Atomic Force Microscopy of Connexin40 Gap Junction Hemichannels Reveals Calcium-dependent Three-dimensional Molecular Topography and Open-Closed Conformations of Both the Extracellular and Cytoplasmic Faces*

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Atomic force microscopy was used to study the three-dimensional molecular topography and calcium-sensitive conformational changes of Connexin40 hemichannels (connexons) reconstituted in 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine lipid bilayers. Two classes of objects were observed that differed in their protrusion heights above the bilayer (2.6 lipid bilayers. Two classes of objects were observed that differed in their protrusion heights above the bilayer (2.6 versus 4.2 nm). Comparison to reconstituted connexons containing Connexin40 truncated to eliminate most of its C-terminal cytoplasmic domain showed that the two height classes corresponded to the shorter extracellular and taller cytoplasmic aspects of the hemichannels and that the C-terminal tail of Connexin40 contributes ~1.6 nm in thickness. Hemichannels imaged in solutions containing <10 μM Ca2+ showed 3.1–3.2 nm depressions (openings) in 30% of the cytoplasmic faces and 65% of the extracellular faces, and high-resolution three-dimensional topography of extracellular or cytoplasmic aspects of some connexons was observed. After addition of 3.6 mM Ca2+, >75% of the connexons in either orientation adopted closed conformations. In contrast, hemichannels imaged in the presence of 0.1 mM EDTA showed large (5.6- to 5.8-nm diameter) openings in nearly all hemichannels regardless of orientation, and detailed topography was visible in many connexons. Real-time imaging following the addition of 3.6 mM Ca2+ showed transitions of both extracellular and cytoplasmic orientations from “open” into “closed” conformations within several minutes. These studies provide the first high-resolution topographic information regarding a connexin with a large cytoplasmic domain and suggest that the extramembranous portions of Connexin40 contribute to a channel entrance that is relaxed by chelation of residual divalent cations.

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Gap junctions are specialized plasma membrane regions containing intercellular channels. They are made of apposing hemichannels (connexons) containing hexameric subunits from a family of proteins called connexins (Cx), which has 21 members in the human and mouse genomes (1, 2). There is substantial similarity among the connexin sequences, especially in their transmembrane and extracellular regions. However, the major intracellular domains (cytoplasmic loop and carboxy-terminal tail) are connexin-specific.

Our understanding of the structures of these molecules (particularly the conserved regions) has recently been substantially advanced. An atomic resolution structure was determined from crystals of Cx26, a connexin with a very short C-terminal domain (3). These data confirmed and extended many of the observations that had previously been made in studies of a C-terminally truncated Cx43 (4). There has been substantially less information regarding the structures of connexins containing large cytoplasmic domains or connexons in dispersed (non-crystalline) arrangements. Cx40 and Cx43 are among such connexins. They are found in cardiac gap junctions that have been noted to have a “fuzzy layer” on their cytoplasmic surfaces in electron micrographs (5–7). Atomic force microscopy offers a powerful approach that has recently been exploited to analyze such issues for Cx43 hemichannels reconstituted into lipid bilayers (8). However, ultrastructural details of the cytoplasmic face were not obtained in this study. The investigators also attribute this to a “blurring effect induced by the flexible C-terminal-domain” (8).

We have used a similar approach to study Cx40, a connexin found in a variety of cell types and especially in the cardiovascular system, where it is present in the atrium, conducting system, and endothelium.

This approach also offers the possibility to examine structural alterations that accompany treatments that affect channel gating. We imaged reconstituted Cx40 hemichannels in fluid phase DOPC bilayers and studied them using an approach similar to the previous AFM study of Cx43 hemichannels (8). We also examined the effects of calcium on Cx40 hemichannels. Calcium is known to regulate the gating of gap junction chan-
**EXPERIMENTAL PROCEDURES**

**Expression of Cx40**—HeLa cells were grown in minimal essential medium supplemented with 10% fetal bovine serum, 0.1 mM non-essential amino acids, 2 mM l-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin (Invitrogen). Rat Cx40 DNA was cloned into pcDNA3.1/hygro (Invitrogen), and an influenza HA epitope tag (YPYDVPDYA) was appended to its C terminus using PCR methods. A truncated version of rat Cx40 in which this amino acid 248 was replaced with an HA tag and a stop codon (Cx40tr248) was generated by PCR and subcloned into pcDNA3.1/hygro. Cx40tr248 corresponds to a construct previously used to study the roles of the Cx40 C terminus in pH- or voltage-dependent gating and conductance (15, 16). Cells were stably transfected with linearized DNA using Lipofectamine (Invitrogen). Stable clones were selected by growing in medium containing hygromycin (150 µg/ml streptomycin (Invitrogen). Clones were screened for Cx40 expression by immunofluorescence and immunoblotting. Selected clones were maintained in medium supplemented with 75 µg/ml hygromycin.

**Affinity Purification of HA-tagged Cx40**—Solubilization of connexons with detergent was performed as described previously (17–20) with a few modifications. Briefly, cultured cells were harvested in PBS (pH 7.4) containing mini EDTA-free buffer containing 75 mM sodium chloride, and 80 mM octylglucoside. A fifth wash was performed using PBS, heated at 37 °C for 40 min to fuse liposomes into planar bilayers, rinsed again five times with PBS, and finally layered with imaging buffer containing 5 mM Tris (pH 7.4) and 50 mM sodium chloride. The concentration of free calcium in reagents was measured by comparison to a standard curve generated using 1–10 µM calcium chloride solution made from anhydrous reagent using the calcium-sensitive probe Fura-2 (Invitrogen) and an analytical spectrophotometer (FluoroMax-3, Horiba Scientific, NJ).

**AFM Imaging and Data Analysis**—The AFM measurements were carried out in the tapping mode using a Multimode Nanoscope IIIa (Veeco Instruments, Santa Barbara, CA) using oxide-sharpened silicon nitride cantilevers with nominal spring constants of 0.32 Newton/m and 0.06 Newton/m (NPS-TT, Veeco Probes, Santa Barbara, CA). To generate histograms of the AFM protrusion height measurements, curve fitting was performed using Origin 8.5 data analysis and graphing software (OriginLab, Northampton, MA).

**RESULTS**

To produce Cx40 hemichannels needed for reconstitution into lipid bilayers and subsequent AFM imaging experiments, we expressed this protein by transfection of HeLa cells. The 100,000 × g supernatant of a cellular homogenate solubilized with 80 mM octylglucoside contained abundant Cx40 (“starting material” in Fig. 1, A and B). Cx40 hemichannels were affinity-purified using a strategy similar to the one we utilized previously to isolate connexons formed of various connexins (19, 20). The affinity purification of Cx40 was facilitated by incorporation of an HA tag after its C terminus that allowed binding to and elution from beads coupled to anti-HA antibodies. The

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nals (9, 10). Moreover, high concentrations (~1–2 mM) of calcium ions in the external medium may close non-junctional gap junction hemichannels (Cx43 (11), Cx46 (12, 13), and Cx26 (13)). Previous AFM studies showed Ca^{2+}-dependent structural changes of the extracellular faces of Cx26 and Cx43 connexons (8, 14). In the current study, we have observed real-time, conformational changes of the cytoplasmic (as well as the extracellular) faces of individual Cx40 connexons in different ionic environments.
results of this purification were monitored by SDS-PAGE followed by immunoblotting (Fig. 1A) and by detection of all proteins by silver staining (B). Analysis of the wash fractions and eluate showed that the Cx40 was essentially purified to homogeneity.

Cx40 hemichannels were reconstituted into DOPC liposomes and then deposited onto cleaved mica surfaces. Height profile measurements of AFM images showed that the thickness of the supported lipid bilayers was $\approx 5$ nm (Fig. 2A, inset), consistent with the expected thickness of a single DOPC bilayer. Separate experiments showed that the bilayer could be dissected by the AFM probe at high force, leaving a square depression, and the measured thickness of the surrounding lipid in these preparations was also consistent with that of a single bilayer (data not shown).

Although a bilayer containing only DOPC exhibited a smooth and featureless surface (Fig. 2A), a bilayer formed from DOPC containing reconstituted hemichannels exhibited a relatively “bumpy” surface containing randomly distributed protrusions (B). The root mean squared roughness for a pure DOPC single lipid bilayer measured 0.217 nm, whereas that of a DOPC single lipid bilayer containing reconstituted hemichannels measured 1.369 nm (over a $250 \times 250$ nm scan size). The density of the protrusions increased in proportion to the amounts of connexons reconstituted into the DOPC, confirming that they corresponded to the added Cx40 hemichannels (data not shown). We developed preparations that consistently resulted in hemichannels inserted fully into the bilayer as evidenced by the vast majority of “bumps” exhibiting protrusion heights of 2–5 nm above the plane of the surrounding bilayer.

Our examination of bilayers containing Cx40 connexons indicated the presence of two different populations of oligomeric particles that protruded above the monolayer with differing heights (examples are indicated by red and green circles in Fig. 3A). Fig. 3B shows a representative section analysis plot (a single-line AFM profile trace) in which connexons of the two different height classes can be clearly distinguished. Height analysis of 244 oligomeric particles from four different prepa-
rations is summarized in the histogram shown in Fig. 3. These data could be fit quite well with two Gaussian distributions with peaks at 2.56 ± 0.02 nm and 4.15 ± 0.03 nm (mean ± S.E.). A small number of connexons had protrusion heights that exceeded 6 nm (Fig. 3, A and C). We interpret these particles as corresponding to unincorporated or stacked/docked connexons (dodecamers) and large aggregates. We excluded those particles from further analysis.

In a previous AFM study, detection of binding of antibodies that recognize specific connexin domains demonstrated that short and tall protrusions represented the extracellular and cytoplasmic faces of Cx43 connexons (16). Because Cx40 has a similar topology to Cx43 with substantially more cytoplasmic than extracellular mass, we anticipated that the short and tall particles observed in this study also represented the extracellular and cytoplasmic orientations of Cx40 connexons, respectively.

We tested these predictions about the identities of particles with different heights by studying reconstituted connexons produced after expression of a Cx40 construct (Cx40tr248) that had been truncated to eliminate most of its carboxyl-terminal cytoplasmic domain (missing the last 109 amino acids). Bilayers with reconstituted Cx40tr248 contained only connexons with rather similar heights (green circles in Fig. 3D and height profile scan in E). Fig. 3F shows a histogram containing the height measurements of 100 Cx40tr248-containing particles. These data were fit with a single Gaussian distribution with a peak at 2.49 ± 0.03 nm (mean ± S.E., n = 100).

Comparison of the heights of Cx40 and Cx40tr248 connexons suggests that the tall and short objects in Fig. 3, A–C represent wild-type Cx40 connexons with protruding faces from their cytoplasmic and extracellular regions, respectively, because removal of the large cytoplasmic carboxyl-terminal domain eliminated the tall population. They also suggest that the C-terminal domain contributes ~1.6 nm in height to the connexon.

We initially performed AFM imaging on the reconstituted Cx40 hemichannels in a solution corresponding to that used for the isolation and purification (5 mM Tris (pH 7.4) and 50 mM sodium chloride). Although we added no divalent cations to this solution, our measurements suggest that it contained low...
concentrations of calcium (\(< 10 \mu M\)). Some of the Cx40 connexons appeared as relatively featureless protrusions (Figs. 3A and 4A). However, many of the cytoplasmic (30%) and extracellular (65%) faces exhibited depressions of their surfaces consistent with openings. Examples of “closed” and “open” connexons are shown in Fig. 4, B–E. The rim diameters of the entrances of the extracellular and cytoplasmic surfaces were 3.2 \(\pm 0.3\) nm (mean \(\pm\) S.E., \(n = 13\)) and 3.1 \(\pm 0.5\) (\(n = 7\)), respectively.

Unlike previous studies of another connexin with a long C-terminal cytoplasmic domain (Cx43), we were able to observe detailed topographic features of the cytoplasmic surface of some Cx40 connexons (Fig. 4, A, C, and E). In some cases, a hexameric structure was clearly evident (Fig. 4E).

Because addition of high concentrations of calcium ions has previously been observed to lead to the closure of non-junctional gap junction hemichannels in a variety of different systems as well as conformational changes of Cx26 (14) and Cx43 (8) connexons as detected by AFM, we examined the appearance of Cx40 connexons after addition of 3.6 mM \(Ca^{2+}\) (Fig. 4F). Under these conditions, most of the hemichannels appeared closed (Fig. 4, F–H). A minority showed some evidence of a surface opening. A few connexons contained central depressions of similar dimensions to those seen in low calcium (Fig. 4, I and J).

When the residual divalent cations were chelated by imaging in imaging buffer containing 0.1 mM EDTA, the connexons appeared strikingly different (Fig. 4K). Discrete subunits were visible in the hemichannels, and they contained large central openings (Fig. 4, L and M). Similar conformational changes were observed after addition of EDTA concentrations as low as 25 \(\mu M\). We observed central surface openings in 100% of extracellular and cytoplasmic connexons with entrance diameters of 5.6 \(\pm 0.2\) (\(n = 24\)) and 5.8 \(\pm 0.2\) (\(n = 21\)) nm, respectively.

We examined this apparent divalent cation-dependent conformational change of Cx40 connexons by imaging individual connexons in the presence of 1.0 mM EDTA and then again within 10 min. after addition of 3.6 mM \(CaCl_2\) to the AFM liquid chamber. Three examples of the observed conformational changes are shown in Fig. 5. When images were acquired in EDTA-containing solution, connexons viewed from either the cytoplasmic (boxes in Fig. 5, A and C) or extracellular face (box in E) had distinct central surface openings. Addition of
calcium led to loss of these openings (Fig. 5, B, D, and F). Height profiles (Fig. 5, insets) confirmed these surface depressions, and they showed some increase in heights of both cytoplasmic and extracellular protrusions for the examples shown (from 4.50 to 4.90 nm, A to B; 3.80 to 4.50 nm, C to D; and 2.95 to 3.27 nm, E to F. This experiment also confirmed the reversibility of the EDTA-induced effects; reversible because examination of \( \geq 20 \) connexons in each orientation showed that \( > 75\% \) of both surfaces were closed by readdition of \( \text{Ca}^{2+} \).

**DISCUSSION**

In this study, we used AFM to produce the first structural data regarding connexons containing Cx40, a gap junction protein that is present in a variety of cells and tissues (such as atrial cardiac myocytes and endothelial cells). Our observations complement and extend prior observations made in studies of other connexins. As in a prior study of Cx43, we detected two different groups of Cx40 hemichannels with differing protrusion heights above the bilayer. The Cx40 monomer has a large C-terminal cytoplasmic tail. Therefore, the cytoplasmic region of Cx40 hemichannels should have a much greater exposed mass of protein than the extracellular face. In prior AFM studies of Cx43 connexons reconstituted into bilayers, binding of domain-specific antibodies was used to demonstrate that the tall connexons represented ones with exposed cytoplasmic regions (15). Extrapolation to Cx40 (which has a similar sequence, topology, and domain lengths to Cx43) suggests that the tall connexons had protruding cytoplasmic faces and the short ones had protruding extracellular faces. This hypothesis was confirmed when we observed that particles containing C-terminally truncated Cx40 only contained a single population of short (2.5 nm) connexons. This observation also suggests that the C-terminal tail of Cx40 contributes \( \sim 1.6 \text{ nm} \) in height to the cytoplasmic extramembranous protrusion of this connexon.
We have made two major novel findings. We obtained architectural details of the subunit organization on the cytoplasmic face of a connexon with a large cytoplasmic face, and we observed calcium-dependent conformational changes of both faces of the connexons. We observed changes between very low calcium concentrations and physiologic (or supraphysiologic) concentrations, and we observed changes in response to chelation of any residual divalent cations.

When scanned in solutions containing low (< 10 μM) Ca\(^{2+}\), 30% of the cytoplasmic connexon faces and 65% of the extracellular faces showed evidence of a central depression or surface entrance with average diameters of 3.1 and 3.2 nm, respectively. Although these measurements are consistent with previous AFM and electron diffraction assessments of the surface entrances to the pores formed by other connexins (8, 14), they are wider than the diameter of the channels’ interior pores buried deeper within the bilayer. Our interpretation is that the surface openings measured by AFM are formed by the extracellular portions of the hemichannels and that the functional pores and their size dimensions remain inaccessible to the finite AFM tip. Our measurements of the sizes of the pores had a rather high variability between connexons (S.E., 0.3–0.5 nm), likely reflecting the substantial flexibility of the structure adjacent to the hemichannel pore in response to the low imaging forces of AFM, as has been suggested previously (14, 22, 23).

After addition of Ca\(^{2+}\), most of the Cx40 hemichannels were closed, and very few of the channels had definite surface openings (viewed from either face). This change is consistent with conformational changes that may accompany the Ca\(^{2+}\)-dependent channel closure that has previously been observed for many different kinds of gap junction channels and hemichannels (9–13). It may result from movement of the individual connexins within the membrane. Electron micrographic studies of isolated rat liver gap junctions have shown two alternative configurations of subunits for preparations isolated in the presence of calcium ions as compared with ones from which calcium ions were removed by dialysis or chelation (24, 25). These observations were used to suggest that Ca\(^{2+}\) ions might cause a rotation of the subunits that closed the pore in a manner analogous to the closing of a camera iris. We observed apparent conformational changes of both cytoplasmic and extracellular faces of the Cx40 connexons. Such structural changes would not have been expected in the rigidly structured extracellular regions of complete gap junction channels (3). However, as previously suggested, it appears that the extracellular domains of these undocked (and dispersed) hemichannels are structurally different from docked hemichannels (contained within gap junctional plaques) (7, 8). Indeed, physiological studies of connexin hemichannels expressed in Xenopus oocytes have shown Ca\(^{2+}\)-dependent gating that was attributed to calcium at or near both extracellular and cytoplasmic aspects of the channel (26, 27).

When divalent cations were chelated from our preparations by addition of EDTA, all of the hemichannels assumed an open conformation. In the presence of EDTA, we observed that the surface openings within the cytoplasmic or extracellular faces dilated by 87 and 75%, respectively. These conformational changes are larger and lead to wider dimensions than were previously observed in studies of the pores or surface openings of other connexins. They reveal an astonishingly high degree of ion-dependent structural flexibility of these Cx40 hemichannels that were randomly dispersed within the bilayer and free of the steric constraints that exist in close-packed two-dimensional crystalline arrangements of some previously studied hemichannels (14). These large “openings” suggest that the extramembranous domains can form a surface entrance to the hemichannels. They also suggest that divalent cations can contribute to conformational changes of the extramembranous portions of the connexin in a manner that reduces the size (or accessibility) of the entrance. These structural changes may relate to alterations in hemichannel function because several investigators who have detected connexin hemichannels (using dye uptake, ATP release, or voltage clamp) have included EDTA or EGTA in their extracellular bath solutions (11, 28–31).

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