Calcium-dependent Open/Closed Conformations and Interfacial Energy Maps of Reconstituted Hemichannels*

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Using an atomic force microscope, we have studied three-dimensional molecular topography and calcium-sensitive conformational changes of individual hemichannels. Full-length (non-truncated) Cx43 hemichannels (connexons), when reconstituted in lipid bilayer, appear as randomly distributed individual particles and clusters. They show a lack of preferential orientation of insertion into lipid membrane; in a single bilayer, connexons with protrusion of either the extracellular face or the large non-truncated cytoplasmic face are observed. Extracellular domains of these undocked hemichannels are structurally different from hemichannels in the docked gap junctional plaques examined after their exposure by force dissection or chemical dissection. Calcium induced a reversible change in the extracellular pore diameter. Hemichannels imaged in a physiological buffer with 1.8 mM Ca$^{2+}$ had the pore diameter of ~1.8 nm, consistent with the closed channel conformation. Reducing Ca$^{2+}$ concentration to ~1.4, 1, and 0 mM, which changes hemichannels from the closed to open conformation, increased the pore diameter to ~2.5 nm for ~27, 74, and 100% of hemichannels, respectively. Thus, open/close probability of the hemichannel appears to be [Ca$^{2+}$]-dependent. Computational analysis of the atomic force microscopy phase mode imaging reveals a significantly higher interfacial energy for open hemichannels that results from the interactions between the atomic force microscope probe and the hydrophobic domains. Thus, hydrophobic extracellular domains of connexins regulate calcium-dependent conformational changes.

Gap junctions are composed of two hemichannels (or connexons) in the plasma membranes of apposing cells. They allow the diffusion-driven transfer of ions and small cytoplasmic molecules between interconnected cells, synchronize electrical activity, and regulate metabolic homeostasis, cell growth, and differentiation (for reviews, see Refs. 1 and 2). Intercellular permeability and conductivity via gap junctions are regulated by physiological factors, including voltage, pH, calcium, and metabolites (1–6). Ultrastructural studies of intercellular channels using gap junctional plaques show that the smaller connexons (e.g. liver gap junctions) are hexagonally packed in a crystalline pattern. Each connexon contains six connexins (e.g. Cx32, Cx26), is roughly cylindrical, ~7.5 nm tall and ~7 nm in diameter with a 1.5–2-nm pore (Refs. 7–9, and see Ref. 10 for review). Cx43 connexons from heart (11) have a large cytoplasmic tail (the “cytoplasmic fuzz”) (12), which is non-rigid and labile and thus renders them less suitable for high resolution studies. Ultrastructural studies of recombinant Cx43 gap junctions with truncated cytoplasmic tail, crystallized in its closed state, show that the transmembrane domains share structural similarity with Cx32 gap junctions (13). No structural study is, however, available on non-truncated Cx43 or other larger connexons, and especially, non-crystalline connexons in their native environment.

Recent studies support the presence and activity of hemichannels in non-junctional plasma membrane regions (14–25). In mammalian tissues, non-junctional connexons composed of major connexins (e.g. Cx43, Cx26, and Cx32) are usually inactive (but see Ref. 26), and many connexins, including Cx46, Cx50, Cx45, and Cx35, when expressed in Xenopus oocyte and in mammalian cell expression systems, induce conductance through cell plasma membrane. Unlike intercellular gap junctional channels (exposed to only intracellular ion milieu), activity of non-junctional hemichannels (exposed to both intra- and extracellular milieus) appears to be modulated in a manner similar to other conventional ion channels (for a review, see Ref. 25). From the cytoplasmic side, non-junctional connexons can be gated by experimental manipulations (e.g. by pH, [Ca$^{2+}$], membrane potential, metabolites, and cyclic nucleotides) similarly as for intercellular gap junctional channels. From the extracellular side, non-junctional connexons are not gated by pH and nucleotides but by calcium (see Refs. 16, 22, 27, and 28 for a review), metabolic inhibition (22, 29, 30), and membrane depolarization (15). AFM images of Cx26–dependent conformational change on the cytoplasmic side of Cx26 connexons present as crystalline patches in the gap junctions and on the extracellular side exposed after the force dissection of the gap junctions are available (9). However, no such study is reported for connexons consisting of connexins with larger extramembranous mass (e.g. Cx43) and especially for pre-docked or undocked randomly distributed Cx43 hemichannels, as would occur in the non-junctional plasma membrane region. Moreover, despite the known functional dose-dependent hemichannel gating, no structural study is available to distinguish graded versus all-or-none channel opening and closing.

In the present study, using atomic force microscopy, we examined three-dimensional molecular surface topography as

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1 The abbreviations used are: AFM, atomic force microscopy; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphatidyl-choline; CT, cytoplasmic tail; EM, electron microscopy.
well as the effect of different divalent cations on the conformations of individual non-crystalline, non-truncated Cx43 hemichannels reconstituted in lipid membranes. Calcium induced conformational changes (closing/opening) in the channel pore in [Ca^{2+}]-dependent fashion. We further used AFM phase mode imaging and computational analysis (31) for the first time on membrane channels to reveal that extracellular hydrophobic domains of connexons regulate calcium-dependent conformational changes.

MATERIALS AND METHODS

**Hemichannel Isolation**—Wild type BICR-M1Rk (Marshall) cell line expressing Cx43 was obtained from Dr. D. Hulser and was grown as described (22). Connexons were isolated by published methods (32). Briefly, cells were collected and homogenized in Hanks’ buffer containing 20 mM HEPES (pH 7.4), 1 mM MgCl2, dithiothreitol, and protease inhibitor mixture at 4 °C. Lysate was centrifuged at 1,000 x g for 5 min, and supernatant was then centrifuged at 100,000 x g for 1 h. Pellets were resuspended in Hanks’ buffer with 0.25 μm sucrose, loaded on a 1:2.2/0.8 sucrose step gradient, and centrifuged at 100,000 x g for 2.5 h. The plasma membrane fraction containing connexons between 0.25 and 1.8 M sucrose was collected and solubilized with buffer S (20 mM HEPES, 50 mM NaCl, 50 mM octylglucoside, pH 7.4). The solution was centrifuged at 100,000 x g for 30 min, and the supernatant was incubated with an anti-peptide CT23 (DQRPSSRASSRSSRPPRDDELI) antibody coupled to Sepharose 4B beads for 2 h under gentle stirring. The beads were then transferred onto a column and washed with 50 ml of buffer S containing 1 mg of phosphatidylcholine/ml. Connexons were eluted with buffer S containing 5 μg peptide CT23 and 1 mg of phosphatidylcholine/ml. The purity of extracted connexons was evaluated by silver staining and Western blot following SDS-polyacrylamide gel electrophoresis as described (19, 33). A silver stain gel of the purified proteins and the Western blot of purified Cx43 are shown below (see Fig. 1C).

**Hemichannel Reconstitution**—1.2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC, Avanti Inc.) was dissolved in chloroform, dried with argon gas, and resuspended in HEPES or phosphate-buffered saline buffer (pH 7.2–7.4) at a concentration of 1 mg/ml. Unilaminar liposomes were formed with DOPC by extrusion using Lipofast from Avestin Inc. (Ottawa, Canada). To reconstitute connexons into liposome membrane, purified connexons were then mixed with the liposomes (~1,000 w/w protein:lipid) and bath-sonicated for 15 min. The liposomes were then deposited on freshly cleaved mica for 20 min, rinsed with buffer (HEPES or phosphate-buffered saline), and heated at 37–39 °C for 2–3 min to fuse liposomes into planar bilayers (34).

**AFM Imaging and Data Analysis**—The specimens were imaged by AFM as described (32, 34, 35). Briefly, AFM images were collected using a NanoScope III Multimode AFM (Digital Instruments). The AFM probe was oxidize-sharpened silicon nitride (Si3N4) tips and were operated at the resonance frequencies of 5–55 kHz with free oscillating amplitudes of ~20–35 nm. Most of the imaging was carried out in tapping mode or low force contact mode with scanning speeds from 1 to 7 Hz that allowed simultaneous collection of the height and phase data. All AFM imaging of planar lipid bilayers with or without reconstituted Cx43 connexons were conducted at room temperature (22–24 °C) in physiological buffer solution (pH 7.2–7.4) as described (22). To confirm the cytoplasmic versus extracellular side and also to provide additional assurance for the specimen purity, we imaged anti-Cx43 antibody-connexon complexes. These experiments were not intended for high resolution imaging, rather only for obtaining qualitative distinction between cytoplasmic and extracellular sides. First, we imaged connexons and identified them with their exposed cytoplasmic or extracellular side using previously defined criteria. Then, monoclonal antibody against the cytoplasmic tail of Cx43 (CT23) was perfused on-line, and its preferential binding (or non-binding) was used to distinguish the sidedness.

For examining calcium-dependent conformational changes, each reconstituted membrane and hemichannel constituted its own internal control, i.e. they were imaged before and after ionic perturbations. Different solutions (Ca^{2+}-free, 1 mM Ca^{2+}, 1.4 mM Ca^{2+}, 1.5 mM Ca^{2+}, 1.8 mM Ni^{2+} (without Ca^{2+}), or 1.8 mM Mg^{2+} (without Ca^{2+})) 10 mM HEPES buffer) were perfused into liquid cells alternately to examine conformational changes (closing/opening) in the channel pore with 50 ml of buffer S containing 1 mg of phosphatidylcholine/ml. Unilaminar liposomes were resuspended in Hanks’ buffer containing 0.25 M sucrose, loaded on a 1.2/2.0 M sucrose step gradient, and centrifuged at 100,000 x g for 2.5 h. The plasma membrane fraction containing connexons between 0.25 and 1.8 M sucrose was collected and solubilized with buffer S (20 mM HEPES, 50 mM NaCl, 50 mM octylglucoside, pH 7.4). The solution was centrifuged at 100,000 x g for 30 min, and the supernatant was incubated with an anti-peptide CT23 (DQRPSSRASSRSSRPPRDDELI) antibody coupled to Sepharose 4B beads for 2 h under gentle stirring. The beads were then transferred onto a column and washed with 50 ml of buffer S containing 1 mg of phosphatidylcholine/ml. Connexons were eluted with buffer S containing 5 μg peptide CT23 and 1 mg of phosphatidylcholine/ml. The purity of extracted connexons was evaluated by silver staining and Western blot following SDS-polyacrylamide gel electrophoresis as described (19, 33). A silver stain gel of the purified proteins and the Western blot of purified Cx43 are shown below (see Fig. 1C).

**Phase Imaging and Numerical Simulation**—AFM phase and height mode images were obtained simultaneously in tapping mode. Phase shift is a function of probe-sample interactions and can reveal sample properties, such as elasticity, charge distribution, surface energy, or energy dissipation (36–38). Phase sensitivity is also influenced by probe parameters such as the oscillation amplitude and set point. Attempts to quantify the absolute phase shift provided inconclusive quantitative results about the sample material properties so far (36, 39–41). However, the relative phase change, when only one (or a few) of the surface properties varies, while other unknown parameters are kept invariant for different surface areas, allows quantitative interpretation (31). Thus, the tip-sample contact force is described by the Maugis continuum mechanics and is a function of the normalized tip-sample curvature, the normalized elastic modulus, the interfacial energy, and the interatomic distance. For our study, the variation of the interfacial energy is the dominant parameter, and the effects of the changes in other parameters are negligible (31). Interfacial energy is defined as the work needed to separate two contacting surfaces and is affected by the surrounding medium (air, liquid). In an aqueous medium, the interfacial energy is reduced by the energy of immersion (related to the surface tension), and for hydrophobic surfaces, it is increased by the energy of solution. Thus, a significant increase in the observed interfacial energy in water would indicate hydrophobic interactions between the probe-sample surfaces (44).

RESULTS

**Structure of the Cytoplasmic and Extracellular Domains**—The thickness of the bilayer without any reconstituted connexons is ~5.0 ± 0.5 nm, as determined from isolated membrane patches and cross-sections of the force-dissected bilayers. The bilayer thickness is consistent with previous AFM measurements of DOPC lipid bilayer in similar aqueous imaging environments (34). The surface topography of these bilayers appears to be smooth and featureless (Fig. 1A). Connexons reconstituted in bilayer appear as structures protruding out from the bilayer surface in a randomly distributed diffused pattern (Fig. 1B, few examples, blue circles). The outer diameter of these connexons is 8.27 ± 0.8 nm.

Connexons appear to fall within two groups with distinct features including differences in the extramembranous protrusion height and structural details. Three-dimensional segmentation analysis of 100 randomly distributed connexons indicates that ~60% of the connexons are of height 1.29 ± 0.44 nm (Fig. 2A, red circles), and ~40% of the connexons are of height ~4.71 ± 0.45 nm (white circles). Height mode images of the first group (1.29 nm in height) exhibit better structural details. Six subunits representing constituent connexons are visible in many connexons, and there is an unambiguous presence of a pore with an indentation depth up to ~0.8 nm when imaged in nominal calcium-free buffer. The second group (4.71 nm in height) shows less defined substructures, and in most cases, do not show a pore (Fig. 2A). Fig. 2B shows a cross-section along the line in the figure; three left structures represent the first group (red arrows), whereas the fourth represents...
the second group (green arrow). The right shoulder on the fourth structure is the side of a hemichannel near the line (Fig. 2A). The height difference is apparently due to the larger cytoplasmic tail (CT) that protrudes out considerably farther from the membrane. Thus, a clear distinction of the sidedness of imaged connexons is achieved that is consistent with previous biochemical and structural studies of Cx43 connexons (19, 33, 46, 47).

In addition, images of the anti-Cx43 antibody (CT23)-connexon complexes show that antibody bound preferentially to the structures that have the features (greater height, lack of ultrastructural details, etc.) characteristic of the cytoplasmic side (Fig. 2, C–E). The volumes of the extracellular and cytoplasmic sides were $\approx 0.5-2$ and $\approx 4-5.5$ nm from the membrane plane, respectively. Hemichannels protruding $\sim 0.5-2$ nm (few examples; red circles) show clear central pores with six subunits, whereas hemichannels protruding $\sim 4-5.5$ nm (few examples; white circles) show no such structural detail. Imaging was performed in Ca$^{2+}$-free buffer. These two populations represent the extracellular (lower protrusion) and the cytoplasmic (higher protrusion) faces of connexons, respectively. Scan size for A and B: 250 nm. C, anti-Cx43-antibodies specific for a cytoplasmic epitope (red circle) absorbed on mica surface. A fork-like structure is visible for many of these individual antibodies. Image size: 470 nm. D1–4 show two individual extracellular-sided connexons (red circles) before ($D_1$ and $D_3$) and after ($D_2$ and $D_4$) antibody incubation. No antibody binding was observed, and the overall structural features on each connexon remained same. Scan size: 20 nm. E1–4 show two sets of clusters of cytoplasmic-sided connexons (red and green circles) in the lipid membrane before ($E_1$ and $E_3$) and after ($E_2$ and $E_4$) antibody binding. Antibody binding increased the overall volume of the bound complexes. Scan size is 20 nm. The height scale in $E_2$ and $E_4$ is about twice as that of $D_2$ and $D_4$ antibody binding.
whole junctional channels; there is simply not enough volume change to account for two apposed hemichannels being isolated and reconstituted together.

Effect of Calcium on the Hemichannel Extracellular Face—A detailed analysis of the Ca\(^{2+}\)-sensitive conformational change was performed only for those structures that resembled the characteristics of hemichannels, namely, six subunits and a height of <2 nm from the outer membrane surface. Particles with the diameter of connexons but no substructure were excluded from the analysis. Such strict criteria for selecting hemichannels for conformational studies ensured that we were not looking at non-connexon structures, including its associative protein macromolecules, and thus minimized any potential bias in our data pool versus automated selection process; the same structural features would have been used to define the automated selection process. Moreover, each reconstituted membrane and hemichannel constituted its own internal control, i.e. they were imaged before and after ionic perturbations. 

The most definitive evidence that the imaged structures indeed are Cx43 hemichannels would require labeling them on-line with anti-Cx43 antibody subsequent to their imaging. In the absence of such definite evidence, structures imaged in this study could be termed “putative hemichannels.”

For calcium-dependent conformational changes, hemichannels were first selected in their open conformation with clearly visible pore (i.e. when imaged in the absence of calcium) to ensure that any calcium-dependent change is observed for only those structures that have features and calcium-sensitive characteristics of hemichannels. Then, [Ca\(^{2+}\)] was increased on-line to a desired level (Ca\(^{2+}\)-free, 1 mM Ca\(^{2+}\), 1.4 mM Ca\(^{2+}\), 1.8 mM Ca\(^{2+}\), 1.8 mM Ni\(^{2+}\) (without Ca\(^{2+}\)), or 1.8 mM Mg\(^{2+}\) (without Ca\(^{2+}\)), and the relative changes in the structural morphology were imaged. It should be mentioned that although the quantitative analysis for calcium-dependent conformational changes were performed on fully open channels and their closure when increasing the calcium concentration, we also performed experiments wherein channels were opened from their closed state to the open state (after reducing the calcium concentration from 1.8 mM).

In Ca\(^{2+}\)-free buffer, AFM images of the extracellular face show a well defined donut-like structure consisting of six subunits and a central pore-like depression (Fig. 3A). Based on the cross-section analysis of the height mode images (performed on 50 connexons), for open channels, the pore diameter is 2.5 ± 0.4 nm (Fig. 3J). The depth of the pore from the rim of the hemichannel reached by AFM tip is 0.8 nm. It should be noted that the depth underestimation is likely due to the constraints imposed by the tip radius of curvature and tip shape. The pore diameter did not change appreciably by an addition of 1.8 mM Mg\(^{2+}\) or Ni\(^{2+}\) in the absence of Ca\(^{2+}\) (Fig. 3, B, C, and J). Subunits and their arrangements show some changes, reflecting a possible subtle role of Ni\(^{2+}\) and Mg\(^{2+}\) in the stability of the extracellular loops.

The addition of 1 mM [Ca\(^{2+}\)] to the Ca\(^{2+}\)-free bathing buffer had no appreciable effect on the structure of the majority (~74%) of the connexons (Fig. 3D). However, for the remaining 26%, the topography changed significantly. The inner rim of the connexons became more constricted, the average pore diameter was reduced to 1.8 ± 0.3 nm, and the central indentation decreased to ~0.1 nm, indicating that these connexons were in the closed conformation (Fig. 3F). Increasing [Ca\(^{2+}\)] further to 1.4 mM, the majority of the connexons (~73%) appeared to be closed with an inner pore diameter of ~1.8 nm, whereas the remaining 27% had the pore diameter of ~2.5 nm (Fig. 3E). When [Ca\(^{2+}\)] was raised to 1.8 mM, the inner pore diameter of all connexons was reduced to ~1.8 nm (Fig. 3J), and the subunits appeared less well delineated (Fig. 3G). The Ca\(^{2+}\)-induced channel closing was reversible; removing Ca\(^{2+}\) from buffer returned the connexons to the open conformation. The open and closed channel diameters (2.5 and 1.8 nm) are similar to those predicted from an earlier EM study by Unger et al. (13) but quite different when compared with the open and closed states of connexons.
closed diameters (1.5 and 0.6 nm) for isolated Cx26 gap junctional channels (9). This further indicates that the structure of Cx43 hemichannel described here is different from Cx26 hemichannel in docked whole junction.

The distribution of the inner rim diameter for the open and closed connexons is shown in Fig. 3J. The ratio of open channels for various cationic concentrations is summarized in Fig. 3K. The two-state distribution of the inner pore diameters (−2.5 and −1.8 nm) indicates a two-[Ca^{2+}] mechanism that opens and closes connexons. This is consistent with previous functional studies of hemichannel and its physiological role (17, 22, 27); in response to extracellular calcium, a single hemichannel switches between open and closed steady states with the open/close probability being [Ca^{2+}]-dependent.

In ~10% of open or closed connexons, we were able to resolve subunit topology of individual connexins (Fig. 3, H and I). Significantly, ultrastructural details are more pronounced in the open conformation. For the open conformation (Fig. 3H), 10–12 extramembranous protrusions around a central pore could be identified. Their approximate alignments (tentatively indicated in Fig. 3, H and I, by the numbers 1–6) suggest that they are likely to be the extracellular loops E1 and E2 for each of the six connexin subunits forming a hemichannel. For the closed conformation (Fig. 3I), the number of extramembranous protrusions was not so well defined and only few distinct subunits could be identified and they appear to overlap considerably and thus are more tightly folded in the closed conformation. A more defined and limited number of domains in the closed conformation suggests a possible refolding of the loops when calcium is removed from E1 and E2.

Effect of Calcium on the Phase Imaging-Interfacial Energetics of Open/Closed Hemichannels—As described under “Materials and Methods” earlier, the interfacial energy maps in our present study were used to determine the hydrophobic versus hydrophilic surfaces, both on the extracellular loops as well as in the pore mouth. Fig. 4 shows representative height (A) and phase (B) images of open connexons. On the cytoplasmic side, the height mode image shows no structural detail (Fig. 4A, white circles); however, the pore can be identified in the phase image (Fig. 4B, white circles). For closed connexons, phase imaging did not resolve much structural features.

When a connexon is in the closed conformation (Fig. 4C,

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**Fig. 4.** Simultaneous height mode and phase mode images of the extracellular and cytoplasmic sides of connexons. A, three-dimensional height image in the absence of calcium. B, the corresponding three-dimensional inverted phase image. Examples of extracellular (red circles) and cytoplasmic (white circles) sides in the same bilayer patch are present. Height difference is used to distinguish extracellular and cytoplasmic portions of the hemichannels. C−F, 10 × 10 nm zoom on representative individual connexons in the three-dimensional height (C and E) and phase (D and F), images in the presence of 1.8 mM [Ca^{2+}] (C and D) and in the absence of Ca^{2+} (E and F), respectively. G−H, corresponding overlaid cross-sections of topography (black trace) and phase shift (red) for closed (G) and open (H) states of a connexon (taken from C−F). I shows an overlaid diagram of normalized phase shifts (relative to the membrane plane) of individual connexons in the presence and absence of Ca^{2+} with or without Ni^{2+} or Mg^{2+}; 1.8 mM [Ca^{2+}] (red), Ca^{2+}-free (green), and Ca^{2+}-free with 1.8 mM [Ni^{2+}] (black) or [Mg^{2+}] (blue).
Ca$^{2+}$-dependent Conformations and Energetics of Hemichannels

Previous structural studies correlating gap junction channel gating have largely focused on the cytoplasmic domain of whole intercellular channels comprising docked connexons from the apposing membranes. For docked connexons, the extracellular side is inaccessible to physiological perturbations (1). In general, structural features (e.g. subunit stoichiometry and pore size) on the extracellular face of undocked Cx43 connexons in the present study are similar to the extracellular face of docked connexons exposed after force dissection of both Cx43 (19) and Cx26 (9) gap junction plaques, consistent with the conserved extracellular and membrane-spanning epitopes of the majority of connexins. However, as described below, there were marked differences in the structural details on the extracellular loops of the reconstituted connexons in this study in comparison with those from preformed gap junctions.

Imaging Purified Connexons—In addition to biochemical analysis of our sample preparation that showed Cx43 in the purified sample, Ca$^{2+}$-dependent conformational changes were examined only for those structures that correspond to known structural features of connexons, namely, six subunits, rotational symmetry, and a height of ~1.3 nm (extracellular side) and ~4.7 nm (cytoplasmic side) from the membrane surface. These characteristics were initially shown by freeze-fracture/negative stain EM and confirmed by EM and x-ray diffraction analysis and are also maintained under “wet” conditions as imaged by AFM (Ref. 19, and see Ref. 28 for review). Particles with the diameter of connexons but no substructure were not included in our data. Moreover, our antibody binding study (Fig. 2) provides clear evidence that the structures observed in our study are indeed connexons and that there are two distinct populations of the extracellular and cytoplasmic side-facing connexons inserted in the membrane. Such strict criteria for selecting connexons for conformational studies ensured that we excluded non-connexon structures, including its associated protein macromolecules, and minimized any potential bias in our data pool versus automated selection process; the same structural features would have been used to define the automatic selection process. The purity of the preparation would merely affect the frequency at which we can observe hemichannels. Moreover, connexons in a field served as their own internal control, i.e. the same connexons were imaged before and after ionic perturbations. The most definitive evidence that these imaged structures are indeed Cx43 hemichannels would require labeling them on-line with anti-Cx43 antibody subsequent to their AFM imaging, currently being undertaken in our laboratory. In the absence of such definite evidence, structures imaged in this study could still be termed putative hemichannels.

Cytoplasmic versus Extracellular Side of Reconstituted Cx43 Hemichannel—Previous high resolution EM studies used crystalline plaques of Cx43 connexons with truncated CT-domains (13, 49, 50). The truncation allowed crystallization and was necessary to limit the blurring effect induced by the flexible CT-domain (47). We used the presence of the CT-domain to our advantage; extracellular and cytoplasmic faces were distinguished by a clear height difference, by the differential labeling with anti-CT antibody, and by poor resolution on the cytoplasmic face. Due to the poor resolution for the cytoplasmic side, a detailed analysis of the Ca$^{2+}$-sensitive conformational change was examined only for the extracellular face.

Connexin subunits with two (E1, E2) loops each on the extracellular side were visible, but detailed features on the E1 and E2 loops were not resolved, i.e. extracellular loops in undocked hemichannels have a less defined ultrastructure (versus the well defined structure seen for chemically dissociated hemi-plaques that contain highly packed and docked connexons and imaged by EM). Earlier AFM studies on hemiplaques exposed after force dissection of docked connexons were unable to show more than one extramembranous protrusion for each of the six connexin subunits (8, 9, 19), suggesting a tightly folded conformation in the docked connexons. It is highly unlikely that such difference in the structure of the reconstituted hemichannels and force-dissected hemichannels is an imaging artifact due to the force dissection itself. Hemichannels, if naturally flexible in the closely packed hemiplaques, should retain their original conformation once the force is reduced (after the force dissection is over) and normal imaging is resumed. Moreover, in our study, connexons seem softer and show considerable height variation (versus uniform height shown in crystalline patches of force-dissected docked hemiplaques). It is likely that in undocked hemichannels, even the inter-connexin interactions are ill defined and which further compromises the structural details. In an earlier study on docked gap junctional hemichannels, the “double barrel” composed of β strands in the extracellular loops (“β-zip model” (51)) was derived from interloop interactions among apposing connexons. This model might not be applicable for undocked connexons. In our study of undocked connexons, extracellular loops are tightly packed (a smaller pore) in the closed state. In the open state, extracellular loops at least partly unfold (a larger pore, a slightly larger outer diameter, and well resolved E1 and E2 loops), i.e. connexons seem to undergo refolding to expose hydrophobic domains when calcium is removed from the extracellular binding domains (potentially removing the charge screening). Thus, our results strongly suggest that extracellular loops in non-junctional hemichannels are structurally different when compared with those in the gap junctional hemichannels.

All-or-None Calcium-sensitive Conformational Change—The pore diameter changes from 2.5 ± 0.4 to 1.8 ± 0.3 nm when the channel changes from the open to the closed conformation. This change in the structural feature is statistically significant and reversible, i.e. for individual connexons, the pore diameter changes reversibly by changing the calcium concentration. Moreover, the pore diameter did not change appreciably by an addition of 1.8 mM Mg$^{2+}$ or Ni$^{2+}$ in the absence of Ca$^{2+}$ (Fig. 3, B, C, and J), although a subtle change in the subunit arrangements was visible. The closed pore diameter remained statistically unaltered for a series of calcium concentrations and was unlikely affected by the noise in the AFM imaging; we only analyzed structures for which repeatable images were collected. The two-state distribution of the inner pore diameters (~2.5 and ~1.8 nm) suggests an all-or-none opening mechanism and is consistent with previous functional studies of hemichannel and its physiological role (17, 22, 27); a single hemichannel switches between open and closed states in an all-or-none manner, and the open/close probability is [Ca$^{2+}$]-dependent. It is possible that statistically non-significant fluctuations in the pore diameter (Fig. 3J) could reflect channel subconductance states. However, this seems highly unlikely in our study. As described under “Materials and Methods,” the
next complete image after changing the calcium concentration was collected in a minute or so, and thus, we have imaged only the steady-state conformations. Because the measurement was for a steady state, any significant occupancy of a state of intermediate (subconductance) diameter would have been observed in the distribution of channel diameters. Our experimental paradigm and data would not preclude the presence of any subconductance state in transition (or with a brief residency) from closed-to-open states and vice versa. For the same reason, we do not believe that the change in pore diameter by 0.7 nm would result in the residual channel state as the same calcium concentrations were sufficient to block dye transfer at whole cell level, which is the key indicator of hemichannel open/closed states (22).

Our results indicate that the opening and closing of non-junctional (undocked) connexons must involve conformational changes at the pore entrance. On the force-dissected extracellular (undocked) connexons must involve conformational which is the key indicator of hemichannel open/closed states (22).

result in the residual channel state as the same calcium concentration was collected in a minute or so, and thus, we have imaged only the steady-state conformations. Because the measurement was for a steady state, any significant occupancy of a state of intermediate (subconductance) diameter would have been observed in the distribution of channel diameters. Our experimental paradigm and data would not preclude the presence of any subconductance state in transition (or with a brief residency) from closed-to-open states and vice versa. For the same reason, we do not believe that the change in pore diameter by 0.7 nm would result in the residual channel state as the same calcium concentrations were sufficient to block dye transfer at whole cell level, which is the key indicator of hemichannel open/closed states (22).

Our results indicate that the opening and closing of non-junctional (undocked) connexons must involve conformational changes at the pore entrance. On the force-dissected extracellular (undocked) connexons must involve conformational which is the key indicator of hemichannel open/closed states (22). Our study shows a significantly different Ca\(^{2+}\)-sensitivity in the gating of Cx43 connexons; within the range of 1.8–0 mM Ca\(^{2+}\), the number of open connexons increases gradually, and at \(-1.4\) mM, two-thirds of all connexons are open. Differences in the Ca\(^{2+}\)-sensitive gating of Cx26 (9) and Cx43 connexons (this study) could be due to the three-dimensional structural and functional differences, especially since they share extensive homology among their extracellular domains and differences in their tissue distribution (e.g. Cx26 for liver, Cx43 for heart) and their physiological functions (e.g. synchronization of heart beat versus metabolic homeostasis). The cooperativity of neighboring Cx26 connexons present in the crystalline hemiplaques could also affect the gating sensitivity (9). In addition, the dissection-exposed crystalline Cx26 hemiplaques are presumably hard (51) and are functionally different from the undocked connexons in the non-junctional domains.

The closed state for connexons under normal physiological extracellular conditions (1.8 mM [Ca\(^{2+}\)], pH 7.4, and a large transmembrane potential 60–70 mV) is consistent with the gating of the whole intercellular (gap junctional) channels. Intercellular channels joining neighboring cells normally are open with unitary conductances because of the favorable physiological cytoplasmic condition (nano-to-micromolar [Ca\(^{2+}\)], pH 7.2, and no transjunctional potential difference) and are closed at high [Ca\(^{2+}\)] or at a large transjunctional potential difference. Open hemichannel with unitary conductance has been reported (26, 30), and Ca\(^{2+}\)-dependent dye uptake and cell volume modulation have been reported for Cx43-expressing cells (22). Thus, we believe that the Ca\(^{2+}\)-sensitive conformational change observed in the present in vitro study would provide a basis for the Ca\(^{2+}\)-dependent gating of native hemichannels in vivo.

The gradual change in the ratio of the open and closed connexons within a certain range of Ca\(^{2+}\) concentration (1.0–1.8 mM) is compatible with its expected physiological function and with the role of calcium as a modulator of hemichannel activity (17, 22, 27). Consistent with the dye uptake and volume regulation in cells (22), our present findings would predict that, at the whole cell level, the strength of cytoplasmic-extra cellular communication and resulting cellular physiology/pathophysiology will depend on the number of open (or closed) connexons in the non-junctional plasma membrane region. Connexons are proposed as an exquisite sensor of extracellular calcium (53) and thus could regulate cell function and growth in a well-defined way.

The Ca\(^{2+}\)-dependent connexon opening was unaffected by the presence of 1.8 mM Ni\(^{2+}\) or Mg\(^{2+}\). This is consistent with previous physiological studies of the effects of Ca\(^{2+}\), Mg\(^{2+}\), and Ni\(^{2+}\) on gap junctions in cardiac cells (52) and on hemichannels in Cx43-expressing cell lines (22). Interestingly, Mg\(^{2+}\) and Ni\(^{2+}\) did not alter the open channel diameter but allowed a better resolution of subunits, suggesting some subtle role of Ni\(^{2+}\) and Mg\(^{2+}\) in the stability of the extracellular loops.

Surface Energetics and Pore Hydrophobicity—On the extracellular side, phase images show a homogenous negative shift (when compared with the bilayer) over the entire region of closed connexons. However, a positive shift is observed at the pore region of the open hemichannels (Fig. 4). Numerical simulation of the contrast mechanism of AFM phase images shows that the positive phase shift is determined mainly by: (i) sample elasticity, (ii) electrostatic field, (iii) topography-induced artifact, and (iv) interfacial energy.

For the imaging parameters we used, the sample elasticity has no effect on the phase shift (38). Low amplitude tip oscillation limits deep penetration into the sample, and thus, the repulsive elastic force (proportional to the depth) is minimal and acts for a very short fraction of the oscillating period. The contact attractive forces derived from the interfacial energy do not depend on the amplitude of the probe tip. The role of electrostatic interaction in our ionic buffer, if any, is minimal. First, there is no difference in the phase shift on the connexon rim when compared with the membrane surface for open or closed states. Second, the presence of the shielding Stern layer makes long range electrostatic effects quite unlikely (54). Third, there is no difference between the relative intensity of the phase shift measured in different electrolyte concentrations that have been used to examine the role of electrostatic forces on AFM imaging (55). The topography-induced artifact is possible due to different tip-sample contact geometries. In our case, however, even highly exaggerated estimates of the change in the contact area would be too small to account for the observed phase shift. The tip is not “buried” in the pore; the actual difference in the penetration depth falls into the order of angstroms. Furthermore, the tip slightly deforms the top of the connexon, so the actual contact area is the whole top surface (this elastic interaction, however, has only a negligible effect on the phase at low amplitude tapping (31)).

The most likely interpretation of our results is that the phase images reveal different interfacial energy at the mouth of the open hemichannel. Using the known surface energy value of the lipid bilayer (56), the interfacial energy at the pore region is estimated to be 0.118 J/m\(^2\) for the closed and 0.163 J/m\(^2\) for the open channels, respectively. Thus, the connexon opening is associated with a significant increase in the interfacial energy in the pore mouth region. As described under “Materials and Methods,” in an aqueous medium, such an increase in the observed interfacial energy would indicate the presence of hydrophobic surfaces, that is, hydrophobic domains exposed at the pore vestibule.

Geometrical Analysis of the Tip-Surface Interaction—To define the pore-lining region responsible for the increased interfacial energy and thus the putative hydrophobic portions, we reconstructed model surfaces contours of open and closed connexons. The convolutions of tip and the model surfaces resemble their measured cross-section contours (Fig. 5). The channel topography observed in our images is the surface contour effectively traced by the apex of the AFM probe. The tip-sample contact zone often does not directly follow the measured contour profile. When the tip reaches into the connexon pore mouth, the probing depth is limited by the tip dimension whereby the side of the tip is in contact with the channel mouth, a vestibule-like structure. In our analysis, we assumed a spherical tip shape with a diameter of 10 nm, which is consistent with results of blind tip reconstruction from our experimental AFM images.
Our model shows that when the probe is positioned at the center of the pore, the tip is in contact with different regions of a connexon in its open versus closed conformations. For a closed connexon, the tip only probes ~0.1 nm of the extramembranous region of the pore mouth. For an open connexon, as the probe scans over the central pore region, the tip contacts deeper in the side wall of the pore mouth. A probing depth of 0.8 nm for an open connexon corresponds to the tip interacting with the vestibul-lining side wall. Using a different tip diameter (5–15 nm) does not qualitatively alter the conclusion of our analysis.

The observation of hydrophobic domains exposed to the extracellular milieu at the open vestibule suggests a gating mechanism based on hydrophobic interactions. Protein tertiary structure can be unbalanced by a strong electrical field, like Ca^{2+} ions binding to the extramembranous parts of the channel. The polarizing effect will weaken the secondary bonds that stabilize the folding (57), and thus, the domains not directly incorporated in the membrane (e.g. domains forming the pore mouth) would gain a higher mobility. Those domains, presumably the parts of the E1 and E2 loops, have ~17 and ~33% hydrophobic residues, respectively, and thus, long range forces arising from the hydrophobic interaction (44) can become a dominant radial drag across the channel. Such drag can rearrange the molecule in a more compact conformation; parts of the vestibule and the six pore-lining domains will close together, hiding the hydrophobic domains and thus physically closing the channel for mass flow (Fig. 5, lower panels). The reversibility and [Ca^{2+}] dependence of this conformational change suggest that the binding takes place via weak interactions such as physisorption or chelation chemistry. Refolding and rearrangement of portions of connexin molecules due to the radial drag across the channel would be consistent with the earlier hypothesis brought forward by Unwin and Zampighi (7) about the movement (sliding) of transmembrane helices to change gap junctional channel (closed and opened) conformations. Our data are limited to account for the whole transmembrane portions of connexins that were used in deriving the earlier model (7), although it seems reasonable to speculate that portions of connexin molecules act like a spring stabilized by a strong electrical field (like Ca^{2+} ions binding to the extramembranous parts of the channel), and reducing this field (for example, by the removal of Ca^{2+} ions) would make them labile enough to rearrange (potentially moving apart to open the channel and thus allowing molecular transport). Moreover, as the water and ions begin flowing inward the pore, they would further exert pressure to move apart peptides lining the apposing walls of the pore.

In conclusion, by combining topographic and phase mode AFM imaging results, we were able to examine the structure of large wild type connexons (e.g. Cx43) with the complete non-truncated cytoplasmic domains. Calcium-dependent open and closed conformations follow an all-or-none approach. The gradual change in the number of open channels as a function of calcium concentration predicts that, at the whole cell level, the strength of the cytoplasmic-extracellular communication and the resulting cellular behavior will depend on the number of open (or closed) hemichannels present in the non-junctional cell membrane regions. Phase mode imaging of open connexon shows an increased interfacial energy, which results from hydrophobic interactions between the AFM tip and the vestibular mouth region of connexon pore and suggests a refolding of the extracellular loops exposing their hydrophobic domains.
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