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Functional and Morphological Correlates of Connexin50 Expressed in Xenopus laevis Oocytes

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Abstract. Electrophysiological and morphological methods were used to study connexin50 (Cx50) expressed in Xenopus laevis oocytes. Oocytes expressing Cx50 exhibited a new population of intramembrane particles (9.0 nm in diameter) in the plasma membrane. The particles represented hemichannels (connexin hexamers) because (a) their cross-sectional area could accommodate 24 ± 3 helices, (b) when their density reached 300–400/μm², they formed complete channels (dodecamers) in single oocytes and assembled into plaques, and (c) their appearance in the plasma membrane was associated with a whole-cell current, which was activated at low external Ca²⁺ concentration ([Ca²⁺]₀), and was blocked by octanol and by intracellular acidification. The Cx50 hemichannel density was directly proportional to the magnitude of the Cx50 Ca²⁺-sensitive current. Measurements of hemichannel density and the Ca²⁺-sensitive current in the same oocytes suggested that at physiological [Ca²⁺]₀ (1–2 mM), hemichannels rarely open. In the cytoplasm, hemichannels were present in ~0.1-μm diameter “coated” and in larger 0.2–0.5-μm diameter vesicles. The smaller coated vesicles contained endogenous plasma membrane proteins of the oocyte intermingled with 5–40 Cx50 hemichannels, and were observed to fuse with the plasma membrane. The larger vesicles, which contained Cx50 hemichannels, gap junction channels, and endogenous membrane proteins, originated from invaginations of the plasma membrane, as their lumen was labeled with the extracellular marker peroxidase. The insertion rate of hemichannels into the plasma membrane (80,000/s), suggested that an average of 4,000 small coated vesicles were inserted every second. However, insertion of hemichannels occurred at a constant plasma membrane area, indicating that insertion by vesicle exocytosis (60–500 μm² membranes/s) was balanced by plasma membrane endocytosis. These exocytic and endocytotic rates suggest that the entire plasma membrane of the oocyte is replaced in ~24 h.

Key words: connexins • hemichannels • gap junctions • exocytosis • endocytosis

Introduction

A family of integral membrane proteins, called connexins, forms specialized cell–cell channels that serve as intercellular pathways of chemical and electrical information transmission. The cell–cell channel is assembled from the docking of two hemichannels (i.e., connexin hexamers) located in the plasma membrane of adjacent cells. This mechanism of cell–cell channel assembly suggests that there may be pools of hemichannels in the plasma membrane of participating cells. Although the physiological significance of undocked hemichannels in cell permeability and homeostasis is unclear, evidence has accumulated that hemichannels can function independently as non- or cation-selective, large conductance ion channels whose activity is dependent on the extracellular calcium concentration and intracellular pH (DeVries and Schwartz, 1992; Ebihara, 1996). Cloning of the connexins, together with the introduction of efficient expression systems such as Xenopus laevis oocytes, opened new experimental avenues to study connexin hemichannels. While the expression of all connexins in oocytes leads to the formation of functional cell–cell channels by paired oocytes (e.g., Werner et al., 1985; Dahl et al., 1987; Steiner and Ebihara, 1996; Wang and Peracchia, 1998), only a few connexins form functional hemichannels in the plasma membrane (Paul et al., 1991; Ebihara and Steiner, 1993; Gupta et al., 1994; Ebihara et al., 1995; Ebihara, 1996; Trexler et al., 1996).

Here, we have combined electron microscopic and electrophysiological methods (Zampighi et al., 1995) to study mouse connexin50 (Cx50) expressed in Xenopus oocytes. Our approach combines hemichannel functional assays and the determination of the number of hemichannels in the plasma membrane of the same cell to assess the contribution of these channels to cell conductance. We show that expression of Cx50 in oocytes led to the insertion of functional hemichannels into the plasma membrane. Measurements of the total...
number of hemichannels in the plasma membrane and the Cx50 current in the same oocytes suggested that hemichannels rarely open at physiological calcium concentrations. When the density of hemichannels in the plasma membrane reached 300–400/μm², they formed complete channels (dodecamers) and assembled into gap junctional plaques. The morphological studies further allowed the identification of hemichannels in vesicles shuttled to and from the plasma membrane. In the cytoplasm, hemichannels were present in small (~0.1-μm diameter) "coated" and in larger (0.2–0.5-μm diameter) vesicles. The coated vesicles fused with the plasma membrane, whereas the larger vesicles originated from endocytosis of the plasma membrane. Trafficking of Cx50 hemichannels took place without changing the area of the plasma membrane, indicating that the rate of vesicles insertion into the plasma membrane was balanced by the rate of vesicle retrieval.

**Materials and Methods**

**Oocytes**  
*Mature Xenopus laevis* oocytes were injected with 50 nl distilled water or 50 nl 1 μg/μl mouse Cx50 cRNA (White et al., 1992) and incubated at 18°C in Barth’s medium (mM: 88 NaCl, 1 KCl, 0.33 Ca(NO₃)₂, 0.41 CaCl₂, 0.82 MgSO₄, 2 NaHCO₃, and 10 HEPES/Tris, pH 7.4) containing 0.1 mg/ml gentamicin (Parent et al., 1992; Zampighi et al., 1995).

**Electrophysiology**  
Electrophysiologically experiments were performed with a two-electrode voltage-clamp technique (Loo et al., 1993). Oocytes were placed in a NaCl buffer (mM: 100 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, and 10 HEPES/Tris, pH 7.5) and the membrane potential was held at −50 mV. The whole-cell Cx50 currents were induced by varying the concentration of external calcium ([Ca²⁺]ₒ). The Ca²⁺ concentration was adjusted as desired by adding appropriate amounts of CaCl₂ and EGTA (Findlay et al., 1985). To obtain current–voltage (I–V) relations, the pulse protocol consisted of 1,000-ms voltage steps from a holding potential (Vₜ) of −50 mV to a series of test voltages from +50 to −150 mV in 20-mV decrements (pCLAMP; Axon Instruments). The voltage clamp had a settling time of 0.2–0.8 ms. Currents were low-pass filtered at 500 Hz, and sampled at 1 ms per point. Membrane capacitance measurements were used to estimate the total surface area of the plasma membrane (using 1 μF/cm²; see Loo et al., 1993; Zampighi et al., 1995). The capacitance was determined by applying 10-20-mV test pulses (Vₜ) of 30–100-ms duration from the holding potential (−50 mV). At each test voltage, the charge (Q) was obtained by integration of the capacitive transient. In both control and Cx50-expressing oocytes, capacitive transients showed a single time constant (~1 ms), and Q was a linear function of the voltage step (Vₜ − Vₜ). The slope of the Q versus (Vₜ − Vₜ) relation was the membrane capacitance Cₘ. In oocytes expressing Cx50, capacitance measurements were made at 3 mV [Ca²⁺]ₒ, to avoid hemichannel activation. Membrane conductance was obtained as either the slope of the steady state I-V relationship or, where indicated, as a chord conductance (e.g., see Fig. 9). The reversal potential (Vₑ) was determined by subtracting the endogenous currents at 5 mM [Ca²⁺]ₒ. All experiments were performed at 21 ± 1°C.

**Fixation**  
After functional measurements, oocytes were fixed at room temperature and prepared for morphological studies (Zampighi et al., 1995). The fixative solution contained 3–3.5% glutaraldehyde in 0.2 M Na-cacodylate (pH 7.35).

**Thin Sectioning**  
Oocytes were postfixed in 1% OsO₄ in 0.2 M Na-cacodylate buffer for 90 min at room temperature. They were washed in 0.1 M Na-acetate buffer (pH 5.0) and block-stained in 0.5% uranyl acetate in 0.1 Na-acetate buffer (pH 5.0) overnight at 4°C. They were dehydrated in ethanol, passed through propylene oxide, and embedded in Polybed 812. Sections were cut in a Sorval MT5000 ultramicrotome (Dupont), collected on single-hole formvar-coated grids, and stained with uranyl acetate and lead (Zampighi et al., 1988).

**Peroxidase**  
Control oocytes and oocytes injected with cRNA were incubated for 1 h in Barth’s solution containing 0.5–1.5 mg/ml of peroxidase (Sigma Chemical Co.), and were then fixed in 3–3.5% glutaraldehyde in 0.2 M Na-cacodylate (pH 7.35) for 2 h at room temperature. They were washed and incubated in 0.1 mg/ml diaminobenzidine in 0.1 M Tris/ HCl (pH 7.5) and H₂O₂, and then prepared for thin sectioning.

**Freeze Fracture**  
Fixed oocytes were infiltrated with 25% glycerol in 0.2 M Na-cacodylate (pH 7.35) for 1 h (21 ± 1°C). Each oocyte was cut into six to eight small pieces (~1 mm³) to obtain replicas from different regions of the surface of the oocyte. The pieces were placed on Balzers specimen holders with the external surface (i.e., the vitelline membrane) facing upward. This orientation yielded extensive P face areas of the plasma membrane, which allowed us to determine the density and size of the newly inserted particles (Zampighi et al., 1995; Eskandari et al., 1998). The specimens were frozen by immersion in liquid propane maintained just above freezing in a liquid nitrogen bath. The frozen specimens were transferred to a Balzers 400 K freeze-fracture-etch apparatus, fractured at either ~150° or ~120°C at ~10⁻³ mbar of partial pressure. The fractured surfaces were coated with platinum at 80°C and carbon at 90°C. Replication at 80°C decreased the length of the shadow of the particle, which greatly facilitated the density quantification and the measurement of the particle diameter. The specimens were coated with 0.5% collagen in amyl acetate to avoid fragmentation of the replica, and cleaned in a solution of bleach to dissolve the organic material. The replicas were washed in distilled water and deposited on formvar-coated, single-hole copper grids. The collagen was removed by immersion in amyl acetate (Zampighi et al., 1988).

**Sampling**  
The large area of the oocyte plasma membrane (~3 × 10⁴ μm²) introduced sampling difficulties since only a small percentage of the area (6–8 × 10³ μm²) was imaged and used for the determination of the particle density. To address this, P faces collected from three to four replicas from different regions of the plasma membrane of each oocyte were imaged at 25,000×. Approximately 100 negatives were collected from each replica. From this pool, ~20 negatives per replica free of optical aberrations and containing an area of 1–2 μm² of uninterrupted P fracture face were printed and used for quantification (Table I). Altogether, the data were obtained by studying over 1,000 negatives from
particles covered by plaques per 100 clecs was estimated using two hemichannels.

Steady state properties of Cx50 hemichannels. The density of endogenous P face particles in the plasma membrane of control oocytes was similar to that of other regions of the plasma membrane. The number in parentheses corresponds to the number of P face areas used in the quantification.

The images were enlarged to 75,000×, digitized, and analyzed using IMAGE software (National Institutes of Health). In each image, we measured the area of the P face and the total number of particles contained in that area. The particle density in the microvilli plasma membrane was quantified independently to ensure that it was similar to that of other regions of the plasma membrane. The diameter of the intramembrane particles was measured perpendicular to the direction of the shadow directly from negatives using a Profile Projector (6C; Nikon Inc.) at a final magnification of 250,000× (Eskandari et al., 1998).

The number of hemichannels in gap junction plaques (n gj) was estimated using n gj = K A1 2d, where K is the fractional area covered by plaques per 100 μm² of plasma membrane, A1 is the total area of the plasma membrane calculated from the capacitance, d is the mean density of particles in plaques (9.196 ± 694/μm²), and 2 because each gap-junction particle is composed of two hemichannels.

Resolution of the Method Used to Determine Particle Density

The density of endogenous P face particles in the plasma membrane of control oocytes was 196 ± 9 particles/μm² (mean ± SEM, n = 8, n refers to the number of P face regions from which the particles were sampled). To detect the expression of Cx50, the particle density had to increase at least to 250/μm²; i.e., 54 particles/μm² (two SDs) above the mean particle density of control oocytes, which represents a confidence level of ~98%. The highest density at which each particle could be resolved without interference from the shadows of neighboring particles was ~10,000 particles/μm² (approached in gap junctional plaques).

Results

Electrophysiology

Steady state properties of Cx50 hemichannels. The electrophysiological properties of Xenopus laevis oocytes injected with Cx50 cRNA were dependent on [Ca²⁺]o. When measured 3 d after injection, in 1 mM external Ca²⁺, the resting membrane potential of Cx50-expressing oocytes was significantly different from that of control oocytes. Control oocytes had a resting potential of −50 ± 5 mV (mean ± SEM, n = 5) and a membrane conductance of 1.7 ± 0.2 μS (n = 5), while the resting potential of oocytes injected with Cx50 cRNA was −33 ± 2 mV (n = 7), and the membrane conductance was 7.2 ± 0.8 μS (n = 7). When the [Ca²⁺]o was elevated to 5 mM, the resting membrane potential and conductance of Cx50-expressing oocytes were restored to those of the controls (not shown).

The dependence of the membrane conductance of oocytes expressing Cx50 on [Ca²⁺]o was studied under voltage-clamp conditions. Fig. 1 A (top) shows a continuous current record from an oocyte expressing Cx50 and maintained at a holding potential (Vh) of −50 mV. Reduction in [Ca²⁺]o from 1 mM to 10 μM induced an inward current of ~1,450 nA. This inward current was reversibly blocked (~65%) by intracellular acidification by addition of acetate (50 mM equimolar Na-acetate replacement for NaCl, pH 7.5) to the bathing medium. The current returned to baseline after restoration of [Ca²⁺]o to 1 mM.

The response of control oocytes to the same experimental protocol is illustrated in Fig. 1 A (bottom). Reduction of [Ca²⁺]o from 1 mM to 10 μM induced a small outward current (9 ± 3 nA, n = 27), which was insensitive to intracellular acidification. This experiment was repeated in more than 70 control oocytes, and in none was there a Ca²⁺-sensitive current as that detected in the Cx50-expressing cells. This suggests the absence of endogenous connexin hemichannels in our control oocytes.

A feature of gap junction channels is their inhibition by octanol, heptanol, and halothane (Johnston et al., 1980; for review, see Spray and Burt, 1990). We tested the effect of 1-octanol on the currents activated by low [Ca²⁺]o. Fig. 1 B shows a current trace from an oocyte
Expression of Connexin50 in Oocytes

held at −50 mV. Reduction in \([\text{Ca}^{2+}]_o\) to 10 μM led to an inward current of ~900 nA that was reversibly inhibited by ~95% by addition of 1 mM octanol to the bathing medium. Octanol had no effect on the holding current of control oocytes (not shown).

We further studied the \(\text{Ca}^{2+}\) sensitivity of the inward current at −50 mV (Fig. 1 C). From 10^{-2} to 10^{-6} M \([\text{Ca}^{2+}]_o\), there were small incremental increases in the holding current; however, when \([\text{Ca}^{2+}]_o\) was lowered to 10^{-7} M, the current increased by ~3.3-fold (Fig. 1 C). In \(\text{Ca}^{2+}\)-free solutions (10 mM EGTA and 0 \(\text{Ca}^{2+}\)), the Cx50 currents were too large for reliable control of the membrane potential.

Voltage dependence of Cx50 hemichannels. In control oocytes, when the membrane potential was stepped from the holding (−50 mV) to test voltages, the capacitive transient decayed to a steady state with a time constant of ~1 ms (Fig. 2 A; Parent et al., 1992). This relaxation was independent of the \([\text{Ca}^{2+}]_o\) (data not shown). The steady state I–V relations in 5 mM or 10 μM external \(\text{Ca}^{2+}\) were similar (Fig. 2 A). Membrane conductances in 5 mM and 10 μM external \(\text{Ca}^{2+}\) in the oocyte in Fig. 2 A were 1.2 ± 0.1 and 1.3 ± 0.1 μS.

The current response of Cx50-expressing oocytes after step changes in membrane potential depended on \([\text{Ca}^{2+}]_o\) (Fig. 2 B). In 5 mM \([\text{Ca}^{2+}]_o\), the currents were not appreciably different from control oocytes, and the conductance was 2.4 ± 0.1 μS. In 10 μM \([\text{Ca}^{2+}]_o\), large Cx50 currents resulted that, after the initial capacitive transient, decayed slowly to a steady state (Fig. 2 B). At steady state, the inactivation was dependent on \([\text{Ca}^{2+}]_o\) (not shown) and the magnitude of the voltage step. It was most prominent at low \([\text{Ca}^{2+}]_o\) and at the largest voltage steps (Fig. 2 B). At the smallest voltage steps, there was no apparent inactivation as the relaxation of the current transient was not appreciably different from that of the capacitive transient, but at the largest voltage steps (~100 mV), the time constant of inactivation ranged from 220–300 ms.

The Cx50 I–V relationships were measured at 10 \(I_{\text{initial}}\) and 980 \(I_{\text{steady state}}\) ms after the onset of the voltage pulse (Fig. 2 C). \(I_{\text{initial}}\) was proportional to the size of

![Figure 1](image.png)

**Figure 1.** Cx50 hemichannels are sensitive to \([\text{Ca}^{2+}]_o\), intracellular pH, and octanol. (A) In a Cx50-expressing oocyte voltage-clamped at −50 mV (top), reduction of \([\text{Ca}^{2+}]_o\) from 1 mM to 10 μM induced a large inward current \(I_{\text{Cx50}}\). Intracellular acidification by the addition of 50 mM Na-acetate to the bathing medium resulted in an ~65% reversible inhibition of \(I_{\text{Cx50}}\). In control oocytes (bottom), reduction of \([\text{Ca}^{2+}]_o\) or intracellular acidification had no appreciable effect on the current. (B) \(I_{\text{Cx50}}\) was inhibited by addition of 1 mM 1-octanol to the bathing medium. (C) The magnitude of \(I_{\text{Cx50}}\) depended on \([\text{Ca}^{2+}]_o\). Measurements were made 48 h after cRNA injection.
the voltage step, resulting in a near linear I–V relationship. $I_{\text{steady-state}}$ deviated from linearity and at the extreme voltage steps ($-150$ to $-90$ mV and $+30$ to $+50$ mV), the conductance was negative. Membrane conductance was $41 \pm 1 \mu S$ for $I_{\text{initial}}$ ($-150$ to $+50$ mV) and $35 \pm 1 \mu S$ for $I_{\text{steady-state}}$ ($-70$ to $+30$ mV). Both $I_{\text{initial}}$ and $I_{\text{steady-state}}$ reversed at the same membrane potential ($-25$ mV), indicating that they were carried by the same ionic species. Equimolar (100 mM) replacement of external Na$^+$ with choline shifted $V_{\text{rev}}$ for both currents to more negative values (approximately $-35$ mV). $V_{\text{rev}}$ was not significantly affected by replacement of external Cl$^-$ with gluconate or Mes (not shown) or by variations in $[\text{Ca}^{2+}]_o$ (Fig. 2 D).

Therefore, in oocytes expressing Cx50, the functional and pharmacological properties of the current induced at low $[\text{Ca}^{2+}]_o$ were consistent with the expression of a functional connexin hemichannel and, henceforth, this current will be referred to as $I_{\text{Cx50}}$.

Time course of Cx50 expression and membrane capacitance. $I_{\text{Cx50}}$ was used as a functional assay of the presence of Cx50 in the plasma membrane. The magnitude of $I_{\text{Cx50}}$ was dependent on the length of time after cRNA injection (Fig. 3 A). $I_{\text{Cx50}}$ appeared as early as $\sim 3$ h after injection (data not shown), increased steadily up to the third day and, thereafter, decreased (Fig. 3 A, ●). Although this time course was very reproducible, the magnitude of $I_{\text{Cx50}}$ was dependent on the amount of cRNA injected and the frog from which the oocytes were harvested. Control oocytes ($V_h = -50$ mV) did not exhibit a Ca$^{2+}$-sensitive current over this time course (Fig. 3 A, ○).
The membrane capacitance ($C_m$) of oocytes injected with Cx50 cRNA was not different from that of control oocytes up to 5 d (300–600 nF). In both groups of oocytes, $C_m$ remained unchanged up to the third day and decreased thereafter (Fig. 3 B). Thus, Cx50 expression occurred without altering the plasma membrane area.

**Electron Microscopy**

Thin-section studies of oocytes expressing Cx50. We focused on the organization of the plasma membrane and the neighboring protoplasmic region (“cortical” region). The plasma membrane of control oocytes contained numerous microvilli, folds, and invaginations that increased its surface area by approximately ninefold compared with a smooth spherical cell of similar diameter (Zampighi et al., 1995). The cytoplasm of the cortical region contained secretory granules closely associated with the plasma membrane (labeled “CG” in Fig. 4 A), small (~0.1 μm) and large (0.2–0.5 μm) diameter vesicles, mitochondria, and endoplasmic reticulum. Oocytes expressing high levels of Cx50, as assessed from the magnitude of $I_{Cx50}$ and the density of the plasma membrane particles (see below), exhibited gap junctions that appeared as pentalamellar structures 16–18 nm in overall thickness (Figs. 4 B and 5 A). The gap junctions were either continuous (“reflective”) or discontinuous (“annular”) with the plasma membrane. Reflective gap junctions were formed by the association of the plasma membrane with a microvillus or a fold (Fig. 4 B), or in membrane invaginations (Fig. 5 A, closed arrow). Annular gap junctions appeared as vesicles in the cytoplasm and most likely originated from invaginations of the plasma membrane, which later pinched off into the cytoplasm (Fig. 4 B, △, and Fig. 5 A).

Freeze-fracture micrographs of the reflective and annular gap junctions revealed the P face of the plasma membrane contained plaques of particles, and the complementary E (external) face contained the corresponding pits (Fig. 5 B). The plaques contained Cx50 hemichannels assembled into gap junctions. The height of the fracture step separating the junctional membranes (E-to-P transition, Fig. 5 B, open arrows) was 6–7 nm, and the extracellular space between the two membranes was 1–2 nm, which is consistent with that found in gap junctions. Annular gap junctions appeared as concave and convex surfaces with plaques of particles and complementary pits also separated by fracture steps of small height (Fig. 5 C).

Gap junctions were not observed in control oocytes (Fig. 4 A), in oocytes expressing low levels of Cx50 (<300 P face particles/μm²), or in oocytes over-expressing other heterologous membrane proteins (in the absence of Cx50), such as the Na⁺/glucose cotransporter (SGLT1), occludin, aquaporin-0, aquaporin-1, or Shaker K¹ channel. We have observed gap junction formation in oocytes expressing connexin46 (Zampighi, unpublished observations).

Freeze-fracture studies of oocytes expressing Cx50. In thin sections, oocytes expressing high levels of Cx50 were identified by the presence of gap junctions, which appeared 48–72 h after cRNA injection (Figs. 4 B and 5 A). In freeze fracture, expression of Cx50 was evident ~24 h after cRNA injection by the appearance of a distinct population of intramembrane particles in the
The newly inserted (Cx50) particles increased the density of particles in the P face, and the Cx50 particle density was correlated with the magnitude of ICx50 (see Figs. 8 and 9). To characterize the particles that appear after the expression of Cx50, we compared the size and shape of the P face particles in control oocytes and those expressing Cx50 using frequency histograms. In the P fracture face of control oocytes, ~93% of the endogenous proteins had a mean diameter of ~7.5 nm, and the rest had a mean diameter of ~11 nm (Eskandari et al., 1998). The density of the P face particle was 196 ± 6 particles/μm² (mean ± SEM, n = 8). The complementary E face of the plasma membrane of control oocytes contained ~13-nm particles at a density approximately four times higher than the particle density in the P face (886 ± 36 particles/μm²). The density of the E face particles did not change after Cx50 expression.

The size (diameter) frequency histograms of the particles in oocytes expressing Cx50 exhibited a population of 9.0 ± 0.4 nm (mean ± SEM, n = 544) diameter particles (Fig. 7 A), in addition to the endogenous integral membrane proteins (hatched region in Fig. 7 A, and a smaller population at 11 nm). The newly inserted particles exhibited a symmetrical cross-sectional geometry that contrasted sharply with the size and shape of the endogenous particles (Fig. 6, A–G). When the density of the newly inserted particles reached 300–400/μm², they formed gap junction plaques (see below). Size frequency histograms also showed that the particle in the gap junction plaques exhibited the same size and shape as the newly inserted particle population (Fig. 7 B). Therefore, expression of Cx50 induced a population of intramembrane particles of distinct size and shape (hexamers), which assembled into plaques of complete channels (dodecamers) at a critical density.

We previously characterized the population of newly inserted P face particles induced by the expression of other heterologous membrane proteins, such as aquaporin-0, aquaporin-1, SGLT1, and opsin. Particle size
analysis showed that each protein was represented by a particle with a distinct size and shape whose cross-sectional area predicts the number of alpha helices in the transmembrane domain (Eskandari et al., 1998). An analysis of the cross-sectional area of the particle induced after the expression of Cx50 predicted that each Cx50 particle could accommodate 24 \( \pm 3 \) \( \alpha \) helices (1.4 nm\(^2\)/helix; Eskandari et al., 1998). Therefore, based on the size and cross-sectional area of the particles, and their ability to form gap junctions, we conclude that Cx50 is inserted into the plasma membrane as hemichannels (hexamers).

The density of Cx50 hemichannels was correlated with \( I_{\text{Cx50}} \). The density of Cx50 hemichannels in the P face (excluding those in gap junction plaques) was examined at various times after Cx50 cRNA injection (Fig. 8 and Table II). To relate the density measurements to functional ones, \( I_{\text{Cx50}} \) (at \(-50\) mV and 10 \( \mu \)M \([\text{Ca}^{2+}]_o\)) and \( C_m \) were also measured in the same oocytes (Table II).

Quantification of the P face particles in oocytes examined at different times after injection showed that the density of hemichannels was proportional to \( I_{\text{Cx50}} \) (Table II). At \( \sim 6 \) h after injection, the current was small (approximately \( \sim 10 \) nA) and the density of particles did not increase significantly above that of endogenous particles measured in control oocytes (Fig. 8 B). At 24 h, the magnitude of \( I_{\text{Cx50}} \) increased to 415 nA and
the hemichannel density to ~200/μm² above background (Fig. 8 C and Table II). At 48 h, ICx50 and hemichannel density increased approximately twofold (Fig. 8 D and Table II). At this level of Cx50 expression, hemichannels assembled into gap junction plaques of P face particles (Fig. 8, D and E, GJ). ICx50 and the density of Cx50 hemichannels peaked at 72 h (Fig. 8 E and Table II); however, they decreased by ~25% with longer incubation times (Table II). The initial rate of Cx50 expression (Table III) indicates that hemichannels were inserted into the plasma membrane at a rate of 80,000 copies/s.

The area of the oocyte plasma membrane was estimated from whole-cell capacitance measurements (assuming 1 μF/cm²; Table II), which together with ICx50 (at −50 mV and 10 μM Ca²⁺) obtained from the same oocyte, allowed for the calculation of the specific membrane conductance (in picosiemens per square micrometer). The specific membrane conductance plotted as a function of the Cx50 hemichannel density (per square micrometer) from the same oocytes, yielded a linear relationship, the slope (2.7 ± 10⁻³ pS) of which corresponded to the product of the open probability (Pₒ) and the single channel conductance (g) of the Cx50 hemichannel (Fig. 9).

Cx50 hemichannels assembled into gap junctions in single oocytes. Gap junction plaques were first observed 48 h after cRNA injection when Cx50 hemichannel density reached 300–400/μm² and, thereafter, occupied a fairly constant area of the plasma membrane (1.3–1.7%). At peak expression, the area covered by plaques contained 8.2–9.2 × 10⁹ hemichannels, 35–40% of the total number of Cx50 hemichannels in the plasma membrane (~2.6 × 10¹⁰; Table III).

Identification of intracellular vesicles containing Cx50 hemichannels. We took advantage of the distinct size and shape of the Cx50 hemichannel particles, as well as their ability to form gap junctions in single oocytes, to identify the vesicles involved in the insertion/retrieval pathway to and from the plasma membrane. Two types of vesicles within the cytoplasm contained hemichannels (Figs. 10–12). One was located close to the plasma membrane and to organelles such as the Golgi complex, the endoplasmic reticulum, and lysosomes (Figs. 10, A–D, and 12). These vesicles measured ~0.1 μm in diameter and contained slender projections, spaced ~20-nm apart, which protruded from the vesicle surface (Fig. 10 A, arrows). Some coated vesicles were

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Expression of Connexin50 in Oocytes

fused with the plasma membrane through a narrow neck 25–30 nm in length and a diameter ranging from 20 to 40 nm (Fig. 10, B and C). In freeze fracture, these vesicles appeared as concave and convex surfaces and, depending on the expression level, contained 5–40 Cx50 hemichannels (Fig. 10, E–I). These vesicles also contained the 13-nm endogenous particles found on the E face.

The other type of vesicle with Cx50 hemichannels exhibited a larger diameter (0.2–0.5 μm) and an irregular shape (Figs. 11 and 12). Markers of the extracellular space, such as peroxidase, showed electron-dense reaction products in the lumen of these vesicles (Fig. 13). Therefore, the larger vesicles were either connected to the extracellular space or originated from invaginations of the plasma membrane that ultimately pinched off into the cytoplasm (Fig. 11 A).

In freeze fracture, the large vesicles were characterized by the presence of Cx50 hemichannels; i.e., 9-nm particles in the P face and the complementary pits in the E face (Fig. 12). Some large vesicles contained hemichannels at a density similar to that in the gap junction plaques described in the plasma membrane (Figs. 11 C and 12, A and B), and most likely correspond to the annular gap junction seen in thin sections (Figs. 4 B and 5 A). Other large vesicles contained
hemichannels at lower densities or in small clusters located in highly curved regions of the vesicle (Fig. 12, A, C, and D). These large vesicles may correspond to early endosomes or the trans-Golgi network.

The E fracture face of the vesicles provided another important clue regarding their origin. These faces contained the complementary pits of the Cx50 hemichannels at a density similar to plaques (Fig. 12 E), or arranged in small clusters (Fig. 12 E, □). In addition to the hemichannels, the E face of the large vesicles also contained large 13-nm-diameter particles (Fig. 12 E, arrows). These E face particles correspond to a population of endogenous proteins in the plasma membrane of control oocytes (Zampighi et al., 1995). Therefore, the vesicles identified in this study contained Cx50 hemichannels intermingled with endogenous proteins of the oocyte plasma membrane, suggesting that trafficking of endogenous and heterologous proteins uses a common pathway.

discussion

Injection of Cx50 cRNA into oocytes leads to the appearance of a new population of particles (Cx50 hemichannels) in the plasma membrane, whose density is directly correlated with a whole-cell Ca$^{2+}$-sensitive current ($I_{Cx50}$). Several observations suggest that the newly inserted particles represent Cx50 hemichannels, and that $I_{Cx50}$ is in fact mediated by Cx50 hemichannels. First, the newly inserted particles exhibited a circular geometry with a diameter of 6.5 ± 0.5 nm (after correcting for the thickness of the platinum-carbon film; 2.4 nm) (Eskandari et al., 1998). The cross-sectional area, 34 ± 4 nm$^2$ ($\pi r^2$), can accommodate 24 ± 3 $\alpha$ helices (Eskandari et al., 1998). Both the particle diameter and the cross-sectional area observed here are consistent with the reported values from two-dimensional crystals of gap junction membrane channels (Unger et al., 1997), and ordered arrays of hemichannels (Perkins et al., 1997). The size and shape of the Cx50 particle suggest a hexameric hemichannel composed of four $\alpha$ helices per subunit.

Second, at a density of 300–400/μm$^2$, Cx50 particles formed reflective gap junctions in the plasma mem-

### Table III

| Oocyte* | Total area of the plasma membrane (μm$^2$) | Percent area covered by plaques | Number of hemichannels (single) | Number of hemichannels (in plaques) | Total number of hemichannels** |
|---------|---------------------------------|-------------------------------|-------------------------------|-----------------------------------|-------------------------------|
| 24      | 3.5 × 10^7                      | 0                             | 0.7 × 10^10                   | 0                                 | 0.7 × 10^10                   |
| 48      | 3.5 × 10^7                      | 1.3                           | 1.4 × 10^10                   | 8.4 × 10^9                        | 2.3 × 10^10                   |
| 72      | 3.1 × 10^7                      | 1.6                           | 1.7 × 10^10                   | 9.2 × 10^9                        | 2.6 × 10^10                   |
| 96      | 2.6 × 10^7                      | 1.7                           | 1.2 × 10^10                   | 8.2 × 10^9                        | 2.0 × 10^10                   |

*The duration of incubation after cRNA injection is given in hours. †The area of the plasma membrane was estimated from the oocyte capacitance (using 1 μF/cm²) (Table II). ‡The percentage area covered by gap junction plaques was estimated from the fraction of the area occupied by plaques in 100 μm² of P face of the plasma membrane (see Materials and Methods). §The number of single hemichannels was the product of the mean density of Cx50 P face particles times the total area of the plasma membrane. ¶The number of cell-to-cell channels in the reflective gap junctions was the product of the total area of the plasma membrane, the fractional area covered by plaques, the mean density of hemichannels in plaques, and two (see Materials and Methods). **The total number of hemichannels was the sum of the single particles plus the particles in gap junction plaques.
brane of single oocytes. This property has not been observed for endogenous proteins in control oocytes, or in oocytes expressing a wide variety of other heterologous membrane proteins.

Third, oocytes expressing Cx50 demonstrated a current (I_{C50}) that was activated at low [Ca^{2+}]_o. The properties of I_{C50} suggest that it is mediated by a nonselective cation channel. I_{C50} could be blocked by octanol or intracellular acidification, other characteristics of gap-junction channels and hemichannels (e.g., Li et al., 1996; Steiner and Ebihara, 1996; Trexler et al., 1998; Wang and Peracchia, 1998).

Finally, at low [Ca^{2+}]_o and large voltage steps, there was a time-dependent inactivation of Cx50 macroscopic currents, such that the steady state I–V relationship displayed regions of negative slope. Similar voltage-dependent characteristics have been observed for other connexin hemichannels (DeVries and Schwartz, 1992; Ebihara and Steiner, 1993; Ebihara et al., 1999).

Altogether, the functional, pharmacological, and morphological characteristics observed in Cx50-expressing oocytes point to the expression of connexin hemichannels.

Estimation of Cx50 Unitary Properties

A unique aspect of this study was that the total number of hemichannels inserted into the plasma membrane was estimated in the same oocytes in which I_{C50} was measured. This allowed us to estimate the unitary properties of Cx50 hemichannels.

observed in control oocytes. (B) Freeze-fracture view of an invagination of the plasma membrane that also ended in a round vesicle of ~100 nm in diameter. The convex E face (E) of the vesicle exhibited pits complementary to the Cx50 hemichannels, and also contained the endogenous 13 nm diameter particles (small dark arrows). (C) Freeze-fracture view of a long tubular invagination continuous with the plasma membrane. The plasma membrane P face (P) contained single as well as plaques of Cx50 hemichannels (open arrow). Similarly, the invagination contained both single hemichannels and hemichannels in plaques (dark arrows). CYT, cytoplasm. Scale bar, 0.1 μm.

Figure 11. Thin section and freeze-fracture electron microscopy of invaginations of the plasma membrane containing Cx50 hemichannels. (A) Thin section of an infolding (~0.3 μm in length) of the plasma membrane that ended in a small rounded vesicle. Regions of close membrane apposition often occurred near the plasma membrane (arrows). Similar structures were also
The increase in membrane conductance due to expression of Cx50 hemichannels ($G_{\text{Cx50}}$) is proportional to the number of hemichannels ($n$), the single hemichannel conductance ($g$), and the channel open probability ($P_o$) ($G_{\text{Cx50}} = P_o \cdot g \cdot n$). Combined freeze-fracture electron microscopy and electrophysiological measurements were used to determine the total number of hemichannels ($n$) and the membrane conductance ($G_{\text{Cx50}}$) in the same oocytes. At $-50 \text{ mV}$ and $10 \mu\text{M} \text{[Ca}^{2+}]_o$, $P_o \gamma$ for Cx50 hemichannels was $-2.7 \times 10^{-3}$ pS (Fig. 9). Using 30 pS for Cx50 single channel conductance (obtained under physiological extracellular and intracellular Na$^+$ and K$^+$ concentrations and symmetrical 100 nm [Ca$^{2+}$]; Eskandari, S., and D.D.F. Loo, manuscript in preparation), we estimate that at $10 \mu\text{M} \text{[Ca}^{2+}]_o$, Cx50 $P_o$ is $-9 \times 10^{-5}$; i.e., only $\sim 1$ in 10,000 hemichannels is open at any given time. In light of the observed Ca$^{2+}$ sensitivity of $G_{\text{Cx50}}$, we predict that Cx50 hemichannels would rarely open at physiological [Ca$^{2+}$]$_o$ (1–2 mM). These data suggest that the whole-cell conductance induced at peak expression by lowering [Ca$^{2+}$]$_o$ to $10 \mu\text{M}$ (46 μS; Table II) results from the opening of $\sim 1.5 \times 10^6$ hemichannels ($\gamma = 30 \text{ pS}$), representing $\sim 0.009\%$ of the total number of single hemichannels in the plasma membrane (1.7 $\times$ 10$^{10}$; Table III). This suggests that, in the oocyte, there is one conducting hemichannel every $\sim 20 \mu\text{m}^2$ of the plasma membrane. At a more physiological [Ca$^{2+}$]$_o$ (1–2 mM),
Physiological \( \text{Ca}^{2+} \) estimate that there would be two conducting hemi-
coated vesicles (0.1 \text{m} \text{m}) diameter) with the plasma
membrane, and that each vesicle contains 5–40 Cx50
hemichannels, we predict that Cx50 over-expression
should increase the plasma membrane by 63–503 \mu \text{m}^2/ \text{s}
(5.4–43.4 \times 10^6 \mu \text{m}^2 in 24 h). In spite of such a sizable
rate of insertion, there was no increase in the area of
the plasma membrane (Fig. 3; Table III). Thus, exocy-
tosis was balanced by endocytosis. It is interesting to
note that at peak Cx50 expression, these rates of exocy-
tosis and endocytosis would lead to the replacement of
the entire plasma membrane of the oocyte in \( \sim 24 \text{ h} \! \).
In contrast, expression of other heterologous mem-
brane proteins such as SGLT1 (Zampighi et al., 1995;
Hirsch et al., 1996) and aquaporin-0 (Chandy et al.,
1997) increased the area of the plasma membrane of
oocytes up to fourfold. In the case of SGLT1, the in-
crease in the plasma membrane area occurs concur-
rently with an increase in the number of transporters in
the plasma membrane, and the rate of SGLT1 insertion
is comparable to that observed here (Hirsch et al.,
1996; Wright et al., 1997). Therefore, it appears that the de-
livery of some endogenous membrane proteins, as well
as heterologous proteins such as SGLT1 and Cx50, to
the plasma membrane involves the same mechanism.
The difference in the trafficking of the heterologous
membrane proteins appears to involve the retrieval
mechanism. In the case of Cx50, endocytosis of large
Cx50-containing vesicles balances the rate of small ves-
icle insertion. In oocytes expressing SGLT1, however,
this pathway does not balance the rate of vesicle exocy-
tosis, resulting in a continuous increase in the area of
the plasma membrane.

Cx50 was inserted into the plasma membrane as
functional hemichannels. No gap junctions were ob-
served until the density of hemichannels in the plasma
membrane reached a threshold of 300–400/ \mu \text{m}^2.
Therefore, the plaques of channels were assembled
from the recruitment of the Cx50 hemichannels in the
plasma membrane. Once maximal expression levels
were reached, the fraction of hemichannels in non-
junctional and junctional membrane regions remained
relatively constant (Table III), indicating that there was
a steady state between the insertion of hemichannels
into the plasma membrane and their entry into junc-
tional plaques. Retrieval occurred through endocytosis
of both hemichannels and complete channels assem-
bled in junctional plaques (see below). Assuming a
steady state at maximal expression levels (72 h), we esti-
mate that the half-life of a single hemichannel (junc-
tional and nonjunctional combined) in the plasma
membrane was \( \sim 3.7 \text{ d} \). This is longer than the meta-
bolic half-life estimated for other connexins in other
expression systems (<5 h; e.g., Fallon and Goode-
nough, 1981; Beardslee et al., 1998).

We identified Cx50 hemichannels in small (\( \sim 100 \text{ nm} \)) and large (0.2–0.5 \mu \text{m}) diameter vesicles. We in-

\[ G_{\text{Cx50}} \text{ decreases by } \geq 20\text{-fold, which would correspond}
\text{to one open hemichannel for every } \sim 400 \mu \text{m}^2 \text{ of the}
\text{plasma membrane. If these findings in oocytes are ex-
trapolated to a smaller cell, such as a liver hepatocyte,}
\text{and a density of 500 hemichannels/ } \mu \text{m}^2 \text{ is assumed, we}
\text{estimate that there would be two conducting hemi-
channels per cell. Therefore, our data suggest that at}
\text{physiological } [\text{Ca}^{2+}]_o, \text{Cx50 hemichannels play a small}
\text{role in single cell permeability and homeostasis, and the}
\text{potential physiological significance of this hemi-
channel would be confined to large cells possessing a}
\text{high density of hemichannels, conditions which favor}
\text{channel opening (e.g., low } [\text{Ca}^{2+}]_o, \text{ or pathophysio-
logical states that may result in apoptosis.)}

Trafficking of Cx50

After Cx50 cRNA injection, the total number of hemi-
channels in the plasma membrane reached a steady
state \( (2.3–2.6 \times 10^{10}) \) at 72 h (Table II), which corre-
related with the electrophysiological assay of hemi-
channel function (Figs. 3 and 8). Based on the number of
hemichannels in the oocyte plasma membrane 24 h af-
er cRNA injection (Table II), the initial insertion rate
of Cx50 into the plasma membrane was \( \sim 80,000 \) hemi-
channels/s. The observation that Cx50 hemichan-
nels were colocalized in the same vesicles with endog-
enous proteins of the oocyte suggests that Cx50 over-
expression does not alter the oocyte’s constitutive
translation and trafficking machinery.

Assuming that insertion is via fusion of the small
coated vesicles (0.1 \mu \text{m} \text{ diameter}) with the plasma

\[ \frac{z}{520} \text{ Expression of Connexin50 in Oocytes} \]
interpret these vesicles as components of the trafficking machinery involved in Cx50 insertion into and retrieval from the plasma membrane. Three observations suggested that the larger vesicle originates from plasma membrane invaginations and is a component of the retrieval pathway. First, when the hemichannel density reached 300–400 μm² in the plasma membrane, the invaginations assembled gap junctions that pinched off and appeared as annular gap junctions in the cytoplasm (Figs. 4 B, 5 A, and 11 A; Larsen et al., 1979). Second, the extracellular marker, peroxidase, labeled the lumen of the large, but not the small, vesicles. Third, these larger vesicles contained hemichannels intermingled with an endogenous plasma membrane protein of the oocyte (i.e., the 13-nm particle in the E face). Altogether, these results suggest that Cx50 hemichannel retrieval shares a pathway with endogenous proteins of the plasma membrane of the oocyte. In this scheme, the large vesicles represent early endosomes formed by endocytosis of large invaginations of the plasma membrane.

The origin and fate of the smaller coated vesicles containing the hemichannels were difficult to determine. One possibility is that they insert the hemichannels into the plasma membrane, as their lumen was not observed to be labeled with peroxidase. This interpretation is likely because these vesicles were observed to be associated with Golgi-resembling flattened membrane sacs. An alternative possibility is that the coated vesicle is involved in the endocytosis of hemichannels only (not gap junctions) from the plasma membrane, and subsequently delivers them to an early endosomal compartment. While additional information will be required to distinguish between the two hypotheses, our

![Diagram showing the trafficking of Cx50 hemichannels to and from the plasma membrane of oocytes occurs along the constitutive (default) pathway. We propose that Cx50 is inserted into the plasma membrane as a hemichannel (connexin hexamer). Insertion into the plasma membrane occurs at an initial rate of 80,000 hemichannels/s. Hemichannel insertion is via exocytosis of small coated vesicles at a rate of 2,000–16,000 vesicles/s, corresponding to the addition of 63–503 μm²/s of membrane to the plasma membrane. Retrieval occurs through the formation of membrane invaginations that eventually pinch off and form large vesicles in the cytoplasm. At a hemichannel density < 300/μm², the Cx50 hemichannels are retrieved, intermingled with some endogenous plasma membrane proteins, in the same large vesicles. At a density > 300 hemichannels/μm², the large vesicles form reflective gap junctions that pinch off and appear as annular gap junctions in the cytoplasm. The principal characteristic of this pathway is that the area of the plasma membrane does not change during hemichannel insertion, indicating that plasma membrane endocytosis balances the rate of vesicle insertion.](image-url)
identification of vesicles containing hemichannels represents a necessary first step for a more accurate description of the trafficking of heterologous proteins to and from the plasma membrane of oocytes.

In summary, our results led to the hypothesis that Cx50 hemichannel insertion into the plasma membrane was via exocytosis of small coated vesicles (Fig. 14). This pathway was shared by both endogenous and Cx50 proteins. This process steadily increased the density of Cx50 hemichannels in the plasma membrane until a steady state level was reached at 72 h. Retrieval of Cx50 hemichannels from the plasma membrane occurred via endocytosis of large vesicles. At low density (<300/μm²), retrieval of hemichannels started in invaginations of the plasma membrane that pinched off and formed large cytoplasmic vesicles. This pathway was also shared by both endogenous and Cx50 proteins. At higher densities (>300/μm²), the large invaginations formed reflective gap junctions that pinched off and appeared as annular gap junctions in the cytoplasm. The principal characteristic was that, despite the ongoing insertion and retrieval processes, the area of the plasma membrane remained constant.

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