Heteromeric, but Not Homomeric, Connexin Channels Are Selectively Permeable to Inositol Phosphates*

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Previous work has shown that channels formed by both connexin (Cx)26 and Cx32 (heteromeric Cx26/Cx32 hemichannels) are selectively permeable to cAMP and cGMP. To further investigate differential connexin channel permeability among second messengers, and the influence of connexin channel composition on the selectivity, the permeability of inositol phosphates with one to four phosphate groups through homomeric Cx26, homomeric Cx32, and heteromeric Cx26/Cx32 channels was examined. Connexin channels were purified from transfected HeLa cells and from rat, mouse, and guinea pig livers, resulting in channels with a broad range of Cx26/Cx32 aggregate ratios. Permeability to inositol phosphates was assessed by flux through reconstituted channels. Surprisingly, myo-inositol and all inositol phosphates tested were permeable through homomeric Cx32 and homomeric Cx26 channels. Even more surprising, heteromeric Cx26/Cx32 channels showed striking differences in permeability among inositol phosphates with three or four phosphate groups and among isomers of inositol triphosphate. Thus, heteromeric channels are selectively permeable among inositol phosphates, whereas the corresponding homomeric channels are not. There was no discernible difference in the permeability of channels with similar Cx26/Cx32 ratios purified from native and heterologous sources. The molecular selectivity of heteromeric channels among three inositol triphosphates could not be accounted for by simple connexin isoform stoichiometry distributions and therefore may depend on specific isoform radial arrangements, some of the channels were permeable to cAMP, and a much larger fraction of the channels was permeable to cGMP. This selectivity between highly similar biological signaling molecules was unanticipated from dye permeability studies of these channels. The ability of connexin channels to discriminate among second messengers is likely to be a biologically important property. Therefore, we asked whether the selective permeability among second messengers extended to inositol phosphates (IPs).

IP permeability is difficult to assess in cells because of the biological effects of manipulation of the levels of these compounds and their short lifetimes in cytoplasm. For example, the lifetime of inositol 1,4,5-triphosphate ((1,4,5)-IP3) in cytoplasm ranges from 9 to 60 s, depending on cell type (26, 27). For this reason, we utilized a well characterized liposome-based technique for assessing permeability of functional connexin channels (20, 28–34). Permeability to each IP was determined by direct assessment of its loss and/or retention in liposomes containing functional connexin channels. In this way, the identity of the permeable molecular species was unambiguously determined.

The use of IPs as permeability tracers has several advantages. First, they are endogenous cytoplasmic molecules available for permeation through gap junction channels in vivo. Second, several IPs, in addition to (1,4,5)-IP3, have been shown to be biologically active (35–41). Third, the availability of IPs with different numbers of phosphate groups at several positions around the inositol ring allows exploration of the structural correlates of any selective permeability observed.

To reveal the effect of connexin channel composition on the selective permeability, two strategies were employed to obtain homomeric Cx26 and Cx32 channel populations, and heteromeric channel populations with a wide range of aggregate ratios of the two connexins, from both native and heterologous sources. We report here the surprising ability of heteromeric, but not homomeric, connexin channels to distinguish among biologically active IPs, including those with the same molecular
weight and charge. The selectivity is not a simple function of the content of one or the other of the connexins but is a function of the heteromeric character of the channels. This has clear implications for the biological role of heteromeric channels and for modulation of intercellular molecular signaling.

**EXPERIMENTAL PROCEDURES**

Components of the “Tet-On” connexin expression system (30) were from BD Biosciences. Dulbecco’s modified Eagle’s medium, G418 sulfate, hygromycin, and doxycycline were from Invitrogen. Agarose-conjugated and unconjugated anti-HA clone HA-7 mouse IgG were from Sigma. Monoclonal anti-Cx26 antibody was from Zymed Laboratories Inc. Monoclonal M12.13 anti-Cx32 mouse antibody (42) was conjugated to CNBr-Sepharose (Amersham Biosciences). Lipids (egg phosphatidylethanolamine, bovine brain phosphatidylserine, and lissamine rhodamine B-labeled egg phosphatidylethanolamine (rhodamine-PE)) were from Avanti Polar Lipids Inc. (Alabaster, IL). Monoclonal M12.13 anti-Cx32 mouse antibody (42) was conjugated and unconjugated anti-HA clone HA-7 mouse IgG were from BD Biosciences. Dulbecco’s modified Eagle’s medium, G418 sulfate, hygromycin, and doxycycline were from Invitrogen. Agarose-conjugated M12.13 anti-Cx32 monoclonal mouse antibody (42). Bead washing and connexin elution were carried out as described for tagged connexins.

From solubilized mouse and guinea pig membranes, the channels purified are heteromeric Cx26/Cx32. The proportion of Cx26 recovered is greater from guinea pig than from mouse livers, consistent with the composition in the livers themselves (46-49).

**Obtaining Different Aggregate Cx26/Cx32 Ratios from Native Tissue**—To obtain different ratios from a single native source, we took advantage of the fact that purification was on the basis of the presence of Cx32. After the connexin was bound to the anti-Cx32 immunobeads, we found that the earliest elution fractions contained the highest proportion of Cx26, presumably due to the lower number of Cx32 epitopes per channel. For example, from solubilized rat liver membranes, the bulk of connexin channels purified are homomeric rCx32. However, when small (~200 μl) elution fractions are used instead of the usual ~600-μl fractions, the first several fractions contained heteromeric rCx26/Cx32 channels with significant Cx26 content, which elute more easily from the antibody matrix. The later fractions contain only homomeric rCx32 channels. This allows a range of isofrom stoichiometries to be obtained from a single native source.

**Channel Reconstitution**—Immunopurified connexins (~1 mg/ml connexin in 80 mM OG, 459 mM urea, and 10 mg/ml phosphatidylcholine/phosphatidylserine/rhodamine-PE at a molar ratio of 2:1:0.01; 200 μl) were reconstituted into unilamellar liposomes using a glass HR10/30 chromatography column (bed volume ~24 ml) packed with Bio-Gel A-0.5m 100–200 mesh media (exclusion limit 500 kDa) in chilled, de-gassed 10 mM HEPES, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 459 mM urea (500 mosm/kg). This solution is referred to below as “urea buffer.” Flow rate was 9 ml/h, and connexin proteoliposomes were eluted in the column void volume.

**Transport-specific Fractionation Activity Assay**—Transport-specific fractionation (TSF) was used to assess the molecular selectivity of reconstituted connexin hemichannels (21, 28, 34, 50). TSF fractionates liposomes containing reconstituted connexin channels into two populations within an iso-osmolar density gradient, based on channel functionality. Fractionation is based on channel permeability to urea and sucrose, uncharged solutes that permeate open connexin channels and have different density at iso-osmolar concentrations (459 mM urea or 400 mM sucrose; 500 mosm/kg). Equilibration of these solutes across the liposome through an open hemichannel occurs rapidly and increases the density of the liposome. TSF therefore reports all-or-nothing per-
FIGURE 1. Permeability assay, TSF. TSF fractionates liposomes by the exchange of light intraliposomal and heavier extraliposomal solutes through reconstituted hemichannels (see “Experimental Procedures”). It fractionates liposomes into two populations based on channel-mediated permeability to urea (U) and sucrose (S), one population of liposomes with functional channels and another in which the liposomes do not contain functional channels. Connexin channels are incorporated into the membranes of unilamellar liposomes and tracer molecules of interest entrapped in the liposomes. When centrifuged in an iso-osmolar density gradient, movement of gradient solutes (typically urea and sucrose) through open channels causes liposomes to become denser and move to a position deep in the gradient. Liposomes without functional channels remain in the upper part of the gradient. Tracers that are permeable through the channels are lost from the liposomes, and those that are impermeable are retained. The average number of channels per liposome is less than 1, so there is always an upper band without channels to serve as a control of tracer loading and nonspecific leakage. Selective permeability among the tracers was determined by direct comparison of the tracer per liposome retained by the two populations of liposomes.

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Tracer Recovery—Upper and lower liposome bands in ~100-μl volumes recovered from TSF density gradients were analyzed for myoinositol or inositol phosphate content. Liposomes were diluted 1:1 v/v with high pressure liquid chromatography-grade methanol and vortexed. The mixture was slowly passed through a Sep-Pak tC18 solid phase extraction cartridge (Waters Associates), pre-equilibrated in methanol, followed by 50% v/v methanol in water to elute the tracer. These samples were dried under argon. As an index of lipid removal, fluorescent rhodamine-PE lipid was completely removed from tracer samples by this technique. Residual myoinositol or inositol phosphate did not bind to the tC18 matrix solid phase extraction cartridge, as quantitative recovery of unentrapped tracer mixed with liposomes versus untreated tracer samples was obtained (p < 0.05; n = 3).

Tracer Analysis, Enzymatic—Inositol phosphates were dephosphorylated to myoinositol, which was detected using the method of Maslanski et al. (51). Briefly, fresh alkaline phosphatase (45 μl of 200 units/ml in 0.1 M Tris-HCl, pH 9.0, 0.1 mM ZnCl2) was added to the dry sample and incubated overnight at room temperature. The enzyme was inactivated at 100 °C for 4 min, and tubes were cooled to 37 °C. To remove possible contaminating traces of glucose, hexokinase (10 μl of 200 units/ml in 50 mM Tris, 10 mM MgCl2, 0.1 M ATP, 0.02% w/v BSA, pH 9.0) was added to each sample and incubated at 37 °C (1 h). The hexokinase reaction was stopped by 100 °C for 3 min. Tubes were cooled to room temperature; NAD+ (10 μl of 0.1 M) and inositol dehydrogenase (10 μl of 1 unit/ml in 10 mM phosphate, 0.02% w/v BSA, pH 6.8) was then added, and incubation was continued at room temperature for 15 min. The pH was then lowered to ~6.5 by addition of 5 μl of 0.80 M HCl. Resazurin (10 μl of 20 μM, in 1.0 mM phosphate, pH 6.5) and diaphorase (5 μl of 10 units/ml in 20 mM phosphate, 0.02% w/v BSA, pH 6.8) were added to each sample and incubated at room temperature for 15 min in the dark. Resazurin is oxidized by NADH, the product of the reaction between inositol dehydrogenase, NAD+, and myoinositol to the fluorescent compound resorufin. For measurement of the resorufin product, 0.1 M Tris-HCl, pH 9.0 (920 μl), was added to each
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sample and thoroughly vortexed, and the fluorescence was measured (λ_ex 565 nm and λ_em 585 nm).

Sample blanks were treated identically to test samples, except the inositol dehydrogenase was omitted. Blank fluorescence was subtracted from the sample fluorescence. Measurement of resorufin fluorescence enabled the amount of myoinositol or dephosphorylated inositol phosphate in the samples to be determined by interpolation from an inositol and tracer standard curve. A separate standard curve was constructed for each tracer compound used. This method was applied identically for each of the IPs tested.

Tracer Analysis, Radioimmunoassay—A second assay was used, specific to (1,4,5)-IP₃ (Biotrak TRK 1000-assay kit; Amersham Biosciences). In brief, (1,4,5)-IP₃ recovered from TSF bands competes with a fixed amount of (1,4,5)-[³H]IP₃ for a fixed number of sites on bovine adrenal (1,4,5)-IP₃-binding protein. The amount of unlabeled (1,4,5)-IP₃ in the dried sample was determined by interpolation from a standard curve. Direct side-by-side comparison of the enzymatic and radioimmunoassay for (1,4,5)-IP₃ gave identical results (p < 0.05).

Protein Recovery for SDS-PAGE, Western Blotting, and Gold Staining—Immunopurified connexin preparations or connexin recovered from TSF bands (52) were separated by SDS-PAGE. Western blots on polyvinylidene difluoride membranes were stained with specific monoclonal antibodies to Cx26, Cx32, or the HA(HN)₆ epitope, which were detected with alkaline phosphatase-conjugated secondary antibody and nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Pierce). For gold staining, nitrocellulose membranes were blocked (10 mM phosphate-buffered saline, pH 7.4, 0.3% v/v Tween 20) for 30 min at 37 °C. Following washing, membranes were stained with Colloidal Gold Total Protein Stain (Bio-Rad). The blots were washed with double-distilled water and air-dried. The Cx26/Cx32 ratio of the reconstituted channels in the lower TSF liposome band, i.e. the population of functional channels, did not differ from that in the upper liposome band, i.e. nonfunctional channels, or the nonreconstituted bead elution fractions.

Data Analysis—The ratio of myoinositol or inositol phosphate to liposome fluorescence (rhodamine-PE) was determined for each sample and corrected for the amount of tracer bound nonspecifically to liposomes. The tracer/rhodamine-PE fluorescence ratio of the upper band reflected the net amount of tracer entrapped per liposome prior to TSF and serves as an internal control for tracer trapping and nonspecific leakage from the liposomes. This was normalized as 100% retention of tracer. For the lower band (liposomes containing functional channels), the tracer/rhodamine-PE fluorescence ratio was normalized to that of the upper band. The data are presented as percentages of liposomes that are permeable to a tracer. Permeability data are expressed as mean ± S.E. Statistical significance was tested by compared means using one-way analysis of variance with p < 0.05.

Correction for More than One Channel per Liposome—For a given ratio (λ) of functional channels to lipid in the liposomes, a Poisson distribution describes the fraction of the liposomes that have functional channels (34). λ is estimated from the maximum activity (percentage (p) of liposomes with n active channels; Equation 1).

\[ p(n) = e^{-\lambda} \cdot \frac{\lambda^n}{n!} \cdot 100 \]  

(Eq. 1)

As λ increases, the percentage of liposomes with two or more channels increases. Therefore, the tracer retention/loss in the TSF lower band (the liposomes containing functional channels) may not accurately reflect the tracer permeability per channel (e.g. if there are two channels, both would have to be impermeable to a tracer for it to be retained, potentially resulting in greater permeability per liposome than per channel). By using the Poisson distribution, λ was used to calculate the distribution of n channels in the liposome population, which was used to compensate for the error introduced by some liposomes containing more than one functional channel. This calculation transforms the fraction of tracer-permeable liposomes in a population into an index of tracer-permeable channels.

Modeling of Isoform Stoichiometry Distributions—Assuming independent association of connexin monomers during hemichannel formation, the frequency of each heteromeric channel stoichiometry formed by two connexins is given by a Bernoulli distribution (Equation 2).

\[ a^b n \cdot n! \cdot (\frac{n}{r}) \]  

(Eq. 2)

where a and b are the incorporation probabilities (i.e. relative abundances) of Cx26 and Cx32, and n is the number of subunits in a hemichannel taken r subunits at a time (20, 53). For these calculations, n = 6 (as hemichannels are hexameric) and r = 0 ≤ r ≤ 6.

Molecular Modeling of Permeants—Molecular models of myoinositol and all inositol phosphates used in this study were made using Spartan 04 (Wavefunction, Irvine, CA). When possible, tracerc.pdb files were obtained from a data base of small heteromolecules and built using Spartan 04 if unavailable. Energy-minimized conformations were determined using MMFF94 force field rules (54). Hartree-Fock 6–31G* ab initio models (55), subject to the MMFF94 geometry constraints, were used for predicting molecular orbital structure (0.002 Å³/atomic unit) and chemical behavior, allowing electrostatic potential and local ionization potential to be mapped onto the orbital surfaces.

RESULTS

Populations of Heteromeric Channels with Different Aggregate Ratios of Cx26 to Cx32 Obtained from Heterologous and Native Expression Systems

From HeLa Cells—We recently described an expression strategy for recombinant connexin channels that mimics the natural structural heterogeneity of Cx26 and Cx32 channels (30). Homomeric and heteromeric Cx26 and or rCx32 hemichannels are expressed in communication-incompetent HeLa cells using bi-directional Tet-On-inducible vectors. For homomeric expression, the channels are tagged at the carboxyl terminus (CT) with a cleavable hemagglutinin epitope preceding a (His-Asn)ₖ sequence, a HA(HN)₆ tag. For heteromeric expression, only one connexin isoform is tagged.

Connexin was purified from the cell lines as described under “Experimental Procedures,” using a monoclonal antibody directed against the HA epitope of the tag. This purification protocol yields connexin hemichannels (20, 21, 34, 44, 45). Western blots (lanes indicated by *) and gold stain of the material purified from Cx26Tag and Cx32Tag HeLa lines show purification of homomeric Cx26 or Cx32 (Fig. 2a, lanes 1* and 2 and lanes 7 and 8*, respectively). Immunopurification from different Cx26Tag/Cx32 cell lines (or Cx26/Cx32Tag lines; data not shown) yielded both Cx26 and Cx32. Gold stain densitometry showed that purifications from different clonal heteromeric cell lines yielded channels with different aggregate isoform ratios (fractional Cx26 contents 0.75, 0.50, 0.33, and 0.25; Fig. 2a, lanes 3–6). Thus, each co-expressing cell line expressed the two connexins in a distinct and defined ratio.
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From Native Tissues—Hemichannels were also obtained by immunopurification from OG-solubilized plasma membranes of rat, mouse, or guinea pig livers using a Cx32-specific monoclonal antibody (M12.13 conjugated to Sepharose beads (34)) (Fig. 2, a). Lanes 1 and 2, respectively, are visualized by Western blot (indicated by *) with anti-Cx32 antibody and by gold stain of connexin purified from Cx26 Tag cell line. Lanes 3–6 are gold stains of protein purified from different Cx26Tag/Cx32 cell lines showing co-purification of Cx32 and Cx26Tag in different ratios (fraction of Cx26 shown beneath each lane). The aggregate isoform ratio (fractional Cx26 content) was determined by densitometry of colloidal gold-stained blots. Different cell lines yielded different stoichiometries. Lanes 1 and 2, respectively, are gold stain and Western blot visualized with anti-Cx32 antibody of protein purified from a Cx32Tag cell line. b, gold stained and Western blot of connexin protein purified from rodent livers using M12.13, an anti-Cx32 monoclonal antibody. Protein immunopurified from rat (lanes 9–13) and mouse (lanes 14–16) liver yielded different Cx26/Cx32 stoichiometries in different immunobead elution fractions (see “Experimental Procedures”). Lanes 11 and 16 contained no detectable Cx26, respectively, i.e. channels were homomeric Cx32. Lanes 12 and 13 are Western blots with mouse anti-Cx26 and mouse anti-Cx32, respectively, against immunopurified heteromeric rCx26/Cx32 channels. Protein immunopurified from guinea pig liver (d, lane 17) contained significantly more gpCx26 than gpCx32.

**TABLE 1**

| Molecular characteristics of inositol and inositol phosphates used in this study |
| Da, z | Volume | Area (% polar) | Length x width x height |
|-------|--------|----------------|------------------------|
| Myoinositol | 180, 0 | 150 | 181 (62) | 5.5 × 5.4 × 3.1 |
| (1)-IP | 258, –2 | 187 | 216 (64) | 7.3 × 6.0 × 5.7 |
| (1,4)-IP | 336, –4 | 229 | 272 (70) | 10.6 × 5.4 × 4.0 |
| (1,4,5)-IP | 414, –6 | 272 | 329 (71) | 9.8 × 7.8 × 5.2 |
| (1,4,6)-IP | 414, –6 | 271 | 329 (71) | 10.6 × 6.7 × 6.2 |
| (1,3,4)-IP | 414, –6 | 272 | 329 (72) | 9.8 × 8.1 × 5.5 |
| (1,3,4,5)-IP | 492, –8 | 287 | 392 (73) | 9.6 × 9.9 × 7.9 |

**Inositol Phosphates as Tracer Compounds for Permeability Analysis**

The selectivity of inositol phosphates by homomorphic and heteromeric connexin channels was explored. IPs are involved in many aspects of cell regulation, and their metabolic products may also play essential roles in cellular function (37). In addition, myoinositol and IPs with one to four phosphate groups include an excellent set of probes with which to examine the roles of size, charge, and charge distribution on the molecular selectivity of connexin channels (Table 1 and Fig. 3).

Several factors were taken into consideration in selecting the IPs used in this study. We reasoned that meaningful selectivity among IPs would most likely be revealed using IPs that were known or suspected to have this study. We reasoned that meaningful selectivity among IPs would most likely be revealed using IPs that were known or suspected to have
Furthermore, (1,3,4)-IP$_3$ is generated by the degradation of (1,3,4,5)-IP$_4$ (39) and modulates the function of (3,4,5,6)-IP$_4$, which inhibits calcium-activated chloride efflux (39, 41). For these reasons, (1,4,5)-IP$_3$, (1,4)-IP$_2$, (1,3,4,5)-IP$_4$, and (1,3,4)-IP$_3$ were chosen for this study. In addition, to specifically address structure-function issues, 1-IP was included, along with (1,4,6)-IP$_3$, because of its structural relation to (1,4,5)-IP$_3$ and (1,3,4)-IP$_3$. The structures of these compounds are shown in Fig. 3.

Each IP was tested for permeability through homomeric Cx26, homomeric Cx32, and heteromeric Cx26/Cx32 channels with different fractional Cx26 content. The method used to assess molecular permeability was TSF of liposomes into which connexin hemichannels were reconstituted (see "Experimental Procedures" and Fig. 1). Tracers were loaded into proteoliposomes as they formed. Permeability was assessed after TSF by direct comparison of tracer selectively lost/retained in liposome populations that do and do not contain functional channels (i.e. the lower and upper TSF bands, respectively; see "Experimental Procedures"). Tracer retention in the upper liposomes is an internal control for loading and nonspecific leakage.

Permeability through Homomeric Hemichannels Purified from HeLa Cells

Homomeric Cx26 and homomeric Cx32 channels were permeable to myoinositol and all tested IPs. The percentage of liposomes containing HeLa rCx32 channels permeable to myoinositol were 95 ± 0.07, 93 ± 1.5 to (1,4,5)-IP$_3$, 96 ± 1.7 to (1,4,6)-IP$_3$, 88 ± 1.9 to (1,3,4)-IP$_3$, and 91 ± 1.6 to (1,3,4,5)-IP$_4$ (Fig. 4a, open bars). The percentage of liposomes containing functional homomeric HeLa rCx32 channels permeable to myoinositol were 96 ± 4.5, 96 ± 4.8 to (1,4,5)-IP$_3$, 93 ± 4.1 to (1,4,6)-IP$_3$, 96 ± 5.2 to (1,3,4)-IP$_3$, and 98 ± 2.3 to (1,3,4,5)-IP$_4$ (Fig. 4a, solid bars). There is no statistically significant difference among the permeabilities to myoinositol and IPs (p < 0.05; n = 5). Homomeric HeLa rCx32 channels were also permeable to myoinositol and all IPs tested. There is no statistically significant difference with regard to permeability of any of the tested permeants (p < 0.05; n = 5). This result was somewhat unexpected, because there is substantial evidence that homomeric Cx26 channels are narrower than homomeric Cx32 channels (9, 10, 20, 57, 58).

Permeability through Heteromeric Channels Purified from Native Tissue

Heteromeric Cx26/Cx32 hemichannels purified from rodent livers, irrespective of aggregate isoform ratio, were permeable to myoinositol. The percentage of liposomes permeable were 100 ± 5.9 for rCx26/Cx32 (Fig. 4b, open bars; aggregate ratio 0.25 rCx26), 98 ± 4.3 for mCx26/Cx32 (Fig. 4b, solid bars; 0.33 mCx26), and 100 ± 2.9 gpCx26/Cx32 (Fig. 4b, shaded bars; 0.80 gpCx26).

Heteromeric Cx26/Cx32 channels purified from rodent livers, irrespective of species, were largely permeable to (1,4,5)-IP$_3$, but substantial fractions of these channel populations were impermeable to other IPs, including other isomers of inositol triphosphate. The percentages of liposomes with rCx26/Cx32 channels permeable to (1,4,5)-IP$_3$ were 98 ± 4.0, with 36 ± 3.5 permeable to (1,4,6)-IP$_3$, 45 ± 1.9 permeable to (1,3,4,5)-IP$_4$, and 36 ± 3.5 permeable to (1,3,4)-IP$_3$. If pdb files were unavailable. Energy-minimized conformations were determined using the MMFF94 force field rule, and molecular orbital structures (0.002 Å³/atomic unit; mesh framework) shown in the figure tracer compounds are as follows: a, myoinositol; b, (1)-IP; c, (1,4)-IP$_2$; d, (1,4,5)-IP$_3$; e, (1,4,6)-IP$_3$; f, (1,3,4,5)-IP$_4$; and g, (1,3,4)-IP$_3$. Elemental carbon is black; oxygen is red, and phosphorus is yellow. Hydrogen atoms are not shown. C-1 and C-4 notations on these structures refer to the carbon atom positions of the inositol ring. Models are viewed from above the inositol ring (left), and en face (right, with C-5 and C-6 carbons toward viewer).
(1,3,4)-IP$_3$, and 35 ± 1.8 permeable to (1,3,4,5)-IP$_4$. mCx26/Cx32 channels also showed similar IP selectivity. Percentages of liposomes permeable to (1,4,5)-IP$_3$ were 80 ± 4.3, with 18 ± 4.5 permeable to (1,4,6)-IP$_3$, 34 ± 5.1 permeable to (1,3,4)-IP$_3$, and 37 ± 2.5 permeable to (1,3,4,5)-IP$_4$. Permeability of heteromeric gpCx26/Cx32 channels was 75 ± 3.5 for (1,4,5)-IP$_3$ and 25 ± 5.6 for (1,4,6)-IP$_3$. These differences in permeability compared with each other and with myoinositol were significant ($p < 0.05; n = 5$). The ability of the channels to differentiate among highly similar molecules is striking. These data show that heteromeric channels are selectively permeable among inositol phosphates, whereas the corresponding homomeric channels are not. This suggests a unique role for heteromeric channels in biological signaling utilizing IPs.

**Permeability through Heteromeric Hemichannels Purified from HeLa Cells**

To determine whether the selectivity was specific to native channels, the same assessment was made using heteromeric channel populations purified from the HeLa cell lines. HeLa rCx26/Cx32 channels with fractional content 0.33 rCx26, the same aggregate ratio as mCx26/Cx32 channels (in Fig. 4b, solid bars), were also fully permeable to myoinositol and showed similar selective permeability among IPs. The percentages of liposomes permeable to myoinositol were 97 ± 3.2 and 66 ± 3.5 to (1,4,5)-IP$_3$, 36 ± 3.1 to (1,4,6)-IP$_3$, 27 ± 4.8 to (1,3,4)-IP$_3$, and 49 ± 3.8 to (1,3,4,5)-IP$_4$ (Fig. 4c, solid bars). The difference between the permeability of myoinositol and tested IPs was statistically significant ($p < 0.05; n = 5$). (1,4,5)-IP$_3$ was permeable through more of the channels than (1,3,4)-IP$_3$, which in turn was permeable through more of the channels than (1,4,6)-IP$_3$.

Heteromeric HeLa rCx26/Cx32 channels with fractional content of 0.80 Cx26, the same stoichiometric ratio as gpCx26/Cx32 (in Fig. 4b, shaded bars), were also permeable to myoinositol and selectively permeable among the other tested IPs. The percentage of liposomes permeable to myoinositol were 100 ± 3.6 and 81 ± 4.8 to (1,4,5)-IP$_3$, 35 ± 4.6 to (1,4,6)-IP$_3$, 43 ± 3.4 to (1,3,4)-IP$_3$, and 61 ± 2.8 to (1,3,4,5)-IP$_4$ (Fig. 4c, shaded bars). This is statistically significant ($p < 0.05; n = 5$), as are permeability differences among the three isomers of inositol trisphosphates ($p < 0.05$).

The results were the same whether the carboxyl-terminal purification tag was present on the Cx26 or the Cx32 or whether the tag was cleaved ($p < 0.05$; data not shown).

These data show that there is little difference in the selective permeabilities as a function of the source of the channels (native versus heterologous) or, surprisingly, as a function of the differences in the
aggregate Cx26/Cx32 ratio tested. The data are plotted as functions of Cx26/Cx32 ratio in Fig. 5, a (native) and b (HeLa). The differences in permeability over the range of heteromeric ratios tested were not significant.

The permeability of inositol, 1-IP, and (1,4)-IP2 through purified connexin hemichannels was also assessed using the same techniques. We found that inositol (Fig. 5, a and b, black line), 1-IP, and (1,4)-IP2 (data not shown) were permeable through all tested hemichannels with no statistically significant difference between them at any fractional Cx26 content (p < 0.05; n = 5).

Permeation of (1,4,5)-IP3 and (1,4,6)-IP3 through Channels of Different Stoichiometry from the Same Native Source

The data presented thus far from rodent channels compares channel populations with different aggregate Cx26/Cx32 ratios from different species. To determine whether species differences are a factor in the differences in IP selectivity, rat and mouse channel populations with a range of aggregate Cx26/Cx32 ratios were obtained by the selective elution technique described under “Experimental Procedures.” The permeabilities to (1,4,5)-IP3 and (1,4,6)-IP3 were assessed and compared with those from HeLa cells.

Homomeric Cx32 and Cx26/Cx32 hemichannels with fractional content of 0.25 rCx26, 0.50 rCx26, and 0.66 rCx26 (data not shown) were permeable through all tested hemichannels with no statistically significant difference between them at any fractional Cx26 content (p < 0.05; n = 5).

Permeation of (1,4,5)-IP3 and (1,4,6)-IP3 through Channels of Different Stoichiometry from the Same Native Source

The data presented thus far from rodent channels compares channel populations with different aggregate Cx26/Cx32 ratios from different species. To determine whether species differences are a factor in the differences in IP selectivity, rat and mouse channel populations with a range of aggregate Cx26/Cx32 ratios were obtained by the selective elution technique described under “Experimental Procedures.” The permeabilities to (1,4,5)-IP3 and (1,4,6)-IP3 were assessed and compared with those from HeLa cells.

Homomeric Cx32 and Cx26/Cx32 hemichannels with fractional content of 0.25 rCx26, 0.50 rCx26, and 0.66 rCx26 were purified from rat liver (Fig. 2b, lanes 9–11). The fractions of liposomes permeable to (1,4,5)-IP3, were 97 ± 1.1 (homomeric), 98 ± 4.0, 60 ± 1.5, and 76 ± 1.4, respectively, for the different fractional contents of rCx26 (Fig. 6a, blue line). For the same channel populations, the fractions of liposomes permeable to (1,4,6)-IP3 were 100 ± 3.6 (homomeric), 36 ± 3.5, 31 ± 2.5, and 36 ± 3.0, respectively (Fig. 6b, blue line).

Homomeric Cx32 and Cx26/Cx32 hemichannels with fractional con-
tent of 0.25 mCx26, 0.33 mCx26, 0.50 mCx26, and 0.75 mCx26 were purified from mouse liver (Fig. 2c, lanes 14–16). The fractions of liposomes permeable to (1,4,5)-IP₃ were 91 ± 1.8 (homomeric), 77 ± 2.1, 80 ± 4.3, 64 ± 1.0, and 82 ± 1.3, respectively (Fig. 6a, red line). For the same channel populations, the fractions of liposomes permeable to (1,4,6)-IP₃ were 94 ± 1.6 (homomeric), 26 ± 2.4, 18 ± 4.5, 23 ± 1.0, and 30 ± 1.6, respectively (Fig. 6b, red line).

From different HeLa lines, homomeric Cx26, homomeric Cx32, and heteromeric Cx26/Cx32 hemichannels with fractional Cx26 content of 0.25, 0.33, or 0.80 (Fig. 2a, lanes 2–7) were purified, matching the aggregations Cx26/Cx32 ratios of channels purified from rodent livers. The fractions of liposomes permeable to (1,4,5)-IP₃ were 96 ± 4.8 (homomeric Cx26), 93 ± 1.5 (homomeric Cx32), 82 ± 1.9, 66 ± 3.5, and 81 ± 4.8, respectively, for these different fractional contents of Cx26 (Fig. 6a, black line). The same channels permeable to (1,4,6)-IP₃ were 93 ± 4.1 (homomeric Cx32), 96 ± 1.7 (homomeric Cx32), 25 ± 1.6, 36 ± 3.1, and 35 ± 4.6, respectively (Fig. 6b, black line). These data show that species differences do not account for the differences in selectivity.

**Heteromeric Channel Stoichiometry Does Not Correlate with Molecular Permeation of (1,4,5)-IP₃ or (1,4,6)-IP₃**

The absence or weak dependence of the selective permeability on Cx26/Cx32 ratio over the heteromeric range tested suggests that selectivity to IPs is not a simple function of this ratio. To test this explicitly, the relationship between specific molecular permeation and predicted channel isoform stoichiometry was examined. By assuming independent probability of occupancy of each radial channel position, stoichiometry distributions over a range of aggregate isoform ratios were calculated using Bernoulli trials (Fig. 7a; Equation 2). Curves were then generated reflecting the fraction of channels with specific stoichiometries less than a given value, as a function of aggregate ratio (Fig. 7b) (20, 53). If the content of a specific connexin isoform determined the permeability of a compound, e.g. if n or fewer of a particular connexin in a channel is required for a specific permeability or impermeability, the permeability distribution as a function of fractional Cx26 content should follow one of these curves.

The experimental data of Figs. 5 and 6 were imposed onto the summed statistical distributions (Fig. 7c, using channels from rodent livers; Fig. 7d, using channels from HeLa cells; Fig. 7e, using (1,4,5)-IP₃ data; and Fig. 7f, using (1,4,6)-IP₃ data). Under the given assumptions, the permeability to IPs could not be accounted for by simple stoichiometry distributions. However, (1,4,5)-IP₃ was permeable through fewer of the heteromeric hemichannels as the aggregate ratio of Cx26/Cx32 approached 1:1 and was permeable through more of the channels when either isoform predominated. (1,4,6)-IP₃ was permeable through fewer of the heteromeric hemichannels than (1,4,5)-IP₃ at all aggregate isoform ratios.

This analysis does not consider differences in the arrangements of connexin monomers around the pore at each isoform stoichiometry. The inability of this analysis to account for the permeabilities suggests that they may be functions of specific isoform radial arrangements in the channels (59). No matter what the structural mechanism, for such finely tuned molecular discrimination among IPs to occur only through heteromeric channels suggests that recognition sites for these molecules must exist, i.e. are formed by Cx26 and Cx32.

**Molecular Modeling of Myoinositol and Inositol Phosphates**

The ability of the heteromeric channels to discriminate (1,4,5)-IP₃ from (1,4,6)-IP₃ and (1,3,4)-IP₃ suggests that discrimination is not on the basis of molecular size, charge, or polarity but rather on the basis of specific thermodynamic interaction with a discrete site(s) within different heteromeric hemichannel pores.

To help support this inference, equilibrium geometries and energy-minimized conformations for myoinositol and all IPs used were calculated using MMFF94 force field molecular mechanics (54). Electron density models, i.e. size surfaces of the energy-minimized structures (Fig. 3), were generated for these structures using the ab initio Hartree-Fock self-consistent field method (55). For isomers of IP₃ (Fig. 3, d–f), molecular modeling shows that volume, surface area, and area of polar regions nonpolar surfaces are identical for (1,4,5)-IP₃, (1,3,4)-IP₃, and (1,4,6)-IP₃ (Table 1). However, the molecular shape is clearly different when viewing the structure from above the inositol ring and along the C-1 to C-4 axis (i.e. the location of phosphate groups common to all isomers).

Knowledge of the electron density surface allowed graphical representation of predicted three-dimensional chemical characteristics. The electrostatic and local ionization potentials were mapped onto the electron density surface. The electrostatic potential (Fig. 8, a and c) describes regions of the molecule acting as Lewis acids or bases, i.e. areas of negative (red) or positive charge (blue). For the three isomers of IP₃, negative charge is present at the three phosphate groups, and the relative differences in charge of the common C-1 and C-4 phosphate groups on each of the isomers are minor. Local ionization potential (Fig. 8, b and d) reflects regions from which electrons are most easily lost, i.e. sites that are most susceptible to electrophilic attack, i.e. are likely to become ionized (red), and conversely sites that are more susceptible to nucleophilic attack (blue).

By using this latter graphical representation, we noticed striking differences between and at the C-1 and C-4 phosphates of all isomers; the C-4 phosphate group of (1,4,5)-IP₃ is predicted to be less readily ionized than the C-4 phosphate group of (1,4,6)-IP₃ or (1,3,4)-IP₃. In the TSF tracer study, (1,4,5)-IP₃ was the most permeable through heteromeric channels. Thus, the distinctly different ionizability of the C-4 phosphate group of (1,4,5)-IP₃ may be involved in the discrimination among the IP₃ isomers.

**DISCUSSION**

The essential findings of this study are as follows: (a) that connexin channels can discriminate among highly homologous inositol phosphates, most strikingly among inositol triphosphates, and (b) that only heteromeric channels were capable of this discrimination, with the corresponding homomeric channels being nonspecific. These findings have implications for biological signaling and for the mechanisms of connexin channel molecular selectivity.

It was believed for many years that connexin channels were so wide that they would be nonspecific among ions and molecules small enough to enter the pore. A large body of data now shows that homomeric channels formed by each connexin isoform are selectively permeable among different molecules of similar size, or by the same molecules at different rates (5–13). Of particular biological importance is the differential permeability of cytoplasmic molecules, including second messengers (6, 20, 21, 25, 60–62).

Our previous work, using native channels composed of Cx26 and/or Cx32, indicated that heteromeric channels could discriminate between cAMP and cGMP (20, 21). The data suggested that channel composition determined permeability to cyclic nucleotides and discrimination among them. Also in the prior work, we observed a trend between impermeability of (1,4,5)-IP₃ and the presence of Cx26 in heteromorphic Cx26/Cx32 channels (20).

Because this earlier work suggested that the stoichiometry of Cx26 and Cx32 within heteromeric channels was a determining factor in the selectivity, it was a goal of the present study to explicitly explore the role...
of isoform stoichiometry. Ideally, such experiments would be carried out with populations of heteromeric channels of single, defined stoichiometries. Unfortunately, such a system is not available.

In this study, populations of native hemichannels with a range of defined aggregate ratios of Cx26/Cx32 were obtained by immunoaffinity purification from livers of different rodent species and from HeLa cells transfected to co-express two connexins. Purifications from rat, mouse, and guinea pig livers contain different proportions of Cx26 as follows: from rat typically ~25% rCx26, mouse livers ~33% mCx26, and guinea pig livers ~80% gpCx26. Channel populations with different aggregate Cx26/Cx32 ratios were also obtained by the judicious use of specific elution fractions from the immunoaffinity matrix, as described under “Experimental Procedures.” Homomeric Cx26, homomeric Cx32 channels, and heteromeric Cx26/Cx32 were also obtained from a HeLa expression system using a carboxyl-terminal purification tag. Because the purification tag was on only one of the co-expressed connexins in...
Hartree-Fock 6-31G* ab initio models, subject to MMFF94 equilibrium geometry constraints, were used for predicting molecular orbital structure (0.002 Å³/atomic units; see Fig. 3); electrostatic potential and local ionization potential were mapped onto the orbital surfaces by Spartan 04. The electrostatic potential (a and c) describes regions of the molecule that carry negative (red) or positive charge (blue). Local ionization potential (b and d) reflects regions sites are most susceptible to electrophilic attack, i.e. are likely to become ionized (red) and, conversely, that are more susceptible to nucleophilic attack (blue). Tracers shown, from top to bottom, are (1,4,5)-IP$_3$, (1,4,6)-IP$_3$, and (1,3,4)-IP$_3$. a and b, IPs are represented with the same orientations and scale as in Fig. 3, as observed from above the inositol ring and en face. c and d, structures are viewed lengthways along C-1 to C-4 of the inositol ring. c, C-4 is in the foreground; d, C-1 is in the foreground. The molecular orbital meshwork structures (0.002Å³/atomic unit) for these viewpoints are also shown.
Connexin Channel Selectivity among Inositol Phosphates

heteromeric channels, we were able to purify channels by their Cx26 or Cx32 content, biasing the purification for one or the other connexin. In addition, cell lines were used that expressed the two connexins in different ratios. By use of these methods, we were able to compare the permeability properties of channel populations over the same range of aggregate stoichiometries from each species, from different species, and from a heterologous expression system.

The data show that homomeric Cx32, homomeric Cx26, and heteromeric Cx26/Cx32 channels, irrespective of Cx26/Cx32 aggregate ratio, were permeable to myoinositol, (1)-IP, and (1,4)-IP\textsubscript{2}. Selectivity was observed only when more than two phosphate groups were present on the myoinositol ring and only for the heteromeric channels. In each of the populations of heteromeric channels, the fraction of heteromeric channels permeable to (1,4,6)-IP\textsubscript{3}, (1,3,4)-IP\textsubscript{3}, or (1,3,4,5)-IP\textsubscript{3} was far less than the fraction permeable to (1,4,5)-IP\textsubscript{3}. In the case of (1,4,5)-IP\textsubscript{3}, the channels were most selective when the aggregate Cx26 to Cx32 was close to 1:1. This was not true for (1,4,6)-IP\textsubscript{3}, which had approximately the same permeability through all heteromeric Cx26/Cx32 channels, regardless of stoichiometry. The permeabilities to the IPs were not significantly different for channels purified from the different rodent species or purified from HeLa cells with the same aggregate stoichiometries.

The selectivities we report here could not have been inferred or anticipated from the studies of connexin channel permeability using the many fluorescent tracers that have been used by our group and others (7, 8, 10, 57, 60, 63). The selectivities were revealed using the biological signaling molecules themselves as probes of the connexin pore. It seems that connexin channels have been finely tuned to select among the cytoplasmic molecules to which they are exposed in situ, and that selectivities among nonbiological tracers are secondary consequences.

The ability of heteromeric Cx26/Cx32 channels to differentiate among different inositol triphosphates (1,4,5)-IP\textsubscript{3}, (1,4,6)-IP\textsubscript{3}, and (1,3,4)-IP\textsubscript{3} was surprising. These isomers have the same molecular weight and charge; the difference is the location of the phosphate groups on the myoinositol ring, which affects shape and charge distribution, and the chemical reactivity of the phosphate groups. This finding emphasizes the importance of the spatial configuration of phosphate groups on the biological selectivity of permeation through connexin channels.

The high degree of selectivity among very similar permeants suggests there are specific sites where permeants interact with the pores. The data are most consistent with a mechanism in which, at least for Cx26/Cx32 channels and this class of second messengers, a molecule must interact appropriately with a highly selective but likely low affinity binding site in the pore in order to permeate, and that molecules that do not cannot permeate. This is analogous to the mechanism for selectivity for K\textsuperscript+ over Na\textsuperscript+ in K\textsuperscript+ channels, only for signaling molecules instead of monovalent atomic cations.

The requirement for heteromericity suggests that such a site involves structural contributions from more than one monomer and from both Cx26 and Cx32 (59). Examination of known inositol phosphate-binding sites did not reveal amino acid sequence similarity with Cx26 or Cx32 and specifically no correspondence with conserved residues in the (1,4,5)-IP\textsubscript{3} receptor-binding core or the IP\textsubscript{3}-coordinating residues (64). This is not surprising given the requirement for heteromericity and presumed low affinity. In this context it is intriguing to note that an engineered α-hemolysin pore, which does not contain a canonical IP\textsubscript{3} binding site, was able to bind IP\textsubscript{3} at high affinity, other inositol phosphates with lower affinity, and cAMP not at all (65).

The most unexpected finding was that the homomeric Cx26 channels were also permeable to all the IPs. This is inconsistent with the trend toward more restrictive permeability with increased Cx26 content that we had noted in previous work (20, 21). One possibility is that Cx26/Cx32 channels, because of their heteromeric nature, have an irregular or narrowed lumen relative to both types of homomeric channels, perhaps because of lack of axial registration of the pore-lining segments of the two isoforms, and these features enable the observed selectivity.

The results make the important point that the earlier work with cyclic nucleotides was not a singular result, and native channels consisting of these two isoforms can be selectively permeable to two classes of second messengers and can distinguish among the members of each class. From the present data we cannot draw a detailed relation between aggregate isoform stoichiometry and the selectivities, but the data strongly indicate that dynamic control of isoform composition of connexin channels in cells (66–68) can dramatically modulate intercellular signaling mediated by the two major classes of second messengers. Specifically, for Cx26 and Cx32, which are often expressed in the same cells and in the same channels (20, 21, 69, 70), the data suggest that cells could decrease selectivity among IPs by down-regulating either connexin and can enhance selectivity by making both proteins in equal amounts.

CONCLUSION

Our findings suggest a unique functional role of heteromeric channels, with implications for connexin channel cell biology. The emerging theme is that heteromeric connexin channels can have a remarkable range of selectivities among cytoplasmic molecules that is not predicted from conventional dye permeability studies. The size and charge of a permeant alone do not alone determine permselectivity because the heteromeric pore can have a significant selectivity among biological permeants with the same size and overall charge. Our results provide a ready explanation as to why more than one connexin is often expressed in the same cells and why they form heteromeric channels. Heteromeric connexin hemichannels can be restrictive and selective, at least regarding IP\textsubscript{3} and cyclic nucleotide permeability, whereas the corresponding homomeric channels are not. The specific selectivities to IPs depend on connexin channel composition and likely isoform arrangement within channels. The next major challenge lies in determining the structural basis of the striking selectivity of different connexin channels.

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