

Open Pore Block of Connexin26 and Connexin32 Hemichannels by Neutral, Acidic and Basic Glycoconjugates

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The mechanisms of molecular discrimination by connexin channels are of acute biological and medical importance. The availability of affinity or open-pore blocking reagents for reliable and specific study of the connexin permeability pathway, would make possible the rigorous cellular and physiological studies required to inform, in molecular terms, the underlying role of intercellular communication pathways in development and disease. Previous work utilized a series of glucosaccharides labeled with an uncharged fluorescent aminopyridine (PA-) group to establish steric constraints to permeability through connexin hemichannels. In that work, the smallest probe permeable through homomeric Cx26 and heteromeric Cx26–Cx32 channels was the PA-disaccharide, and the smallest probe permeable through homomeric Cx32 channels was the PA-trisaccharide. The larger impermeable probes did not block permeation of the smaller probes. Building on this work, a new set of glucosaccharide probes was developed in which the label was one of a homologous series of novel anthranilic acid derivatives (ABG) that carry negative or positive formal charge or remain neutral at physiological pH. When the PA-label of the smallest impermeant PA-derivatized oligosaccharides was replaced by ABG label, the resulting probes acted as reversible, high-affinity inhibitors of large molecule permeation through connexin pores in a size and connexin-specific manner.

Keywords. Anthranilic acid, channel, connexin, glycoconjugate, maltosaccharide, open-pore block

INTRODUCTION

Connexin channels mediate intercellular ionic current and have remarkable selective permeability among cytoplasmic molecules. The importance of this selectivity is made clear by the developmental and physiological defects caused by connexin mutations and by the lack of full compensation achieved

by other connexins, even when the mutant connexin is replaced by a fully functional but different connexin isoform (23, 31)

One may look to the naturally occurring, disease-producing connexin mutations for clues as to the determinants of pore selectivity. However, for the most part, the phenotypes do not inform the molecular basis of the erroneous intercellular permeation that is

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the root cause of the pathology. Despite best efforts, the set of rules that govern permeant selectivity and how that selectivity is achieved by different connexin channels remains undefined.

To understand the basis of this molecular selectivity requires the ability to gain detailed functional and structural information about the interior of the connexin pore. For many other channels, highly specific toxins and affinity reagents that enter or occlude the pore have been of inestimable value in this regard (5, 11, 12, 26, 32). Teleologically, inaccessibility of the pore from the extracellular space, and the cytoplasmic location of the pore entrances of junctional channels, makes them comparatively poor targets for toxins, for which a primary attribute is rapid action.

For the most part, the pores of connexin channels have been empirically characterized by the use of a heterogeneous group of charged tracers (7, 19). Uncharged probes have been utilized for only a few connexins and offer the only way to separate the influence of molecular size from other parameters affecting permeability (1, 21). In addition, the selective permeability of some connexins to cytoplasmic molecules has been characterized (1, 8, 9, 10, 17, 20, 24). Together these studies reveal that for any given connexin, discrimination among large permeants has little relation to unitary conductance or to charge preference among atomic ions (29, 30). That different methods and probes were used lends credence to these summative conclusions.

Previous work utilized a set of oligoglucosaccharides (maltosaccharides) labeled with a small uncharged fluorescent moiety to explore the steric limits of pore permeation for hemichannels formed by heteromeric Cx26–Cx32 and homomeric Cx32 (1).

To determine the influence of charge on the permeation of these saccharide tracers, a new set of small homologous fluorescent labels of differing formal charge was synthesized and conjugated to the maltosaccharides. To our surprise, the new labeling moiety appears to enable open-pore block of connexin channels in a size and connexin-specific manner.

METHODS

The overall approach to connexin hemichannel immunoaffinity purification, reconstitution into liposomes and assessment of channel activity has been described in previous publications (1, 25). In the present work, homomeric Cx26, heteromeric Cx26–Cx32, homomeric Cx32 and homomeric Cx43 hemichannels were obtained from hepatocytes and/or mammary epithelium (1, 18).

Transport Specific Fractionation

There are powerful limitations on the direct physiological study of intercellular channels. On the other hand, investigations of hemichannels are profitable because intercellular channels behave, for the most part, as if each hemichannel is an independent functional unit acting in series with the other docked hemichannel. There is only occasional or minor divergence from this notion, though the superimposition of two hemichannel properties can give rise to unexpected phenomena (4, 22, 27). The TSF (Transport-Specific Fractionation) assay used here gives information about connexin channels that is, ironically, unavailable by *in situ* study of junctional channels.

TSF separates liposomes, into which connexin channels have been reconstituted, into populations within a density gradient based on their permeability to urea and sucrose, osmolytes that pass through open connexin channels. The TSF gradient is formed from urea and sucrose, which have different densities at iso-osmolar concentrations (13, 14) (Figure 1). Unilamellar liposomes are formed in, and entrap, urea-containing solution, the less dense osmolyte. During TSF centrifugation, urea and sucrose exchange through the open connexin pore, equilibrating the aqueous solution density inside and outside the liposome. This makes the phospholipid membrane the sole determinant of liposome density. Since the density gradient is constructed so that the density of phospholipid is near the bottom, those liposomes with and without functional channels

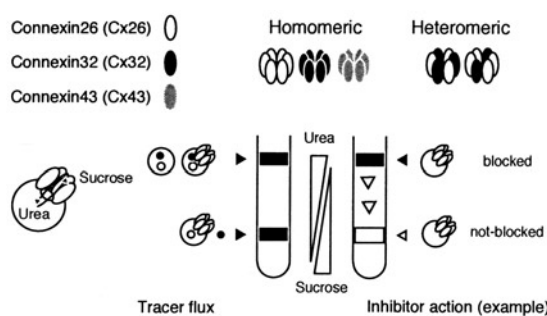


Figure 1. *Transport-Specific Fractionation (TSF)*. See METHODS for explanation.

band in the lower and upper regions of the gradients, respectively. The banding positions of liposomes are monitored via rhodamine-labeled lipid fluorescence making it possible to visualize, collect and quantitate the liposomes and their contents.

Molecular permeability is assessed by the direct comparison of intravesicular tracer compounds selectively lost or retained by the liposomes in the upper and lower TSF bands, the upper liposomes serving as an internal control for loading and leakage. The time taken for the liposomal contents to reach equilibrium with the external solution through a hemichannel is brief (msec); therefore, any non-zero permeability will result in complete loss of tracer from a liposome. With exposure to a potential channel modulator, a change in liposome distribution between the upper and lower band positions, relative to control, reflects a change in the fraction of channels that is permeable to the osmolytes. Such a change can be due to pore block or to a reduction to zero of channel open probability. Since osmolyte exchange is rapid, TSF reports only all-or-nothing permeability and modulatory effects, independent of variation in the number of channels reconstituted.

RESULTS AND DISCUSSION

Selective Permeability of Connexin Channels to Aminopyridine-Glycoconjugates

Steric exclusion limits of homomeric Cx26, Cx32, Cx43 and heteromeric Cx26–Cx32 hemichannels

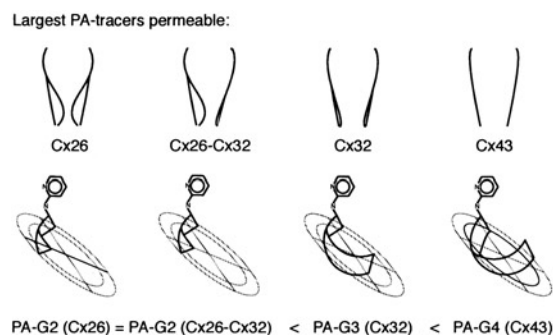


Figure 2. *Schematic of Connexin Hemichannels and Largest Impermeant PA-Sugars*. The chemical moiety at the upper end of the probes is the fluorescent aminopyridyl moiety. This moiety is not drawn to scale.

were defined by a size-indexed series of $\alpha(1 > 4)$ -linked glucosaccharide helices (maltosaccharides; maltose [G2] through maltopentaose, [G5]) derivatized with a small uncharged fluorescent 2-aminopyridine (PA-) group (15) (Figure 2) at the reducing end. These maltosaccharides form rigid helical structures in solution, stabilized by internal hydrogen bonding, with approximately six glucosaccharide residues per full turn (16). Consequently, for up to six saccharide units, the dimension presented to a pore increases approximately 0.2\AA in the minor axis of the van der Waals envelope (cross-sectional dimension) per additional saccharide. The PA label is small enough that it is not the limiting size element of the derivatized sugar.

These probes revealed differences in the steric barriers to permeation among connexin hemichannels. PA-G2 (maltose) was the only tracer permeable through all channels tested. PA-G3 was permeable through homomeric Cx32 pores, but not through any pores that contained Cx26. PA-G4 was permeable through homomeric Cx43 pores, but not pores composed of Cx26 and/or Cx32. PA-G5 was impermeable through all connexin pores tested. The impermeable probes (*e.g.*, PA-G4 for homomeric Cx32) did not block to the permeability of the smaller probes (*e.g.*, G2-PA and G3-PA).

Novel Glycoconjugates Show High Affinity, Connexin-Specific Open Channel Block

To resolve the influences of molecular size and charge on connexin permeant selectivity, it was desired to derivatize maltosaccharides with a family of structurally similar small fluorescent moieties that carry either a single positive charge, a single negative charge, or remain uncharged. The PA label is not amenable for addition of charge, so three novel fluorescent derivatives of anthranilic (aminobenzoic) acid amide (ABG) were synthesized and used initially to impose formal charge upon the same size-indexed series of maltosaccharides used for the previous size selectivity studies.

When ABG labels were derivatized to G4, which as PA-derivative was the smallest tracer unable to pass through homomeric Cx32 channels, each G4-glycoconjugate acted as a reversible high-affinity inhibitor of channel permeability to both urea and sucrose, reliable indicators of large molecule permeation. ABG derivatization of G2 and G3, which as PA-conjugates were permeable through Cx32 channels, were without inhibitory effect. Similarly, heteromeric Cx26–Cx32 pores were inhibited by exposure to ABG-derivatized G3, which was the smallest PA-tracer impermeant through these pores, but not to ABG-derivatized G2 (smaller, permeable) or G4 (larger, impermeable).

The correlation between the number of saccharide units of the ABG-oligosaccharide required for block and the relative size of the pore determined with the uncharged PA-sugars strongly indicates that the site of interaction for these novel ABG-glycoconjugates with connexin is within the pore. Furthermore, it suggests involvement in the block of the same physical size-selectivity elements of the connexin molecules that contribute to the open pore characteristics. ABG free label did not cause pore block. That the PA-G3 and PA-G4 sugars do not block the connexin channels, yet the corresponding ABG-derivatized sugars do, suggests that a specific structure-activity relation of the ABG labels is required for the effects observed and that open pore

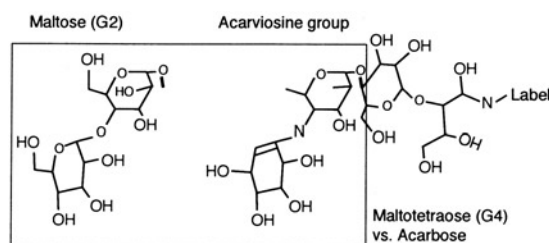


Figure 3. Maltotetraose (G4) and the Analog Acarbose.

block demands their conjugation to a saccharide of appropriate size.

The role of the saccharide in the block was further explored by ABG derivatization of acarbose (3), a competitive inhibitor of α -glucosidase (“amylase”), the degradative enzyme for $\alpha(1 > 4)$ -linked glucosaccharides. In acarbose, the first two glucose residues at the non-reducing end of the maltosaccharide backbone (*i.e.*, the end not available for labeling) are replaced by nitrogen-linked pseudo-saccharide analogs (acarviosine; Figure 3). These pseudo-glucose analogs drastically reduce the ability of the molecule to form internal and external hydrogen bonds. Despite this, acarbose adopts an identical conformation to G4 (which as ABG derivative blocked Cx32) in solution at neutral pH (2). ABG-acarbose was ineffectual at Cx32 pore block. This finding suggests that the ability of maltosaccharides to form hydrogen bonds enhances the block or the stability of the blocking agent within connexin pores.

Structure-Activity Relationships for Glycoconjugate Block

Traditionally, the study of channel selectivity using electrophysiological methods focuses on the ability to distinguish among atomic ions. We show that the open pore barrier of homomeric and heteromeric Cx26 and Cx32 channels can be assessed using large, nonelectrolytes of glucose. Our preliminary findings infer that the new anthranilamide aglycone contributes significantly to glycoconjugate-connexin binding, with the open pore block likely

being effected by the bulk saccharide backbone. Our speculations on the mode of action of these reagents are guided by studies of the interaction of carbohydrate and similar amphipathic ligands with other channels and proteins.

The binding of amphipathic ligands in a bacterial efflux pore (33) repositions buried amino acid side chains between transmembrane α helices as the hydrophobic head weakly associates with the pore; this movement in the order of tenths of Angstroms reorganizes the local environment in terms of available charge and hydrogen bond pairings to create a high affinity binding site for the hydrophilic bulk. A similar synergy of binding may occur for the ABG reagents in which the anthranilamide aglycone enables high affinity associations of the appropriate saccharide backbone within the connexin pore.

Maltosaccharide translocation by the bacterial maltoporin ("maltopermease") involves the en face interaction of stacked glucopyranose residues with the so-named "greasy slide" of aromatics and the "polar track" of charged residues within the pore (6, 28). The interactions with the "polar track" occur in part through hydrogen bonding, which helps thread the sugar through the pore and keeps it in close contact with the slide during translocation. G4 also interacts by hydrogen bonding with α -glucosidase through at least two hydrogen bonds per glucose to aromatic residues and through the glycosidic bond oxygen to carboxylate residues (3). The reduced hydrogen bonding capability of acarbose may account for the inability of ABG-acarbose to interact with the physical size-selectivity elements of the pore and block Cx32 pores vs. ABG-G4.

The conformational and topographical requirements for the carbohydrate-connexin interactions are being systematically explored and are guiding the design of improved molecular probes that can be used for open pore block of connexin channels in living cells. These probes are useful tools with which to investigate connexin pore structure, and modulate connexin channel permeability in vivo as well as in vitro.

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