand the distinct biological consequences that follow from intercellular junctions composed of different kinds of connexin channels, it is important to be able to compare the ability of a given compound to permeate channels formed by different connexins. Similarly, it is important to know how well different cytoplasmic molecules permeate a given type of connexin channel. Differences in permeation are undoubtedly the major determinants of the unique signaling functions that each type of connexin channel seems to serve. Information on the relative magnitudes of molecular permeability, rather than all-or-none information, requires consideration of a multitude of factors. The best way to obtain such information is to determine the junctional permeability coefficient for each compound and each connexin, derived from the rate of junctional flux and the concentration in the donor cell. Junctional permeability must then be normalized to the number of channels, so that the permeability per channel can be determined.

This ideal type of measurement is difficult to obtain. It requires monitoring or determining the concentration of compound in the donor cell, and determining the rate of appearance of the compound in the receiver cell. These measurements can be complicated if the concentration of free compound in the donor cell is not constant over the time course of the measurement, due to leakage, metabolism, or junctional transfer itself, unless that concentration is monitored as a function of time. In addition, calculation of junctional permeability can be compromised by cytoplasmic binding, spatial "buffering" or inhomogeneities of the compound within the donor cell. Measurement of junctional flux into the receiver cell also requires that attention be paid to the potential for leakage and metabolism over the time course of the measurement. Unless the total amount of compound entering the receiver cell is measured, variation in cell volume and inhomogeneities of compound distribution can introduce errors, as can binding to cytoplasmic components. Finally, to convert the junctional permeability to channel permeability, the number of functional channels must be determined. This is typically done by dividing the macroscopic junctional conductance by the unitary

conductance of the junctional channels. This index of channel number can be compromised by the existence of subconductance states, by channel open probabilities that are less than unity, and by the fact that for large plaques of active channels, the conductance does not linearly reflect channel number, due to the overlapping access resistance domains of closely packed channels (Wilders and Jongsma, 1992; Ramanan et al., 1994; Hall and Gourdie, 1995).

It is important to note, as hinted above, that relative permeabilities to endogenous compounds can be affected by metabolic degradation, a factor that does not affect assessment of permeability to nonbiological molecules. Consider the case where a biological compound is degraded at a rate within an order of magnitude of the rate of junctional flux, or faster. The detection of its rate of accumulation in the receiving cell will not accurately reflect the rate of junctional flux, giving an artifactually low apparent junctional permeability. Rates of degradation can differ between cells, and be dependent upon metabolic state.

Given the above considerations, measurement of relative permeabilities may seem impossible to perform with confidence. However, each of the issues raised above can be addressed and controlled for in various ways. In some cases, absolute numbers for junctional permeability are not obtained, but instead indices of junctional permeability are empirically correlated, such as the number of cells surrounding a source cell in which a compound reaches a threshold level after a certain length of time.

Two recent studies show how most of these concerns can be successfully addressed (though not necessarily by the specific approaches summarized above).

In the work of Bedner et al. (2003, 2006), cAMP was released photolytically in the donor cell, and activity of CNG channels in the receiver cell reported the transjunctional accumulation of cAMP. Activity of CNG channels was monitored by the Ca signal of Fluo-4. The raw data showed no correlation between Ca signal in the receiver cell with the number of gap junction channels as assessed by junctional conductance. This demonstrated the need for strict normalization for cell volume, expression of the sensor (in this case, the CNG channels) and nonlinearity of response of the sensor (the response of the CNG channels to increased cAMP, and the response of Fluo-4 to the increased Ca signal). The correction for these factors was to only use data in which the cAMP to Fluo-4 signal in the receiver cell due to junctional transfer was equal to that obtained in the same cell by direct photolysis with variable flash duration. Once equivalence of this signal from the two sources (photolytic release of cAMP in the receiver cell and the cAMP signal due to junctional flux from the receiver cell) was obtained, the cAMP flux could be compared for cells expressing different connexins, when normalized to the number of channels. Control experiments excluded potential cytoplasmic bridges, and other studies showed that Ca was not the junctional signal that caused Ca to increase in receiver cells following cAMP uncaging in the donor cell.

Since the peak amplitude of the Fluo-4 response was taken as the index of cAMP flux, without the careful internal controls performed, this measurement could be compromised by variation in the kinetics of the response in the receiver cell as a function of the connexin expressed—perhaps different levels of cAMP hydrolysis or even kinetics of the CNG channel itself. But in this study, these factors are controlled for by the internal calibration for each cell pair. One potential source of error is the possibility of different substate occupancies of the different connexin channels, which would introduce error into the calculation of the number of junctional channels. Another is that since the error introduced by access resistance mentioned above varies with the size of the junctional plaque (more specifically, the number of closely packed functioning channels), data from cell pairs with high and low junctional conductances may not be consistent, because of errors in determining the number of junctional channels in the two cases.

Hernandez et al. (2007) used novel ratiometric fluorescent sensors to directly assess junctional flux of cAMP and of IP3. Use of such sensors simplifies the measurement in eliminating the intermediate transduction step in the previous case from change in second messenger level to change in Ca influx. The sensors used were FRET-conjugates of proteins that are sensitive to the respective second messengers. For cAMP, the sensor was based on Epac, a guanine nucleotide exchange factor (Ponsioen et al., 2004) and for IP3 was based on the ligand-binding domain of an IP3 receptor (Tanimura et al, 2004). Each sensor was genetically expressed in the cells, and the responses calibrated in those cells via patch electrode dialysis with solutions of known concentrations. The donor cell was patch-clamped with a pipette containing a known amount of compound (cAMP or IP3). The total ratiometric FRET fluorescence was determined for each cell, and the volume of each cell determined from through-focus z-axis sequences of confocal images. The junctional transfer rate was calculated from the

FRET signals in the donor and receiver cells only during the interval when the FRET signal was known to linearly reflect the concentration of the compound being sensed. Immediately afterward, junctional conductance was determined by patch-clamp of the receiver cell, to permit estimation of the number of functional junctional channels. The possibility of the exogenous compounds affecting junctional conductance during the experiment was controlled for by maintaining the dual-cell patch for long enough to see if there was a tendency for the junctional conductance to change. Typically, the junctional conductance was found to trend downward, so the value used for calculation of number of channels was estimated by extrapolation back to the time interval during which the FRET measurements were made. The two considerations mentioned above regarding possible error in estimation of number of junctional channels due to gating or subconductance states, and due to access resistance, apply here as well.

3. The published data

Tables 2 and 3 summarize data published to date that are informative regarding permeation by cytoplasmic molecules through connexin channels, where there is reasonable identification of both the connexin channel composition and the permeant. Many other studies demonstrate specific second messenger permeation through unidentified connexin channels, or permeation by unidentified molecules through identified connexin channels, but the data from these studies are not included.

The two table s contain the same information, but are organized differently. Table 2 organizes the data according to type and composition of connexin channel. Table 3 organizes the same data by molecular permeant. For each report, some of the relevant elements of the measurements are indicated, according to the key below, which may be helpful in assessing the results in light of the discussion above. Note that not all the listed controls are applicable to all the studies.

Annotations for Table 2 and Table 3
ϕ: positive evidence for the compound being impermeant
IP3: inositol-1,4,5-triphosphate

For junctional channels

Channel identity	1a. explicit evidence against cytoplasmic continuity
	1b. control for paracellular pathway for ATP/IP3/Ca flux
Compound identity	2. Ca signaling used to infer Ca/IP3 permeation

For hemichannels

Channel identity	3a. used connexin channel blockers
	3b. correlated flux with connexin expression or flux blocked by connexin antisense
	3c. flux dependent on low Ca or mechanical stimulation
	3d. flux blocked by connexin mimetic peptides
	3e. controlled for flux through purinergic receptor activated channels
	3f. immunopurified and reconstituted connexin
Compound identity	(not an issue for hemichannel studies)

These Tables are on the side of being inclusive; any study of interest should be examined closely, keeping in mind the points raised in Section 2 above. Entries have been combined whenever possible. For simplicity, data from intercellular spread of Ca waves are represented as indicating junctional permeability to IP3 (rather than for IP3 and/or Ca), unless involvement of IP3 is excluded (see discussion above). There are two kinds of data, that for permeation without indication of relative magnitude, and that for relative permeability. In the tables, the latter follow the former, and values for relative permeabilities, when available, follow each permeant in parentheses, normalized to the compound in the series with the highest permeability.

These data are at once exciting and disappointing. They are exciting in that many types of channels and permeants have been investigated, and there is positive evidence that a wide variety of important cytoplasmic molecules permeate the various types of channels. The data are disappointing in that the information, with the notable exceptions of Cx26, Cx32, Cx26/Cx32 and Cx43 channels, do not yet allow one to ascertain or infer *differences* in molecular signaling mediated by the different channel types. To do so, a larger dataset is needed for absolute permeabilities, and for relative permeabilities across connexins and across permeants.

The studies that generated this information employed a wide range of techniques and degrees of control and rigor. It is important to reiterate that the single most serious potential source of error is the nature of the permeability pathway through plasma membrane when assessed by release of compounds from cells, whether assessed directly or via Ca waves (where junctional communication is excluded), due to possible participation of pannexin/P2X7 receptor-activated channels.

Taken at face value, almost all the studied connexin channels seem permeable to cAMP and IP3. The most informative work is that which yields information on the *relative* permeabilities of different connexins or different molecules, but as described above, obtaining such information is a complex and tedious task. However, the unique strength of those studies is that they offer direct comparison of data on permeants or

Table 2
Permeabilities organized by channel type and composition

26h	ATP (Tran Van Nhieu et al., 2003)	3a 3b 3e (purinergic block had only partial effect)
	IP3 (Locke et al., 2004; Ayad et al., 2006)	3f
26h or 26/30h	ATP (Zhao et al., 2005)	3a 3c
26h or 43h	ATP (Gomes et al., 2005)	3a 3c 3d [a]
26h or 30h or 26/30h or 43h	Aspartate (Ye et al., 2003)	3a 3c 3e [a]
	Glutamate (Ye et al., 2003)	3a 3c 3e [a]
26/32h	cAMP (Bevans et al., 1998; Ayad et al., 2006)	3f (not permeable through all the heteromeric channels)
	cGMP (Bevans et al., 1998; Ayad et al., 2006)	3f (not permeable through all the heteromeric channels)
	IP3 (Gomes et al., 2005)	3f (not permeable through all the heteromeric channels)
	IP3 (Ayad et al., 2006)	3f (not permeable through all the heteromeric channels; differential permeability among inositol triphosphate isoforms)
26j	cAMP (Bedner et al., 2003, 2006)	1a
	IP3 (Niessen et al., 2000)	1b 2
	IP3 (Niessen and Willecke, 2000)	2
	IP3 (Paemeleire et al., 2000)	1b 2
	IP3 (Beltramello et al., 2005)	1a 1b 2
	IP3 = cAMP (Hernandez et al., 2007)	1a 1b
26j or 26/30j	IP3 (Zhang et al., 2005)	1a 1b 2 (Ca injection used to rule out Ca flux)
26/32j	siRNA 22mer (Valiunas et al., 2005)	1a
	ϕ RNA 12mer (Valiunas et al., 2005)	1a
26/32j or 32j	IP3 (Niessen and Willecke, 2000)	2
	IP3 (Clair et al., 2001)	1a 2 (Ca flux ruled out as causing Ca signaling)
32h	ATP (Cotrina et al., 1998)	3a 3b 3c (connexin block had only partial effect)
	ATP (Cotrina et al., 2000)	3b 3c
	ATP (Belliveau et al., 2006)	3a 3b
	ATP (De Vuyst et al., 2006)	3a 3b 3d 3e (peptide work in question; cytoplasmic peptide had effect from extracellular medium)
	cAMP (Bevans et al., 1998; Locke et al., 2004)	3f
	cGMP (Bevans et al., 1998; Locke et al., 2004)	3f
	Glutamate (Takeuchi et al., 2006)	3a 3d
	IP3 (Locke et al., 2004; Ayad et al., 2006)	3f
32j	cAMP (Bedner et al., 2003, 2006)	1a
	Ca++ (Sáez et al., 1989)	2 (connexin identity inferred)

Table 2 (*continued*)

	IP3 (Sáez et al., 1989)	2 [a]
	IP3 (Niessen et al., 2000)	1b 2
	IP3 (Paemeleire et al., 2000)	1b 2
	Adenosine > glucose(0.73) > ADP/ATP(0.50) > glutathione(0.37) > AMP(0.19) > glutamate(0.12) (Goldberg et al., 2002)	1b [b]
	Glutathione = glutamate > ADP/ATP(~0.7) (Goldberg et al., 1999)	1a [b]
36j	cAMP (Bedner et al., 2003, 2006)	1a
37j or 37/43j or 43j	Ca++ (Christ et al., 1992)	1a 2 [a]
38h	ATP (Bahima et al., 2006)	3a 3b 3c
43h	ATP (Cotrina et al., 1998)	3a 3b 3c (connexin block had only partial effect)
	ATP (Cotrina et al., 2000)	3b 3c
	ATP (Romanello and D'Andrea, 2001)	3a 3c 3e
	ATP (Stout et al., 2002)	3a 3b 3c
	ATP (Pearson et al., 2005)	3a 3d 3e
	ATP (Belliveau et al., 2006)	3a 3b
	ATP (Eltzschig et al., 2006)	3a 3d
	ϕ ATP during paracellular propagation of Ca waves (Suadicani et al., 2006)	
	cAMP (Kam et al., 1998)	3f
	IP3 (Kam et al., 1998)	3f
	IP3 (Romanello and D'Andrea, 2001)	3a 3c 3e
	NAD+ (Bruzzone et al., 2001a, b)	3a 3b 3c 3f
	Prostaglandin E2 (Jiang and Cherian, 2003; Cherian et al., 2005)	3a 3b 3c 3e
43j	ADP/ATP (Goldberg et al., 1998)	1a
	ϕ adenosine (Goldberg et al., 1999)	1a
	cAMP (Bedner et al., 2003, 2006)	1a
	cAMP (Ponsioen et al., 2007)	1a
	IP3 (Venance et al., 1997)	1a 1b 2 [a]
	IP3 (Niessen et al., 2000)	1b 2
	IP3 (Paemeleire et al., 2000)	1b 2
	IP3 (Romanello and D'Andrea, 2001)	1a 1b 2
	Mobile pH buffers (Swietach and Vaughan-Jones, 2004)	1a [a,c]
	Peptides up to 10mers (Neijssen et al., 2005)	1a
	siRNA 22mer (Valiunas et al., 2005)	1a
	ϕ dsRNA 12mer (Valiunas et al., 2005)	1a
	ADP /ATP = glutathione = glutamate (Goldberg et al., 1999)	1a [b]
	ADP/ATP = glucose > glutathione(0.46) > AMP(0.38) > glutamate(0.29) ≫ adenosine (~0) (Goldberg et al., 2002)	1b [b]
	RNA 12mer > 16mer > 24mer (Valiunas et al., 2005)	1a
43/46j	cAMP (Qu and Dahl, 2002)	1a (not permeable through substate)
45j	cAMP (Bedner et al., 2003, 2006)	1a
46h	cAMP (Qu and Dahl, 2002)	3b (not permeable through substate)
47j	cAMP (Bedner et al., 2003, 2006)	1a
pan1h	ATP (Bao et al., 2004a; Locovei et al., 2006)	

[a]Connexin identity inferred.

[b]Due to metabolic issues, in this study there is some ambiguity of the identities of the compounds that actually permeated the junctional channels. ATP could contribute to the measurement of ADP, AMP and adenosine, and glutamate could contribute to the glutathione measurement. Therefore the relative permeabilities reported may be artifactually skewed toward the downstream metabolic products, resulting in the appearance of lesser relative permeability to ADP/ATP and to glutamate.

[c]Mobile pH buffers are phosphate, homocarnosine, taurine, and acetylated derivatives of anserine, carnosine and histidine (Vaughan-Jones et al., 2002).

Table 3
Permeabilities organized by molecular permeant

ATP	26h (Tran Van Nhieu et al., 2003)	3a 3b 3e (purinergic block had only partial effect)
	26h or 26/30h (Zhao et al., 2005)	3a 3c
	26h or 43h (Gomes et al., 2005)	3a 3c 3d[a]
	32h (Cotrina et al., 1998)	3a 3b 3c (connexin block had only partial effect)
	32h (Cotrina et al., 2000)	3b 3c
	32h (Belliveau et al., 2006)	3a 3b
	32h (De Vuyst et al., 2006)	3a 3b 3d 3e (peptide work in question; cytoplasmic peptide had effect from extracellular medium)
	38h (Bahima et al., 2006)	3a 3b 3c
	43h (Cotrina et al., 1998)	3a 3b 3c (connexin block had only partial effect)
	43h (Cotrina et al., 2000)	3b 3c
	43h (Romanello and D'Andrea, 2001)	3a 3c 3e
	43h (Stout et al., 2002)	3a 3b 3c
	43h (Pearson et al., 2005)	3a 3d 3e
	43h (Belliveau et al., 2006)	3a 3b
	43h (Eltzschig et al., 2006)	3a 3d
	ϕ 43h during Ca waves (Suadicani et al., 2006)	
	pan1h (Bao et al., 2004a; Locovei et al., 2006)	
ADP or ATP	43j (Goldberg et al., 1998; Goldberg et al., 1999)	1a[b]
	43j (Goldberg et al., 2002)	1b[b]
	43j $\gg$ 32j($\sim$0.01) (Goldberg et al., 1999)	1a[b]
	43j > 32j(0.14) (Goldberg et al., 2002)	1b[b]
AMP		
	43j $\gg$ 32j (Goldberg et al., 2002)	1b[b]
	43j > 32j(0.14) (Goldberg et al., 2002)	1b[b]
Adenosine	32j (Goldberg et al., 2002)	1b[b]
	ϕ 43j (Goldberg et al., 2002)	1b[b]
Aspartate	26h or 30h or 26/30h or 43h (Ye et al., 2003)	3a 3c 3e[a]
cAMP	26h (Locke et al., 2004)	3f
	26j (Hernandez et al., 2007)	1a 1b
	26/32h (Bevans et al., 1998; Locke et al., 2004)	3f (not permeable through all the heteromeric channels)
	32h (Bevans et al., 1998; Locke et al., 2004)	3f
	43h (Kam et al., 1998)	3f
	43j (Ponsioen et al., 2007)	1a
	43/46j (Qu and Dahl, 2002)	1a (not permeable through substate)
	46h (Qu and Dahl, 2002)	3b (not permeable through substate)
	43j > 26j(0.34) > 45j(0.21) = 32j(0.18) > 47j(0.14) $\gg$ 36j(0.03) (Bedner et al., 2003, 2006)	1a
cGMP	26h (Locke et al., 2004)	3f
	26/32h (Bevans et al., 1998; Locke et al., 2004)	3f (not permeable through all the heteromeric channels)
	32h (Bevans et al., 1998; Locke et al., 2004)	3f
Ca^{2+}	32j (Sáez et al., 1989)	2[a]
	37j or 37/43j or 43j (Christ et al., 1992)	1a 2[a]
Glucose		
	43j > 32j(0.23) (Goldberg et al., 2002)	1b[b]
Glutamate	26h or 30h or 26/30h or 43h (Ye et al., 2003)	3a 3c 3e[a]
	32h (Takeuchi et al., 2006)	3a 3d
	43j > 32j(0.12) (Goldberg et al., 2002)	1b[b]
	43j $\gg$ 32j(0.03) (Goldberg et al., 1999)	1a[b]
Glutathione		
	43j > 32j(0.23) (Goldberg et al., 2002)	1b[b]
	43j $\gg$ 32j(0.03) (Goldberg et al., 1999)	1a[b]
IP3	26h (Locke et al., 2004; Ayad et al., 2006)	3f
	26j (Niessen and Willecke, 2000)	2

Table 3 (*continued*)

	26j (Paemeleire et al., 2000)	1b 2
	26j (Beltramello et al., 2005)	1a 1b 2
	26j (Hernandez et al., 2007)	1a 1b
	26j or 26/30j (Zhang et al., 2005)	1a 1b 2 (Ca injection used to rule out Ca flux)
	26/32h (Locke et al., 2004)	3f (not permeable through all heteromeric channels)
	26/32h (Ayad et al., 2006)	3f (not permeable through all the heteromeric channels;differential permeability among inositol triphosphate isoforms)
	26/32j or 32j (Niessen and Willecke, 2000)	2
	26/32j or 32j (Clair et al., 2001)	1a 2 (Ca flux ruled out as causing Ca signaling)
	32h (Locke et al., 2004; Ayad et al., 2006)	3f
	32j (Brehm et al., 1989)	2[a]
	32j (Paemeleire et al., 2000)	1b 2
	43h (Kam et al., 1998)	3f
	43h (Romanello and D'Andrea, 2001)	3a 3c 3e
	43j (Venance et al., 1997)	1a 1b 2[a]
	43j (Paemeleire et al., 2000)	1b 2
	43j (Romanello and D'Andrea, 2001)	1a 1b 2
	32j > 43j > 26j (Niessen et al., 2000)	1b 2 (normalized by Mn spread, not junctional conductance)
Mobile pH buffers	43j (Swietach and Vaughan-Jones, 2004)	1a[a,c]
NAD+	43h (Bruzzone et al., 2001a, b)	3a 3b 3c 3f
Peptides ⩽ 10mers	43j (Neijssen et al., 2005)	1a
Prostaglandin E2	43h (Jiang and Cherian, 2003; Cherian et al., 2005)	3a 3b 3c 3e
RNA 12mer	ϕ 26/32j (Valiunas et al., 2005)	1a
	43j (Valiunas et al., 2005)	1a
RNA 16mer	43j (Valiunas et al., 2005)	1a
RNA 24mer	43j (Valiunas et al., 2005)	1a
dsRNA 12mer	ϕ 43j (Valiunas et al., 2005)	1a
siRNA 22mer	ϕ 26/32j (Valiunas et al., 2005)	1a
	43j (Valiunas et al., 2005)	1a

[a]Connexin identity inferred.

[b]Due to metabolic issues, in this study there is some ambiguity of the identities of the compounds that actually permeated the junctional channels. ATP could contribute to the measurement of ADP, AMP and adenosine, and glutamate could contribute to the glutathione measurement. Therefore the relative permeabilities reported may be artifactually skewed toward the downstream metabolic products, resulting in the appearance of lesser relative permeability to ADP/ATP and to glutamate.

[c]Mobile pH buffers are phosphate, homocarnosine, taurine, and acetylated derivatives of anserine, carnosine and histidine (Vaughan-Jones et al., 2002).

connexins within the same experimental paradigm. A discussion of the potential biological importance of the specific relative permeabilities discovered to date is in Bedner et al., 2006.

Post-translational modifications may affect permeability, either by direct modification of the permeation pathway or by altered gating leading to occupation of conductance substates (Moreno et al., 1992; Qu and Dahl, 2002; Ek-Vitorin et al., 2006). This means that the rank orders of metabolite permeation may vary depending on the cell system used, due to different post-translational modifications of the connexin.

The most unexpected finding is that siRNAs, which one would expect to be too large to permeate junctional channels, can in fact do so (Valiunas et al., 2005). This property is connexin-specific, and provides proof-of-principle for an exciting and potentially very important avenue of intercellular regulation.

It has been suggested that hemichannel permeability differs from that of junctional channels formed by the same connexin(s). There is no evidence for this in the data published to date; these data show good correspondence between hemichannel and junctional channel permeabilities for cytoplasmic compounds, subject to the uncertainty about involvement of panx1 channels previously noted.

The work with reconstituted heteromeric Cx26/Cx32 hemichannels shows that some of these channels were permeable to IP3 and some were not, whereas all the inositol triphosphates tested permeated the

corresponding homomeric hemichannels (Ayad et al., 2006). This suggests that heteromeric channels can have higher degrees of selectivity than homomeric channels. Given the ubiquitous distribution of heteromeric channels in vivo, this may be biologically important, and offers a unique role for heteromeric connexin channels.

This work points out a caveat for the study of the permeability of heteromeric channels. Since there is no way, yet, to produce heteromeric channels of a single stoichiometry or radial arrangement of different connexins, any population of heteromeric channels is likely to be structurally heterogeneous, and therefore likely to contain channels with a variety of permeabilities and impermeabilities. This means that a permeability determined for a population of heteromeric channels is likely to be a mixture of different all-or-none selectivities and of relative permeabilities, making it difficult to assign a specific permeability to a specific heteromeric structural form.

4. Potential mechanisms of permeation and selectivity

What are the possible mechanisms and determinants of the observed selectivities among biological molecules?

4.1. Evidence for specific affinities, or, Connexin channels are not molecular sieves

One historical view has been that large channels, including connexin channels, act as molecular sieves that discriminate among potential permeant molecules largely on the basis of size. In this simple view, all molecules able to enter a pore and squeeze past its narrowest internal diameter will permeate. Charge may be expected to play a role, with charged permeants interacting with the pore vestibule and the lumen of the pore via electrostatic field effects. The degree to which charge influences permeation would depend on the number of charges involved and the degree to which steric factors determine the distance between charge(s) on the permeant and charge(s) on the lumen walls (e.g., with the charges of larger permeants more closely approaching lumen walls than those of smaller permeants; see Zambrowicz and Colombini, 1993). It is often presumed that the charge selectivity of a pore is reflected in the charge selectivity of the currents that pass through the channels as assessed by ionic replacement and reversal potentials.

When one compares the pore diameters assessed by uncharged molecules (Cx43 > Cx32 > Cx26 = Cx26/Cx32 > Cx37 > Cx46; see Section 1) with the biological permeants that have been shown to pass through them one is struck by the absence of correlation.

Even the narrowest pore in this series (Cx46) is permeable to cAMP. Cx26, Cx32 and Cx43 are all permeable to cAMP, ATP and IP3. Cx46 is permeable to cAMP yet some Cx26/Cx32 channels are impermeable to cAMP, even though Cx46 ostensibly has the narrower pore. On the basis of the relative permeability data for Cx32 and Cx43 channels, one would not conclude that Cx32 is the narrower pore, which is clearly the case.

A wide variety of cation/anion selectivities have been described for connexin channels, ranging from highly cation selective (Cx40, Cx45) to slightly anion selective (Cx32) (Neyton and Trautmann, 1985; Harris et al., 1992; Veenstra et al., 1995; Veenstra, 1996; Beblo and Veenstra, 1997; Wang and Veenstra, 1997; Suchyna et al., 1999; Trexler et al., 2000; Verselis and Veenstra, 2000; Harris, 2001).

For Cx32, since permeation of adenosine is greater than that of the anions ADP/ATP (Goldberg et al., 2002), one might infer that increased negative charge and size decreases the permeation. However, increased negative charge should *favor* permeation through this anion-preferring pore, and AMP is smaller and less negative, yet permeates less well. In the case of Cx43, ADP/ATP permeate better than adenosine (Goldberg et al., 2002), making the largest and most negatively-charged molecules the most permeant through this cation-preferring channel. The most highly cation-selective connexin channel (Cx45) (Veenstra et al., 1995) is permeable to the anion cAMP. The relative permeabilities of the weakly anion selective channel (Cx32) do not correlate with the magnitude of negative charge.

The anion cAMP permeates best through the wide Cx43 channel (Wang and Veenstra, 1997), next best through the narrow, cation-preferring Cx26 channel, followed by both the most highly cation-selective channel (Cx45) and the wide anion-preferring channel (Cx32) (Bedner et al., 2003, 2006), which are equally permeable to it. In addition, the fact that some of the Cx26/Cx32 channels are impermeable to some inositol

triphosphates and permeable to others (Ayad et al., 2006) makes clear that pore diameter and permeant charge are not the key determinants of the selective permeability.

A recent study of permeability of cation-preferring Cx26 channels are essentially equally permeable to cAMP and IP3 (Hernandez et al., 2007), even though the latter has six times the negative charge. This is a striking example of the inability of charge selectivity derived from fluxes of atomic ions to predict charge selectivity for biological molecules.

The exception is that the relative efficacy of permeation of the highly anionic IP3 through Cx32, Cx26 and Cx43 channels does correlate to some extent with the degree of cation selectivity, with Cx32 being the most permeable, Cx43 less permeable, and Cx26 being the least permeable (presumably due to its narrower pore; Cx43 and Cx26 being equally and only moderately cation selective) (Niessen et al., 2000). From a purely biological perspective, it is worth noting that the data show that Cx26 is more permeable to cAMP than is Cx32, and that Cx32 is more permeable to IP3 than is Cx26. Speculations on the consequences of this inverse permeability preference are in Bedner et al. (2006).

These examples make clear that the molecular sieve model does not account for the data on cytoplasmic permeants (they also do not account for the data on non-biological tracers; Veenstra et al., 1995; Veenstra, 1996; Nicholson et al., 2000; Goldberg et al., 2004).

The significant differences in permeability cannot be readily accounted for by differences in permeant size or charge—it is difficult to account for the observed selectivity without invoking some kind of *specific* affinity between the channels and specific biological permeants. Based on computations, such interactions have been proposed for non-biological permeants as well (Nitsche et al., 2004; Weber et al., 2004).

4.2. Specific affinities in other large pores

Are the data on molecular permeation through other wide pores informative on this issue? There is a growing literature on metabolite-specific permeabilities of wide pores. Like connexin channels, these other channels conduct ions and a variety of small molecules. However, in each of the cases discussed below, the permeability to certain biological molecules is specifically enhanced, and in some cases, reduced, to certain homologous nonbiological permeants.

The maltoporins (*aka* LamB channels) favor the flux of maltose over that of other oligosaccharides (Dutzler et al., 1996; Wang et al., 1997; Nikaido, 2003). In these channels, the spatial configuration of lumenal hydrophobic residues that spiral down the length of the pore form a "greasy slide" whose structure is optimized for maltose.

Voltage-dependent anion channel (VDAC) was originally characterized as a chloride selective channel (Schein et al., 1976). It turns out to be the primary pathway for metabolite flux through the outer membrane of mitochondria. It is conductive to ATP in its open state, but not the "closed" state, which is really just a lower conductance state, even though the hydrodynamic size of ATP is only about one-half that of the closed state diameter (Rostovtseva and Colombini, 1996; Rostovtseva and Bezrukov, 1998; Rostovtseva et al., 2002a). Single channel studies show that a variety of nucleotides enter the pore equally well, but they interact with an intra-pore binding site with affinities that range over a factor of 40, in the order: NADPH $>$ ATP/GTP $>$ NADH $>$ NAD $=$ ADP $>$ AMP $\gg$ UTP. The ability to distinguish ATP from UTP suggests a specificity for purine bases (Rostovtseva et al., 2002a, b).

In recent work, the role of overall charge in this selectivity was assessed. Genetic removal of a set of positive charges within the pore resulted in a channel that was cation rather than anion selective, and that had only about half the unitary conductance. In spite of this change, the permeability to ATP was unaltered (Komarov et al., 2005)—one would have expected the permeability to ATP to decrease with the change in charge selectivity. It was concluded that ATP flux through VDAC channels is determined by specific sites within the channel, not by the overall charge density of the pore, which is reflected in the cation/anion selectivity. Thus, the general electrostatic interactions inferred from conductance measurements and reversal potentials of atomic ions do not play a crucial role in the *molecular* flux through this channel. This has direct implications for similar inferences about connexin channel permeability, as suggested by the discussion of Cx43 and Cx32 permeability above.

In a different way to probe the basis of the selectivity, the ability of synthetic molecules of similar size and charge as ATP to permeate VDAC channels was assessed (Rostovtseva et al., 2002a). The anions tetraglutamate and 1-hydropene-3,6,8-trisulfonate were excluded from the pore, again making the point that highly specific interactions—in this case those of natural metabolites—are key determinants of selectivity.

A third example, and perhaps the most illuminating, is provided by the interaction of penicillin antibiotics with the bacterial porin OmpF. This channel is the primary pathway by which β-lactam antibiotics, including penicillin, gain entry into bacteria (Delcour, 2003; Nikaido, 2003). A series of penicillins, selected on basis of size and charge, were assessed for ability to permeate the OmpF pore (Nestorovich et al., 2002; Danelon et al., 2006). The results from experiments and molecular dynamics simulations show that among this set of highly similar molecules the ability to permeate the pore is crucially dependent on specific charge distributions and molecular flexibilities that permit complementation of the charge distribution at the narrowest part of the pore. In other words, a successful permeant must interact in a highly specific manner with the selectivity filter. In close analogy to interaction of a K ion with the selectivity filter of K channels, it was found that the strength of the interactions of the permeants at this site correlated with enhanced flux—if the interactions are sufficiently weak, the molecule does not permeate.

From these studies, it is clear that wide channels can have selectivity mechanisms that are highly specific for certain permeants, and whose properties cannot be extrapolated in a straightforward way from estimates of pore size, conductance or charge selectivity, or even from known permeants.

4.3. *Where is the molecular selectivity filter?*

None of the channels mentioned above, or connexins, have canonical binding sites for the indicated molecules. The efficacy of non-canonical sites is demonstrated by the intriguing finding that providing a ring of arginines in the lumen of the α-hemolysin pore confers upon it the ability to bind IP3 with low nanomolar affinity, but not another negatively charged second messenger, cAMP (Cheley et al., 2002).

The lack of canonical sites is understandable because the canonical sites for these molecules have affinities too great to allow rapid flux. The issue of the relationship between intrapore molecular affinities and efficiency of transfer has been investigated computationally (Schwarz et al., 2003; Berezhkovskii and Bezrukov, 2005a, b; Bauer and Nadler, 2006).

To localize the key sites of interaction with cytoplasmic permeants within connexin channels, one might look to examples where point mutations have affected the permeability to second messengers without appearing to grossly affect other permeability properties. In Cx26, the V84L mutation was found to affect the ability to propagate intercellular Ca signaling (e.g., altered permeability to IP3) without affecting either the unitary conductance or the permeability to Lucifer Yellow (Beltramello et al., 2005). This striking result suggests that V84 is pivotal for IP3 permeation, specifically. However, this residue is in the middle of the M2 transmembrane helix, and shows only weak and inconsistent reactivity to thiol reagents (Skerrett et al., 2002). M2 is not inferred to be pore-lining on the basis the existing cryo-EM map (Fleishman et al., 2004), which may be of the closed state of the channel. If V84 is not pore lining, perhaps the modest difference in volume involved in the V84L substitution results in a change that propagates sterically or allosterically to alter topography and/or charge exposure in the pore lumen.

Also in Cx26, in another study, V84L, V95M and A88S (all in M2), and a T5M mutation of Cx30 (in the N-terminal domain), affected the ability to support intercellular Ca signaling without effects on junctional conductance (Zhang et al., 2005). For these mutants, the junctional permeability to propidium iodide was affected, however, but single channel measurements were not made. Accessibility experiments suggest that the two residues just beyond A88 may be exposed to the lumen (Skerrett et al., 2002), and there is reason to think that the region of the N-terminal domain near T5 may have access to the pore under some conditions, based on its effects on voltage gating (Purnick et al., 2000).

In spite of the intriguing effects of these mutants, there is not much of a basis, yet, upon which to speculate regarding the structures, connexin segments or specific sites of connexin channels that define the observed molecular permeabilities.

5. Signaling consequences of different molecular permeabilities

From Tables 2 and 3, the largest permeability differences are:

For Cx32 junctional channels, a 8.3-fold difference in permeability between adenosine and glutamate (Goldberg et al., 2002).
For Cx43 junctional channels, a 3.4-fold difference between ADP/ATP and glutamate (Goldberg et al., 2002).
For ADP/ATP, a 100-fold and a 7.1-fold difference between Cx43 and Cx32 junctional channels (Goldberg et al., 1999, 2002).
For cAMP, a 33-fold difference between Cx43 and Cx36 junctional channels (Bedner et al., 2003, 2006).
For glucose, a 4.3-fold difference between Cx43 and Cx32 junctional channels (Goldberg et al., 2002).
For glutamate, a 33.3-fold and a 8.3-fold difference between Cx43 and Cx32 junctional channels (Goldberg et al., 1999, 2002).
For glutathione, a 33.3-fold and a 4.3-fold difference between Cx43 and Cx32 junctional channels (Goldberg et al., 1999, 2002).

If one presumes that a later measurement of the same permeabilities by the same group is the more accurate one, the largest fold-differences range from 33 to 3.4.

The question arises: What is the biological utility of an intercellular pathway with differences in permeability to second messengers within this range or even less? For example, what difference would it make whether cAMP permeates a junctional channel several times better than IP3? The naive view would be that these molecules will easily equilibrate within a population of well-coupled cells, so relative differences in junctional permeability are unimportant, and all that matters is whether the molecules can permeate at all.

This view neglects several factors. The profile of steady-state levels of a compound within a population of coupled cells critically depends upon the specific relations among the rates of junctional flux, synthesis, degradation and diffusion in cytoplasm of the compound. If the relations between these rates are different for another compound, its profile will be different.

Second messenger molecules typically have constrained lifetimes and diffusional persistence. The average lifetimes of cAMP and IP3 in cytoplasm are ~60 s (Bacskai et al. 1993) and <10 s (Kasai and Petersen, 1994; Wang et al., 1995; Fink et al., 2007), respectively. This five-fold difference in lifetime is a major determinant of effective range and steady state concentration of these molecules. If a cell is producing high levels relative to its coupled neighbors, the junctional site becomes a point source of the compound for the coupled cells. It is easy to see that for a signaling molecule with restricted lifetime and diffusion a relative increase in junctional permeability could substantially affect the level reached at any particular location within the population of receiver cells, and how fast (see below). It has been estimated that the effective range of IP3, a function of its cytoplasmic lifetime and diffusion constant, is ~24 μM (Allbritton et al., 1992). If its diffusion is constrained by junctional transfer, its effective range is less, and the degree to which its intercellular diffusion is constrained (e.g., its relative permeability) directly affects its effective range. The corresponding number for cAMP is ~81 μM (Bacskai et al. 1993; Huang and Gillette, 1991). Thus, the relative junctional permeabilities to cAMP and IP3 determine their relative ranges of action. Computational modeling shows that differences in levels of junctional molecular permeability over a 10-fold range—within that seen in the published data—can produce profound differences in tissue response to periodic release of second messengers (Ramanan et al., 1998).

Whenever there is a change in any of the relevant rates (junctional flux, synthesis, degradation; e.g., in response to hormone receptor activation) at a particular location, the profile of concentration of a compound will change with kinetics that are functions of all the rates, including junctional permeability. If two compounds have different junctional permeabilities, that is, if the rates of junctional flux differ, the rates of change will be accordingly different. Essentially, for two compounds with different junctional permeabilities the system behaves as if it is better coupled with regard to one compound than the other. The consequence is that not only are the steady state profiles of the compounds different, but the *kinetics of change* in those levels are different for each compound. Thus, the relative junctional permeabilities are key factors in any signaling or

regulatory system that has a kinetic component—where the rate of change or oscillation in the level of a compound is important, for which there are many examples.

A large literature supports the idea that oscillatory changes in signaling molecules (e.g., Ca, cAMP) convey information distinct from changes in steady-state levels (Hajnóczky et al., 1995; Jafri and Keizer, 1995; Dupont et al., 2000a; Breitwieser, 2006), and that such oscillations occur across systems of coupled cells (cf., Nathanson et al, 1995; Robb-Gaspers and Thomas, 1995; D'Andrea and Vittur, 1996; Rottingen et al., 1997; Tordjmann et al., 1997; Dupont et al., 2000b, Ravier et al., 2005; Haddock et al., 2006). In many cases, intercellular spread of the oscillatory signal has been shown to be dependent on junctional, as opposed to paracrine, communication.

The rates at which the signaling molecules permeate junctions have a defining effect whether the oscillatory effects are transmitted and the nature of the oscillations themselves. Oscillation frequency is a function of agonist dose (e.g., vasopressin in liver; Rooney et al., 1989) and therefore the ability to follow or appropriately propagate the oscillatory signal depends on the kinetics of junctional flux. Calculations show that the transitions from unsynchronized to harmonically locked Ca oscillations occur within a 10-fold change of junctional permeability, and the transition from harmonically locked oscillations to phased locked oscillations occur with a 7-fold change (Höfer, 1999). Other computational work shows that the range of propagated Ca waves varies strongly with changes in junctional permeability of less than 5-fold (Höfer et al., 2001, 2002). The roles of signal kinetics and of the degree of junctional molecular flux in defining intercellular oscillatory signaling has been described computationally in other work as well (Schuster et al., 2002; De Blasio et al., 2004; Perc and Marhl, 2004; Tsaneva-Atanasova et al., 2006; Ullah et al., 2006).

These studies show that even modest differences or changes in selectivity at cellular junctions—within the range reported in the published work—can have major impact on the strength, character and location of the intercellular signaling, under both steady-state and kinetic conditions.

6. Conclusions

The fundamental finding is that, as a rule, connexin channels are permeable—to some degree—to the expected cytoplasmic molecules. The most important findings are: (1) in the few cases where they have been examined directly, there are dramatic, unanticipated and connexin-specific differences in the magnitudes of channel permeability to cytoplasmic molecules, particularly among those thought to directly mediate intercellular signaling (i.e., cAMP, IP3). (2) Intercellular movement of siRNAs can be mediated by gap junction channels, in a connexin-specific manner. (3) In a few cases, there appear to be all-or-none differences in permeability among closely related signaling molecules, and this occurs primarily in heteromeric channels. The data strongly indicate that certain biological molecules have highly specific interactions within connexin pores that enable surprising degrees of selective permeability that cannot be predicted from simple considerations of pore width or charge selectivity, nor extrapolated from known permeabilities to other biological molecules or non-biological tracer compounds.

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