

# Connexin Specificity of Second Messenger Permeation: Real Numbers At Last

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The discovery many years ago that ions and cytoplasmic molecules could diffuse between cells via gap junction channels inspired excited speculation about the roles such intercellular communication could play in development, physiology, and pathology. This view was reinforced when it later emerged that there are many types of gap junction channels, with  $\sim 20$  functionally distinct vertebrate isoforms of the component protein (connexin), which can combine in homomeric and heteromeric channel structures that have distinct conductance, dye permeability, and gating properties. The unitary conductances range from 10 pS to 300 pS; their cation/anion permeability ratios ( $P_{K^+}/P_{Cl^-}$ ) range from  $\sim 8.0$  to  $\sim 0.8$  and their permeabilities to fluorescent tracers are highly disparate. None of these parameters correlate with each other (Harris, 2001).

What is all this variability good for? It seems unlikely to be all about electrical coupling; it makes more sense to think that the distinct pore/permeability properties are important and perhaps designed for the ability to define, mediate, and dynamically modulate the vocabulary of intercellular molecular signals.

In fact, evidence for the importance of gap junction as conduits of intercellular molecular signals is growing. In recent years it has been demonstrated, in mostly qualitative ways, that different types of connexin channels have different effective permeabilities to specific cytoplasmic molecules, and that the permeability differences among connexin channels cannot be readily inferred from other known functional features. This work has increased speculation that (a) the pores of connexin channels are fine tuned for selectivity among specific cytoplasmic molecules, and (b) the connexin channels expressed at a particular cellular or tissue location are functionally selected for the ability to mediate highly specific intercellular signaling (i.e., for permeability to a specific second messenger, or for a specific spectrum of permeabilities among a set of second messengers).

Several studies have inferred or determined per-channel permeabilities to second messengers, but quantitative data directly comparing the permeabilities of different junctional connexin channels to a second

messenger have been lacking. The paper by Kanaporis et al. in this issue (see p. 293) provides such information; it reports the relative and absolute per-channel permeabilities to cAMP of junctional channels formed by three different connexins (Cx26, Cx40, and Cx43). In addition, they provide, for each connexin studied, a quantitative relation between junctional conductance and cAMP permeability and between permeability to the dye Lucifer yellow (LY) and permeability to cAMP. This information permits junctional conductance or LY permeability to be used to quantitatively estimate the corresponding junctional permeability to cAMP (which of course is far more difficult to measure). In addition, the cAMP/ $K^+$  and LY/ $K^+$  permeability ratios are used to estimate the relative limiting diameters of the three types of channels, with results that tend to confirm that there is real permeant-pore specificity when it comes to permeation by second messengers.

The experiments use a cyclic nucleotide-modulated channel (SpIH) as a sensor of cytoplasmic cAMP, which is used to report junctional cAMP flux from a donor cell that is whole-cell patched with a pipette containing a high concentration of cAMP. Junctional conductance is measured continuously during transfer, and the degradation and synthesis of cAMP are inhibited. The cAMP flux into the recipient cell is reported by the increasing magnitude of the SpIH tail currents. The linear portion of the rate of increase is expressed as a function of junctional conductance and as a function of the number of junctional channels. The results show that the per-channel permeability of Cx43 junctional channels to cAMP is 3.2 times higher than Cx26 channels and 5.2 times higher than Cx40 channels. These numbers are further analyzed to yield actual cAMP flux rates per channel and flux rates normalized to cation flux. These results are then compared with analogous data for LY flux, which show the same qualitative trend, but lower per-channel permeabilities than to cAMP.

Measurements like this are difficult and prone to all sorts of confounding factors; both ends of the pore are inside of cells, so the health of the cells must be maintained while altering the levels of a signaling molecule that can be degraded (and synthesized) during the experiment, the junctional conductance must be

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monitored or controlled for, as must differences in cell volume, all while controlling or monitoring the levels of the second messenger in the donor cell, and recording its rate of change in the recipient cell. In the present work, all these factors were explicitly controlled for, or data were presented to indicate that they had little effect. cAMP concentration in the recipient cell was determined from calibration of the SpIH channel currents. The cAMP level in the donor cell was calculated from a model of cAMP diffusion into the cell from the pipette during the time between going whole-cell and saturation of the CNG channel response in the recipient cell. This estimate represents the upper limit of the actual concentration in the donor cell during loading period, and thus the absolute numbers for cAMP permeability derived from it are lower limits.

This work therefore is unique in providing direct and meaningful comparisons of the permeability of junctional channels formed by different connexins to a biological second messenger. This information will be important for understanding the distinct roles of different connexins in biological signaling, and, in time, for understanding the molecular determinants that modulate connexin channel permeability to cAMP.

#### What Do We Know about Selective Permeability of Connexin Channels among Second Messengers?

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). Many studies, beginning in the mid-1960s, later demonstrated the permeation of exogenous tracers of various kinds through gap junction channels. Evidence that gap junctions could mediate intercellular movement of endogenous metabolites (then called “metabolic cooperation”) soon followed (Subak-Sharpe et al., 1966; Gilula et al., 1972), as did the seminal demonstrations of movement of cAMP through junctional channels (Tsien and Weingart, 1974; Lawrence et al., 1978).

Since these initial findings, gap junction channels have been shown to be permeable to a wide variety of cytoplasmic molecules, including inositol phosphates, nucleotide triphosphates, cyclic monophosphates, nucleotides, amino acids, glutathione, calcium ion, glucose and its metabolites, cytosolic pH buffers, small RNAs, and small peptides (for review see Harris, 2007).

The first studies to compare transjunctional movement of a set of cytoplasmic molecules employed a “metabolite capture” system in which donor cells were metabolically radiolabeled and the transferred compounds found in the recipient cells analyzed (Goldberg et al., 1998, 2002). These studies were the first to indicate that different connexin channels had differential permeability among cytoplasmic compounds, and to make clear that the differences did not correspond to

what we thought we knew about the channels from studies using nonbiological molecules as tracers. Normalization to single channel properties was difficult and indirect in this system, but the data suggested that Cx43 channels are more permeable to ATP, glutathione, glutamate, and glucose than are Cx32 channels, while Cx32 channels are more permeable to adenosine.

About the same time, Niessen et al. (2000) performed a study to determine the relative ability of different connexins to mediate junctional IP<sub>3</sub> flux, as reported by propagation of intercellular Ca<sup>2+</sup> waves. The data were not normalized to number of junctional channels, but to relative degree of neurobiotin and Mn<sup>2+</sup> coupling. Given these caveats (indirect and qualitative measure of IP<sub>3</sub> permeability, number of channels inferred from permeation of small tracers), the data suggested that Cx32 channels were more permeable to IP<sub>3</sub> than were Cx43 channels, and much more so than Cx26 channels (IP<sub>3</sub>: Cx32 > Cx43 >> Cx26).

The first study to directly detect junctional second messenger flux employed the cystic fibrosis transmembrane conductance regulator (CFTR) as a sensor (Qu and Dahl, 2002). Interestingly, this study showed that transjunctional voltage essentially eliminated the permeability to cAMP of Cx43 channels but had only moderate effect on the electrical coupling. This was attributed to the voltage-driven occupancy of a subconductance state with a narrower pore, and made the point that intercellular molecular signaling could be modulated while maintaining electrical signaling.

Recently, Hernandez et al. (2007) used novel genetically expressed ratiometric fluorescent sensors to directly assess junctional flux of cAMP and of IP<sub>3</sub> through Cx26 channels, providing the first quantitative data comparing permeabilities of two second messengers through the same junctional channels. The sensors were FRET conjugates of proteins sensitive to the respective second messengers. The sensor for cAMP was based on Epac, a guanine nucleotide exchange factor and for IP<sub>3</sub> was based on the ligand-binding domain of an IP<sub>3</sub> receptor. The cAMP or IP<sub>3</sub> was introduced into the donor cell via a patch pipette. The junctional transfer rate was calculated from the FRET signals in the donor and recipient cells during the interval when the FRET signal was shown to linearly reflect the concentration of the compound being sensed. Junctional conductance was determined immediately afterward. The results were that the per-channel permeabilities to cAMP and IP<sub>3</sub> through Cx26 channels were quite similar, and much greater than that to LY.

Bedner et al. (2003, 2006) used the activity of CNG channels as reflected in a Fluo-4 Ca<sup>2+</sup> signal to report and compare flux of cAMP through several types of junctional channels. cAMP was released photolytically in the donor cell. In this study it was necessary to correct for expression of the cAMP sensor (the CNG channels)

and nonlinearity of the reported response of the sensor (the combined  $\text{Ca}^{2+}$  response of the CNG channels and the response of Fluo-4 to the  $\text{Ca}^{2+}$  signal). Due to the difficulty of making this correction in a rigorous manner, in the end this study could report only the number of junctional channels of each connexin type that was required to mediate a similar amount of junctional cAMP flux, and did not allow calculation of per-channel permeabilities. The results give the following relative order of cAMP permeability through junctional channels formed by different connexins:  $\text{Cx43} > \text{Cx26} > \text{Cx45} = \text{Cx32} > \text{Cx47} \gg \text{Cx36}$ .

These findings, if put together with those of Nissen et al. above suggest that Cx32 is more permeable to  $\text{IP}_3$  permeation than is Cx26, and Cx26 is more permeable to cAMP than is Cx32. However, Hernandez et al. reported that the per-channel permeabilities, based on direct sensing of the two compounds, was essentially identical for Cx26 channels, an apparent contradiction.

It further has been reported, in abstract form, that Cx26 channels are more permeable to cAMP than are Cx43 channels (also an apparent contradiction with the Bedner et al. data), and that Cx43 channels were more permeable to AMP, ADP, and ATP than were Cx26 channels (Toloue and Nicholson, 2007). It was specifically noted that Cx43 channels were many times more permeable to AMP than to cAMP. This study used radiolabeled tracers, however a full report has not been published, so the analysis cannot yet be evaluated.

The Kanaporis et al. (2008) paper in this issue combines most of the best aspects of each of the studies summarized above: direct quantitative sensing of the second messenger, direct measurement of junctional conductance and unitary conductance, controls for degradation/synthesis of the second messenger and for effects of the second messenger on the junctional channels, and obtaining quantitative data for several connexins. The normalization to  $\text{K}^+$  conductance and to LY permeability allowed for additional analysis/interpretation.

#### What Difference Does It Make for Cell Signaling?

The Bedner et al. studies indicated that some connexin channels are many times more permeable to cAMP than others; the Kanaporis et al. paper establishes this point quantitatively. Are these differences in permeability biologically meaningful? What difference would it make whether cAMP permeates one type of junctional channel several times better than another? One view would be that any permeant will rapidly 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.

For a second messenger, this is like saying that if cells are electrically coupled at all then all of the coupled cells will eventually reach the same membrane potential, and the degree of electrical coupling makes no difference.

In other words, this view neglects the effective “lifetime” or diffusional persistence of the second messenger in cytoplasm, and the importance of kinetic aspects of molecular signaling. Indeed, because the profile of steady-state levels of a second messenger within a population of coupled cells critically depends on the relationships among the rates of its junctional flux, synthesis, degradation, and diffusion in cytoplasm; the rate of junctional flux will be an important determinant of the distribution.

The average lifetime of cAMP in cytoplasm is  $\sim 60$  s (Bacskai et al., 1993) (that of  $\text{IP}_3$  is much less; Kasai and Petersen, 1994; Wang et al., 1995). For a signaling molecule with restricted lifetime, a difference in junctional permeability could substantially affect the level reached at any particular location within a population of recipient cells, and how rapidly (see below). If junctional transfer is reduced, the effective range will be less; the relative junctional permeabilities to cAMP, together with the intrinsic rates of synthesis and degradation, therefore determine its relative range of action. Computational modeling shows that differences in levels of junctional molecular permeability approximating the range observed in Kapaoris et al. can produce profound differences in tissue response to periodic release of second messengers (Ramanan et al., 1998).

The *kinetic* consequences of even three to fivefold differences in per-channel junctional permeability are likely to be much more dramatic. A cellular system coupled by Cx43 channels will behave as if it is much better coupled with regard to cAMP than a system coupled by Cx40 channels. The data in Kanaporis et al. show that for the same junctional electrical conductance, the junctions in a Cx43-coupled system will be 10-fold more permeable to cAMP than those in a system that is coupled by Cx40 channels. The importance of this will be obvious to anyone familiar with the consequences of differences in electrotonic coupling for intercellular transmission of dynamic electrical signals (e.g., action potentials) (Bennett, 1966; Bennett et al., 1970). When the “stimulus” (the change in cAMP level, in this case) and/or the responding elements (the signaling systems sensitive to cAMP) have kinetic features, the linkage between them (the junctional flux) will be a determining factor of the character of the signal generated in the coupled cell(s) and of the response of systems in them that receive the signal.

For example, when there is a change in any of the relevant rates (e.g., cAMP or  $\text{IP}_3$  increase in response to hormone receptor activation; change in connexin channel composition) 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 the rates of junctional flux differ, the rate and character of the change will be accordingly different.

Thus, 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 signaling compound is important, for which there are many examples. A large literature supports the idea that oscillatory changes in the concentrations of signaling molecules convey information distinct from changes in steady-state levels (Hajnóczky et al., 1995; Jafri and Keizer, 1995; Dupont et al., 2000; Breitwieser, 2006), and that such oscillations occur across systems of coupled cells (Nathanson et al., 1995; Robb-Gaspers and Thomas, 1995; Tordjmann et al., 1997; Ravier et al., 2005).

The rates at which the signaling molecules permeate junctions have a defining effect on whether oscillatory signals are transmitted and the nature of the oscillations themselves. The oscillation frequency can be a function of agonist dose (e.g., Rooney et al., 1989) and the ability to follow or appropriately propagate an oscillatory signal therefore would be expected to depend on the kinetics of the junctional flux, as predicted from computational studies (Hofer, 1999). Further computational studies show that the range of propagated  $\text{Ca}^{2+}$  waves varies strongly with changes in junctional permeability of less than five-fold (Hofer et al., 2001, 2002). The roles of signal kinetics and of magnitude of junctional molecular flux in defining intercellular oscillatory signaling have been described computationally in other systems as well (De Blasio et al., 2004; Schuster et al., 2002; Perc and Marhl, 2004; Tsaneva-Atanasova et al., 2006; Ullah et al., 2006).

Taken together, these studies show that even modest differences or changes in selectivity at cellular junctions, within the range reported in Kanaporis et al. for the three connexins studied, can have major impact on the strength, character, and location of the intercellular signaling, under both steady-state and kinetic conditions.

#### What Are the Implications for Understanding the Character of the Connexin Pore?

Permeation through connexin channels, whether by atomic ions or molecules, has been difficult to understand. In some cases, the selectivity sequences of connexin channels among atomic ions are unremarkable, accounted for by aqueous mobility or by simple interaction with a charged site within the pore (Trexler et al., 1996). In other cases, such as for Cx43 and Cx40, the selectivity sequences suggest that selectivity is influenced by interaction between anions and cations within the pore (Beblo and Veenstra, 1997; Wang and Veenstra, 1997; Hu et al., 2006). It has been suggested that in these cases anion permeation/occupancy could reduce cation permeation, in apparent violation of the independence principle that underlies many analytic formalisms for ion permeation through large diameter “non-selective” channels, which may be a consequence of fact that both anions and cations can simultaneously enter and occupy the pore of these channels. Similar effects have been

noted also in certain other channels that allow both cation and anion entry (Borisova et al., 1986; Franciolini and Nonner, 1994a,b).

Kanaporis et al. highlight an area of concern related to molecular permeability. They point out that the Cx43 and Cx40 channels are significantly more cation selective than are Cx26 channels ( $P_{\text{Cl}^-}/P_{\text{K}^+}$  is 0.13, 0.14, and 0.38, respectively). The unitary conductances are 55, 125, and 110 pS, respectively, yet the permeability to cAMP and to LY follows the order  $\text{Cx43} > \text{Cx26} \gg \text{Cx40}$ . Thus, the most cation selective, lowest conductance channel has the highest permeability to large, anionic molecules. (Cx37 channels provide a similar example; they have a unitary conductance of  $\sim 300$  pS yet are among the most size restrictive.) This leads to the idea that perhaps the channels with large conductances and high size selectivity (Cx43 and Cx40 in this case) have short but narrow pores (see Finkelstein, 1975).

To explore his idea, Kanaporis et al. calculate the hydrodynamic diffusion of particles in narrow pores as a function of the ratio of permeant size to the pore diameter (Levitt, 1975). This calculation was originally applied by Dwyer et al. (1980) to estimate the diameter of the end-plate acetylcholine receptor channel. Because this formula considers hydrodynamic factors only, it would be expected to best account for permeability ratios when the charge of the tested permeants is identical. This analysis was previously applied to connexin channels, using monovalent cation permeability ratios, to estimate that Cx43 channels had approximately the same diameter as Cx40 channels (Beblo and Veenstra, 1997; Wang and Veenstra, 1997). Kanaporis et al. now use the same approach to estimate pore diameters for the three connexin channels they studied. Using the  $\text{LY}/\text{K}^+$  permeability ratio, the calculations suggest that Cx26 and Cx40 have approximately the same diameter, and that Cx43 is a little wider. If the small difference in the calculated values (1.09 nm vs. 1.25 nm) is considered to be significant, one could argue either that pore is indeed a bit wider, or that the difference is due to the way that the charge, chemistry, or shape of the LY molecule interacts with the different pores relative to how  $\text{K}^+$  interacts with them; and further, that the “match” with Cx43 facilitates its diffusion within the pore relative to that for the other connexins.

The pore diameter estimates using the  $\text{cAMP}/\text{K}^+$  permeability ratios are more intriguing. The  $\text{cAMP}/\text{K}^+$  permeability data (a) yielded narrower pore diameters for each connexin channel than did the  $\text{LY}/\text{K}^+$  data, (b) indicated that Cx26 and Cx40 have similar diameters ( $\sim 0.67$  nm), but (c) the difference between them and Cx43 (1.12 nm) was much greater.

#### What Does It Mean that the cAMP Data Suggest Narrower Pores than the LY Data?

If the only difference between the permeant species was their minor diameter, the channel size estimates from

the two sets of data would be expected to be similar. Therefore one would presume that the difference is due to other factors, the obvious ones including permeant charge, charge distribution, and chemical interactions (e.g., hydrogen bonding ability).

That such factors would influence permeation is not surprising. However, the data suggest that cAMP moves through the pores as if they experience a narrower pore than that experienced by the much larger LY (which has nearly twice the minimal diameter). This suggests the possibility that this small biological permeant interacts more strongly with the pore than (the much larger) exogenous tracer molecule, which the pores never see in nature. In other words, there is the possibility for selectivity that is specific for cytoplasmic, as opposed to nonbiological, permeants.

The disjuncture between permeation properties of ions, tracers, and cytoplasmic molecules was made very clear a few years ago by Beltramello et al. (2005). They found that a point mutation from valine to leucine in Cx26 produced no change in unitary conductance or permeability to LY, but severely compromised junctional IP<sub>3</sub> flux, as assessed by intercellular Ca<sup>2+</sup> waves. Other data also directly show that connexin channels can be highly selective among biological permeants in ways not revealed by tracers (e.g., Bevans et al., 1998; Ayad et al., 2006).

#### What Does It Mean that the cAMP Data Yield a Much Bigger Calculated Difference between the Pore Width of Cx43 and that of Cx26 and Cx40 than Do the LY Data?

Put simply, cAMP reveals a difference in the permeation characteristics among these channels that LY does not. This could be regarded as indicating that the cAMP interacts more strongly with the pores of Cx26 and Cx40 than it does with that of Cx43, and that LY cannot tell the difference, suggesting that there can be specificity to the match between biological permeants and specific connexin channels, and that this biologically relevant interaction is undetectable by at least some nonbiological probes of connexin pore properties.

Not surprisingly, the relevant probes for connexin channel function seem to be the molecules that they see in nature, and for whose selective permeation the channels may be optimized. It will be interesting to see whether this conjecture is borne out by studies of other biological permeants. Of course, unresolved issues include the structures and molecular mechanisms that underlie what are apparently specific and discriminating interactions of biological permeants with the pore. Are there discrete sites at which the molecules must interact in order to permeate, in rough analogy to the selectivity mechanisms of ion-specific channels, or is the selectivity like that of substrate-specific porins, in which permeation of a particular molecule is specifically and substantially facilitated (or perhaps hindered) over that of others? Meaningful investigation of these questions must await

not only greater structural resolution of the pore itself, but also more studies like those of Kanaporis et al. to help identify those cytoplasmic molecules that interact most strongly or specifically with connexin pores.

The author regrets not being able to specifically cite much of the large body of work that contributed to the topic discussed in this Commentary.

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