Permeation of Calcium through Purified Connexin 26 Hemichannels

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Background: Indirect evidence suggests that connexin hemichannels are permeable to Ca²⁺, but direct demonstration is lacking.

Results: Calcium moves into liposomes containing purified Cx26 in response to a concentration gradient.

Conclusion: Cx26 hemichannels are permeable to Ca²⁺.

Significance: Cx26 hemichannels may play a role in Ca²⁺ influx into cells under conditions that lead to hemichannel activation, such as ischemic damage.

Gap junction channels communicate the cytoplasms of two cells and are formed by head to head association of two hemichannels, one from each of the cells. Gap junction channels and hemichannels are permeable to ions and hydrophilic molecules of up to M_r 1,000, including second messengers and metabolites. Intercellular Ca²⁺ signaling can occur by movement of a number of second messengers, including Ca²⁺, through gap junction channels, or by a paracrine pathway that involves activation of purinergic receptors in neighboring cells following ATP release through hemichannels. Understanding Ca²⁺ permeation through Cx26 hemichannels is important to assess the role of gap junction channels and hemichannels in health and disease. In this context, it is possible that increased Ca²⁺ influx through hemichannels under ischemic conditions contributes to cell damage. Previous studies suggest Ca²⁺ permeation through hemichannels, based on indirect arguments. Here, we demonstrate for the first time hemichannel permeability to Ca²⁺ by measuring Ca²⁺ transport through purified Cx26 hemichannels reconstituted in liposomes. We trapped the low affinity Ca²⁺-sensitive fluorescent probe Fluo-5N into the liposomes and followed the increases in intraliposomal [Ca²⁺] in response to an imposed [Ca²⁺] gradient. We show that Ca²⁺ does move through Cx26 hemichannels and that the permeability of the hemichannels to Ca²⁺ is high, similar to that for Na⁺. We suggest that hemichannels can be a significant pathway for Ca²⁺ influx into cells under conditions such as ischemia.

Gap junction channels (GJCs)² are aqueous channels that communicate the cytoplasms of adjacent cells (1–3). They are formed by head to head association of hemichannels (HCs, connexin hexamers, connexons), one from each of the neighboring cells, that are permeable to ions and hydrophilic molecules of up to M_r 1,000 (1–3).

In addition to their known permeability to inorganic monovalent ions, it is well established that the GJC and HC pores are sufficiently large to allow permeation of many larger compounds including cAMP, cGMP, IP₃, ATP, and glucose, as well as Ca²⁺ (4–8). GJCs and HCs display significant selectivity for molecules of very similar properties (e.g., cAMP versus cGMP, IP₃ isomers) because of major interactions of the permeants with the pore, which prevents simple extrapolation from apparent pore size to permeabilities of specific molecules (1, 9–11).

Connexin-mediated intercellular Ca²⁺ signaling can occur by movement of second messengers such as IP₃ and/or Ca²⁺ through GJCs (1, 12–20) or by a process that involves release of ATP through HCs (paracrine pathway) (1, 4, 8, 17, 21). In the latter, ATP release through HCs increases Ca²⁺ influx and intracellular [Ca²⁺] in response to activation of purinergic receptors in neighboring cells. There are studies that support roles of IP₃ and/or Ca²⁺ permeation through GJCs (12–20), as well as a major role of HCs in the paracrine pathway (4, 8, 17, 21). However, unambiguous identification of the pathways and mediators associated with an effect in cells is possible but often difficult.

Physiological levels of extracellular [Ca²⁺] decrease HC activity (22–24). However, HCs can be activated in the presence of millimolar extracellular [Ca²⁺] under a number of conditions, including ischemia, inflammation, connexin dephosphorylation, and extracellular alkalinization (25–28). Understanding Ca²⁺ permeation through Cx26 HCs is also important because increased Ca²⁺ permeability may constitute the bases

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‡ The abbreviations used are: GJC, gap junctional channel; AF350, Alexa Fluor 350; AF647, Alexa Fluor 647; DDM, n-dodecyl-β-D-maltoside; Cx26; con

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for the association of the G45E mutant with the keratitis ichthyosis deafness syndrome (29).

Previous studies suggested Ca\(^{2+}\) permeation through GJCs or HCs (1, 13, 14, 18, 20, 30), but those studies are indirect, and therefore Ca\(^{2+}\) permeation through open HCs was considered likely but remained unproven. Here, we show that Ca\(^{2+}\) actually permeates purified and reconstituted Cx26 HCs and that HC Ca\(^{2+}\) permeability is high, similar to that for Na\(^{+}\).

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification**—We expressed and purified wild-type human Cx26 followed by a poly-\(\text{His}\) tag (six His) at the C-terminal end (Cx26). Human Cx26 was amplified by PCR using pOcyt-Cx26 as template (23), with the addition of the poly-\(\text{His}\)-tag and EcoRI and XbaI flanking sequences for cloning into the baculovirus transfer vector pFastBac (Invitrogen). Recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen) following the manufacturer’s instructions. The viruses were produced in Sf9 insect cells grown at 26 °C in Grace’s medium supplemented with 10% fetal calf serum and 0.05 mg/ml gentamycin.

Proteins were expressed in Sf9 cells in suspension, grown in 2-liter baffled flasks containing 700 ml of HyClone CCM3 medium supplemented with 10 μg/ml of gentamycin. The cells grown at 26 °C, shaken at 200 rpm, were infected at a concentration of ~2 × 10\(^5\) cells/ml, using a multiplicity of infection of 2. When cell viability, determined by Trypan blue staining, was ~40%, ~48 h post-infection, the cells were harvested by centrifugation at 1,000 × g for 10 min. This and all subsequent steps were performed at 4 °C unless indicated otherwise. Purification started by resuspending fresh pellets or pellets stored at −80 °C in a 1 mM bicarbonate solution containing 1 mM PMSF, and the cells were lysed with a Dounce homogenizer. The membranes were alkali-extracted by addition of NaOH to 60 mM, followed by incubation on ice for 30 min. Alkali-extracted samples were centrifuged at 26,000 × g for 30 min, and the membrane pellet was solubilized with 2.5% \(\text{n-\text{dodecyl-\(\beta\)-d-maltoside (DDM)}}\), in 2 M NaCl, 2 mM EGTA, 2 mM DTT, 1% glycerol, 1 mM PMSF, and 10 mM glycine/NaOH, pH 8, at a protein concentration of <2 mg/ml. The suspension was sonicated with a probe sonicator and then incubated for 2 h with gentle rotation. After ultracentrifugation at 100,000 × g for 30 min, the solubilized material was diluted with 2.5 volumes of 5 mM imidazole, 10% glycerol, 1 mM PMSF, 0.03% DDM, and 50 mM HEPES/NaOH, pH 8.0. The diluted samples were then loaded onto a Talon Co\(^{2+}\) column (Talon Superflow, Clontech) pre-equilibrated with the dilution buffer, at a rate of 0.5 ml/min. After washing with 10 column volumes of 10 mM KCl, 10% glycerol, 20 mM imidazole, 0.03% DDM, and 10 mM HEPES/KOH, pH 7.4, elution proceeded with the same buffer but increasing imidazole to 250 mM.

**Analysis of the Oligomeric State of Purified Cx26**—The oligomerization of solubilized Cx26 was determined by gel filtration on a Superdex 200HR 10/300 GL column (GE Healthcare) using an ÄKTA FPLC system (GE Healthcare). For these experiments, Cx26 (500 μl at ~0.5 mg/ml) in 0.03% DDM, 150 mM NaCl, 10% glycerol, and 10 mM HEPES/NaOH, pH 7.4, was injected into the column equilibrated with the same buffer and run at a flow rate of 0.5 ml/min. Apparent molecular mass was calculated from the linear relationship between partition coefficient \(K_p\) and the log of the molecular mass, using the following standards, run under the same conditions: 440 kDa (ferritin), 158 kDa (aldolase), 43 kDa (ovalbumin), and 13.7 kDa (ribonuclease A); blue dextran 2000 (~2,000 kDa) was used to determine the void volume. Highly purified Cx26 (peak collected from the gel filtration run) was also analyzed by dynamic light scattering measured at 90 degrees using a Brookhaven Instruments BI-200SM with an avalanche photodetector (Brookhaven, CT).

**Reconstitution of Cx26 HCs**—Reconstitution was performed in a mixture of phosphatidylcholine (PC) and phosphatidylserine (PS) at a 2:1 ratio (w/w), as described for Cx43 HCs (31), with some modifications. Lipids from chloroform stocks, mixed at the indicated ratio, were lyophilized overnight under argon. The dry lipid film was rehydrated in 75 μl of buffer/mg of lipid, and the suspension was warmed to 37 °C until it became transparent. For all experiments, except the sucrose transport assay (see below), the buffer contained 100 mM KCl, 0.1 mM EGTA, 2.3% \(\text{n-octyl-\(\beta\)-d-glucopyranoside}\), and 25 mM HEPES, pH 7.6. Solubilized Cx26 was added to the lipid-detergent mixture and dialyzed through a Spectra/Pro 6,000–8,000 molecular mass cutoff membrane (Spectrum Laboratories, Rancho Dominguez, CA) for 36 h at room temperature against 2 liters of detergent-free buffer containing 10 ml of a 50% (w/v) suspension of Biobeads SM-2 (Bio-Rad). Unilamellar liposomes and proteoliposomes were produced by extrusion (Mini-Extruder; Avanti Polar Lipids, Alabaster, AL). The proportion of HCs with the extracellular domains facing out was estimated from thrombin digestion experiments. For these studies, we engineered a human Cx26 with the addition of a thrombin cleavage site between the end of the Cx26 sequence and the poly-\(\text{His}\) tag (Cx26T). The thrombin site was introduced by exchange of the 742-bp BamHI/XbaI fragment of Cx26 with an identical PCR fragment, except for the addition of the sequence for the thrombin cleavage site LVPRGS. Cx26T reconstituted in liposomes (typically 20 μg of protein) was incubated with 0.1 units of restriction grade thrombin (EMD Biosciences) at room temperature, for 16 h, and the reactions were stopped by adding SDS gel sample buffer. The percentage of HCs with the extracellular domains facing out was estimated from densitometry analysis of the signal from immunoblots probed with an anti-\(\text{His}\)-tag antibody (anti-Penta-\(\text{His}\); Qiagen).

**Secondary Structure Determination by Infrared Spectroscopy**—The secondary structure of purified Cx26 was determined at 20 °C by Fourier transform infrared spectroscopy using a Tensor 37 infrared spectrometer equipped with a liquid N\(_2\)-cooled photovoltaic MCT detector with a BaF\(_2\) window (Bruker, Billerica, MA). Purified Cx26 in DDM was studied on a 7-\(\mu\)m path length transmission cell (AquaSpec; Bruker) at a concentration of 1–5 mg/ml in the gel filtration buffer described above, whereas reconstituted Cx26 (200 μg at 1:50 protein/lipid, w/w) was analyzed on an attenuated total reflectance cell (BioATR II; Bruker). The spectra collected between wave numbers of 3,100 and 1,000 cm\(^{-1}\), with a resolution of 4 cm\(^{-1}\), were used to calculate secondary structures from the difference spectra (sol-
ubilized Cx26 minus buffer or proteoliposomes minus liposomes), using the Bruker protein library.

Sucrose Transport—Sucrose permeability of liposomes containing purified Cx26 was determined by the transport-specific fractionation technique (31–33). For these experiments, Cx26 was reconstituted in liposomes containing traces of phosphatidylethanolamine labeled with lissamine rhodamine B (PE-R; PC:PS:PE-R ratio of 2:1:0.05, w/w/w), using a buffer that contained 10 mM KCl, 0.1 mM EGTA, 459 mM urea, and 10 mM HEPES, pH 7.6. Basically, liposomes containing PE-R were layered onto a linear iso-osmolar sucrose gradient (0–400 mM sucrose with a reverse urea gradient), and the gradient was centrifuged on a swinging bucket rotor (Beckman SW32Ti) at 125,000 × g for 5 h at 4 °C. The location of the liposomes was determined from direct visualization and from the fluorescence of fractions collected from the top to the bottom of the gradient. Rhodamine B fluorescence was measured at 590 nm, with excitation at 560 nm (spectrofluorometer F-7000; Hitachi).

Transport of Fluorescent Dyes—Liposomes and proteoliposomes were loaded with either Alexa Fluor 350 (AF350, 250 μM) or AF 647 (AF647, 125 μM) in 100 mM KCl, 0.1 mM EGTA, 25 mM HEPES/KOH, pH 7.6. Loading was accomplished by four freeze-thaw cycles, each by placing the samples 1 min in liquid N2 followed by 5 min in a bath at 27 °C. Loading by extrusion was less efficient, but the functional data were similar, indicating that the HCs were not affected by the freeze-thaw procedure. After removal of most extraliposomal dyes by gel filtration on PD10 columns (GE Healthcare), the samples were run at 21–22 °C on a 3-ml Superdex HR200 5/150 GL gel filtration column (GE Healthcare), on a LabAlliance APLC system equipped with absorbance and multiwavelength fluorescence detectors (LabAlliance, State College, PA). The position of the liposomes was determined by the absorbance at 280 nm or the fluorescence from PE-R. Retention of dye trapped in liposomes was detected at excitation and emission wavelengths of 345 and 445 nm for AF350 and 650 and 670 nm for AF647.

ATP Transport—Experiments were performed as described in the previous section, but the liposomes and proteoliposomes were loaded with 1 mM ATP. After removal of most extraliposomal ATP by gel filtration on PD10 columns (GE Healthcare), 250 μl of the samples were combined with 5 μl of a mix containing luciferase (Quantilum Photinus pyralis recombinant luciferase; Promega, Madison, WI), luciferin (GoldBio, St. Louis, MO), EDTA, and MgSO4, to yield final reaction concentrations of 18 nM, 610 μM, 0.5 mM, and 5 mM, respectively. Luminescence was measured on a Turner 20/20n luminometer (Sunnyvale, CA), and ATP retained in the liposomes was determined as the difference in luminescence between total ATP (after addition of Triton X-100 to 0.4%) and extraliposomal ATP (accessible to luciferase in the absence of Triton X-100).

Transport of Ca2+, Na+, and H+ Equivalents—For the Ca2+ transport experiments, the low affinity Ca2+-sensitive probe Fluo-5N (pentadecane-7,13-diylbis[5-methoxy-6,2-benzofurandiyl]is(SBFI, tetrammonium salt, 25 μM; Invitrogen) in a buffer devoid of Na+ (replaced with N-methyl-d-glucammonium, NMDG); 70 mM KCl, 0.1 mM EGTA, 30 mM NMDG, and 25 mM HEPES/KOH, pH 7.6. Extraliposomal [Na+] was increased to 15 mM by replacing the NMDG in the solution above with Na+. Excitation was at 340 nm, and emission was measured through a 400-nm-long pass filter. The assay for H+ transport was based on the pH sensitivity of fluorescein emission; we used liposomes containing traces of the fluorescein-labeled phospholipid N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine (DHPE-Fl, head group-labeled; Invitrogen). Purified Cx26 was reconstituted in a mix of lipids containing PC, PS, and DHPE-Fl at a PC:PS:DHPE-Fl ratio of 2:1:0.005 (w/w/w). For the experiments, starting with 100 mM KCl, 0.1 mM EGTA, 25 mM HEPES/KOH, pH 7.6, the pH was reduced by HCl addition until the one to one mixture of the final solution had a pH of 6.4. The detection conditions were as described for Fluo-5N. All of the experiments were performed at 20 °C.

Analysis of the Changes in Fluorescence—Multiexponential functions were used to fit the changes in fluorescence of the stop flow experiments. The need for multiexponential fits was not surprising for a number of reasons, depending on the probe, including the effects of Ca2++, and potentially pH, on HC activity, the “mixed” orientation of the HCs, and the nonlinear dependence of Fluo-5N and SBFI emission on [Ca2+] and [Na+], respectively. Half of the HCs are in the extracellular domains-out orientation (see “Results”), and therefore a potential inhibition at 500 μM Ca2+ and any effect of decreasing pH may occur first in the HCs with the extracellular domains facing out and only later in HCs with the extracellular domains facing in; for example, exposure of the extracellular side of the HCs to 500 μM Ca2+ will occur in <0.5 ms for HCs in the extracellular domains-out orientation, but it will take seconds for Ca2+ to reach inhibitory concentrations inside the liposomes. In addition, the apparent Keq of Fluo-5N for Ca2+ under our experimental conditions was 132 ± 10 μM (n = 3), and therefore Fluo-5N emission changes linearly with [Ca2+] only to ∼40 μM [Ca2+], ∼25% of the fluorescence at 500 μM [Ca2+]. A similar nonlinear dependence of SBF emission on [Na+] exists because its apparent Keq for Na+ under the conditions of our experiments is ∼15 mM. In contrast, the decrease in the fluorescence from the fluorescein-labeled phospholipids is linear with lowering pH from 7.6 to 6.4. The initial rates of change in concentrations were calculated for the 0–10-ms period using the faster exponential rate, expressed over a 1-s period. We argue that this simplified calculation of initial transport rates is justified for the purpose of our studies, despite a minor underestimation. The faster rates were 2.55 ± 0.43, 2.42 ± 0.49, and 13.91 ± 0.97 s−1 for Ca2+, Na+, and H+, respectively, and the components with these rates accounted for 85–95% of the fluorescence change in the 0–10-ms period. The ratio of the faster
to the second faster rates were 13 ± 1 (n = 20), 16 ± 2 (n = 6), and 14 ± 1 (n = 6), for Ca$^{2+}$, Na$^+$, and H$^+$, respectively. Fits to three-exponential functions were performed with the Applied Photophysics Pro-Data Viewer analysis software, and the goodness of fit was determined from the random residual distribution, which showed no structure and chi-squared values near unity.

Other Techniques—Protein concentrations were determined with the BCA protein assay reagent (Bio-Rad), using bovine serum albumin as standard. For Western blots, we used anti-Cx26 antibodies directed against the intracellular loop and N-terminal region (Zymed Laboratories Inc., South San Francisco, CA) or His tag (anti-Penta-His; Qiagen). Detection was by imaging (Odyssey infrared imager; Li-Cor Biosciences, Lincoln, NE) of goat anti-rabbit IRDye 800 (Li-Cor Biosciences) for the anti-Cx26 antibodies or goat anti-mouse Alexa Fluor 680 (Invitrogen) for the anti-His antibody.

Statistics—The data shown are the means ± S.E. Statistically significant differences were assessed by Student's t test for unpaired data or one-way analysis of variance, as appropriate.

RESULTS

Biochemical Characterization of the Purified Cx26—Cx26 purified by a combination of immobilized metal affinity chromatography (based on the C-terminal His tag) and size exclusion chromatography shows a high degree of purity, as evaluated in gel filtration chromatograms (Fig. 1A) and Coomassie Blue-stained gels of detergent-solubilized Cx26 (Fig. 1B). The latter and Western blots probed with anti-Cx26 antibodies (see Fig. 1C for an example of reconstituted Cx26) show monomers and some oligomers stable in SDS, which run faster than expected. Only one absorbance peak was observed in size exclusion chromatograms, corresponding to an apparent molecular mass of 235 ± 12 kDa (n = 18). This is compatible with an Cx26 hexamer with ~30% (w/w) associated detergent. The hydrodynamic radius from dynamic light scattering determinations was 5.4 ± 0.2 nm (n = 6). From the difference between this value and the hydrodynamic radius of 4.4 nm, calculated for the Cx26 HC structure (34), we estimated that the protein-detergent complex contains 40–45% (w/w) of detergent. The apparent size of the protein-detergent complex indicates that Cx26 solubilized in DDM is present as hexamers (HCS), as opposed to dodecameric GJCs.

The purified Cx26 was highly structured, as determined from its infrared spectrum (Fig. 1D). The calculated α-helix content averaged 59 ± 1% for DDM-solubilized Cx26 (n = 20), in reasonable agreement with the recent crystal structure of Cx26 (54% α helix) (35). The calculated percentage of α-helix of reconstituted Cx26 was large but smaller than that of detergent-solubilized Cx26 (43 ± 2%, n = 3, p < 0.05). This difference could indicate that the structure of detergent-solubilized Cx26 is different from that of the protein in a lipid bilayer. However, we are not certain about the significance of this result because there are no libraries of protein standards acquired on an ATR cell, and therefore we had to analyze the ATR-FTIR spectra using a library of spectra acquired on the transmission cell.

To assess the proportion of HCs in the extracellular domains-out orientation, we used a Cx26 with a thrombin cleavage site between the end of the Cx26 sequence and the poly-His tag (Cx26T). Densitometry analysis was employed to determine the change in the signal arising from the His tag, from immunoblots probed with an anti-His tag antibody. Fig. 1E shows that essentially all the His tag signal disappeared when Cx26T solubilized in 0.03% DDM (det) was subjected to thrombin digestion (90 ± 3%, n = 4), whereas from the reconstituted Cx26T HC data (lipo), we estimated that 51 ± 2% of the HCs were in the extracellular domains-out orientation (n = 4).

Permeability of Cx26 Hemichannels to Large Solutes—In the previous section, we showed that the hydrodynamic properties of Cx26 purified from insect cells are consistent with the presence of HCs. Here, we performed functional studies to determine whether the reconstituted Cx26 has permeability properties that resemble those of Cx26 HCs. First, we determined whether reconstituted Cx26 HCs were permeable to sucrose using the transport-specific fractionation technique developed by Harris and co-workers (32, 33) to separate sucrose-permeable from sucrose-impermeable liposomes (31). The method is based on the migration of liposomes upon centrifugation in a linear iso-osmolar sucrose gradient, with [sucrose] increasing
from top to bottom, and a reversed urea gradient to maintain the osmolarity constant. The liposomes remain in the upper part of the tube, because the denser lipid is buoyed up by the entrapped urea solution of lower density. The heavier, sucrose-loaded liposomes containing sucrose-permeable HCs migrate as a narrow band to a lower position in the tube. Only liposomes containing Cx26 migrated down the gradient, and the fraction of sucrose-permeable liposomes was proportional to the protein to lipid reconstitution ratio (Fig. 2A). Measurement of rhodamine B fluorescence in the gradient fractions allows for quantitation of the proportion of sucrose-permeable and impermeable liposomes (Fig. 2B). A summary of the sucrose permeability data is shown in Fig. 2C. For a 1/50 (w/w) protein/lipid reconstitution, we estimated the fraction of impermeable liposomes at 23 ± 4% (n = 6), which can be explained by an average of 1.5 functional HCs per liposome, as calculated from the Poisson distribution. All of the transport assays described below were performed using a 1/100 (w/w) protein/lipid reconstitution, where <5% of the liposomes are expected to contain more than one functional HC.

Second, we evaluated the permeability of Cx26 HCs to other molecules. Based on available information in cells (1), we expect purified and reconstituted Cx26 HCs to be permeable to AF350 (349 Da molecular mass) but not to AF647 (1,300 Da molecular mass, respectively). To determine which dyes were permeable through Cx26 HCs, we loaded the probes into the liposomes, and their association with the liposomes was determined after separation by size exclusion chromatography. The typical size exclusion chromatogram in Fig. 2D shows that the liposomes can be easily separated from the extraliposomal free dyes (AF350 or rhodamine B fluorescence) and expressed the data as the means ± S.E. relative to the liposome values (n = 5 for AF350; n = 3 for AF647). The asterisk denotes p < 0.001 compared with the liposome values.

Permeability of Cx26 Hemichannels to Ca2+—As mentioned in the introduction, there is indirect evidence for Ca2+ permeability through HCs (1, 13, 14, 18, 20, 30). To directly demonstrate Ca2+ permeation through Cx26 HCs, we determined Ca2+ influx into liposomes containing purified and reconstituted HCs. For these assays, we loaded the liposomes with the low affinity Ca2+-sensitive fluorescent probe Fluo-5N and then removed most of the extraliposomal probe by gel filtration. As expected from its molecular mass (958 Da), Fluo-5N was removed most of the extraliposomal probe by gel filtration. As expected from its molecular mass (958 Da), Fluo-5N was retained inside the liposomes after size exclusion chromatography in an ATP-free buffer to remove extraliposomal ATP. The results in Fig. 3 show that ATP also permeates through purified Cx26 HCs.
trace) but not into those without HCs (black trace). The addition of Ca\(^{2+}\) produced a fast (mixing-limited) increase in fluorescence that is likely due to binding of Ca\(^{2+}\) to traces of extraliposomal Fluo-5N remaining after the gel filtration. We ascribe the subsequent slower increase in fluorescence, observed only in the proteoliposomes (red trace), to influx of Ca\(^{2+}\) through Cx26 HCs. Near the end of the experiments, the addition of the Ca\(^{2+}\) ionophore ionomycin (1 \(\mu\)M) increased intraliposomal [Ca\(^{2+}\)] to similar values in liposomes and proteoliposomes. The increase in Fluo-5N fluorescence elicited by Ca\(^{2+}\) was reversed by the addition of EGTA (Fig. 4B). This is likely the result of the combination of Ca\(^{2+}\) efflux from the liposomes and EGTA influx into the liposomes. A summary of the data is presented in Fig. 4C.

The rate of increase in Fluo-5N emission from experiments like those in Fig. 4A is not accurate because mixing was done manually. This problem was addressed by performing similar experiments on a stop-flow rapid mixing device. In these experiments, complete mixing occurs in \(0.5\) ms, and changes in fluorescence after mixing can be followed accurately. Because the rapid changes caused by binding of Ca\(^{2+}\) to extraliposomal

FIGURE 3. Permeability of purified Cx26 HCs to ATP. Liposomes and Cx26 proteoliposomes loaded with 1 mM ATP were run on the size exclusion column to separate free extraliposomal ATP from ATP retained inside. ATP retained was measured by luminescence using a luciferin/luciferase assay, as the difference between total ATP (inside plus outside, measured after the addition of Triton X-100 to 0.4%) and extraliposomal ATP (before Triton X-100). The data are expressed as the means \pm S.E. \((n = 3, \text{ two different purifications})\) of the ATP retained/total counts. The asterisk denotes \(p < 0.001\) compared with the liposome values.

FIGURE 4. Permeability of purified Cx26 HCs to Ca\(^{2+}\). A, Ca\(^{2+}\) influx into liposomes containing purified Cx26 HCs. The red trace (Cx26) corresponds to proteoliposomes with Fluo-5N trapped inside, where intra- and extraliposomal Ca\(^{2+}\) proceeded to near equilibration upon increasing outside free [Ca\(^{2+}\)] from \(<10\) nM to 500 \(\mu\)M (Ca\(^{2+}\) arrow). The black trace corresponds to liposomes without Cx26 (No Cx26). The Ca\(^{2+}\) ionophore ionomycin (1 \(\mu\)M) was added at the labeled arrow to increase intraliposomal [Ca\(^{2+}\)] to similar values in liposomes and proteoliposomes. The data were normalized to the fluorescence after addition of ionomycin. B, reversible changes in Fluo-5N fluorescence elicited by sequential additions of Ca\(^{2+}\) and EGTA to Cx26 proteoliposomes. First arrow, addition of 600 \(\mu\)M CaCl\(_2\); second arrow, addition of 500 \(\mu\)M EGTA; third arrow, addition of 1 mM CaCl\(_2\). C, summary of near steady-state Ca\(^{2+}\) influx data. The values were normalized to the fluorescence in the presence of ionomycin. D, rate of Ca\(^{2+}\) influx into liposomes containing purified Cx26 HCs. Red trace (Cx26, 500 \(\mu\)M [Ca\(^{2+}\)]), proteoliposomes exposed to a 500 \(\mu\)M [Ca\(^{2+}\)] gradient; blue trace (No Cx26, 500 \(\mu\)M [Ca\(^{2+}\)]), liposomes exposed to a 500 \(\mu\)M [Ca\(^{2+}\)] gradient. Records from Cx26 proteoliposomes or liposomes in the absence of [Ca\(^{2+}\)] gradient were indistinguishable and did not show a change in fluorescence (similar to the blue trace). The black line is a multieponential fit to the data.
Relative H\(^{+}\), Na\(^{+}\), and Ca\(^{2+}\) permeabilities of liposomes containing Cx26 HCs

The data are presented as the means ± S.E., and \(n\) is the number of measurements. \(\Delta C_i/\delta t\) is the initial rate of increase in intraliposomal concentration of ion \(x\) (first 10 ms). Normalized \(\Delta C_i/\delta t\) is the rate of change in ion \(x\) concentration normalized for a 1 ms concentration gradient. The relative permeabilities were calculated from the normalized rates.

| \(\Delta C_i/\delta t\) (in mM s\(^{-1}\)) | \(\text{Na}^{+}\) | \(\text{Ca}^{2+}\) |
|------------------------------------------|-----------------|-----------------|
| 222 ± 15                                 | 35 ± 7          | 1.9 ± 0.3       |
| Normalized \(\Delta C_i/\delta t\) (in s\(^{-1}\)) | 19 ± 1\(^{a}\)  | 2.4 ± 0.5       | 3.8 ± 0.6\(^{b}\) |
| Relative permeability                     | 7.9             | 1               | 17\(^{a}\)       |
|                                        | 17 from 6 experiments | 39 from 6 experiments | 68 from 20 experiments |

\(^{a}\) \(p < 0.001\) compared with the \(\text{Na}^{+}\) value. 
\(^{b}\) Not statistically different compared with the \(\text{Na}^{+}\) value.

Fluo-5N (Fig. 4A, black trace) occur in <0.5 ms, they become part of the base line, and only subsequent slower changes are detected. Typical records of fast mixing experiments are shown in Fig. 4D, where an increase in Fluo-5N emission was observed upon increasing free \([\text{Ca}^{2+}]\) from <10 mM to 500 \(\mu\)M only in the proteoliposomes (red trace). Increasing \([\text{Ca}^{2+}]\) in liposomes did not elicit net \(\text{Ca}^{2+}\) influx (blue trace). A multieponential function was necessary to fit the increase in Fluo-5N emission in proteoliposomes (red trace), and we used the faster rate constant to calculate the rate of increase in intraliposomal \([\text{Ca}^{2+}]\) (Table 1).

To assess \(\text{Ca}^{2+}\) permeability relative to other ions, we determined the rates of increase in \([\text{Na}^{+}]\) and [protonated HEPES] (transport of \(\text{H}^{+}\) equivalents) in response to \(\text{Na}^{+}\) and pH gradients across the liposome bilayer. The basic principle of the \(\text{Na}^{+}\) transport experiments was the same as that of the \(\text{Ca}^{2+}\) transport experiments described earlier, using the \(\text{Na}^{+}\)-sensitive probe SBFI instead of Fluo-5N. The liposomes loaded with SBFI (~907 Da) were made in a nominally \(\text{Na}^{+}\)-free buffer (NMDG). Typical records in response to a rapid increase in extraliposomal \([\text{Na}^{+}]\) from zero to 15 mM are shown in Fig. 5A. Consistent with the \(\text{Ca}^{2+}\) data, an increase in SBFI fluorescence was observed only in the proteoliposomes exposed to a \([\text{Na}^{+}]\) gradient (red trace). The rate of increase in intraliposomal \([\text{Na}^{+}]\) was computed essentially as described for the \(\text{Ca}^{2+}\) experiments (see “Experimental Procedures”).

\(\text{H}^{+}\) transport was evaluated in liposomes containing traces of a phospholipid labeled with fluorescein at the head group. In pilot experiments in a fluorometer (Fig. 5B), lowering pH produced a rapid decrease (mixing limited) in fluorescence in both liposomes (black trace) and proteoliposomes containing Cx26 HCs (red trace); the decrease in fluorescence was approximated twice in the proteoliposomes but reached similar levels after lipidosome permeabilization with Triton X-100. We interpret these results as being due to quenching of fluorescence from fluorescein bound to the outer leaflet, similar in liposomes and proteoliposomes (~50% of the total), and inner leaflet, only in the proteoliposomes following \(\text{H}^{+}\) permeation through the HCs. To assess the \(\text{H}^{+}\) transport more quantitatively, we performed rapid mixing experiments in a stop-flow cell. Fig. 5C shows that lowering pH from 7.6 to 6.4 produced a rapid decrease in fluorescein emission, only in the proteoliposomes exposed to the pH gradient (red trace). The fluorescence decreased linearly with pH in the range of our experiments, and from the fluorescence time courses, we calculated a decrease in intraliposomal pH from 7.60 to 7.45 ± 0.01 in 10 ms (\(n = 17\) from six independent experiments). We then calculated the protonated [HEPES] from the Henderson-Hasselbalch equation, using a \(pK_a\) of 7.55, and calculated the rates in Table 1 from the differences in protonated [HEPES].

Millimolar extraliposomal \([\text{Ca}^{2+}]\) at the time of decreasing pH to 6.4 prevented pH equilibration. When \([\text{Ca}^{2+}]\) was increased to 5 or 10 mM, intraliposomal pH never reached the final pH of 6.4 during the 20 s of the experiment. The pH remained at 6.91 ± 0.05 (\(n = 15\) from three independent experiments) and 7.19 ± 0.09 (\(n = 4\) in 1 experiment) for 5 and 10 mM \([\text{Ca}^{2+}]\), respectively. These results were expected from the known inhibitory effect of millimolar \([\text{Ca}^{2+}]\) on HCs. A summary of the calculated rates of change in intraliposomal concentrations and relative permeabilities is presented in Table 1.
DISCUSSION

Recombinant Cx26 HCs expressed in Sf9 insect cells has been used for structural studies without parallel functional analysis (37–39), with exception of a recent publication focused on single-HC recordings (40). Previous electron microscopy results clearly point to Cx26 purified as HCs (hexamers), as opposed to GJCs (dodecamers) (37–39, 41), and our measurements of hydrodynamic radius by dynamic light scattering and apparent molecular mass by size exclusion chromatography fully agree with that notion.

The purified Cx26 HCs reconstituted in liposomes displayed permeability to Alexa Fluor 350 (349 Da, net charge −1) and sucrose (342 Da, uncharged) but were impermeable to Alexa Fluor 647 (~1,300 Da, −2 net charge), SBFI (907 Da, −4 net charge), and Fluor-5N (958 Da, −5 net charge). As expected, the purified HCs were also permeable to Na+ and H+ (H+ equivalents). This permeability pattern is as predicted from dye permeability studies in cells (1) and suggests that our purified Cx26 is properly folded in the bilayer, yielding the expected permeability properties: it shows size-selective solute permeability.

The most important findings of the present studies were the direct demonstrations of ATP and Ca2+ permeation through the purified Cx26 HCs. ATP (507 Da; net charge of was approximately −3.75) was permeable through purified Cx26 HCs. This result agrees with previous indirect evidence for ATP permeation through Cx26 HCs in cells (4, 8) and supports the suggested role of HC-mediated ATP secretion by the supporting cells in the control of hearing sensitivity (36). As mentioned in the introduction, a number of indirect studies in cells suggested significant Ca2+ movement through GJCs or HCs (1, 13, 14, 18, 20, 30), but direct demonstration was missing. For this, it is essential to use a purified and reconstituted preparation of HCs where there is no possibility of Ca2+ transport through parallel unidentified pathways. Our results support previous interpretations that suggested a role of Ca2+ fluxes through GJCs and HCs in physiological and pathophysiological conditions (17, 21, 25–28).

Ca2+ permeability is relatively high, similar to that for Na+. In a previous study, the relative K+/cAMP and K+/Lucifer yellow permeability ratios were estimated at ~40 and 230, respectively (7), which is not surprising because cAMP and Lucifer yellow are larger than atomic ions. In this context, it is likely that permeability of Cx26 HCs to HEPES is lower than that to Na+, and therefore the high permeability to H+ equivalents is likely to reflect a high H+/Na+ permeability ratio.

Even though the Ca2+ permeability is high, cell to cell Ca2+ fluxes may not be physiologically important because cytosolic [Ca2+] and the cell-to-cell concentration gradients are low. Therefore, significant cell-to-cell Ca2+ fluxes via GJCs depend critically on the number of open permeable channels. Although most cells have fewer HCs than GJCs at the plasma membrane, the much higher extracellular [Ca2+] suggests that HCs can have a significant role in Ca2+ influx, at least under certain circumstances (17, 21, 25–28) or in disease-causing mutants that display higher Ca2+ permeability (G45E mutant associated with keratitis ichthyosis deafness syndrome) (29). In summary, our results showed that Cx26 HCs are permeable to Ca2+ and suggest that HCs can be a pathway for Ca2+ influx into cells under conditions such as ischemia.

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