Green Fluorescent Protein Changes the Conductance of Connexin 43 (Cx43) Hemichannels Reconstituted in Planar Lipid Bilayers*

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Background: Connexin 43 hemichannels participate in many cellular processes. To elucidate their location and function within living cells, they were labeled with GFP.

Results: Recombinantly expressed Cx43 and Cx43-GFP form conducting hemichannels in reconstituted planar membranes. Their conductance states and voltage dependence differ.

Conclusion: Fusion of GFP to Cx43 significantly affects the electrophysiological behavior of Cx43 hemichannels.

Significance: GFP can significantly alter channel activities.

In mammalian tissues, connexin 43 (Cx43) is the most prominent member of the connexin family. In a single lipid bilayer, six connexin subunits assemble into a hemichannel (connexon). Direct communication of apposing cells is realized by two adjacent hemichannels, which can form gap junction channels. Here, we established an expression system in Pichia pastoris to recombimantly produce and purify Cx43 as well as Cx43 fused to green fluorescent protein (GFP). Proteins were isolated from crude cell membrane fractions via affinity chromatography. Cx43 and Cx43-GFP hemichannels were reconstituted in giant unilamellar vesicles as proven by fluorescence microscopy, and their electrophysiological behavior was analyzed on the single channel level by planar patch clamping. Cx43 and Cx43-GFP both showed an ohmic behavior and a voltage-dependent open probability. Cx43 hemichannels exhibited one major mean conductance of 224 ± 26 picosiemens (pS). In addition, a subconductance state at 124 ± 5 pS was identified. In contrast, the analysis of Cx43-GFP single channels revealed 10 distinct conductance states in the range of 15 to 250 pS, with a larger open probability at 0 mV as compared with Cx43, which suggests that intermolecular interactions between the GFP molecules alter the electrophysiology of the protein.

Processes such as development, differentiation, and maintenance of nutrients in tissues of vertebrates rely on a controlled exchange of intracellular solutes. These diverse tasks are succeeded by intercellular or junctional channels, known as gap junctions that connect adjacent cells with each other to form a synergistic entity. In vivo, gap junctions are assembled into larger arrangements that consist of several hundred channels, so-called gap junction plaques (1). Functional channels participate in embryonic development, synchronous contraction of muscle cells, and bone remodeling as well as homeostasis in tissues and organs (2). Gap junction proteins are composed of two connexin hemichannels in juxtaposed membranes. Each connexin hemichannel (connexon) is built by a hexameric unit of six connexin subunits. In humans, 21 isoforms of the connexin multigene family have been identified (3). All connexins share analogous structural motives and consist of four transmembrane α-helices, two extracellular loops, and one intracellular loop; thereby the N and C termini are exposed to the cell interior (2). These structural motives were recently confirmed by solving the crystal structure of the connexin 26 gap junction channel (4).

The major isoform of the connexin family is connexin 43 (Cx43) (5), which consists of 382 amino acids (Fig. 1A) (6). Cx43 obtains versatile functional properties reflected by its distribution in at least 34 different tissues as well as 46 cell types (7), including cardiomyocytes, keratinocytes, astrocytes, retinal glia cells, and osteoblasts (5). For instance, it mediates the transfer of electrical impulses to facilitate synchronous contraction of the heart muscle (8). Cx43 is also the main connexin in bone, where gap junctions transport nutrient and waste between osteocytes, which are separated by a few micrometers in the stiff bone matrix (9). It also acts as a transducer for the anti-apoptotic effect of bisphosphonates in osteocytes (10). A further hallmark of the broad functional spectrum of Cx43 is the rare disease oculodentodigital dysplasia, which causes the
abnormalities of ocular, nasal, and dental structures as well as a few neuronal dysfunctions (11).

The synthesis, maturation, and trafficking of connexin isoforms have been visualized in several studies by tagging the green fluorescent protein to the C-terminal end of connexins (12, 13). Investigation of the influence of this large fluorescent protein was started by Contreras and co-workers (14, 15). However, the characterization was restricted to Cx43 and Cx43-eGFP in HeLa cells. Other studies elucidate the essential role of the C-terminal tail of Cx43 and its interaction with the L2 region (amino acid residues 119–144) of the intracellular loop to maintain protein activity (16).

Here, we managed to express and reconstitute Cx43 and Cx43 fused to green fluorescent protein (Cx43-GFP; GFP with the point mutation S65T (17)) in planar membranes to study the electrophysiological properties of the formed hemichannels. Heterologous expression of Cx43 and Cx43-GFP was achieved in a Pichia pastoris expression system resulting in high levels of biomass, which is related to high protein levels. Purified protein was reconstituted into giant unilamellar vesicles (GUVs). These protein-containing GUVs were applied to a planar patch-clamp setup (Fig. 1B), which facilitates the study of purified ion channels as well as whole cell recordings (18–24). With this setup, we were able to elucidate the influence of the
GFP tag at the C terminus of Cx43 on its electrophysiological behavior of Cx43. Our results provide evidence that GFP attached to each connexin monomer alters the observed conduction states significantly, which might be a result of intermolecular GFP interactions.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of Recombinant Proteins**—Mouse Cx43 and GFP-tagged Cx43 (Cx43-GFP) were produced by heterologous overexpression in *P. pastoris*. The cDNA coding for Cx43 and Cx43-GFP was recloned from transfected HeLa cells (kindly provided by Prof. Willecke, University of Bonn, Germany) and ligated into the EcoRI and XbaI sites (Cx43) and EcoRI and SalI sites (Cx43-GFP) of the pPICZ-B vector (Invitrogen). The following primers were used: Cx43, 5’-AATAATGAACTCGAGGGATCCGGATCG-3’; 5’-ATAATTCTAGAATCTCCAGGTCATCAGGCCGAGGTCT-3’; Cx43-GFP, 5’-AATAATGAATTCCGAAAGATGGGTGACCTGGAGC-1 and 5’-ATATAATTCTAGAATCTCCAGGTCATCAGGCCGAGGTCT-3’; and Cx43-GFP, 5’-AATAATGAACTCGAGGGATCCGGATCG-3’ and 5’-ATATAATTCTAGAATCTCCAGGTCATCAGGCCGAGGTCT-3’.

**P. pastoris** (Sxa1) was transformed with the plasmid containing the c-Myc epitope and a hexahistidine (His) tag. Cx43-GFP was expressed with a hexahistidine tag. The cloning and expression procedure was carried out according to established procedures (25). Briefly, *P. pastoris* X33 cells were grown for 2 days at room temperature in YPD (yeast extract–peptone–dextrose) medium (0.2 liter). This preculture was utilized for inoculation of the fermentation stage (6 liters). After 24 h of batch fermentation, the nutrition source glycerol was fed for 4 h to increase the amount of biomass prior to the induction of protein expression. Expression of the transfected protein was induced by switching to methanol feeding (methanol-fed batch phase). After 24 h of methanol feeding, cells were harvested by centrifugation and stored at −80 °C. Wet cells were resuspended in 50 mM Tris, pH 7.0, and lysed by high pressure homogenization using a French press at 4 °C. Membrane fractions were separated by centrifugation at 16,000 × g at 4 °C for 1 h, collected, and stored at −80 °C.

**Solubilization, Purification, and Western Blot Analysis**—*P. pastoris* membrane pellets were resuspended for 2 h at 4 °C in 1 mM bicarbonate buffer, pH 8.0, containing 1 mM PMSF and homogenized by sonification for 30 s. The purification protocol was adapted from a previously reported procedure (24). First, the membrane fragments were solubilized for 2 h at 4 °C in detergent buffer containing 3% dodecyl maltoside (DDM), 1 mM NaCl, 1 mM PMSF, 0.005% NaN3, and 10 mM HEPES, pH 7.4. After removal of insoluble material, the supernatant was incubated with a Ni-NTA-agarose resin (Novagen) overnight at 4 °C in 0.2% DDM, 1 mM NaCl, 0.005% NaN3, and 10 mM HEPES, pH 7.4. The Ni-NTA-agarose resin was washed with 0.2% DDM, 1 mM NaCl, 0.005% NaN3, and 10 mM HEPES, pH 7.4, containing 10 mM imidazole. Bound protein was eluted with 300 mM imidazole.

**SDS-PAGE and Western Blot Analysis**—The presence of purified Cx43 and Cx43-GFP was confirmed by Western blot analysis after denaturing 12.5% SDS-PAGE, using two monoclonal antibodies that recognize amino acids 241–254 of Cx43 (anti-Cx43, Santa Cruz Biotechnology, Inc.), and a pentahistidine antibody that recognizes the histidine tag (anti-Hisα, Qiagen). The final concentration of protein, between 1 and 2 mg/ml, was determined by UV-visible spectroscopy. The extinction coefficients were calculated by using the ProtParam tool (ExPASy proteomics server) according to Gill and von Hippe (26) with ε280 = 57.340 m−1·cm−1 for Cx43 and ε280 = 79.355 m−1·cm−1 for Cx43-GFP.

**Liquid Chromatography/Mass Spectrometry**—Protein eluted from the Ni-NTA-agarose resin was separated by denaturing 12.5% PAGE and stained with Coomassie, and the appropriate bands were excised for LC/MS analysis. In-gel digestion with trypsin was performed according to the procedure described by Shevchenko et al. (27). The obtained tryptic peptides were loaded on a preparative μ-PrecolumnSM cartridge (P/N 160454, Dionex, Idstein, Germany) and further separated on a nano-HPLC-MS with an analytical capillary column (Dionex P/N 160321) utilizing the UltiMate 3000 HPLC system (Dionex). The peptides were transferred online to the LCQ DecaXP mass spectrometer through electrospray ionization by the use of a PicoTipTM emitter and a spray voltage of 1.5 kV. During the LC gradient the mass spectrometer was cycled through the acquisition of a full MS scan within the mass range of 300 to 1400 Da followed by four data-dependent collision-induced MS/MS spectra of the four most intense ions. The data were collected in the centroid mode, and about 2000 MS/MS spectra were collected during the LC/MS run in an Xcalibur raw data file. The “peak list” for the TurboSEQUEST analysis was created with extract-ms provided with the Bioworks-Browser 3.3.1 package. MS/MS spectra with a total ion current higher than 105 were used for correlation analysis against the protein database entries. The MS data were queried to a nonredundant NCBI database. The following search parameters were used for the TurboSEQUEST analysis: (i) precursor ion mass tolerance, less than 1.4 atomic mass units; (ii) fragment ion mass tolerance, less than 1.0 atomic mass unit; (iii) up to three missed tryptic cleavages allowed; and (iv) fixed cysteine modification by carboxyamidomethylation (plus 57.05 atomic mass units) and variable modification by methionine oxidation (plus 15.99 atomic mass units). Matching peptides have to pass the following filters: (i) cross-correlation scores (Xcorr) over 2.0, 2.5, and 3.0 for peptides of charge states 1, 2, and 3, respectively; (ii) ΔCn of the best peptide matches, at least 0.4; and (iii) primary scores (Sp), at least 600. Protein identification required at least two different peptides matching these criteria, and the degree of completeness of the b and y ion series for each TurboSEQUEST result was checked manually for every protein identified.

**Reconstitution of Purified Cx43 and Cx43-GFP in GUVs**—Cx43-GFP was used to visualize directly the Cx43 reconstituted in GUVs by fluorescence microscopy. Reconstitution in GUVs was performed according to a technique adapted from Martinac et al. (28) starting from proteo-small unilamellar vesicles (SUVs) to achieve large protein densities. 2 mg of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine was dissolved in trichloromethane in a glass test tube and dried under nitrogen for 30 min followed for 3 h under vacuum. The lipid film was hydrated in 2 mM KCl, 20 mg/ml n-octyl-β-D-glucopyranoside,
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and 1 mM MOPS/Tris, pH 8.0, and Cx43-GFP was added to a final concentration of ~40 μg/ml. After 30 min of incubation, 160 mg of wet polystyrene beads (Bio-Beads SM2 Adsorbents, Bio-Rad Laboratories) were added. After 2 h, an additional 160 mg of wet beads were added, and the solution was incubated overnight to remove any remaining detergent resulting in proteo-SUVs. The prepared proteo-SUVs were fused to GUVs during the electroformation process. 25 μl of a 2.6 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine solution in trichloromethane was placed on each of two indium tin oxide (ITO) coated-glass slides and dried for at least 3 h under vacuum. A chamber was then formed with a 1-mm Teflon spacer to separate the two ITO coated-glass slides, and the lipid films were rehydrated in 1.5 ml of 1 mM aqueous sorbitol solution. Up to 25 μl of a proteo-SUV suspension was added, and the chamber was connected by two copper electrodes. For GUV formation, an AC field was applied for 3.5 h with incremented voltage rising every 60 s from 50 mV to 1.6 V at 12 Hz (sinusoidal wave). Finally, the frequency was lowered to 5 Hz for 10 min to detach the proteo-GUVs from the ITO coated-glass slide (square wave). Protein reconstitution was visualized by fluorescence microscopy.

For electrophysiological recordings, only a few proteins need to be reconstituted into GUVs. Thus, purified Cx43 was incorporated into GUVs either by the above mentioned method or after GUV formation from detergent solution (23). In the latter case, GUVs were produced by electroformation (29) using a Vesicle Prep station (Nanion Technologies, Munich, Germany). 10 ml 1,2-dipalmitoyl-sn-glycero-3-phosphocholine/cholesterol (9:1) was dissolved in trichloromethane. ~20 μl of lipid solution was placed on the ITO-glass surface of the Vesicle Prep Pro Station and allowed to dry. The dry lipid film was rehydrated using 250 μl of 1 mM sorbitol in water. Vesicles were formed by electrosessing under the influence of an AC field for 2 h. GUVs were collected and incubated with a protein suspension containing 0.2% DDM and Cx43 at a final concentration of 10 ng/ml for 20 min. Polystyrene beads (40 mg/ml) were added for 4 h to remove the detergent.

Confocal Laser Scanning Microscopy—Fluorescence images of Cx43-GFP- and Cx43-doped GUVs, which were recognized by a FITC-conjugated hexahistidine antibody, were obtained by a FITC-conjugated hexahistidine antibody, were obtained using a confocal microscope (LSM 710, Carl Zeiss, Jena, Germany) equipped with a water immersion objective W Plan-Apochromat 63×/1.0 n.a. (Zeiss). GFP and FITC were excited at λex = 488 nm, and emission was detected at λem = 495–580 nm.

Electrical Recordings—All lipid bilayer recordings were done using the Port-a-Patch® planar patch clamp system (Nanion Technologies). Lipid bilayers containing Cx43 or Cx43-GFP were produced from GUVs prepared as described above using the Port-a-Patch method. Briefly, a droplet of recording solution was placed on each side of a glass chip containing an aperture of approx. 1–2 μm diameter and the glass chip was mounted on the Port-a-Patch. 5 μl of GUV suspension was then added to the upper side. As free-floating GUVs are drawn toward the aperture by suction, they contact the glass substrate and burst, coating a portion of the glass with a lipid bilayer. The resulting free-standing lipid bilayer across the aperture is stable and accessible to electrical recording, and if it contains Cx43 or Cx43-GFP hemichannels, their properties can be measured electrically. Signals were acquired using an EPC patch-clamp 10 amplifier and the data acquisition software PatchMaster (both from HEKA, Lambrecht, Germany) at a sampling rate of 50 kHz. The recorded data were digitally filtered at 3 kHz. All recordings were performed at room temperature. For bilayer experiments, the following solution was employed: 200 mM KCl, 2 mM EDTA, 10 mM HEPES, pH 7.0. The mean conductance values for single channels were obtained from Gaussian fits of all points histograms.

RESULTS

Production and Purification of Cx43 and Cx43-GFP—Mouse Cx43 with a hexahistidine tag at the C terminus for affinity purification was recombinantly expressed in P. pastoris. For visualization of Cx43 in artificial membranes, Cx43 was fused to green fluorescent protein with a single point mutation of S65T in the original GFP sequence to enhance GFP fluorescence emission. P. pastoris cells were cultivated at the fermenter scale. Biomass was increased with a glycerol-fed batch phase such that P. pastoris better adapted to the methanol induction, which increased protein production. After lysis, membranes were separated from the crude cell extract by centrifugation and solubilized in DDM, which is known to be an efficient detergent for connexon isolation from membrane fragments (30). Solubilized Cx43 and Cx43-GFP were then purified by affinity chromatography using a Ni-NTA-agarose resin. Assessments for purity were based on Coomassie-stained denaturing gels in which the bands were identified as Cx43 and Cx43-GFP using Western blot analysis (Fig. 2). The Western blots show only one band at about 45 and 71 kDa, corresponding to the monomers of Cx43 and Cx43-GFP, respectively, which indicates a uniform channel appearance. No protein heterogeneity was found in the protein preparations. The analysis of the elution fraction of Cx43-GFP by LC/MS/MS confirmed the identity of Cx43 from Mus musculus. In the SDS-PAGE, one very strong band was observed between 66 and 90 kDa. By LC/MS/MS, we were able to prove that this band corresponds to the alcohol oxidase (AOX) from P. pastoris. AOX is co-expressed with the protein of interest, as protein expression is induced by methanol, and AOX is responsible for metabolizing methanol as a carbon source. As AOX is not a transmembrane protein, it is removed during the procedure of reconstituting Cx43 into GUVs.

Protein Reconstitution and Visualization of Proteo-GUVs by Fluorescence Microscopy—To visualize reconstituted protein in GUVs by fluorescence microscopy, a rather high protein density is required. Although the reconstitution of large amounts of membrane proteins in lipid vesicles with diameters of 100–200 nm is well established (31), reconstitution of large transmembrane proteins in GUVs is less straightforward. Here, we established a method of reconstituting Cx43 and Cx43-GFP in GUVs in large quantities and in a reproducible manner starting from small unilamellar vesicles. To visualize reconstituted Cx43 in GUVs, we made use of either the fluorescent GFP tag of the protein or a FITC-conjugated antibody. Confocal laser scanning fluorescence images of Cx43-GFP reconstituted into GUVs clearly show a bright fluorescence of the lipid bilayer.
demonstrating that the protein is embedded in the GUV membrane (Fig. 3A). The incorporation of nonfluorescent Cx43 in GUVs was visualized by the addition of a FITC-conjugated antibody against the C-terminal hexahistidine tag. Although there is some fluorescence at the outside of the GUVs from the antibody in solution, an increased fluorescence intensity can be observed clearly at the membrane interface, which corroborates the insertion of Cx43 into the lipid bilayer of the GUVs (Fig. 3B).

Electrophysiological Characterization of Cx43 in Planar Lipid Bilayers—The electrophysiological characterization of Cx43 as well as Cx43-GFP was achieved by using the planar patch clamp method. First, the wild-type protein and Cx43-GFP in proteo-SUVs or detergent micelles were fused to giant vesicles. Resulting proteo-GUVs were added to a gigaohm seal voltages. The current-voltage relationship (Fig. 3A) for single Cx43 hemichannels, also determined as shown in Fig. 4B, from event histograms, was positioned at 224 ± 8 pS. The main conductance state at

The electrophysiological characteristics of Cx43 hemichannels in planar lipid bilayers were analyzed on the single channel level under symmetrical buffer conditions (200 mM KCl, 2 mM EDTA, 10 mM HEPES, pH 7.0). Directly after the gigaohm seal resistance had been formed, the activity of Cx43 could be monitored. We measured the conductance levels while varying the holding potential \( V_m \) between −100 and +100 mV. Fig. 4A shows the typical current traces of Cx43 activity at three different voltages. The current-voltage relationship (\( I-V_m \) curve) clearly shows the ohmic behavior of the Cx43 hemichannel (Fig. 4B). From the slope of the \( I-V_m \) curve, the average conductance was determined to be \( 228 \pm 8 \) pS. The main conductance level \( (G_m) \) for single Cx43 hemichannels, also determined from event histograms, was positioned at \( 224 \pm 26 \) pS \( (n = 32) \), where \( n \) is the number of bilayers with activity formed over the aperture of the glass substrate (Fig. 4C). In addition, a subconductance state at \( G_1 = 124 \pm 5 \) pS was observed.

The conductance of junctional channels is known to be sensitive to the applied voltage (32). The voltage dependence, however, does not necessarily arise from the voltage dependence of the conductance level but also can be a function of the open probability. To measure the open probability \( (P_o) \) of the hemichannel under steady state conditions, we applied continuous voltage potentials for more than 180 s for nine lipid bilayer preparations containing Cx43. The open probability of Cx43 was determined from the current traces as a function of applied voltage (Fig. 4D). The data were fit to a Boltzmann equation (Equation 1) according to Mazet et al. (33).

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P_o(V_m) = \frac{P_{o, \max} - P_{o, \min}}{1 + \exp \frac{V_m - V_{50}}{m_{50}}} + P_{o, \min}
\]

\( P_{o, \min} \) is the minimum and \( P_{o, \max} \) the maximum open probability. \( V_{50} \) is the holding potential; \( V_{50} \) is the transmembrane voltage, where \( P_o = 50\% \) and at which the function has a slope of \( m_{50} \). Assuming a symmetrical voltage dependence for Cx43 and \( P_{o, \min} \) results in \( P_{o, \max} = 55\% \) and \( P_{o, \min} = 44\% \), whereas \( V_{50} \) is dependent on the sign of the potential with \( -51 \) and \( +53 \) mV as well as \( m_{50} = -7 \) and \( +4 \) mV\(^{-1} \), respectively. The number of events is also higher for lower voltages, with 5800 events at \( +20 \) mV compared with only 341 events at \( +80 \) mV. Another characteristic of a channel is its open lifetime, which was determined as shown in Fig. 4E. A second order exponential decay was fit to the data, which provided two time constants with \( \tau_1 = 15 \pm 3 \) ms and \( \tau_2 = 75 \pm 5 \) ms.

Electrophysiological Characterization of Cx43-GFP in Planar Lipid Bilayers—Cx43 fused to GFP is frequently used in cellular systems to localize the protein within a living cell. We analyzed the influence of the C-terminal GFP tag on the electrophysiological behavior of Cx43. Channel activity of Cx43-GFP could
be readily monitored at different holding potentials in the range of −100 to +100 mV. Characteristic current traces of Cx43-GFP at holding potentials of +80 and −20 mV are shown in Fig. 5A. Similar to the current-voltage characteristic of Cx43, Cx43-GFP hemichannels exhibit an ohmic behavior (Fig. 5B). In the I-V_m curve presented, a conductance level of 57 ± 3 pS was observed from the slope. The same linear relationship was obtained for other conductance levels. The event histogram (Fig. 5C), however, deviates from that observed for Cx43. The analysis of 3676 events revealed various conductance states of Cx43-GFP in the range of 15 to 250 pS. Conductance states from G_1 to G_6 comprise about 70% of all events in the histogram and reveal multiple subconductance states of Cx43-GFP with an average increment of 14 pS. Furthermore, we were able to identify G_7 = 110 ± 4 pS, G_8 = 145 ± 8 pS, and G_9 = 184 ± 11 pS. Only G_10 = 250 ± 3 pS reaches the conductance level that was observed for wild-type Cx43 of 224 ± 26 pS. Single channel conductance values larger than 200 pS were, however, rarely observed, i.e. in only 1.5% of all events. At a holding potential of −20 mV, a mean conductance state at 184 ± 10 pS was observed. In contrast, G_1 and G_2 were the most prominent conductance states at +80 mV.

The open probability as a function of applied transmembrane potential was also investigated for Cx43-GFP. Voltages were applied in 21 sweeps from −100 to +100 mV in 10 mV steps, and each voltage was recorded for 5 s. Continuous voltage potentials were applied for 100 s to 11 lipid bilayer preparations containing Cx43-GFP. Fig. 5D shows the open probability as a function of V_m. Assuming a symmetrical voltage dependence for P_o, max and P_o, min results in P_o, max = 71% and P_o, min = 48%, whereas V_s, 50 is dependent on the sign of the potential with −41 and +56 mV as well as m, 50 = −6 and +8 mV⁻¹, respectively. For further analysis, the open lifetime (τ) (Fig. 5E) of Cx43-GFP hemichannels was determined. A second order exponential decay was fit to the data, which provided two time constants with τ_1 = 10 ± 1 ms and τ_2 = 81 ± 26 ms.

Modulation of Cx43 and Cx43-GFP Hemichannel Activity—Numerous studies have reported regulation of hemichannels by the alteration of pH (34), especially sulfonic acids such as HEPES and taurine. Here, the activity of the Cx43 hemichannels reconstituted into GUVs was blocked by the addition of mM concentrations of taurine (Fig. 6A). Similarly, the addition of taurine to active Cx43-GFP hemichannels at a holding potential of +80 mV led to an immediate drop in activity from a mean conductance level of 55 ± 4 pS to zero (Fig. 6B). Another proof that indeed Cx43 hemichannels are monitored is blocking the activity by lanthanum ions. Several studies (35−37) have shown the blockade of hemichannel activity by lanthanum. In our setup, the addition of 26 μM La³⁺ clearly blocked the channel activity of Cx43-GFP (Fig. 6C).

To validate the proper function of Cx43 purified from P. pastoris, we also used crude membrane preparations obtained from HeLa cells expressing Cx43 and fused them to bilayers. The conductance state was found to be 211 ± 5 pS (Fig. 7) (n = 5), which is in good agreement with the conductance state observed for Cx43 purified from P. pastoris. The activity of these Cx43 hemichannels was also blocked by 10 mM taurine.

DISCUSSION

For many years, the expression of connexins had been limited to mammalian cell cultures and oocytes, and the preparation of larger protein quantities was restricted to natural
have shown the expression of Cx43 in only a few reconstituted proteins. Such an amount of protein is, insertion of Cx43 from a detergent solution, which resulted in functional manner. The first one relies on the spontaneous study, we used two different methods to reconstitute Cx43 in a been established to reconstitute membrane proteins. In this reconstitution of membrane proteins is still not very straight-forward. During the last two decades, different procedures have proteins were obtained from harvested glutathione-GFP at the C terminus in fragments in rather large amounts. To allow for functionality sources. However, this is sufficient only for biomolecular studies that require small quantities of protein. Biophysical and structural studies rely on larger amounts of purified proteins. During the last two decades, various alternatives for the heterologous overexpression of connexins have been developed. The baculovirus/insect cell expression system was used to synthesize connexins 26, 32, and 43 with high protein yields (30, 38, 39). In vitro expression of functional Cx43 has also been demonstrated as feasible (40–43). However, the protein amounts remain rather low, and protein synthesis is technically demanding. Very recently, Gniddehou et al. (44) have shown the expression of Cx43 in Escherichia coli by fusion to glutathione-S-transferase.

In this study, we demonstrated the successful expression and purification of functional recombinant Cx43 and Cx43 fused to GFP at the C terminus in P. pastoris at the fermenter scale. P. pastoris is a well established heterologous expression system for the production of membrane proteins (45) and solubilized proteins were obtained from harvested P. pastoris membrane fragments in rather large amounts. To allow for functionality studies to be performed on those proteins, i.e. monitoring their channel activities in solvent-free lipid bilayers, the proteins have to be reconstituted into GUVs. In general, functional reconstitution of membrane proteins is still not very straightforward. During the last two decades, different procedures have been established to reconstitute membrane proteins. In this study, we used two different methods to reconstitute Cx43 in a functional manner. The first one relies on the spontaneous insertion of Cx43 from a detergent solution, which resulted in only a few reconstituted proteins. Such an amount of protein is, however, sufficient to monitor single channel activities of the proteins. The method has already been applied to reconstitute Cx26 in GUVs (24). To obtain larger protein amounts, Streicher et al. (46) reconstituted integrins into GUVs by briefly drying preformed proteoliposomes on ITO coated-glass slides. However, the drying process can cause inactivation of the protein. Hence, Martinac et al. (28) developed a gentler approach for the reconstitution of the E. coli-derived ion channel MscL in GUVs. We successfully adapted this procedure to functionally reconstitute Cx43 and Cx43-GFP in GUVs, as shown by their electrophysiological investigation.

Connexin hemichannels have been thought to remain closed until docking with other hemichannels to prevent leakage from the cell. In the last decade, a body of evidence has been accumulated that demonstrates that hemichannels formed by a number of different connexins can open in at least some cells and that their opening can be modulated under various physiological and pathological conditions (47–50). In particular, Cx43 has been implicated in diverse roles such as volume regulation (51) and efflux of NAD + and ATP (49, 50, 52). To study hemichannel activity, as yet only patch clamp experiments on Cx43-expressing cells have been performed. Here, we reconstituted Cx43 in a planar artificial membrane with a well defined membrane composition.

Under the given conditions, Cx43 exhibited a main conductance of 224 ± 26 pS, similar to the fully open state conductance, G = 220 ± 11 pS, of Cx43 hemichannels expressed in HeLa cells (14) and approximately double that of Cx43 gap junctions (53, 54). The ohmic behavior found in Cx43-expressing cells was also reproduced in the reconstituted membrane system. Contreras

**FIGURE 5. Electrophysiological characterization of Cx43-GFP.** A, characteristic current traces of Cx43-GFP hemichannels obtained at holding potentials of +80 mV and −20 mV in 200 mM KCl, 2 mM EDTA, 10 mM HEPES, pH 7.4. The corresponding point amplitude histograms show a conductance of 45 ± 4, 13 ± 2, and 21 ± 1 pS. B, current-voltage (I−V) relationship of Cx43-GFP hemichannels showing its ohmic behavior. The conductance was determined as the slope with 57 ± 3 pS. C, event histogram (3676 events) of Cx43-GFP at holding potentials of +80 and −20 mV. The solid line is the result of fitting 10 Gaussian distributions to the data: G1 = 15 ± 4 pS, G2 = 31 ± 4 pS, G3 = 48 ± 4 pS, G4 = 61 ± 3 pS, G5 = 74 ± 6 pS, G6 = 87 ± 10 pS, G7 = 110 ± 4 pS, G8 = 144 ± 8 pS, G9 = 184 ± 11 pS, and G10 = 250 ± 5 pS (bin width: 4 pS). D, open probability of Cx43-GFP hemichannels obtained from 11 independent experiments (2349 events). Fitting Equation 1 to the data results in the following parameters: $P_{\text{open max}} = 71\%$, $P_{\text{open max}} = 48\%$, $V_{\text{m}} = -41$ mV, $m_{\text{in}} = -6$ mV$^{-1}$ for negative $V_{\text{m}}$, and $V_{\text{iop}} = +56$ mV, $m_{\text{in}} = +8$ mV$^{-1}$ for positive $V_{\text{m}}$. E, open lifetime of Cx43-GFP. The solid line shows the result of fitting a second order exponential decay to 2349 single channel events with $\tau_1 = 10 ± 1$ ms and $\tau_2 = 81 ± 26$ ms (bin width: 20 ms).
GFP Influences Electrophysiological Behavior of Cx43

FIGURE 6. Modulation and blocking of Cx43 and Cx43-GFP hemichannel activity in planar lipid bilayers. A, current trace of Cx43 recorded at +80 mV. The mean conductance was determined to be 236 ± 8 pS from an all-points histogram. Instantaneously after addition of 10 mM taurine (asterisk), the resulting current went to zero. B, current trace of Cx43-GFP obtained at +80 mV. The all-points histogram shows a main conductance of 55 ± 4 pS. The addition of 44 mM taurine (asterisk) results in full blockade of the channel activity. C, current trace of Cx43-GFP recorded at +80 mV. The mean conductance was determined to be 63 ± 3 pS from an all-points histogram. Instantaneously after the addition of 26 μM LaCl3 (asterisk), the resulting current went to zero. All experiments were performed in 200 mM KCl, 2 mM EDTA, 10 mM HEPES, pH 7.4.

et al. (14) report on an additional substate with a conductance of 77 ± 13 pS. We also observed a substate, however, at a slightly larger value of 124 ± 5 pS. As it is well established that connexons are blocked by La3+ and taurine (14, 37), we proved unambiguously that the reconstituted protein channels are functional connexons by blocking Cx43 activity successfully with taurine.

To visualize the position of connexons and gap junctions in living cells, one generally makes use of GFP-tagged variants of connexons (55, 56). As we were able to reconstitute Cx43 as well as Cx43-GFP in planar membranes, we compared their electrophysiological properties under otherwise identical conditions. The same ohmic behavior for Cx43-GFP was observed as for Cx43, and blocking with lanthanum and taurine was also successful. However, the conductance states differed significantly from the wild type. Ten different conductance states could be distinguished from the event histogram (Fig. 5C). Contreras et al. (14) expressed Cx43-eGFP in HeLa cells and found only one mean conductance, \( G = 223 \pm 9 \text{ pS} \), without any subconductance states. Although the GFP attached to Cx43 differ only in one amino acid, the main difference is the expressing cell line in which the GFP-tagged connexons are assembled. We found conductance levels as small as \( G_1 = 15 \pm 4 \text{ pS} \) and \( G_2 = 31 \pm 4 \text{ pS} \). Such small conductance states were also reported by Kang et al. (52) for wild-type Cx43 expressed in C6 cells and astrocytes. For Cx43-eGFP they found, however, only a few channel openings with reduced amplitudes that were still larger than our observed substates of about 15 pS. Intermediate conductance states were also reported for Cx43 and Cx43-GFP expressed in HEK293 cells (48, 35). Taken together, it is unquestionable that not only the wild-type Cx43 but also Cx43-GFP is fully functional in the reconstituted membrane system. However, in our reconstituted membrane system, the number of substates differs considerably between the Cx43 wild type and Cx43-GFP. As GFP is positioned at each connexin monomer, which then assembles as a hexamer within the plane of the membrane, the local GFP concentration in two dimensions becomes quite large. This might favor dimer formation mediated by a hydrophobic patch (amino acids Ala206, Leu221, and Phe223), which is known to occur at rather high protein concentrations in bulk (>6 μM) and is enhanced at high salt concentrations (>100 mM). It is conceivable that such dimers alter the required conformational changes from the closed to the open state of Cx43 hemichannels leading to various substates. It is known that the intermolecular interactions of the C terminus and cytoplasmic loop domains of Cx43 are essential for the hemichannel activity (57).

In our reconstituted membrane system, the open probabilities of Cx43 and Cx43-GFP both follow a bell-shaped curve similar to what has been found for Cx32 (33). In the case of Cx43-GFP the maximum open probability at 0 mV is, however, significantly larger (71%) than that for Cx43 (54%). Although the conductance levels as well as the open probabilities differ for Cx43 and Cx43-GFP, the mean first open time constants for Cx43 and Cx43-GFP of \( \tau_1 = 15 \pm 3 \) and \( 10 \pm 1 \) ms, respectively, agree very well with that of Cx43 expressed in C6 cells (\( \tau = 10.4 \pm 0.8 \text{ ms} (52) \)).

In conclusion, we have demonstrated the successful expression and reconstitution of Cx43 and Cx43-GFP in a well defined artificial planar membrane system. Our results reveal strong evidence that both Cx43 and Cx43-GFP form fully functional hemichannels in these reconstituted membranes. They also suggest that the GFP tag influences the conductance levels as well as the open probability of the hemichannel at low potentials. The reconstitution procedure presented, combined with the planar patch clamp setup, might provide a useful tool for investigating connexin hemichannels and their modulation \textit{in vitro} in a well defined system.

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