Accessibility of cx46 Hemichannels for Uncharged Molecules and Its Modulation by Voltage

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ABSTRACT Excised patches of oocyte membrane containing cx46 hemichannels were used to determine the accessibility of the channels for several uncharged molecules at negative and positive holding potentials. The molecular weights of the test molecules (sugars and polyethylene glycols) ranged from 180 to 666 Daltons, with diameters from 5.8 to 12 Å. Activation of the voltage gate (V_j gate) at positive potentials shifted the accessibility limit for these test molecules to lower sizes indicating that net charge of the test molecule does not determine accessibility. The sugars changed several channel properties: 1), single-channel conductance decreased in an inverse relationship to the size of the test molecule; 2), the apparent open probability of the connexin channels was reduced; 3), the lifetimes of the apparent occupancy states exceeded the expected transit times assuming simple diffusion by orders of magnitude. These results suggest a channel that is not cylindrical and in which test molecules bind or are trapped in the pore or the vestibulum. Furthermore, the effect of sugars and the large difference in transit rates for small ions and larger molecules suggest that they may involve different permeation mechanisms.

INTRODUCTION

Gap junctions are cell-cell channels found in almost all animals, vertebrates as well as invertebrates (Evans and Martin, 2002; Harris, 2001). Each gap junction channel is composed of two hemichannels, called connexons, that reside in apposing cell membranes. A connexon is formed by six subunits called connexins in vertebrates. In a gap junction channel two hemichannels line up, and the two extracellular loops of each subunit extend across the gap to form a hydrophilic path connecting the cytoplasm of the two cells. The intercellular pathway permits the passage of ions and molecules up to 1,000 Daltons, including all known second messengers (e.g., cAMP, IP3, and calcium) and small molecules of intermediate metabolism. Some connexins form open hemichannels when expressed in unpaired *Xenopus* oocytes, conducting currents without having contact with another membrane. Open hemichannels are formed by cx46 and cx50, the native connexins in the lens of the eye, and by cx32E143, a chimeric connexin consisting of cx32 with the first extracellular loop replaced by the corresponding sequence of cx43 (Paul et al., 1991; Pfahnl et al., 1997; Zampighi et al., 1999).

Most vertebrate gap junction channels are regulated by transjunctional voltage, i.e., the potential difference between the cells (Spray et al., 1981). The voltage gate (V_j gate) partially closes the channels. Passage of small molecules (i.e., fluorescent tracer molecules and cAMP) through gap junction channels is selectively excluded by the voltage gate in both cx46/43 gap junction channels and cx46 hemichannels, whereas electrical continuity is preserved (Qu and Dahl, 2002). The closure of the voltage gate appears to involve a conformational change along the pore, which narrows rather than completely occludes the pathway. However, the cutoff limit for small molecules imposed by the voltage gate is presently unknown.

To determine the cutoff limit for small molecules to pass through gap junction channels at different holding potentials, we explored the effect of sugars on conduction in cx46 single hemichannels. To exclude charge effects on single-channel conductance, uncharged test molecules of various molecular weights were tested on excised patches containing cx46 single hemichannels. Nonelectrolytes have been used previously to probe channel dimensions (Bezrukov, 2000; Bezrukov and Vodyanoy, 1993; Bezrukov et al., 1994; Krasilnikov et al., 1992; Merzlyak et al., 1999; Sabirov et al., 1993) including those of gap junction channels (Oh et al., 1997). If nonelectrolytes access the channel, then the presence of these molecules in the channels could reduce the flow of ions and thus single-channel conductance. Nonelectrolytes excluded from the channel should not change the single-channel conductance. In this way we tested sugars and polyethylene glycols (PEGs), from 180 to 666 Daltons with diameters from 5.8 to 12 Å. To detect voltage-induced changes of the pore, currents were measured at both negative and positive potentials. Positive potential activates the voltage gate. We found that changing the membrane potential from negative to positive shifted the size limit beyond which ionic conductance was not affected by the added nonelectrolyte from stachyose (12 Å) to sucrose (8.9 Å). Furthermore, the effects of sugars were more complex than expected and indicate that molecules other than small ions permeate gap junction channels by hindered diffusion.

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MATERIALS AND METHODS

Preparation of oocytes

Oocytes were prepared as described previously (Dahl, 1992). *Xenopus laevis* oocytes were isolated by incubating small pieces of ovary in 2 mg ml^{-1}

collagenase in calcium-free OR2 and stirring at 1 turn/s for 3 h at room temperature. After being thoroughly washed with regular OR2, oocytes devoid of follicle cells and having an uniform pigmentation were selected out and stored in OR2 at 18° C (OR2 solution in mM: 82.5 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 1.0 $CaCl_2$, 1.0 Na_2HPO_4, 5.0 HEPES, antibiotics (Penicillin, 10,000 units/ml; Streptomycin, 10 mg/ml), pH7.5).

In vitro transcription of mRNAs

Cx46 cloned into the expression vector pSP64T was obtained from Dr. D. L. Paul (Paul et al., 1991). mRNA was transcribed by Sp6 RNA Polymerase from 10 μg of *EcoRI*-linearized plasmid using the mMessage mMachine kit (Ambion, Austin, TX). mRNA was quantified by absorbance (260 nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis. 20 nl of mRNA (50 ng/μl) was injected into oocytes. The injected oocytes were then transferred into fresh OR2 medium with elevated Ca^{2+} concentration (5 mM) to keep the gap junction hemichannels closed and incubated at 18°C for 18–24 h. For electrophysiological recordings oocytes were transferred back to regular OR2.

Patch clamp technique

Single cx46 hemichannels were studied by the patch-clamp technique (Hamill et al., 1981) using an Axopatch-1B amplifier (Axon Instruments, Foster City, CA). Currents were filtered at 5 kHz, digitized using a VR-10B digital data recorder, and stored on video tape. The recordings were transferred to a Power Macintosh (Apple, Cupertino, CA) computer using an ITC-18 Computer Interface (Instrutech, Toronto, Canada) and analyzed. Acquisition and analysis were done with the Acquire and TAC programs (both from Bruxton, Chicago, IL).

The vitelline membrane of the oocyte was removed and the oocyte was washed once before being transferred into a new dish containing potassium gluconate solution (KGlu solution: 140 mM KGlu, 10 mM KCl, 5.0 mM TES, pH 7.5). Electrode pipettes made from glass capillary tubing (1.5–0.86 mm, No. GC150F-15, Warner Instrument, Hamden, CT) were pulled using a Flaming-Brown Micropipette Puller (Model P-97, Sutter Instrument, Novato, CA) and polished with a microforge (Narishige Scientific Instruments, Lake Forest, CA) to 0.5–1 μm with a resistance of 10–20 MΩ in KGlu solution. Both the standard pipette and bath solution were KGlu solution. After an inside-out patch was excised from the membrane and a cx46 hemichannel was identified, the patch was transferred into a microperfusion chamber, which was continuously perfused with solution. The perfusion system was driven by gravity at a flow rate of 100 μl/s.

Sugar exclusion test

The sugar to be tested was dissolved in KGlu solution to a sugar concentration of 100 mM and applied to the patch through a microchamber perfusion system. Inside-out patches were excised from cx46 expressing oocytes. After a cx46 channel was identified, the patch was held at either negative or positive potentials, and 100 mM sugar was applied from the intracellular side of the channel for at least 5 min before being washed out with KGlu.

Statistics

Analysis of variance was performed for all data and Bonferroni *t*-tests for difference were applied. Channel activity was analyzed only for patches containing single channels and with data records exceeding 75 s both before and after the application of sugars. For assessment of the effects of the sugars on channel conductance, open probability, and mean open time, paired *t*-tests were performed and the *p* values are indicated in the figures.

RESULTS

Effect of sugars on single-channel currents

Uncharged molecules, including sugars and PEGs, with different molecular weight and diameter, were chosen to determine if their access to single cx46 hemichannels is affected by the voltage gate (Table 1). Based on previous applications of nonelectrolytes to channels (Bezrukov, 2000; Bezrukov and Vodyanoy, 1993; Bezrukov et al., 1994; Krasilnikov et al., 1992; Merzlyak et al., 1999; Sabirov et al., 1993), our expectation was that sugars that are small enough to enter the channel should reduce channel conductance by stochastically occupying part of the channel. If the test molecules are too large, no change of channel conductance should be observed. 100 mM sorbitol, glucose, or sucrose indeed reduced unitary currents relative to control (Fig. 1, *a*, *b*, and *c*). Stachyose, the largest sugar tested, did not lead to a significant change of conductance (Fig. 1 *d*). This result suggests that at negative potentials molecules of the size of stachyose or larger are excluded from the channels. With sugars small enough to access the channel, the change of unitary conductance (γ_{max}) was inversely related to the size of sugar molecules. Sorbitol, the smallest sugar tested, reduced the unitary conductance noticeably compared to control (Fig. 1 *a*, *lower* and *upper traces*). Glucose and sucrose, the larger sugars, reduced conductance to a lesser extent (Fig. 1, *b* and *c*).

To test for accessibility changes imposed by the voltage gate the patch was held at positive potentials. At positive potentials, the cx46 channel mainly dwells in a subconductance state, which is the main consequence of the V_j gate (Trexler et al., 1996; Pfahnl and Dahl, 1998), and rarely stays in the full open state. In the full open state the currents at positive potentials are smaller than at negative potentials, i.e., the channel rectifies. When 100 mM sorbitol or glucose was applied to the cytoplasmic side of the channel, the currents at both rectified full open state and subconductance state were reduced (Fig. 2, *a* and *b*). No effect was observed with sucrose (Fig. 2 *c*).

A quantitative summary of these results is shown in Fig. 3. At negative potentials (Fig. 3 *a*), the largest inhibition (~50%) was produced by sorbitol. Stachyose and sucrose did not reduce channel conductance significantly, whereas the effect of glucose (~20% inhibition) was intermediate among the sugars tested.

TABLE 1 List of uncharged test molecules

Molecules	Diameter (Å)	MW	Formula
D-sorbitol	5.8	182	$C_6H_{14}O_6$
D-(+)-glucose	7.5	180	$C_6H_{12}O_6$
PEG 200	8.0	200	$(C_2H_4O)n \sim 4{\cdot}H_2O$
Sucrose	8.9	343	$C_{12}H_{22}O_{11}$
PEG 400	11.2	400	$(C_2H_4O)n \sim 8{\cdot}H_2O$
Stachyose	12	666	$C_{24}H_{42}O_{21}$

MW, molecular weight.

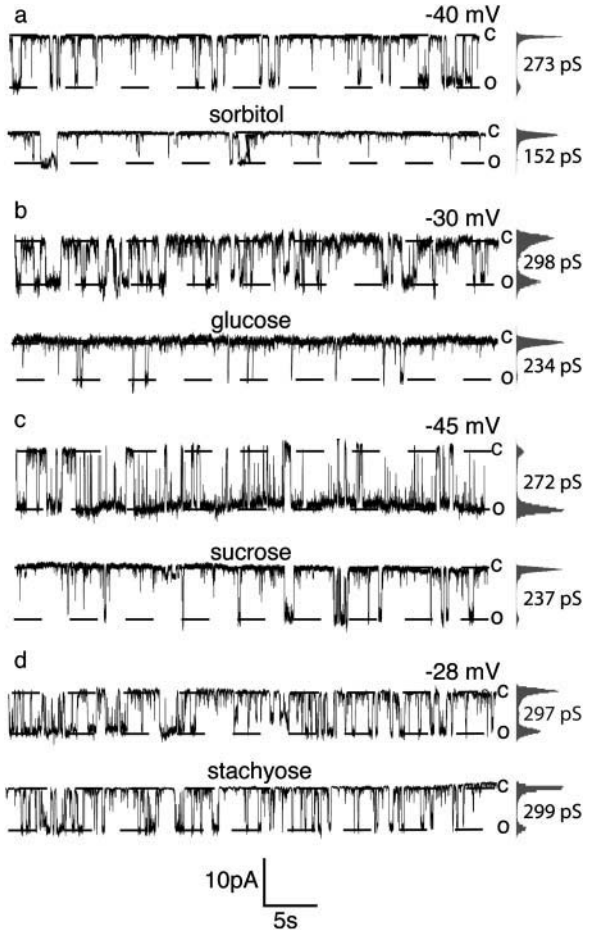

FIGURE 1 Effect of sugars on cx46 single-channel currents at negative holding potentials. Single-channel currents were recorded from inside-out patches excised from oocytes expressing cx46. Channels were held at negative potentials as indicated. Open (*o*) and closed (*c*) states are indicated by dotted lines. Sugars were applied at 100 mM from the intracellular side of the channel for 5 min. Representative records on the same patch before (*upper trace*) and after (*lower trace*) applying sorbitol (*a*), glucose (*b*), sucrose (*c*), or stachyose (*d*) are displayed. All-point amplitude histograms and single-channel conductances are shown on the side of each record.

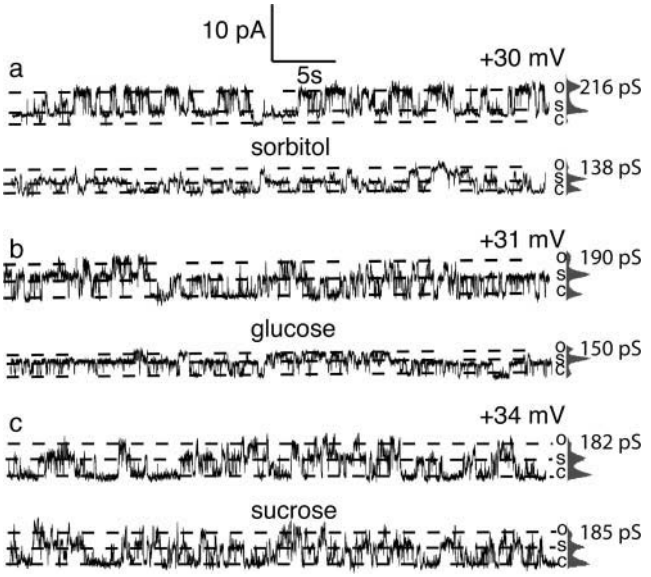

FIGURE 2 Effect of sugars on cx46 single-channel currents at positive holding potentials. Single-channel currents were recorded from inside-out patches held at positive potentials before (*upper trace*) and after (*lower trace*) applying sorbitol (*a*), glucose (*b*), and sucrose (*c*). All sugars were applied at 100 mM from the intracellular side of the channel for 5 min. Representative records are displayed. The channel dwelled in three states, closed state (*c*), subconductance state (*s*), and open state (*o*), as indicated by dotted lines. All-point amplitude histograms and single-channel conductances (γ_{max}) are displayed on the side of each record.

A quantitative analysis of data at positive potentials is shown in Fig. 3 *b*. The reduction of single-channel conductance was found only when sorbitol and glucose were applied. In both cases the rectified conductance and the subconductance were decreased. There was no noticeable change when sucrose or stachyose were applied at positive potentials (Fig. 3 *b*). This result suggests that closure of the voltage gate does not change the size exclusion limit significantly.

Fig. 4 shows that some sugars reduced the conductance of gap junction hemichannels more than bulk conductivity. This finding contrasts the conductance effects of PEG polymers in alamethicin channels (Bezrukov and Vodyanoy, 1993; Parsegian et al., 1995). In addition, channel inhibition in cx46 hemichannels was more pronounced than in alamethicin channels.

The change of single-channel activity after application of sugars was complex in that single-channel conductance was not the only channel property altered by the sugars. Molecules that reduced unit conductance at negative potentials also induced several lower conductance levels. This effect was more pronounced with small sugars than with large sugars. As shown in Fig. 5 *b*, after the application of sorbitol, the sojourns went to different levels besides the full conductance (γ_{max}). There are at least three peaks corresponding to open states discernible on the histogram plot, and perhaps one or two more. With sucrose, as shown in Fig. 5 *c*, at least one additional conductance level was observed besides the full conductance state.

The sugars also reduced the probability of the channels to conduct. As shown in Fig. 6 the P_o of full openings (γ_{max}) was reduced by sorbitol, glucose, and sucrose. Even when full and partial conduction events were pooled together, the P_o was reduced by the same sugars whereas the number of openings was little changed. Stachyose did not affect the P_o significantly. Thus it appears that sugars, besides reducing unit conductance, can also reduce the open probability of hemichannels, possibly by channel block.

In addition to affecting single-channel conductance, sugars also changed the mean open time. Fig. 7 shows the mean open time of cx46 hemichannels held at negative or positive potentials before and during the presence of sugars. At negative potentials (Fig. 7 *a*) mean open time was reduced

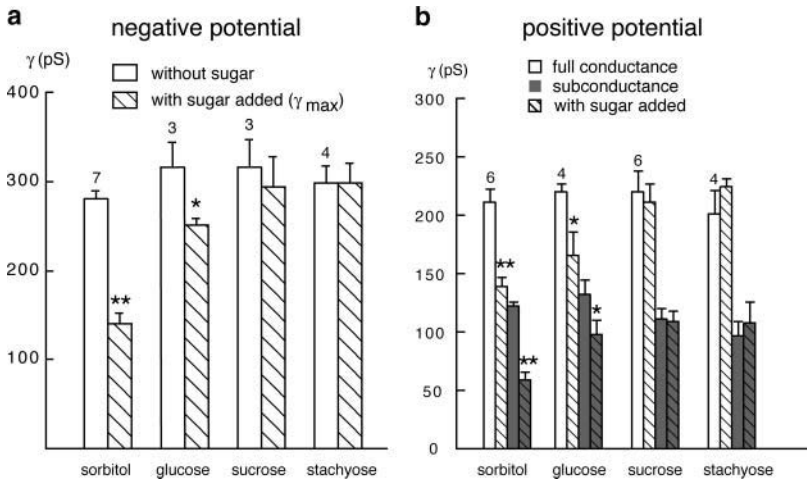

FIGURE 3 Single-channel conductance before and after applying sugars at negative (*a*) and positive (*b*) holding potentials. At negative potentials, reduction of channel conductance was observed when sorbitol or glucose was applied, but not with stachyose. The reduction was inversely related to the size of sugars. At positive potentials, conductance of both full open state and subconductance state were affected by sugars. Although the reduction is seen with sorbitol and glucose, no change is apparent with sucrose and stachyose. Mean ± SE are plotted; *n* is given above the bars. Statistical significance of difference between pre- and postsugar application based on paired *t*-tests is indicated: $*p < 0.05$, $**p < 0.01$.

when sorbitol, glucose, or sucrose was applied, but was unaffected by stachyose. This result parallels effects caused by these sugars on single-channel conductance and open probability, suggesting the accessibility of sorbitol, glucose, and sucrose to the channel and the exclusion of stachyose from the channel at negative potentials. At positive potentials, only sorbitol shortened the mean open time, whereas other sugars did not induce a change (Fig. 7 *b*).

Table 2 summarizes the effects of the sugars on single-channel parameters. It is clear that single-channel conductance should not be the sole criterion for determining the accessibility of sugars to the channel, but that open probability and mean open time are parameters that should be considered, as well.

PEG polymers yielded inconsistent data sets (data not shown). Application of these compounds remained without obvious consequences in some patches whereas in others the effect was similar to that of sugars. This finding is in accordance with a previous report (Oh et al., 1997) about effects of PEG on gap junction channels. The reason for this bimodality is not clear.

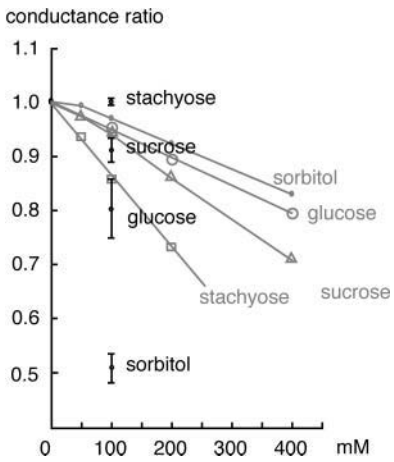

FIGURE 4 Solution conductivity as a function of sugar (sorbitol, glucose, sucrose, or stachyose) content. Bulk conductivity was determined with a Yellow Springs Instrument meter (Yellow Springs, OH). Conductivity ratios of KGlu solution with and without sugar are plotted. For comparison with sugar effects on the channel, data from Fig. 3 are replotted.

DISCUSSION

The present data show that the voltage gate of gap junction channels excludes sugar molecules according to size, similar to the way it excludes charged molecules. Net charge of the test molecule thus is not responsible for exclusion. Instead, steric constraints appear to be imparted by the gate. When the voltage gate is activated at positive potentials glucose still affects channel properties whereas sucrose does not. The voltage-imposed exclusion limit hence lies between the sizes of glucose and sucrose. The test molecules were applied from the intracellular side of the channel, so the accessibility change likely reflects a voltage-induced conformational change around the internal mouth of the channel. Mutational analysis of the voltage gate of gap junction channels also indicates a localization of the voltage gate toward the cytoplasmic portion of the channel (Oh et al., 2000). This voltage-induced pathway change by ~3 Å is sufficient to slow down or occlude the passage of cellular compounds that are permeant without activation of the gate.

The effect of sugars on channel properties was more complex than anticipated. Formally, the situation is similar to that in Coulter counters, commercial devices that determine the number and size of small particles in a capillary by conductance changes (DeBlois et al., 1977; Parsegian et al., 1995). Assuming a regularly shaped, cylindrical channel it is anticipated that occupancy of the channel with nonelectrolytes would reduce channel currents proportionally to molecular size. However, for molecules close to the size of the pore entry should be inversely related to size because of reduced probability of a larger molecule to enter the pore. When the test molecule reaches and exceeds the pore diameter total exclusion should occur. Consequently, with

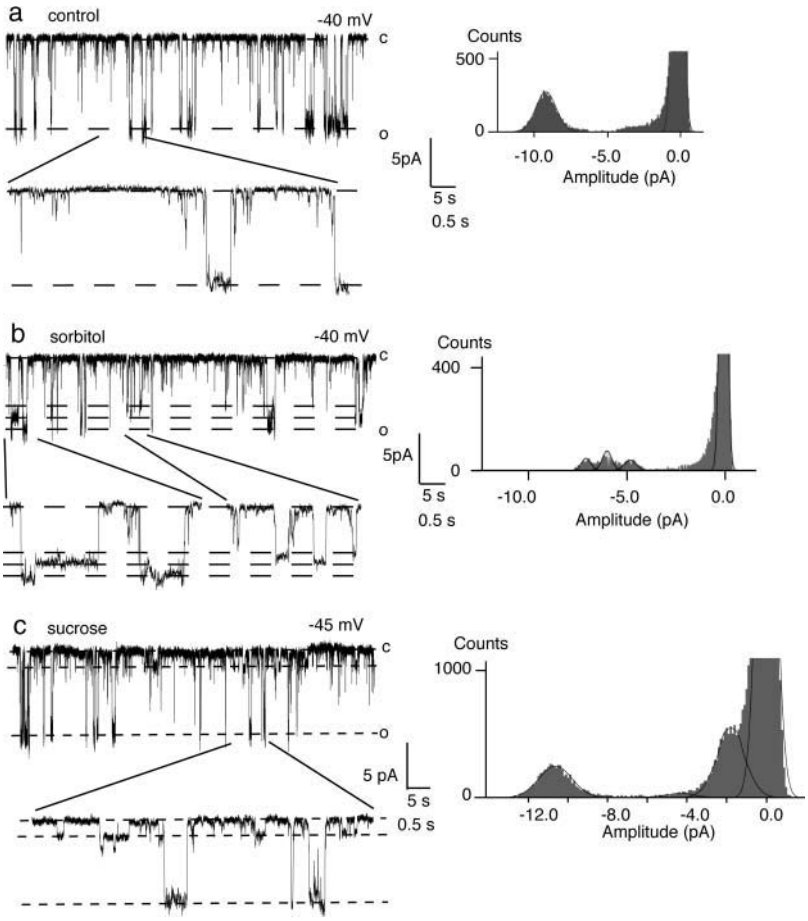

FIGURE 5 Induction of low conductance levels by sugars. Control channels dwell in two states, open and closed (*a*). Different excursion levels of single-channel currents (indicated by *dotted lines*) became apparent when sorbitol (*b*) or sucrose (*c*) was applied at negative potentials. Parts of the records are displayed at extended timescale to illustrate the long dwell times at the lower conductance levels. All-point amplitude histograms are based on 96-s segments of records. The predominant peaks correspond to the closed state; other peaks correspond to the open state at different levels.

increasing size of the test molecules, the inhibition of channel currents should peak, diminish toward the pore size, and the channel currents should remain unaffected when the test molecule exceeds the pore size.

Previous studies using nonelectrolytes in alamethicin channels at least in part fulfilled these expectations. Channel conductance was reduced and channel noise was increased with PEG polymers smaller than the channel pore whereas larger polymers did not affect these channel parameters (Bezrukov and Vodyanoy, 1993; Bezrukov et al., 1994; Krasilnikov et al., 1995; Parsegian et al., 1995). However, inhibition of channel conductance was inversely related over a wide range of polymer sizes as if the probability of polymer access to the channel declined steadily with increasing size. With regard to maximal channel conductance, γ_{max}, the same phenomenon was observed in the present study with sugars on cx46 hemichannels. Sorbitol, the smallest sugar tested, yielded the largest reduction of γ_{max}. In a previous report on application of polymers to gap junction channels, inhibition of conductance by polymers was size independent and polymer exclusion occurred in one step (Oh et al., 1997). Unfortunately, presentation of data in that paper is limited to a conductance-hydrodynamic radius plot and thus comparison with the present data is not possible.

Here we report that sugars not only modify channel properties in terms of channel conductance (γ_{max}), but also induce multiple low conductance levels and affect the apparent open probability. The effect on γ_{max} was inversely related to sugar size as observed for polymers in alamethicin channels. This result would be expected for sugar sizes close to the pore radius, a situation in which reflection would affect the odds of a particle entering the pore. A small molecule will have a better chance to hit the channel head-on whereas a larger molecule will be more likely to strike the channel rim and get reflected back into bulk solution. The observation that the reduction of channel conductance exceeds that of the bulk solution also favors the possibility of a close fit of the particle in the channel.

To our knowledge, the generation of multiple low conductance levels in channels by nonelecrolytes has not been reported. The effect is most pronounced for the smaller sugar, sorbitol, whereas it is only barely detectable with the larger sucrose molecule. Probably this phenomenon is not a true induction of subconductance states of the channel proper due to conformational change of the channel protein, but rather represents a partial obliteration of the channel path by the sugars. Such defined low conductance states are typically not seen in the absence of sugar, although

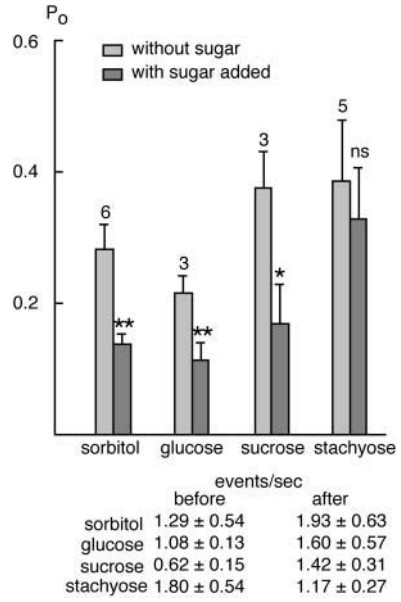

FIGURE 6 Open probability plot for cx46 hemichannels with and without sugars. A significant reduction of the open events was observed with sorbitol, glucose, and sucrose. Stachyose did not change the open probability to a significant extent. For the analysis of all events the detection threshold was set at 0.2 pA above the noise level. Mean ± SE are plotted; n is given above the bars; $*p < 0.05$; $**p < 0.01$.

incomplete openings with a very short lifetime can occur without a definable peak on all-point histograms (see also Trexler et al., 1996; Pfahnl and Dahl, 1998). Thus the different conductance levels probably represent either varying occupancy of the channel by sugar molecules or the residence of the test molecule in different-sized parts of the channel resulting in varying relative block.

Sugars should traverse the channel in the order of nanoseconds assuming they diffuse freely through the channel. However, the lifetimes of the sugar-induced conductance ''states'' can last hundreds of milliseconds, a time that is in the same range as observed for maltose and its transport protein (Bezrukov et al., 2000; Kullman et al., 2002). This finding suggests that the molecules reside in the channel for extended periods of time. Hence there is apparent binding of the sugars to the channel wall. Yet all sugars tested, and even polyethyleneglycol polymers, exerted similar effects on the channel. This lack of specificity makes a binding event unappealing. On the other hand, this does not mean that binding can be excluded altogether.

Although the sugars used in this study are bona fide nonelectrolytes and as such have a net zero charge, dipole moments cannot be neglected. Surface charges on the sugars, for example, could be responsible for interactions with the channel wall. This type of interaction could be responsible for the retention of the sugars within the channel, giving rise to the attenuated conductance levels that last in the order of milliseconds. Disparities in surface charge distribution between sugars could account for subtle differences in their channel effects, although the ''binding'' mechanism would be similar.

The transit rate of charged fluorescent tracers that are in the same size range as the sugars used in the present study through hemichannels and complete gap junction channels is several orders of magnitude lower than that of ions (Table 3; Valiunas, 2002). Thus larger molecules do not permeate the channel by free diffusion. Rather they seem to be trapped inside the channel for extended periods of time. It is conceivable that the channel is not a rigid conduit but is subject to motions that form pockets separated by labile constrictions (Fig. 8). Movement of molecules from pocket to pocket (or movement of the pocket) could account for both the low transit rates and the induction of ''conductance states'' by sugars.

An alternate explanation for the sugar effects on channel properties could be an osmotic collapse of the channel or

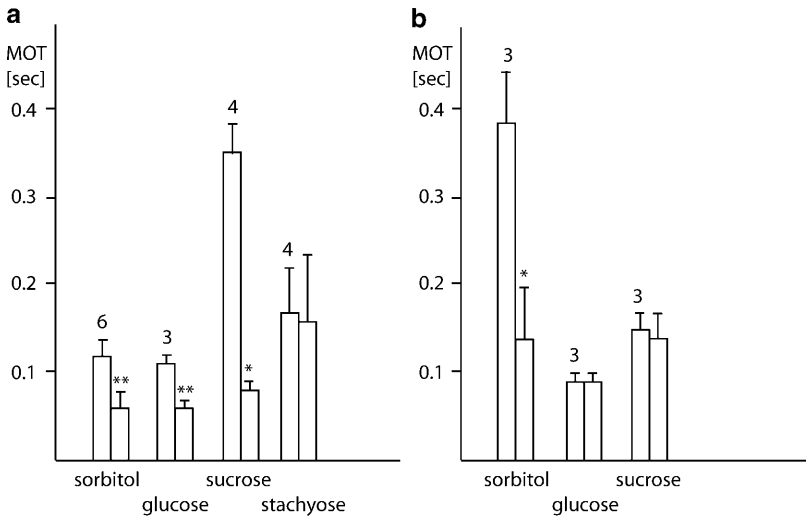

FIGURE 7 Mean open times before and after applying sugars at negative potentials (a) or positive potentials (b). For each sugar, the open-state dwell time of a representative record before (control) and after applying sugar was analyzed in 75-s segments. Detection threshold was set at 50%. Mean open time before and after applying sugar is shown as mean ± SE; n indicates the number of patches examined. Mean ± SE are plotted; n is given above the bars; $*p < 0.05$; $**p < 0.01$.

TABLE 2 Effect of sugar molecules on cx46 single channels

Holding potentials	Sugars	γ_{max}	P_o (γ_{max})	MOT	Accessibility
	Control	1	1	1	+
	Sorbitol	0.50 ± 0.04	0.49 ± 0.06	0.50 ± 0.17	+
Negative	Glucose	0.79 ± 0.02	0.53 ± 0.12	0.55 ± 0.09	+
	Sucrose	0.93 ± 0.11	0.45 ± 0.16	0.23 ± 0.03	+
	Stachyose	1.00 ± 0.07	0.85 ± 0.19	0.94 ± 0.47	–
Holding potentials	Sugars	$\gamma_{full}/\gamma_{sub}$		MOT	Accessibility
	Control	1	1	1	
	Sorbitol	0.66 ± 0.04/ 0.48 ± 0.05	0.76 ± 0.06	0.36 ± 0.15	+
Positive	Glucose	0.75 ± 0.09/ 0.74 ± 0.09	0.90 ± 0.02	1.00 ± 0.11	(+)
	Sucrose	0.95 ± 0.07/ 0.99 ± 0.08	0.81 ± 0.17	0.93 ± 0.20	–
	Stachyose	1.10 ± 0.03/ 1.11 ± 0.18			–

Data from Figs. 3, 6, and 7 for single-channel conductance (γ_{max}, γ_{full}, and γ_{sub}), open probability (P_o), and mean open time are presented here normalized to the values before addition of the sugars (control). MOT, mean open time.

parts of it. The nonelectrolytes were added to the regular bath solution whereas the pipette solution contained only salts. Thus an osmotic gradient was established during the sugar application. Because all sugars were applied at the same concentration a strictly osmotic effect should be independent of the size of the sugar. This was not observed; almost all parameters were changed differently by the various sugars. Furthermore, as studies on potassium channels indicate, much higher osmotic stress is required to collapse channels (Zimmerberg et al., 1990; 3.5 Osmol versus 100 mOsmol in the present study).

Similar to the induction of low-level conductances the change in open probability by the sugars is probably not due to conformational changes of the channel proteins. More likely it represents steric block of the channel. Consistent with such a mechanism channel noise is larger in the presence of the nonelectrolytes. These observations then suggest asymmetry of the channel in agreement with earlier reports (Zhou et al., 1997) and also evidenced by crystallography at 7-Å resolution (Unger et al., 1999). Residence of the sugar molecules in the narrow part of the channel could lead to channel block whereas their presence in vestibular parts of the channel could be responsible for the low conductance levels. With activation of the voltage gate at positive potentials sugars could reduce the full open state and the subconductance state proportionally suggesting that the sugars may not be retained in the gate structure but reside in other parts of the channel where they either block conductance or reduce it.

We hypothesize that a rather narrow and permanent albeit gated channel allows the flux of ions through gap junction channels. Transient formation of cavities by thermal movements of the channel (''channel breathing'') could accom-

TABLE 3 Diffusion rates of ions and dye molecules

Diffusion rate*	−20 mV
Ions	3.75×10^7
Lucifer yellow	6.39×10^4
Cascade blue	7.10×10^4
Calcein	5.63×10^2

Calculations are based on data from an earlier study (Qu and Dahl, 2002). Fluorescence intensity was determined with NIH IMAGE software (http://rsb.info.nih.gov/nih-image/) and calibrated with a series of dilutions of the fluorescent dyes. Number of channels was calculated from the macroscopic membrane conductance at −20 mV with single-channel conductance of 300 ps.

*Number of molecules passing through each cx46 hemichannel per second.

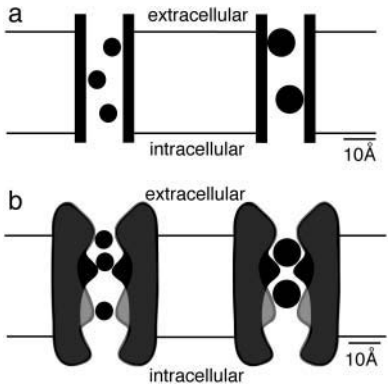

FIGURE 8 Cartoon of permeation of small and large sugar molecules through the channel. (*a*) Permeation of sugars through a cylindrical channel should result in a size-dependent reduction of channel conductance and increase channel noise. (*b*) The channel is hypothesized not to be a rigid but a flexible structure. The channel has transient constrictions that trap solutes in pockets and thereby induce lower conductance levels with lifetimes of milliseconds. For example, the superimposed shapes in grades of gray depict different conformations. Sugars trapped close to the constrictions can lead to channel block and thereby give rise to a reduced ''open probability''. Thus entrapment of different numbers of sugar molecules and/or entrapment in different parts of the channel could lead to the various conductance levels observed. The permeation filter, determining the exclusion limit, may be also a flexible part of the channel or be more rigid. Gating of the channel to the closed state (zero current) is a different conformation and not depicted.

modate larger molecules and shuttle them through the membrane in a fashion akin to peristalsis. Such a mechanism not only would be consistent with the observations made here on sugar accessibility but also could explain the lack of a sharply defined exclusion limit, the several orders of magnitude discrepancy between the transit rates of ions and larger tracer molecules (Valiunas, 2002), and the poor correlation between channel conductance and permeability for larger molecules in channels made of different connexins (Gong and Nicholson, 2001; Veenstra, 1996; Veenstra et al., 1995).

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REFERENCES

Bezrukov, S. M. 2000. Ion channels as molecular coulter counters to probe metabolite transport. *J. Membr. Biol.* 174:1–13.

Bezrukov, S. M., L. Kullman, and M. Winterhalter. 2000. Probing sugar translocation through maltoporin at the single channel level. *FEBS Lett.* 476:224–228.

Bezrukov, S. M., and I. Vodyanoy. 1993. Probing alamethicin channels with water-soluble polymers. Effect on conductance of channel states. *Biophys. J.* 64:16–25.

Bezrukov, S. M., I. Vodyanoy, and V. A. Parsegian. 1994. Counting polymers moving through a single ion channel. *Nature.* 370:279–281.

Dahl, G. 1992. The Xenopus oocyte cell-cell channel assay for functional analysis of gap junction proteins. *In* Cell-Cell Interactions. A Practical Approach. B. Stevenson, W. Gallin, and D. Paul, editors. IRL, Oxford. 143–165.

DeBlois, R. W., C. P. Bean, and R. K. A. Wesley. 1977. Electrokinetic measurements with submicron particles and pores by the resistive pulse technique. *J. Colloid Interface Sci.* 61:323–335.

Evans, W. H., and P. E. M. Martin. 2002. Gap junctions: structure and function (Review). *Mol. Membr. Biol.* 19:121–136.

Gong, X. Q., and B. J. Nicholson. 2001. Size selectivity between gap junction channels composed of different connexins. *Cell Commun. Adhes.* 8:187–192.

Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflugers Arch.* 391:85–100.

Harris, A. L. 2001. Emerging issues of connexin channels: biophysics fills the gap. *Q. Rev. Biophys.* 34:325–472.

Krasilnikov, O. V., R. Z. Sabirov, V. I. Ternovsky, P. G. Merzliak, and J. N. Muratkhodjaev. 1992. A simple method for the determination of the pore radius of ion channels in planar lipid bilayer membranes. *FEMS Microbiol. Immunol.* 5:93–100.

Krasilnikov, O. V., L. N. Yuldasheva, R. A. Nogueira, and C. G. Rodrigues. 1995. The diameter of water pores formed by colicin Ia in planar lipid bilayers. *Braz. J. Med. Biol. Res.* 28:693–698.

Kullman, L., M. Winterhalter, and S. M. Bezrukov. 2002. Transport of maltodextrins through maltoporin: a single-channel study. *Biophys. J.* 82:803–812.

Merzlyak, P. G., L. N. Yuldasheva, C. G. Rodrigues, C. M. Carneiro, O. V. Krasilnikov, and S. M. Bezrukov. 1999. Polymeric nonelectrolytes to probe pore geometry: application to the alpha-toxin transmembrane channel. *Biophys. J.* 77:3023–3033.

Oh, S., C. K. Abrams, V. K. Verselis, and T. A. Bargiello. 2000. Stoichiometry of transjunctional voltage-gating polarity reversal by a negative charge substitution in the amino terminus of a connexin32 chimera. *J. Gen. Physiol.* 116:13–31.

Oh, S., Y. Ri, M. V. Bennett, E. B. Trexler, V. K. Verselis, and T. A. Bargiello. 1997. Changes in permeability caused by connexin 32 mutations underlie X-linked Charcot-Marie-Tooth disease. *Neuron.* 19: 927–938.

Parsegian, V. A., S. M. Bezrukov, and I. Vodyanoy. 1995. Watching small molecules move: interrogating ionic channels using neutral solutes. *Biosci. Rep.* 15:503–514.

Paul, D. L., L. Ebihara, L. J. Takemoto, K. I. Swenson, and D. A. Goodenough. 1991. Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of Xenopus oocytes. *J. Cell Biol.* 115:1077–1089.

Pfahnl, A., and G. Dahl. 1998. Localization of a voltage gate in connexin46 gap junction hemichannels. *Biophys. J.* 75:2323–2331.

Pfahnl, A., X. W. Zhou, R. Werner, and G. Dahl. 1997. A chimeric connexin forming gap junction hemichannels. *Pflugers Arch.* 433:773–779.

Qu, Y., and G. Dahl. 2002. Function of the voltage gate of gap junction channels: selective exclusion of molecules. *Proc. Natl. Acad. Sci. USA.* 99:697–702.

Sabirov, R. Z., O. V. Krasilnikov, V. I. Ternovsky, and P. G. Merzliak. 1993. Relation between ionic channel conductance and conductivity of media containing different nonelectrolytes. A novel method of pore size determination. *Gen. Physiol. Biophys.* 12:95–111.

Spray, D. C., A. L. Harris, and M. V. Bennett. 1981. Equilibrium properties of a voltage-dependent junctional conductance. *J. Gen. Physiol.* 77:77–93.

Trexler, E. B., M. V. Bennett, T. A. Bargiello, and V. K. Verselis. 1996. Voltage gating and permeation in a gap junction hemichannel. *Proc. Natl. Acad. Sci. USA.* 93:5836–5841.

Unger, V. M., N. M. Kumar, N. B. Gilula, and M. Yeager. 1999. Three-dimensional structure of a recombinant gap junction membrane channel. *Science.* 283:1176–1180.

Valiunas, V. 2002. Biophysical properties of connexin-45 gap junction hemichannels studied in vertebrate cells. *J. Gen. Physiol.* 119:147–164.

Veenstra, R. D. 1996. Size and selectivity of gap junction channels formed from different connexins. *J. Bioenerg. Biomembr.* 28:327–337.

Veenstra, R. D., H.-Z. Wang, D. A. Beblo, M. G. Chilton, A. L. Harris, E. C. Beyer, and P. R. Brink. 1995. Selectivity of connexin-specific gap junctions does not correlate with channel conductance. *Circ. Res.* 77:1156–1165.

Zampighi, G. A., D. D. Loo, M. Kreman, S. Eskandari, and E. M. Wright. 1999. Functional and morphological correlates of connexin50 expressed in Xenopus laevis oocytes. *J. Gen. Physiol.* 113:507–524.

Zhou, X. W., A. Pfahnl, R. Werner, A. Hudder, A. Llanes, A. Luebke, and G. Dahl. 1997. Identification of a pore lining segment in gap junction hemichannels. *Biophys. J.* 72:1946–1953.

Zimmerberg, J., F. Bezanilla, and V. A. Parsegian. 1990. Solute inaccessible aqueous volume changes during opening of the potassium channel of the squid giant axon. *Biophys. J.* 57:1049–1064.