GFP influences the electrophysiological behavior of Cx43

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

Significance: GFP can significantly alter channel activities.

SUMMARY

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 recombinantly 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 analyzed on the single channel level by planar-patch-clamping. Cx43 and Cx43-GFP both show an Ohmic behavior and a voltage dependent open probability. Cx43 hemichannels exhibit one major mean conductance of \((224 ± 26)\) pS. In addition, a subconductance state at \((124 ± 5)\) pS was identified. In contrast, the analysis of Cx43-GFP single channels revealed ten distinct conductance states in the range of 15 to 250 pS with a larger open probability at 0 mV compared to Cx43, which suggests that intermolecular interactions between the GFP molecules alter the protein’s electrophysiology.

Processes like development, differentiation and maintenance of nutrients in tissues of vertebrates rely on 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). Junctional channels participate in embryonic development, synchronous contraction of muscle cells, bone remodeling as well as homeostasis in tissues and organs (2). Gap junction proteins are
GFP influences the electrophysiological behavior of Cx43 composed of two connexon hemichannels in juxtaposed membranes. Each connexon hemichannel is built by a hexameric unit of six connexin subunits. In humans, 21 isoforms of the connexin multi-gene 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 (ODDD), 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 connexin's (12,13). Investigation of the influence of this large fluorescent protein was started by Contreras and coworkers (14,15). However, the characterization was restricted to Cx43 and Cx43eGFP in HeLa cells. Other studies elucidated the essential role of the C-terminal tail of Cx43 and its interaction with the L2 region (aa 119-144) of the intracellular loop to remain 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 be able 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. Our results provide evidence that GFP attached to each connexin monomer alters the observed conductance states significantly, which might be a result of intermolecular GFP interactions.

**EXPERIMENTAL PROCEDURES**

**Expression and purification of recombinant proteins** – Mouse connexin 43 (Cx43) and GFP tagged Cx43 (Cx43-GFP) were produced by heterologous overexpression in Pichia pastoris. The cDNA coding for Cx43 and Cx43-GFP was re-cloned 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’-AATAATGAAATTTCGAAACGATGGGTGACTGAGCGCCTTGGGGAAGCTGCTGGA-3’, 5’-ATATATTCTAGAATCTCCAGGTCATCAGGCCGGAGGTCT-3’ and Cx43-GFP 5’-AATAATGAAATTTCGAAACGATGGGTGACTGGAGCGCCTTGGGGAAGCTGCTGGA-3’, 5’-CCATTCTAGAATCTCCAGGTCATCAGGCCGGAGGTCT-3’ and Cx43-GFP 5’-AATAATGAAATTTCGAAACGATGGGTGACTGGAGCGCCTTGGGGAAGCTGCTGGA-3’, 5’-CCATTCTAGAATCTCCAGGTCATCAGGCCGGAGGTCT-3’. Mouse Cx43 was expressed with a C-terminal peptide 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, Pichia pastoris X33 cells were grown for 2 days at room temperature in YPD (yeast extract peptone dextrose) medium (0.2 L). This pre-culture was utilized for inoculation of the fermentation stage (6 L). After 24 hours of batch fermentation, the nutrition source glycerol was fed for 4 hours 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 hours 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 hour, collected and stored at -80 °C.

**Solvabilization, purification and Western blot analysis – Pichia pastoris** membrane pellets were resuspended for 2 hours at 4 °C in 1 mM bicarbonate buffer, pH 8.0 containing 1 mM PMSF, and homogenized by sonication for 30 seconds. The purification protocol was adapted from a previously reported procedure (24). First, the membrane fragments were solubilized for 2 hours at 4 °C in detergent buffer containing 3 % dodecyl maltoside (DDM), 1 M NaCl, 1 mM PMSF, 0.005 % NaN₃, 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 M NaCl, 0.005% NaN₃, 10 mM HEPES, pH 7.4. The Ni-NTA-agarose resin was pre-washed with 0.2 % DDM, 1 M NaCl, 0.005% NaN₃, 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 % polyacrylamide gel electrophoresis (SDS-PAGE), using two monoclonal antibodies, which recognize amino acid 241-254 of Cx43 (anti-Cx43, Santa Cruz Biotechnology, Inc.), and a penta-histidine antibody, which recognizes the histidine tag (anti His₅, Qiagen). The final concentration of protein was between 1-2 mg/mL and was determined by UV/Vis spectroscopy. The extinction coefficients were calculated by the ProtParam tool (ExPASy Proteomics Server) according to Gill et al. (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 % polyacrylamide gel electrophoresis, stained with Coomassie and the appropriate bands were excised for LC/MS analysis. The 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-Precolumn™ Cartridge (Dionex P/N 160454) and further separated on a nano-HPLC-MS with an analytical capillary column (Dionex P/N 160321) utilizing the ultimate 3000 HPLC system (Dionex, Idstein, Germany). The peptides were online transferred to the LCQ DecaXP mass spectrometer through electrospray ionization (ESI) by the use of a PicoTip™ 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 from 300-1400 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 MS2 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 BioworksBrowser 3.3.1 package. MS2 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 non-redundant NCBI database. The following search parameters were used for the TurboSEQUEST analysis: (i) precursor ion mass tolerance less than 1.4 amu, (ii) fragment ion mass tolerance less than 1.0 amu, (iii) up to three missed tryptic cleavages allowed, and (iv) fixed cysteine modification by carboxyamidomethylation (plus 57.05 amu) and variable modification by methionine oxidation (plus 15.99 amu). 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) the 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 manually checked for every protein identified.

**Reconstitution of purified Cx43 and Cx43-GFP in GUVs** – Cx43-GFP was used to directly visualize Cx43 reconstituted in giant unilamellar vesicles (GUVs) by fluorescence microscopy. Reconstitution in GUVs was performed according to an adapted technique of Martinac et al. (28) starting from proteo-small unilamellar vesicles (SUVs) to achieve large protein densities. 2 mg la-pamitoyll-2-oleoyl-sn-glycero-3-phosphocholine (POPC) was dissolved in trichloromethane in a glass test tube and dried under nitrogen for 30 min followed for 3 hours under vacuum. The lipid film was hydrated in 2 mM KC1, 20 mg/mL n-octyl-β-D-glucopyranoside, 1 mM MOPS/TRIS, pH 8.0 and Cx43-GFP was added to a final concentration of approx. 40 μg/mL. After 30 min of incubation,
160 mg of wet polystyrene beads (Bio-Beads SM2 Adsorbant, Biorad Laboratories) were added. After 2 hours, additional 160 mg of wet beads were added and the solution was incubated overnight to remove 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 POPC solution in trichloromethane were placed on each of two indium tin oxide (ITO) glass slides and dried for at least 3 hours under vacuum. A chamber was then formed with a 1 mm Teflon spacer to separate the two ITO glass slides and the lipid films were rehydrated in 1.5 mL of 1 M aqueous sorbitol solution. Up to 25 µL of proteo-SUVs suspension were added and the chamber was connected by two copper electrodes. For GUV formation, an AC field was applied for 3.5 hours with incremented voltage rising every 60 sec 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 glass slide (square wave). Protein reconstitution was visualized by fluorescence microscopy.

For electrophysiological recordings, only few proteins need to be reconstituted into GUVs. Thus, purified Cx43 was incorporated into GUVs by either the above-mentioned method or after GUV formation from detergent solution (23). In the latter case, GUVs were produced by electroformation (29) using the Vesicle Prep Station (Nanion Technologies, Munich, Germany). 10 mM 1,2-diphytanoyl-sn-glycero-3-phosphocholine (DPhPC)/cholesterol (9:1) was dissolved in trichloromethane. Approx. 20 µL of lipid solution were 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 M sorbitol in water. Vesicles were formed by electroswelling under the influence of an AC field for 2 hours. 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 minutes. Polystyrene beads (40 mg/mL) were added for 4 hours to remove the detergent.

Confocal laser scanning microscopy (CLSM) – Fluorescence images of Cx43-GFP and Cx43 doped GUVs, which were recognized by a FITC conjugated hexa-histidine antibody, were obtained with a confocal microscope (LSM 710, Carl Zeiss, Jena, Germany) equipped with a water immersion objective W Plan-Apochromat 63×/1.0 na (Zeiss). GFP and FITC were excited at $\lambda_{ex} = 488$ nm, emission was detected at $\lambda_{em} = 495-580$ nm.

Electrical recordings – All lipid bilayer recordings were done using the Port-a-Patch planar-patch-clamp system (Nanion Technologies, Munich, Germany). 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 were then added to the upper side. As free-floating GUVs are drawn towards 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 with an EPC 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: 10 mM HEPES, 200 mM KCl, 2 mM EDTA, 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 Pichia pastoris. For visualization of Cx43 in artificial membranes, Cx43 was fused to green fluorescent protein (GFP) 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 adapts to the methanol induction, which increases 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 connexin isolation from membrane fragments (30). Solubilized Cx43 and Cx43-GFP were then purified by affinity chromatography using a Ni-
identity of Cx43 from the preparations. The analysis of the elution fraction of Cx43-GFP by LC/MS/MS confirmed the 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 is observed between 66 kDa and 90 kDa. By LC/MS, we were able to prove that this band corresponds to the alcohol oxidase (AOX) from Pichia 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 carbon source. As AOX is not a transmembrane protein, it is removed during the reconstitution procedures of Cx43 into GUVs.

Protein reconstitution and visualization of proteo-GUVs by fluorescence microscopy – To be able to visualize reconstituted protein in GUVs by fluorescence microscopy, a rather high protein density is required. While 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 to reconstitute Cx43 and Cx43-GFP in GUVs in large quantities and in a reproducible manner starting from small unilamellar vesicles. For visualizing reconstituted Cx43 in GUVs, we made use of the fluorescent GFP-tag of the protein or used 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 non-fluorescent Cx43 in GUVs was visualized by the addition of a FITC-conjugated antibody against the C-terminal hexahistidine tag. While there is some fluorescence at the outside of the GUVs from the antibody in solution, an increased fluorescence intensity can be clearly observed at the membrane interface corroborating 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 unilamellar vesicles. Resulting proteo-GUVs were added to a glass chip with a micro-fabricated aperture. The generation of a lipid bilayer on the 1-2 μm sized aperture was forced by suction application resulting in a membrane resistance of $R_m > 1 \ \Omega$.

The electrophysiological characteristics of Cx43 hemichannels in planar lipid bilayers were analyzed on the single channel level in symmetrical buffer conditions (10 mM HEPES, 200 mM KCl, 2 mM EDTA, pH 7.0). Directly after the $\Omega$-seal resistance had been formed, activity of Cx43 could be monitored. We measured the conductance levels while varying the holding potential $V_m$ between $-100 \ \text{mV}$ and $+100 \ \text{mV}$. Fig. 4A shows 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 $(228 \pm 8) \ \text{pS}$. The main conductance level $G_1$ for single Cx43 hemichannels was also determined from event histograms and was positioned at $(224 \pm 26) \ \text{pS}$ ($n = 32$), 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 $G_1 = (124 \pm 5) \ \text{pS}$ was observed.

The conductance of junctional channels is known to be sensitive to the applied voltage (32). The voltage dependency, however does not necessarily arise from the voltage dependence of the conductance level, but can also 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 $180 \ \text{s}$ for $9$ lipid bilayer preparations containing Cx43. The open probability of Cx43 was determined from the current traces as a function of applied potential (Fig. 4D). The data was fit to a Boltzmann equation (eq. 1) according to Mazet et al. (33):

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

$P_{o, \text{min}}$ is the minimum and $P_{o, \text{max}}$ the maximum open probability. $V_m$ is the holding potential, while
$V_{50}$ 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 $P_{o,\ max}$ and $P_{o,\ min}$ results in $P_{o,\ max} = 55$ % and $P_{o,\ min} = 44$ %, while $V_{50}$ is dependent on the sign of the potential with $-51.5$ mV and $+53.1$ mV as well as $m_{50} = -7.4$ mV$^{-1}$ and $+4.0$ mV$^{-1}$, respectively. The number of events is also higher for lower voltages, with 5800 events at $+20$ mV compared to 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 $r_1 = (15 \pm 3)$ ms and $r_2 = (75 \pm 5)$ 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 GFP-tag at the C-terminus on the electrophysiological behavior of Cx43. Channel activity of Cx43-GFP could be readily monitored at different holding potentials in the range of $-100$ mV to $+100$ mV. Characteristic current traces of Cx43-GFP at holding potentials of $+80$ mV 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 presented $I-V_m$ curve, a conductance level of $(57 \pm 3)$ pS is obtained 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 different 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 $G_7 = (110 \pm 4)$ pS, $G_8 = (145 \pm 8)$ pS and $G_9 = (184 \pm 11)$ pS. Only $G_{10} = (250 \pm 3)$ pS reaches the conductance level that was observed for wild type Cx43 of $(224 \pm 26)$ 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 $(184 \pm 10)$ 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 $-100$ mV to $+100$ mV in 10 mV steps, and each voltage was recorded for 5 seconds. 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 $P_{o,\ min} = 48$ %, while $V_{50}$ is dependent on the sign of the potential with $-41$ mV and $+56$ mV as well as $m_{50} = -6.4$ mV$^{-1}$ and $+7.8$ mV$^{-1}$, respectively. For further analysis, the open lifetime $\tau$ (Fig. 5E) of Cx43-GFP hemichannels was determined. A second order exponential decay was fit to the data, which provided two time constants with $r_1 = (10.3 \pm 0.5)$ ms and $r_2 = (81 \pm 26)$ ms.

Modulation of Cx43 and Cx43-GFP hemichannel activity–Numerous studies reported a regulation of hemichannels by alteration of pH (34), especially sulfonic acids like 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 leads to an immediate drop in activity from a mean conductance level of $(55 \pm 4)$ 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 $\mu$M La$^{3+}$ 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 $(211 \pm 5)$ 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 sources. However, this is only sufficient for biomolecular studies that required small quantities of proteins. 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 connexin 26, 32 and 43 with high protein yields (30,38,39). In vitro expression of functional Cx43 has also been demonstrated to be feasible (40–43). However, protein amounts remain rather low and protein synthesis is technically demanding. Very recently, Gnidehou 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 Pichia 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 be able to perform functionality studies on those proteins, i.e. monitoring their channel activities in solvent-free lipid bilayers, the proteins have to be reconstituted into giant unilamellar vesicles (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 results in only few reconstituted proteins. Such 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 proteo-SUVs on indium tin oxide slides. However, the drying process can cause inactivation of the protein. Hence, Martinc et al. (28) developed a more gentle approach for the reconstitution of the Escherichia 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.

Connexon hemichannels have been thought to remain closed until docking with another hemichannel to prevent leakage from the cell. In the last decade, a body of evidence has been accumulated, which 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 exhibits a main conductance of (224 ± 26) pS, which is 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 is also reproduced in the reconstituted membrane system. Contreras et al. (14) reported 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 unambiguously proved 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 makes generally 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 differ 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 $G = (223 ± 9)$ pS without any subconductance states. While 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 $G_1 = (15 ± 4)$ pS, and $G_2 = (31 ± 4)$ 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,
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however only few channel openings with reduced amplitudes that were larger than the 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 is fully functional in the reconstituted membrane system but also Cx43-GFP. However, in our reconstituted membrane system, the number of substates differs considerably between 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 very large. This might favor dimer formation mediated by a hydrophobic patch (amino acids Ala$_{206}$, Leu$_{221}$ and Phe$_{223}$), which is known to occur at rather high protein concentrations (> 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 carboxy-terminus and cytoplasmatic 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 case of Cx43-GFP the maximum open probability at 0 mV is, however significantly larger (71 %) than that for Cx43 (54 %). While 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 $\tau_1 = (15 \pm 3)$ ms and $\tau_1 = (10.3 \pm 0.5)$ ms, respectively, agree very well with that of Cx43 expressed in C6 cells ($\tau = (10.4 \pm 0.8)$ ms (52)).

In conclusion, we could demonstrate the successful expression and reconstitution of Cx43 and Cx43-GFP in a well-defined artificial planar membrane system. Our results revealed 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 presented reconstitution procedure combined with the planar-patch clamp setup might provide a useful tool to investigate connexon hemichannels and their modulation in vitro in a well-defined system.

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FOOTNOTES

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The abbreviations used are: GUVs, giant unilamellar vesicles; SUVs, small unilamellar vesicles Cx43, Connexin43; TAT, transcription transactivator; aa, amino acid; ODDD, oculodentodigital dysplasia; DDM, dodecyl maltoside; AOX, alcohol oxidase.

FIGURE LEGENDS

FIGURE 1. A. Membrane topology model of Cx43 (adapted from Yeager (58) and Sorgen (59)). Each Cx43 subunit contains four transmembrane α-helices, which are connected by two extracellular loops and one intracellular loop. The N- and C-terminus are both exposed to the cytoplasm. The monoclonal antibody D7 (SantaCruzBiotechnology®) recognizes residues of amino acids 241-254 (marked by a black line). B. Schematic representation of a connexon-doped GUV approaching the micro-fabricated glass support to form a planar lipid bilayer.

FIGURE 2. SDS gels and Western blot analysis of Cx43 and Cx43-GFP protein fractions. A. The Coomassie-stained SDS gel (CS) depicts a typical elution fraction of Cx43, while the Western blots (WB anti-Cx43 and WB anti His) show Cx43 at approx. 45 kDa. B. The Coomassie-stained SDS gel (CS) shows a typical elution fraction of Cx43-GFP. The Western blot (WB anti-Cx43) detects Cx43-GFP at about 70 kDa.

FIGURE 3. Confocal laser scanning fluorescence images of Cx43 and Cx43-GFP doped GUVs. A. Cx43-GFP-doped GUVs. B. Cx43-doped GUVs visualized by binding of a FITC-conjugated anti His antibody. Scale bars: 20 µm.

FIGURE 4. Electrophysiological characterization of Cx43. A. Current traces and point-amplitude histograms obtained from reconstituted Cx43 hemichannels. The currents through a single purified Cx43 hemichannel in a planar lipid bilayer (bathed in 10 mM HEPES, 200 mM KCl, 2 mM EDTA, pH 7) were
recorded at the indicated holding potentials. Channel openings, indicated as upward currents, correspond to an conductance increase of 220 pS. B. Cx43 current-voltage ($I-V_m$) relationship. The main conductance was calculated from the slope of the linear regression and was determined to be (228 ± 8) pS. C. Event histogram of 1680 single channel events of Cx43 with a mean conductance $G_m = (228 ± 26)$ pS and a subconductance state at $G_1 = (124 ± 5)$ pS. D. Open probability of Cx43 hemichannels obtained from 32 independent experiments. Fitting eq. (1) to the data results in the following parameters: $P_{o,max} = 55 \%$, $P_{o,min} = 44 \%$, $V_{50} = -51.1$ mV, $m_{50} = -7.37$ mV$^{-1}$ for negative $V_m$ and $V_{50} = +53.06$ mV, $m_{50} = +4$ mV$^{-1}$ for positive $V_m$ values. E. Open lifetime of Cx43. The solid line shows the result of fitting a second order exponential decay to 1647 single channel events with $t_1 = (14.9 ± 3)$ ms and $t_2 = (74.8 ± 5)$ ms [bin width: 20 ms].

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 10 mM HEPES, 200 mM KCl, 2 mM EDTA, pH 7.4. The corresponding point-amplitude histograms show a conductance of (45 ± 4) pS, (13 ± 2) pS and (21 ± 1) pS. B. Current-voltage ($I-V_m$) 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 mV and –20 mV. The solid line is the result of fitting 10 Gaussian distributions to the data: $G_1 = (15 ± 4)$ pS, $G_2 = (31 ± 4)$ pS, $G_3 = (48 ± 4)$ pS, $G_4 = (61 ± 3)$ pS, $G_5 = (74 ± 6)$ pS, $G_6 = (87 ± 10)$ pS, $G_7 = (110 ± 4)$ pS, $G_8 = (144 ± 8)$ pS, $G_9 = (184 ± 11)$ pS and $G_{10} = (250 ± 3)$ pS [bin width: 4 pS]. D. Open probability of Cx43-GFP hemichannels obtained from 11 independent experiments (2349 events). Fitting eq. (1) to the data results in the following parameters: $P_{o,max} = 71 \%$, $P_{o,min} = 48 \%$, $V_{50} = -41$ mV, $m_{50} = -6.4$ mV$^{-1}$ for negative $V_m$ and $V_{50} = 56$ mV, $m_{50} = 7.8$ mV$^{-1}$ for positive $V_m$ values. 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 $t_1 = (10.3 ± 0.5)$ ms and $t_2 = (80.5 ± 25.9)$ ms [bin width: 20 ms].

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 in. The mean conductance was determined to be (63 ± 3) pS from an all-points histogram. Instantaneously after addition of 26 µM LaCl$_3$ (asterisk), the resulting current went to zero. All experiments were performed in 10 mM HEPES, 200 mM KCl, 2 mM EDTA, pH 7.4

FIGURE 7. Current trace of Cx43 channel activity reconstituted from crude membranes (HeLa cells) into lipid bilayers. The main conductance was determined to be (211 ± 5) pS. After addition of 10 mM taurine (asterisk) the channel activity is immediately blocked.
Figure 1

A

B

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GFP influences the electrophysiological behavior of Cx43

Figure 2
GFP influences the electrophysiological behavior of Cx43

Figure 3
GFP influences the electrophysiological behavior of Cx43

Figure 4

A

B

C

D

E
GFP influences the electrophysiological behavior of Cx43

Figure 5

A

B

C

D

E
GFP influences the electrophysiological behavior of Cx43

Figure 6

A

+80 mV / 10 mM taurine

10 pA

1 s

B

+80 mV / 44 mM taurine

4 pA

2 s

C

+80 mV

+80 mV / 26 μM LaCl₃

4 pA

200 ms

+80 mV / 26 μM LaCl₃

100 ms

4 pA

1 s
GFP influences the electrophysiological behavior of Cx43

Figure 7

![Graph showing electrophysiological behavior with a scale of 10 pA and 1 s]