Connexin-Aequorin Chimerae Report Cytoplasmic Calcium Environments along Trafficking Pathways Leading to Gap Junction Biogenesis in Living COS-7 Cells*

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The cytoplasmic calcium environments along membrane trafficking pathways leading to gap junction intercellular communication channels at the plasma membrane were studied. Connexins, the constitutive proteins of gap junctions, were fused at their carboxyl termini to the calcium-sensitive photoprotein aequorin. The cellular location of the chimeric proteins was determined by immunolocalization and subcellular fractionation. The generation of functional gap junctions by the connexin chimerae was monitored by the ability of the cells to exchange small dyes. Although aequorin fused to connexin-26 was nonfunctional, its ability to report Ca\(^{2+}\) and to form functional gap junctions was rescued by replacement of its cytoplasmic carboxyl tail with that of connexin-43. In COS-7 cells expressing these connexin-aequorin chimerae, calcium levels below the plasma membrane were higher (5 μM) than those in the cytoplasm (100 nM); gap junctions were able to transfer dyes under these conditions. Cytoplasmic levels of free calcium surrounding the ERGIC/Golgi reported by connexin-43 chimera (420 nM) were twice those measured by connexin-32 chimera (200 nM); both chimerae measured calcium levels substantially higher than those reported by a connexin-26 chimera (130 nM). Dispersion of the ERGIC and Golgi complex by brefeldin A led to a marked reduction in calcium levels. The results show that the various connexin chimerae were located in spatially different subcellular stores and that the ERGIC/Golgi regions of the cell maintain heterogeneous cytoplasmic domains of calcium. The implications of the subplasma-membrane Ca\(^{2+}\) levels on the gating of gap junctions are discussed.

Gap junctions are pervasive intercellular channels that directly connect cells in tissues and organs allowing electrical coupling and the transfer of molecules and ions (<1 kDa) between cells (1). Gap junction biogenesis requires the oligomerization of protein subunits (connexons) into a hexameric channel (11–14), but the mechanisms are poorly understood. These targeted aequorin constructs reveal differences between gap junction and connexon biogenesis (5).

The implications of the subplasma-membrane Ca\(^{2+}\) levels on the gating of gap junctions are discussed.

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The abbreviations used are: ER, endoplasmic reticulum; Cx-Aeq, connexin-aequorin chimerae; LA, firefly luciferase-apoaequorin chimerae; [Ca\(^{2+}\)]\(_{cyt}\), cytoplasmic free calcium concentration; [Ca\(^{2+}\)]\(_{pl}\), subplasma membrane free calcium concentration; ERGIC, endoplasmic reticulum/Golgi intermediate compartment; TGN, trans-Golgi network; LY, lucifer yellow; PBS, phosphate-buffered saline; aa, amino acid(s); PCR, polymerase chain reaction; EGFP, enhanced green fluorescent protein; WT, wild-type.

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functionally separate calcium stores within the ER (24, 31), results supported by complementary studies using fluorescent calcium-sensitive dyes (32, 33). These findings, taken together with the limited diffusion of calcium in the cytoplasm (34), suggest that heterogeneous calcium microenvironments play an important role in channel organization and trafficking between organelles. Here, we describe the use of Cx-Aeq chimerae to measure levels of free cytoplasmic calcium along protein trafficking pathways and at the cell surface following integration of the chimerae into the plasma membrane and the gap junction.

**EXPERIMENTAL PROCEDURES**

**Construction of Domain-swapped Chimerae—**PCR was used to generate fragments of Cox6 (35) and Csx2 (36) corresponding to the start of the protein to the end of the M4 region (Cox6, aa 1–210; Csx2, aa 1–209) from full-length cDNAs using the oligonucleotides detailed below. Domain boundaries in connexins were deduced from Clustal V sequence alignment and current topological models (1). Following incubation (30 min, 37 °C) with Klenow fragment (Promega), products were fused with PCR-generated fragments corresponding to the carboxyl tail of Cox43 with aequorin attached (Cox43-Aeq, aa 227–573) in a second stage amplification (21) to produce Cox26/43T-Aeq and Csx2/43T-Aeq (see Fig. 1). The chimeric DNA was ligated into the TA cloning vector PCR3 according to manufacturer’s instructions (Invitrogen).

Construcst in which the aequorin was removed from Cox26/43T-Aeq and Csx2/43T-Aeq, thereby creating Cox26/43T and Csx2/43T, respectively (see Fig. 1), were produced using PCR, appropriate forward (sense) primers, and a reverse (antisense) primer (R43B3H, 5’-GCTCTGGTTGCTATGACAGTCAAGTTGCAGCTC-3’). The resultant products were incubated with Klenow fragment and ligated into PCR3 as described above. Sequences of oligonucleotide primers were as follows for Cox26/43T-Aeq (Cox26P1 sense, 5’-CAGCTATACGCTACTAAGGGAGATGGAGGATTGAAGGACAACTC-3’; Cox26M4R antisense, 5’-GGAAACAGGCTACGACTAGCAAGTGTGAGTTGACCTCGTTCTGCAGAC-3’ and Cox26M4F sense, 5’-GACATCTGCTACTAGCAAGTGTGAGTTGACCTCGTTCTGCAGAC-3’) and for Csx2/43T-Aeq (Csx2 (32)P1 sense, 5’-CAGCTATAATACGACTC-3’; Pet-II antisense, 5’-GGAAACAGGCTACGACTAGCAAGTGTGAGTTGACCTCGTTCTGCAGAC-3’) and for Csx2/43T-Aeq (Csx2 (32)P1 sense, 5’-CAGCTATAATACGACTC-3’; Pet-II antisense, 5’-GGAAACAGGCTACGACTAGCAAGTGTGAGTTGACCTCGTTCTGCAGAC-3’). The DNAs encoding the domain-swapped constructs (1 μg) were trancribed and translated in *vitro* using a coupled rabbit reticulocyte TNT system (Promega), *T7* polymerase (Promega), and 35S-labeled methionine and cysteine (Promix, Nycomed-Amersham). Radiolabeled protein products were separated by SDS-polyacrylamide gel electrophoresis (12.5% (w/v) acrylamide) under reducing conditions followed by autoradiography of the gels. Radiolabeled protein products were incubated with Klenow fragment and ligated into the TA cloning vector PCR3 according to manufacturer’s instructions (Invitrogen).

To measure cytosolic domains of Ca2+ in cells expressing Cox-Aeq chimerae, recombinant apoaequorin was converted to the active photoprotein by adding coelenterazine (final concentration, 2 μM) at 37 °C, 10 min prior to experiments. To measure Ca2+ directly under the plasma membrane (*Cox2+im*), the recombinant photoproteins were activated by adding coelenterazine (2 μM) to cells in Ca2+-free medium containing 1 mM EGTA (30). The coverslips were mounted in a perfusion chamber at 37 °C and brought into contact with a fiber optics bundle attached to a photon counting camera (25). Cells were perfused for 30 min at 37 °C in Ca2+-free medium containing 1 mM EGTA and 1 mM CaCl2 (500 μM) were incubated in the dark with 2 μM coelenterazine for 4 h at 4 °C. Light production from each chimera was measured in different Ca2+ buffers (pH 7.2) (Molecular Probes) and calculated as rate constants from the total aequorin activity assessed in saturating concentrations of calcium (25, 45).

**Intracellular Ca2+ Measurements—**After the introduction of recombinant DNA encoding the Cx-Aeq chimerae or LA, COS-7 cells (1 × 106) were trypsinized 48 h later and transferred to glass coverslips. Sodium butyrate was added to cells approximately 18 h prior to commencement of experiments (37). To measure cytosolic domains of Ca2+ in cells expressing Cx-Aeq chimerae, recombinant apoaequorin was converted to the active photoprotein by adding coelenterazine (final concentration, 2 μM) at 4 h prior to experiments. To measure Ca2+ directly under the plasma membrane (*Cox2+im*), the recombinant photoproteins were activated by adding coelenterazine (2 μM) to cells in Ca2+-free medium containing 1 mM EGTA (30). The coverslips were mounted in a perfusion chamber at 37 °C and brought into contact with a fiber optics bundle attached to a photon counting camera (25). Cells were perfused for 30 min at 37 °C in Ca2+-free medium containing 1 mM CaCl2 and then the postnuclear supernatant was applied and the centrifugation continued for a further 2 h. 16 (1-Ml) fractions were collected and density and protein concentration determined. Fractions (500 μl) were incubated in the dark for 2 h with 500 μl of buffer O (in mM: 1 EDTA, 500 NaCl, 5 β-mercaptoethanol, 10 Tris, pH 7.4) and 2 μM coelenterazine (Molecular Probes). Aequorin chemiluminescence in each fraction was analyzed in a luminometer after the injection of saturating amounts of calcium (50 μM).
Cytoplasmic Calcium Domains Detected by Cx-Aeq Chimerae

Cellular Localization of Chimeric Protein—Immunocytochemistry of COS-7 cells expressing Cx-Aeq chimerae stained with aequorin antibodies showed punctate staining at the plasma membrane. Cx32-Aeq, Cx43-Aeq, Cx26/43T-Aeq, and Cx32/43T-Aeq were also located in intracellular regions near the nucleus (Fig. 2, A–D, respectively, indicated with arrows). The intracellular accumulation of chimeric proteins is characteristic of many cultured cells expressing exogenous connexins (21, 55, 61) and, as immunolocalization of wild-type connexins (Table I). The variation in [Ca2+] in different cellular compartments was confirmed by confocal microscopy. Cells stained with markers to ER (calreticulin, Fig. 2E), ERGIC (p58, Fig. 2F), and Golgi (GM130 and TGN46, Fig. 2, G and H, respectively) demonstrated that these intracellular stores of Cx-Aeq chimerae were localized to ERGIC/Golgi compartments. These results were further confirmed by the changes in subcellular localization occurring following brefeldin A treatment. The drug redistributed the ER and Golgi markers (Fig. 2, N and O, respectively) but not ER (Fig. 2M). Brefeldin A redistributed Cx32-Aeq, Cx43-Aeq, and Cx32/43T-Aeq (Fig. 2I, J, and L, respectively) in a similar manner suggesting that these Cx-Aeq chimerae were all localized in the ERGIC/Golgi region of the secretory pathway. Quantification of immunostaining by confocal microscopy indicated that there was a 90% decrease in plasma membrane-associated Cx-Aeq chimerae following brefeldin A treatment. In contrast, the brefeldin A-induced redistribution of Cx26/43T-Aeq was less marked than observed for the other Cx-Aeq chimerae, and some plasma membrane localization remained (Fig. 2K, arrows). Since there was overlap in immunostaining of ERGIC and Golgi compartments in COS-7 cells (Fig. 2, F–H), a biochemical subcellular fractionation approach was adopted to independently analyze the cellular location of the various Cx-Aeq chimerae.

Subcellular fractionation of COS-7 cells expressing the Cx-Aeq chimerae on Nycodenz density gradients resolved the ER, Golgi, and ERGIC regions of the cell (Fig. 3, A, B, and D, respectively) and confirmed the predominant ERGIC/Golgi localization of Cx32-Aeq, Cx43-Aeq, and Cx32/43T-Aeq chimerae (Fig. 3, E, F, and H, respectively). However, Cx26/43T-Aeq colocalized mainly with an ERGIC marker, and only a small proportion accumulated in the Golgi regions of the cell (~20% of total Cx26/43T-Aeq protein was found in the Golgi compared with 76% of Cx32-Aeq and 81% of Cx43-Aeq) (Fig. 3G). Despite its lower efficiency in forming gap junction channels, Cx26/43T-Aeq did not colocalize with an ER marker by subcellular fractionation. The distribution of Cx32/43T-Aeq in the Golgi-containing fractions of the gradient correlated closely with that of Cx32-Aeq. However, Cx32/43T-Aeq also showed increased retention in the ER when compared with either Cx32-Aeq or Cx43-Aeq (subcellular fractionation demonstrated a ratio of ER/Golgi localized chimeric protein of 0.55 for Cx26/43T-Aeq (Fig. 3H) compared 0.2 and 0.15 for Cx32-Aeq and Cx43-Aeq (Fig. 3, E and F, respectively). Overlap was observed between Golgi and plasma membrane markers (Fig. 3, B and C), but densitometric analysis of confocal images demonstrated that only 5–10% of the chimeric Cx-Aeq protein expressed was found at the plasma membrane.

Cytoplasmic Ca2+ Levels along Trafficking Pathways—Biluminescence measured in COS-7 cells expressing recombinant protein was converted into calcium values using rate constants determined for each of the chimera (Fig. 4). Calibration of each chimera at pH 7.2 showed that the addition of various connexin isoforms to the amino terminus of aequorin has little effect on the Ca2+-dependent light emission in vitro when compared with each other or with wild-type aequorin; these results show that the aequorin moiety in all chimerae were folded correctly. It should be noted that in cells reconstituted in 1 mM Ca2+, the aequorin partner in Cx-Aeq chimerae associated with the plasma membrane was consumed and thus cannot contribute to the [Ca2+] reported. When expressed in COS-7 cells reconstituted in calcium-containing media, the cytoplasmically located aequorin moiety of Cx43-Aeq reported a [Ca2+] higher than Cx32-Aeq (0.42 µM ± 0.01 and 0.2 µM ± 0.018, respectively, n = 6); also, these chimerae reported [Ca2+] higher than that detected in the bulk cytoplasm by LA (0.09 µM ± 0.01, n = 6) (Fig. 5A). The variation in [Ca2+] reported by Cx32-Aeq and Cx43-Aeq is strongly indicative that these chimerae reside in different cytoplasmic calcium envi-

![Fig. 1. In vitro synthesis of chimeric Cx-Aeq proteins.](image-url)

**Table I**

| cDNA injected | Dye transfer | % |
|---------------|--------------|---|
| EGFP          |              |   |
| Cx26WT        | 37 ± 1.4     |   |
| Cx32WT        | 47.6 ± 8.3   |   |
| Cx43WT        | 52.4 ± 13.3  |   |
| Cx26/43T-Aeq  | 52.2 ± 11.5  |   |
| Cx26/43T      | 14.3 ± 5.7   |   |
| Cx32/43T-Aeq  | 19.2 ± 8.9   |   |
| Cx32/43T      | 18.5 ± 4.6   |   |
| Cx32-Aeq      | 23.1 ± 9.7   |   |
| Cx26-Aeq      | 4.1 ± 1.3    |   |

*Results are given as percentage of cells transferring dye to two or more neighbors (mean ± S.E., n = 30)* following enhancement of recombinant protein expression with sodium butyrate. Results obtained in the absence of sodium butyrate treatment were 5–10% lower, and sodium butyrate did not affect dye-coupling characteristics of nontransfected HeLa cells.
rons within the Golgi apparatus. Cx32/43T-Aeq reported a [Ca$^{2+}$], (0.19 μM ± 0.02, n = 6) similar to that of Cx32-Aeq and much lower than that of Cx43-Aeq (Fig. 5A). This result also demonstrated that the different [Ca$^{2+}$], reported were unlikely to be a function of the length of the carboxyl tail of the connexin isoform to which aequorin was attached. In contrast, Cx26/43T-Aeq reported a [Ca$^{2+}$], (0.13 μM ± 0.02, n = 6) similar to the value determined by LA in the bulk cytosol (Fig. 5A). This agreed with the subcellular fractionation data showing that the majority of this chimera did not coincide with a Golgi marker. LA did not detect a different [Ca$^{2+}$], when co-expressed with different chimerae (Fig. 5A) or exogenous wild-type Cx32 or Cx43 in COS-7 cells (data not shown); therefore, the different calcium levels reported are unlikely to be due to altered permeabilities to external Ca$^{2+}$ of the channels formed by the chimerae. Furthermore, the calcium levels reported are also unlikely to arise as a consequence of different cytoplasmic pH environments (6.8–7.4; Ref. 52) since aequorin chemiluminescence is insensitive to pH within this range (53, 54). Following treatment with brefeldin A, Cx32-Aeq, Cx43-Aeq, Cx26/43T-Aeq, and Cx32/43T-Aeq detected different calcium environments (0.16 μM ± 0.01, 0.17 μM ± 0.01, 0.14 μM ± 0.01, respectively, n = 6) consistent with the redistribution of the Golgi apparatus shown by immunocytochemistry. After drug treatment, the calcium measurements reported by Cx-Aeq chimerae were closer to those in the bulk cytoplasm reported by LA, which remained unchanged after incubation in brefeldin A (0.089 μM ± 0.009, n = 6) (Fig. 5B). These intracellular calcium measurements suggest that the Golgi apparatus maintains spatially separate cytoplasmic Ca$^{2+}$ domains.
Ca²⁺ Levels at the Plasma Membrane—Using COS-7 cells reconstituted in Ca²⁺-free media (KRH-Ca²⁺), we selectively triggered Cx-Aeq chimeras specifically associated with the plasma membrane by addition of 1.3 mM Ca²⁺ to the perfusion medium (30). This procedure showed that all chimeras reported calcium levels immediately beneath the plasma membrane (≈5 μM) that were approximately 50-fold higher than those detected in the bulk cytosol (Fig. 6D). A small transient Ca²⁺ elevation (≈0.3 μM) of approximately 200 s was detected by cytoplasmically located LA and was attributed to Ca²⁺ influx upon readdition of 1 mM Ca²⁺ to the perfusion medium.

During calcium entry into the cell (between ∼100 and 300 s, Fig. 6, A and C), it was calculated that less than 0.05% of total cytoplasmic LA was consumed, and thus the amount of LA was effectively constant. Therefore, it was assumed that chemiluminescent emission during re-addition of Ca²⁺ to cells expressing Cx-Aeq chimeras was contributed by Cx-Aeq resident at the plasma membrane. Using chemiluminescent activity of plasma membrane-associated Cx-Aeq chimeras after subtraction of basal activity, i.e., before Ca²⁺ was added to the perfusion medium, levels of calcium beneath the plasma membrane were measured during Ca²⁺ influx and, importantly, for several

**Fig. 3.** Subcellular fractionation of COS-7 cells expressing Cx-Aeq chimeras. The gradients were characterized using markers for ER (A), Golgi (B), plasma membrane (C), and ERGIC (D) (see “Experimental Procedures”) and are expressed as normalized units (43). The distribution of chimeric Cx32-Aeq (E), Cx43-Aeq (F), Cx26/43T-Aeq (G), and Cx32/43T-Aeq (H) in the gradient was determined from the chemiluminescent activity of each fraction. Results are given as percentage specific activity (counts per microgram of protein) (mean, n = 3).

**Fig. 4.** Calibration of aequorin in Cx-Aeq chimeras. Calcium dose responses of Cx32-Aeq (•), Cx32-Aeq (○), Cx26/43T-Aeq (○), and Cx26/43T-Aeq (□) at pH 7.2 as described under “Experimental Procedures” generated rate constants (k (s⁻¹)). They are plotted as mean ± S.E. (n = 3).
hundred seconds after entry of calcium into the cell ceased (Fig. 6D). After a further short incubation in EGTA followed by another cycle of addition of Ca\(^{2+}\) to the medium, there was no increase in chemiluminescence activity (data not shown). This demonstrated that, in contrast to results obtained with LA, all chemiluminescent activity of Cx-Aeq chimerae at the plasma membrane was triggered following readdition of Ca\(^{2+}\) to the external medium. The plateau occurring in Fig. 6D represented a steady-state \([Ca^{2+}]_{pm}\) environment in COS-7 cells of approximately 5 \(\mu M\).

To investigate whether gap junctions were in an open configuration, the intercellular transfer of LY was examined. Fig. 7 shows that LY was transferred between cells, indicating that COS-7 cells were extensively coupled by gap junction intercellular channels under identical conditions used to determine \([Ca^{2+}]_{pm}\).

**DISCUSSION**

Ca\(^{2+}\) plays a central role in communication and cell signaling (46) and has been historically implicated in effecting the closure of gap junctions (11, 14). A targeted expression approach was adopted to position aequorin at gap junctions in living cells and thus determine \([Ca^{2+}]_{pm}\).
of gap junction assembly highlight anomalies between transiently expressed in HeLa cells. Such differences in fidelity were formed partially functional intercellular channels when present work it is evident that Cx26/43T and Cx32/43T chimerae allowed electrical coupling (47). However, in the case of Cx32 was replaced with that of Cx43 failed to form gap junctions less efficiently than wild-type connexins, attachment of aequorin or carboxyl-tail domain substitution between connexin isoforms did not appear to modify the trafficking of the chimerae to the plasma membrane and gap junctions. In Xenopus oocytes, expression of a construct in which the carboxyl tail of Cx32 was replaced with that of Cx43 (156 aa).

The results obtained by imaging of the chemiluminescent activity indicated that although these chimerae formed gap junctions less efficiently than wild-type connexins, attachment of aequorin or carboxyl-tail domain substitution between connexin isoforms did not appear to modify the trafficking of the chimerae to the plasma membrane and gap junctions. In Xenopus oocytes, expression of a construct in which the carboxyl tail of Cx32 was replaced with that of Cx43 failed to form gap junctions that allowed electrical coupling (47). However, in the present work it is evident that Cx26/43T and Cx32/43T chimerae formed partially functional intercellular channels when transiently expressed in HeLa cells. Such differences in fidelity of gap junction assembly highlight anomalies between Xenopus oocytes and mammalian model expression systems (48). The lower efficiency of incorporation of these various chimerae into functional gap junction channels in HeLa cells compared with wild-type connexins points to unfavorable interactions that may occur between domains, e.g., the intracellular loop and carboxyl tail of the chimerae or when reporter groups such as aequorin are attached to the carboxyl tail of connexins (37). Interactions occurring between cytoplasmic domains of connexins have been proposed to regulate channel gating (49), and domain substitution experiments show that there are well-established incompatibilities between domains of different connexin isoforms (47). Therefore, it is not surprising that the attachment of aequorin to the carboxyl terminus of connexins or transposing carboxyl tails between connexin isoforms modified to varying degrees the channel-gating mechanisms.

Intracellular stores of Cx-Aeq chimerae in COS-7 cells that reported different cytoplasmic calcium environments were identified by two complementary approaches. The location of these stores varied according to which connexin isoform the aequorin was attached, and since cytoplasmic free calcium may be involved in the trafficking of proteins (6), it follows that cytoplasmic gradients of calcium may also regulate oligomerization of connexins into connexons during gap junction biogenesis. These cytoplasmic calcium gradients were abolished following Golgi disassembly and relocation to areas around cell nuclei induced by brefeldin A. This observation emphasizes the importance of the Golgi in maintaining spatially separate cytoplasmic Ca²⁺ environments. Alternative approaches have demonstrated that cytoplasmic hot spots of Ca²⁺ surround the majority of intracellular organelles (50) but not secretory granules (51). The aequorin moiety in Cx-Aeq chimerae is positioned cytoplasmically and as such is located in environments of pH between 6.8 and 7.4 (52). The chemiluminescent emission from aequorin is not sensitive to pH within this range (45, 53, 54), and thus the calcium levels reported by the chimerae are not influenced by the local concentration of hydrogen ions at these stations on the secretory pathway.

The results obtained in cells expressing Cx26/43T-Aeq are intriguing, for calcium environments in the vicinity of this chimera were different. Also, two complementary cell localization approaches showed that Cx26/43T-Aeq was found at low levels in the Golgi apparatus compared with other Cx-Aeq chimerae. Cx26/43T-Aeq also achieved and maintained a plasma membrane location in brefeldin A-treated cells for a length of time, which this work and that of others (55) have shown leads to the complete removal by turnover of resident recombinant connexins from the plasma membrane. These results, taken together with reports that alternative protein trafficking routes through the cell exist (56, 57) indicate that Cx26/43T-Aeq with its different intracellular localization and Ca²⁺ environment to other chimerae may follow a non-classical trafficking pathway to the gap junction (58).

All Cx-Aeq chimerae examined reported a sub-plasma-membrane cytoplasmic calcium concentration of approximately 5 μM. This elevated level of Ca²⁺ was not due to EGTA-induced membrane damage since cells incubated in EGTA showed unchanged [Ca²⁺]ₘₚₚ, suggesting that no increased entry of Ca²⁺ into the cell occurred. Influx of Ca²⁺ into cells previously maintained in Ca²⁺-free media resulted in a transient elevation in [Ca²⁺]ₘₚ. However, [Ca²⁺]ₘₚ lasted for several minutes and reached an elevated steady-state sub-plasma-membrane cytoplasmic calcium environment of ~5 μM. Previous studies using targeted aequorin have indicated that during addition of Ca²⁺, a transient and dramatic Ca²⁺ elevation occurred at the plasma membrane, which quickly decayed to a basal value that existed before Ca²⁺ influx occurred (21, 29, 30). This decay occurs because consumption of active photoprotein at the plasma membrane may give the impression that there is a decrease in both chemiluminescent activity and [Ca²⁺]ₘₚ. The present data suggest this was not the case and that high [Ca²⁺]ₘₚ occurred in COS-7 cells maintained in a Ca²⁺-containing medium. The mechanisms by which this high [Ca²⁺]ₘₚ is maintained without Ca²⁺ permeating into the bulk cytoplasm remain unclear. Further supporting the present results are those using mutant aequorins with lower affinities for Ca²⁺ that enhance the longevity of active protein in elevated concentrations of Ca²⁺ in cultured A7r5 cells (30) and in the squid.
presynaptic terminal (59). Fluorescent dyes have also been used to monitor calcium under the plasma membrane (33, 60), but difficulties in precise subcellular localization of these dyes together with their calcium buffering capacity compromise their utility; these dyes reported [Ca\textsuperscript{2+}]\textsubscript{pm} of approximately 1–2 μM, which were lower than in studies in which aequorin was used.

Gap junction channels have been proposed to close when cytoplasmic Ca\textsuperscript{2+} is between 0.5 and 2 μM (12, 13). However, the plasma membrane is a heterogeneous organelle, and care should be taken in extrapolating the values of [Ca\textsuperscript{2+}]\textsubscript{pm} to all regions of the plasma membrane, including the gap junction. Since the Cx-Aeq chimerae studied demonstrated punctate staining at the plasma membrane and were targeted to gap junctions that transferred dyes (Fig. 7), does the calculated value of ~5 μM at the plasma membrane represent the [Ca\textsuperscript{2+}]\textsubscript{pm} of the gap junction pore itself? The extensive dye coupling occurring under the conditions used to measure [Ca\textsuperscript{2+}]\textsubscript{pm} indicates that the gap junctions were open.

To explain the present data in the context of the role of calcium in the mechanism of operation of gap junction intercellular channels, two hypotheses can be discussed. In the first, connexon hemichannels in the plasma membrane, prior to assembly into gap junctions, are in a closed configuration since they exist in a high (~5 μM) sub-plasma-membrane Ca\textsuperscript{2+} environment. Other studies have shown that “free” connexon hemichannels in the plasma membrane are close (62). As these hemichannels accrete and dock with partner channels in juxtaposed cells, they enter into or generate a lower intracellular Ca\textsuperscript{2+} environment at this confined gap junction microdomain, which is more favorable for opening the coupled hemichannels. In the second hypothesis, a uniformly high sub-plasma-membrane Ca\textsuperscript{2+} level exists in cells including areas directly beneath gap junctions. Such a relatively high [Ca\textsuperscript{2+}]\textsubscript{pm} would favor a closed configuration of gap junctions (11), and therefore unidentified auxiliary proteins associated with the gap junction may mediate channel opening. The present studies do not distinguish between these two hypotheses, but any mechanism proposed must also take into account other central questions concerning intercellular signaling across gap junctions, especially how Ca\textsuperscript{2+} waves propagate between cells (19).

With regard to the question of whether connexons are in a closed configuration during their intracellular trafficking, it seems unlikely that the hemichannels are shut by the low ambient cytoplasmic Ca\textsuperscript{2+} levels now determined in the ER/ERGIC/Golgi compartments. Other unknown mechanisms may operate to maintain connexons migrating along the secretory pathway in a closed state or to regulate the cytoplasmic and luminal Ca\textsuperscript{2+} environments.

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