Intercellular Calcium Signaling via Gap Junction in Connexin-43-transfected Cells*

Toshihiko Toyofuku‡, Masanori Yabuki, Kinya Otsu, Tsunehiko Kuzuya, Masatsugu Hori, and Michihiro Tada

From the Department of Medicine and Pathophysiology, Osaka University Medical School, Suita, Osaka 565, Japan

In excitable cells, intracellular Ca\(^{2+}\) is released via the ryanodine receptor from the intracellular Ca\(^{2+}\) storing structure, the sarcoplasmic reticulum. To determine whether this released Ca\(^{2+}\) propagates through gap junctions to neighboring cells and thereby constitutes a long range signaling network, we developed a cell system in which cells expressing both connexin-43 and ryanodine receptor are surrounded by cells expressing only connexin-43. When the ryanodine receptor in cells was activated by caffeine, propagation of Ca\(^{2+}\) from these caffeine-responsive cells to neighboring cells was observed with a Ca\(^{2+}\) imaging system using fura-2/AM. Inhibitors of gap junctional communication rapidly and reversibly abolished this propagation of Ca\(^{2+}\). Together with the electrophysiological analysis of transfected cells, the observed intercellular Ca\(^{2+}\) wave was revealed to be due to the reconstituted gap junction of transfected cells.

We next evaluated the functional roles of cysteine residues in the extracellular loops of connexin-43 in gap junctional communication. Mutations of Cys\(^{64}\), Cys\(^{187}\), Cys\(^{192}\), and Cys\(^{198}\) to Ser showed the failure of Ca\(^{2+}\) propagation to neighboring cells in accordance with the electrical uncoupling between transfected cells, whereas mutations of Cys\(^{61}\) and Cys\(^{68}\) to Ser showed the same pattern as the wild type. \(^{1^4}C\)Iodoacetamide labeling of free thiols of cysteine residues in mutant connexin-43s showed that two pairs of intramolecular disulfide bonds are formed between Cys\(^{54}\) and Cys\(^{192}\) and between Cys\(^{187}\) and Cys\(^{198}\). These results suggest that intercellular Ca\(^{2+}\) signaling takes place in cultured cells expressing connexin-43, leading to its own synchronization and that the extracellular disulfide bonds of connexin-43 are crucial for this process.

The intercellular transmission of molecules through channels in a specialized cell membrane structure, the gap junction, is a mechanism for direct signaling between adjacent cells. In the nervous system, intercellular Ca\(^{2+}\) signaling between glial cells may represent a system of widespread non-synaptic communication (1–5). A Ca\(^{2+}\) imaging system has shown that a wave of elevated intracellular Ca\(^{2+}\) level ([Ca\(^{2+}\)]\(_i\)) passes through a confluent monolayer of glial cells is triggered on micropipette stimulation of a single glial cell (2, 4, 5), topical application of glutamate (1, 5), or localized photo-stimulation (5). In the cardiac cells, [Ca\(^{2+}\)]\(_i\), changes periodically during the cycle of excitation-contraction coupling (6, 7), and muscle contraction is induced by thousand-fold increases in [Ca\(^{2+}\)]\(_i\), released through activated ryanodine receptor from sarcoplasmic reticulum. However, no studies to date have demonstrated intercellular propagation of the increased [Ca\(^{2+}\)]\(_i\). If increased [Ca\(^{2+}\)]\(_i\) propagates across cells, this should be a mechanism for the synchronization of excitable cells in a wide range.

Gap junctions are assemblies of cell-cell channels (8–10). Each channel is formed through the docking of two hemichannels located in opposing cell membranes, and each hemichannel is composed of a hexamer of connexin monomers. The gap junction permits the passage of soluble molecules of up to 1 kDa in size (11–13), including cAMP, Ca\(^{2+}\), inositol(1,4,5)-trisphosphate (Ins\(_{1,4,5}\)), ATP, and morphogens (8, 14, 15). The permeability of the gap junction can be reversibly regulated by several factors, including pH, Ca\(^{2+}\), cAMP, and cGMP (16–18).

Since cDNAs encoding the connexin gene family were isolated, two experimental systems have been exploited for functional characterization of isolated connexin genes. Connexin cRNA has been injected into Xenopus oocytes (19–21). Channel properties of gap junctional channels between oocytes placed in close contact has been studied by the dual voltage clamp method. As an alternative to the oocyte expression system, connexin has been expressed in cultured mammalian cells, and its function was assayed as the transfer of a microinjected fluorescent dye or electrophysiologically (22–25). Although the two expression systems yielded comparable results, recent studies demonstrated that there is no distinct correlation between junctional conductance, dye transfer, and/or ion selectivity of gap junctions in either system (26, 27). In a mammalian expression system, differences in the extent of dye transfer have been detected between several types of connexin transfecants, although they showed similar junctional conductance (26, 27). Therefore, analysis of serial changes in [Ca\(^{2+}\)]\(_i\), by a Ca\(^{2+}\) imaging system should be required to study the functional role of gap junction on an intercellular Ca\(^{2+}\) signaling. We designed a cell system in which cells expressing both connexin-43 and ryanodine receptor are surrounded by cells expressing only connexin-43 on the basis of preliminary results: 1) HEK293 cells do not express caffeine-sensitive Ca\(^{2+}\) release channel ryanodine receptor nor functional gap junction, and 2) transfected HEK293 cells express ryanodine receptor and connexin-43 functioning in a proper manner. By using a Ca\(^{2+}\) imaging system, we could observe an intercellular Ca\(^{2+}\) wave from a cell triggering Ca\(^{2+}\) excitation through a confluent monolayer of cells.

A hydrophobicity plot of connexin-43 showed it consists of four hydrophobic membrane spanning domains separated by
hydrophilic segments (9, 28). The two hydrophilic extracellular loops (encompassing amino acids 44–68 and 185–207 of connexin-43) are highly conserved in all connexin isoforms. The most striking feature of these two extracellular loops is the presence of six cysteine residues. Each loop has three cysteine residues with the consensus for the first loop being CXXCXXC (Cys187, Cys192, and Cys198 in connexin-43) and for the second loop CX2CXXC (Cys187, Cys192, and Cys198 in connexin-43). In the structure of the intercellular channel, it is the extracellular domain where the homophilic docking of hemichannels must occur that eventually results in the opening of the cell-cell channel. In general, two cysteine residues at different points on the polypeptide chain but adjacent in the three-dimensional structure of a protein can be oxidized to form a disulfide bond, which stabilizes the correct folding of the protein. In this study, we determined whether the formation of disulfide bonds in the extracellular domain directs the correct assembly of the gap junction.

EXPERIMENTAL PROCEDURES

Construction of Mutant Connexin-43s—The sequence corresponding to rat connexin-43 cDNA was amplified by reverse transcription-polymerase chain reaction using mRNA isolated from rat heart and cloned into the Bluescript KS(+) vector. Polymerase chain reaction primers were designed according to the published sequence (28) with an EcoRI site at the 5'-end to facilitate cloning. The cloned sequences were verified by nucleotide sequencing.

Oligonucleotide-directed mutagenesis was performed by the method of Kunkel (29). Mutations were carried out in specific cDNA fragments, which were ligated into sites in the polylinker in the Bluescript KS(+) vector. Synthetic oligonucleotides containing mutated bases were hybridized to a fragment of cDNA inserted into the single-stranded template to begin mutagenesis. After verifying the nucleotide sequences, the mutated fragment was ligated back into its original position in the cDNA. For mutation of the cysteine residues at positions 54, 61, and 68 to serine residues, the HindIII-HindII fragment of connexin-43 cDNA in the Bluescript KS(+) vector was used as a template to replace these cysteines with serines. For mutation of the cysteine residues at positions 187, 192, and 198 to serine residues, the HindIII-SacI fragment in the Bluescript KS(+) vector was used. For mutation of cysteine residues at positions 260, 271, and 298 to serine residues, the EcoRI-EcoRI fragment was used.

Stable Expression of Connexin-43 in HEK293 Cells—HEK293 cells were grown in Dulbecco's modified Eagle's medium (high glucose) supplemented with 10% fetal calf serum and penicillin at 37 °C under an atmosphere containing 5% CO2. Connexin-43 cDNA was ligated into the EcoRI site of the pcDNA3 vector (Invitrogen), containing the neomycin (G418)-resistant gene as a dominant selectable marker. HEK293 cells were transfected with expression vectors using the calcium phosphate precipitation technique and then the transfected cells were grown for 24–48 h before experimentation.

Northern Blot RNA Analysis—Total cellular RNA from HEK293 cells was prepared from transfected HEK293 cells. Total cellular RNA was prepared from HEK293 cells transfected with pcDNA3 alone (Control), pcDNA3 containing connexin-43 (Cx43) cDNA, or the pmic-ryanodine receptor (RyR) cDNA. Twenty μg of RNA was separated by electrophoresis, blotted onto nitrocellulose, and then hybridized specifically to the 32P-labeled gene probe of Cx43 cDNA or RyR cDNA (indicated in each panel). B, immunoblot analyses of proteins from transfected HEK293 cells. Plasma membrane-enriched protein fractions (left panel) and microsomal membrane protein fractions (middle and right panels) of transfected HEK293 cells were prepared. Ten μg of protein was electrophoresed, transferred to nitrocellulose, and then incubated with monoclonal anti-Cx43, anti-RyR, or anti-InsP3 receptor antibodies (indicated in each panel). C, immunocytochemical localization of connexin-43 and ryanodine receptor in cultured cells. Cells expressing Cx43 were stained with anti-Cx43 IgG antibodies, followed by incubation of biotinylated anti-mouse IgG antibodies and fluorescein isothiocyanate-conjugated streptavidin. Cells expressing ryanodine receptor were stained with anti-RyR IgM antibodies, followed by rhodamine-labeled anti-mouse IgM antibodies.

Fig. 1. Expression of connexin-43, InsP3 receptor, and ryanodine receptor in cultured cells. A, Northern blot analyses of RNA from transfected HEK293 cells. Total cellular RNA was prepared from HEK293 cells transfected with pmic-43 cDNA (Control), pmic-43 containing connexin-43 (Cx43) cDNA, or pmic-ryanodine receptor (RyR) cDNA. Twenty μg of RNA was separated by electrophoresis, blotted onto nitrocellulose, and then hybridized specifically to the 32P-labeled gene probe of Cx43 cDNA or RyR cDNA (indicated in each panel). B, immunoblot analyses of proteins from transfected HEK293 cells. Plasma membrane-enriched protein fractions (left panel) and microsomal membrane protein fractions (middle and right panels) of transfected HEK293 cells were prepared. Ten μg of protein was electrophoresed, transferred to nitrocellulose, and then incubated with monoclonal anti-Cx43, anti-RyR, or anti-InsP3 receptor antibodies (indicated in each panel). C, immunocytochemical localization of connexin-43 and ryanodine receptor in cultured cells. Cells expressing Cx43 were stained with anti-Cx43 IgG antibodies, followed by incubation of biotinylated anti-mouse IgG antibodies and fluorescein isothiocyanate-conjugated streptavidin. Cells expressing ryanodine receptor were stained with anti-RyR IgM antibodies, followed by rhodamine-labeled anti-mouse IgM antibodies.

Protein Immunoblot Analysis—Cells of each type were harvested and pelleted with a microcentrifuge. Whole-cell membrane-enriched protein fractions of HEK293 cells transfected with the pcDNA3 vector alone or pcDNA3 containing connexin-43 cDNA were prepared by lysing the pellets in 0.5% Nonidet P-40, followed by centrifugation at 10,000 × g for 1 h. Microsomal membrane protein fractions of HEK293 cells transfected with the pcDNA3 vector alone or pmic-ryanodine receptor cDNA were prepared by the method previously described (30). Then samples were solubilized in the SDS loading buffer and resolved on 12% SDS-polyacrylamide gels, followed by electrophoretic transfer to nitrocellulose. The nitrocellulose blots were incubated with a monoclonal mouse anti-connexin-43 IgG antibody (Zymed Laboratories Inc.), or a monoclonal mouse anti-ryanodine receptor IgM antibody (Zymed Laboratories Inc.), or a monoclonal mouse anti-InsP3 receptor IgG antibody (American Research Products Inc.). The blots were then washed three times with TBS containing 0.1% Tween 20, incubated with a peroxidase-labeled affinity purified anti-mouse IgG antibody or a peroxidase-labeled whole anti-mouse Ig antibody, washed again, and then developed using an enhanced chemiluminescence system.

Immunofluorescence Analysis—Cells on a glass cover slide were fixed
Intercellular Calcium Signaling in Transfected Cells

Fig. 2. Intercellular Ca\(^{2+}\) wave on cultured cells in response to caffeine. [Ca\(^{2+}\)], maps in a field of cells at 0, 10, and 20 s after the onset of the peak increase in [Ca\(^{2+}\)], in caffeine-responsive cells are shown. [Ca\(^{2+}\)] is indicated by a pseudocolor scale bar. A, [Ca\(^{2+}\)] in HEK293 cells transfected with the ryanodine receptor cDNA in response to caffeine. Caffeine (15 mM) infusion increased [Ca\(^{2+}\)], in several clustered cells located at the periphery of a cell island, which were revealed to be cells expressing the ryanodine receptor. B-1, intercellular Ca\(^{2+}\) propagation across connexin-43-expressing cells transfected with ryanodine receptor cDNA in response to caffeine. Caffeine infusion induced a wave of increased [Ca\(^{2+}\)], that was communicated cell by cell to surrounding cells from caffeine-responsive cells. The peak [Ca\(^{2+}\)], reached approximately 500 nM in this example. Note that the peak [Ca\(^{2+}\)], in surrounding cells appeared to be identical to that in caffeine-responsive cells. B-2, effect of octanol on intercellular Ca\(^{2+}\) propagation across cells. Octanol (500 μM) was added to the same cells as in B-1, and 5 min later caffeine stimulation was performed. The intercellular Ca\(^{2+}\) wave across the cells was markedly decreased in magnitude as compared with B-1. B-3, the reversibility of the octanol effect on intercellular Ca\(^{2+}\) propagation across cells. Octanol was washed out with HEPES buffer, and then caffeine stimulation was performed for the same cells as in B-1. The intercellular Ca\(^{2+}\) wave regained the same spatial pattern as that in B-1. C, immunocytochemical localization of the ryanodine receptor protein in the same cells as in B. Primarily caffeine-responsive cells shown in B were revealed to be ryanodine receptor-expressing cells.

Measurement of [Ca\(^{2+}\)] was determined by measurement of fura-2/AM fluorescence (Molecular Probe Inc.) for 1 h, followed by fluorescein isothiocyanate-conjugated streptavidin (Vector Laboratories Inc.) for 1 h. Anti-ryanodine receptor antibody-antigen complexes were visualized using rhodamine-labeled anti-mouse IgM (Organon Teknika Corp.) for 1 h. Cover slides were then mounted in Mowiol 4–88 (Vector Laboratories Inc.). The cells were photographed on a Olympus Provis AX80 microscope fitted with the appropriate filters.

Inhibitors of gap junctional communication, octanol and doxyl stearic acids (DSA) (33, 34) were examined as to their effects on cell-cell coupling by demonstrating an intercellular Ca\(^{2+}\) wave. Following recovery, the cells were superfused with HEPES buffer containing 500 μM octanol or 50 μM DSA to block the cell-cell coupling. After 5 min, the cells were superfused with the HEPES buffer containing 15 mM caffeine a third time to demonstrate the reversibility of the actions of the drugs.
The role of InsP₃-mediated Ca²⁺ release mechanism on the intracellular Ca²⁺ wave was examined by using activators and inhibitors of InsP₃ production. Histamine and vasopressin activate phospholipase C, leading to a dramatic increase in intracellular InsP₃ production (35). Application of histamine or vasopressin was performed by replacing the control HEPES buffer with a solution containing 10 μM histamine or 0.5 mM vasopressin. U73122 inhibits phospholipase C, leading to a block of intracellular InsP₃ production (36, 37). Cells were first superfused with HEPES buffer containing 15 mM caffeine to establish the presence of cell-cell coupling by demonstrating an intracellular Ca²⁺ wave. Following recovery, the cells were superfused with HEPES buffer containing 0.1 mM trichloride, 500 mM Tris-HCl, pH 8.0, and 2 mM EDTA in the presence of caffeine and histamine or vasopressin. After 5 min, the cells were superfused with the HEPES buffer containing 15 mM caffeine a second time.

Electrophysiology—Gap junctional conductance was measured with the double whole cell patch-clamp procedure (16, 38) using Geneclamp 500 amplifier (Axon Instruments, Inc.). The cells were obtained by freshly dissociating pure populations of confluent cultures on 1-cm diameter glass coverslips. The coverslip was transferred to the stage of a Nikon Diaphot microscope, where experiments were performed at room temperature while exchanging the bath solution (133 mM NaCl, 3.6 mM KCl, 1.0 mM CaCl₂, 0.3 mM MgCl₂, 16 mM glucose, 3.0 mM HEPES, pH 7.2). Each cell of a pair was voltage-clamped using patch-needle pipette made on Narishige NA-9 vertical puller and filled with a solution of 0.5 mM CaCl₂, 2 mM MgCl₂, 5.5 mM EDTA, 5.0 mM HEPES, pH 7.2. High resistance seals (>10⁸ Ωm) were formed on each cell with the aid of gentle suction, and access to the cell interior was then gained by brief strong suction applied to the patch-needle pipette. Cells were voltage-clamped at holding potentials of ~40 mV and applied 10 mV voltage pulses to each of the pair. Junctional current (Ij) was measured as the current evoked in one cell by the voltage step in the other cell (Vj). Junctional conductance (Gj) was calculated by the equation Ij/Vj.

Isolation of Mutant Connexin-43s—Highly enriched connexins were obtained by an alkaline extraction procedure (39). The transfected cells were harvested and pelleted. The pellets were resuspended in 2 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 100 mM glycerine NaCl, pH 10, and the aid of a 22-gauge needle, were sonicated for about 20 s in a bath sonicator. The sample was centrifuged at 100,000 × g for 60 min at 4 °C. The supernatant was concentrated with a Centriprep 30 microconcentrator (Amicon). The concentrated protein was applied to a column packed with Superox 6 prep grade (Pharmacia Fine Chemicals) and eluted with 50 mM HEPES, pH 8.0, 500 mM NaCl, and 5 mM EDTA. Fractions collected from the column were analyzed by Western blotting with a monoclonal antibody against connexin-43. Then the fractions containing connexin-43 were pooled.

Reaction of Isolated Mutant Connexin-43s with [¹⁴C]Iodoaceticamide—By repeated concentration and reutilization in a Centrprep 30 microconcentrator, the pooled protein was equilibrated against 7 M guanidine hydrochloride, 500 mM Tris-HCl, pH 8.0, and 2 mM EDTA in the presence or absence of 0.1 mM tri-n-butylphosphine (TBP), a powerful reagent for the specific cleavage of disulfide bonds in proteins (40). The sample was incubated for 20 min at 60 °C, cooled to 23 °C, and then transferred to a tube containing 20 μl of [¹⁴C]Iodoacetamide (8 μM final concentration), as described (41). The reaction mixture was immuno precipitated with a monoclonal anti-connexin-43 antibody mixed with an affinity purified anti-mouse IgG(Fc) antibody and 50 μl of Protein A-Sepharose in a buffer comprising 50 mM HEPES, pH 7.2, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 μM phenylmethylsulfonyl fluoride, 10 μg/ml of aprotinin, and 10 μg/ml leupeptin. The immuno precipitated samples were washed with 50 mM Tris-HCl, pH 7.0, solubilized in SDS loading buffer, and then resolved on 12% SDS-polyacrylamide gels. The gels were dried and examined by autoradiography.

RESULTS

Expression of Transfected Connexin-43 and the Ryanodine Receptor in Cells—Compared with the trivial amounts of connexin-43 mRNA and protein in HEK293 cells, the transfection of HEK293 cells with connexin-43 cDNA resulted in clones with high levels of its mRNA and protein (Fig. 1, A and B). Furthermore, clones expressing connexin-43 revealed a pattern of dense immunoreactivity at cell-cell interfaces (Fig. 1C), in contrast to those in HEK293 cells which showed no detectable immunoreactive sites between cells (data not shown).

When clones expressing connexin-43 were transfected with ryanodine receptor cDNA, the levels of its mRNA and protein were much greater than those in HEK293 cells (Fig. 1, A and B). The immunocytochemical localization of ryanodine receptor protein in transfected cells revealed that approximately 1–5% of the total cells expressed this protein in the cytoplasmic structures (Fig. 2, C), which appeared to be identical to the previous result (30). Caffeine has been shown to be an activator of the ryanodine receptor in the sarcoplasmic reticulum (31, 32). When caffeine was applied to ryanodine receptor cDNA-transfected cells, caffeine-sensitive Ca²⁺ release was observed in one cell at the periphery of cell clusters, which appeared to be ryanodine receptor-expressing cells (Fig. 2A). This indicated that the expressed ryanodine receptor had formed a functional Ca²⁺ release channel in an intracellular membrane in transfected cells.

Intercellular Propagation of Ca²⁺ in Connexin-43-expressing Cells—When connexin-43-expressing cells were transfected with pMT2 vector containing ryanodine receptor cDNA, application of caffeine increased [Ca²⁺]i, in two separate cells in cell cluster, and then the increased [Ca²⁺]i, was propagated cell by cell in all directions to surrounding cells in culture. The magnitude of the increase in [Ca²⁺]i, reached 500 nM and lasted for 30 s as long as the application of caffeine was continued (Fig. 2B-1). Thus, Ca²⁺ released from an intracellular store through the activated ryanodine receptor triggered large scale Ca²⁺ propagation to surrounding cells.

To determine whether the propagation of Ca²⁺ between cells occurred through gap junctions composed of connexin-43, we next added an inhibitor of gap junctional communication to the superfusion. We used octanol and DSA, which are known to block gap junctional communication rapidly and reversibly in many cell types, including cardiac myocytes (33, 34), and chick lens epithelial cells (42). Five minutes after the start of octanol administration, caffeine was applied to the same cells. In contrast to the extensive Ca²⁺ propagation response without the inhibitor, caffeine application increased [Ca²⁺]i, in two cells, which were the same caffeine-responsive cells in the initial experiment, but the propagation of Ca²⁺ did not occur (Fig. 2B-2). After washing out of octanol for 5 min with HEPES buffer, caffeine induced the propagation of Ca²⁺, which showed the same spatial pattern as that in the initial experiment (Fig. 2B-3). When we used DSA as an alternative inhibitor of gap junctional communication, the same phenomenon was observed (data not shown). After observing the Ca²⁺ levels of cells by the Ca²⁺ imaging system, the ryanodine receptor proteins in transfected cells were localized with the same cells by the immunofluorescence assay (Fig. 2C). The localization of ryanodine receptor-expressing cells appeared to be superimposed on the caffeine-responsive cells. Thus, connexin-43s expressed in HEK293 cells form a functional gap junctional pathway for Ca²⁺ at the sites of intercellular connection.

To determine the conductance properties of expressed connexin-43, whole cell voltage-clamp recordings were obtained from cell pairs as described previously (16, 38) (Fig. 3). Repetitive 10 mV pulses (V1, V2) were applied to a pair of voltage-clamped transfected cells. Upward going responses in current trace I1 and I2 represent current flowing through junctional membranes. Downward going responses are currents flowing through nonjunctional and junctional membranes. Junctional current (Ij) was measured as the current evoked in one cell by the voltage step in the other, divided by the amplitude of the voltage step delivered to the other (Vj) (16). Control HEK293
Intercellular Calcium Signaling in Transfected Cells

**FIG. 3.** Ca\(^{2+}\) response of cultured cells to activator or inhibitor of phospholipase C, [Ca\(^{2+}\)], maps in a field of cells at 0, 10, and 20 s after the onset of the peak increase in [Ca\(^{2+}\)], in primarily responsive cells are shown, [Ca\(^{2+}\)], is indicated by a pseudocolor scale bar. A, effect of histamine (10 \(\mu M\)), an activator of phospholipase C, on [Ca\(^{2+}\)], in connexin-43-expressing cells. Histamine (10 \(\mu M\)) infusion induced a global increase in [Ca\(^{2+}\)], in the cells, [Ca\(^{2+}\)], reaching 500 nM. B-I, intercellular Ca\(^{2+}\) propagation across connexin-43-expressing cells transfected with ryanodine receptor cDNA in response to caffeine. Caffeine infusion induced a wave of increased [Ca\(^{2+}\)], that was communicated from cell to surrounding cells from caffeine-responsive cells. Caffeine application increased [Ca\(^{2+}\)], in one cell, which was same caffeine-responsive cell in the initial experiment, but [Ca\(^{2+}\)], in adjacent cells dramatically decreased with distance from caffeine-responsive cells (Fig. 4B-2).

Electrophysiological analysis showed that the treatment of histamine, vasopressin, or U73122 did not affect the junctional conductance of connexin-43 in transfected cells (Fig. 3), indicating that InP3-mediated process is not involved in the gating properties of gap junction. Thus, the result that U73122 decreased [Ca\(^{2+}\)], in adjacent cells with distance from caffeine-responsive cell without changes in junctional conductance confirmed the notion that InP3 production augments [Ca\(^{2+}\)], in adjacent cells by the activation of InP3-mediated Ca\(^{2+}\) release. It is therefore postulated that InP3, and/or Ca\(^{2+}\) itself, diffusing through gap junction, trigger the InP3-mediated Ca\(^{2+}\) release process cell by cell, leading to the observed intercellular Ca\(^{2+}\) wave.

**Intercellular Propagation of Ca\(^{2+}\) in Mutant Connexin-43-Expressing Cells**—To determine whether the conserved cysteine residues in the extracellular loops play an important structural or functional role in gap junctional communication, we constructed six connexin-43 mutants, in which each of the six cysteine residues in the extracellular loops was replaced by a serine residue, and stably expressed them in HEK293 cells. All connexin-43 mutants were overexpressed in the same amount as that of wild type and localized at the interfaces between cells (Fig. 5). The functional properties of each mutant were determined with a Ca\(^{2+}\) imaging system using fura-2/AM, as described for the wild-type connexin-43. Regarding the three cysteine residues in the first extracellular loop, the Cys\(^{61}\) to Ser mutant responded to caffeine but lost the ability of propagation of Ca\(^{2+}\) across neighboring cells (Fig. 6B). On the contrary, the Cys\(^{61}\) to Cys\(^{68}\) to Ser mutants still showed an extensive Ca\(^{2+}\) propagation response to caffeine, and pretreatment with octanol inhibited this Ca\(^{2+}\) wave rapidly and reversibly (Fig. 6A). Thus, the Ca\(^{2+}\) propagation properties of these mutants appeared to be indistinguishable from those of the wild-type connexin-43. As to the three cysteine residues in the second extracellular loop, the Cys\(^{187}\), Cys\(^{192}\), and Cys\(^{198}\) to Ser mutants lost the ability of propagation of Ca\(^{2+}\) (Fig. 6B). We usually confirmed our results using at least three clones for each mutant. Although the amounts of mutant connexin-43 mRNA and protein varied in individual clones, the spatial patterns of the propagated Ca\(^{2+}\) response to caffeine were indistinguishable among the clones for each mutant.

Electrophysiological analysis was used to test cell-expressing
mutant connexin-43 for the ability to form homotypic intercellular channels. Junctional conductances of Cys61 and Cys68 to Ser mutants have approximately identical values to that of wild-type, whereas those of Cys54, Cys187, Cys192, and Cys198 to Ser mutants are the very low values (Fig. 7). These results indicated that the extracellular cysteine at positions 54, 187, 192, and 198 is crucial for the functional gap junction.

Reaction of Isolated Mutant Connexin-43 with [14C]Iodoacetamide—Of the six cysteine residues present in the extracellular loops, four were essential for the formation of the correct structure of gap junctions. To investigate whether these four cysteine residues form disulfide bonds, isolated mutant proteins were reacted with [14C]iodoacetamide, which is incorporated into the free thiol of cysteine but not into the disulfide bond between cysteines. If the TBP-reduced form of a mutant connexin is a much better substrate for alkylation with iodoacetamide than the nonreduced form, this indicates that cysteine residues in the mutant connexin form disulfide bonds. We first demonstrated that the released [Ca2+]i, in caffeine-responsive cells propagates to surrounding cells only when they express the gap junctional protein, connexin-43. Inhibitors of gap junction such as octanol and DSA rapidly and reversibly inhibited the propagation of Ca2+ across cells. Results of Ca2+ imaging system correspond relatively well to the electrophysiological analysis of transfected cell pairs. We therefore concluded that expressed connexin-43 in cultured cells could form the functional gap junction, which is involved in the intercellular Ca2+ wave.

How the Ca2+ wave crosses a gap junction is unknown, but it could depend upon the diffusion of either Ca2+ itself or InsP3 (14, 44). With regard to the diffusion of these messenger molecules, free or cytosolically buffered Ca2+ exhibits a short range and short lifetimes of less than a second, whereas InsP3 is considered as a long range messenger, showing lifetimes between 1 and 60 s (45). In this study, there was no gradual decrease in peak Ca2+, in surrounding cells compared with that in primarily caffeine-responsive cells during an intercel-
lular propagation of Ca$^{2+}$. We therefore hypothesized that regenerative production and local diffusion of messengers such as Ca$^{2+}$ or InsP$_3$ through gap junctions across cells should take place. The intercellular Ca$^{2+}$ wave may occur through an InsP$_3$-mediated process, because heparin, an antagonist of the InsP$_3$ receptor, has been demonstrated to inhibit the propagation of Ca$^{2+}$ between airway epithelial cells (44). We tested the possibility of InsP$_3$-mediated Ca$^{2+}$ release mechanism in our cell system by using activators and inhibitors of InsP$_3$ production. Activators of phospholipase C such as histamine and vasopressin increased [Ca$^{2+}$]$_i$ in cultured cells, whereas inhibitor of phospholipase C such as U73122 decreased intercellular propagation of Ca$^{2+}$ across connexin-43-expressing cells without affecting junctional conductance. Thus, sequential increases in [Ca$^{2+}$]$_i$ in connexin-43-expressing cells during intercellular Ca$^{2+}$ wave should be due to the activation of InsP$_3$-mediated Ca$^{2+}$ release cell by cell. Although the [Ca$^{2+}$]$_i$ sufficient for induction of InsP$_3$-mediated Ca$^{2+}$ release were not known in this study, there have been evidences that phospholipase C is activated by Ca$^{2+}$ (46, 47) and the InsP$_3$ receptor releases Ca$^{2+}$ from intracellular stores by [Ca$^{2+}$]$_i$ (52). In these cells, ATP released from mechanically stimulated cells acts as an extracellular messenger that diffuses to adjacent cells to increase [Ca$^{2+}$]$_i$, through the activation of the P2-purinergic receptors (53, 54). Therefore, the Ca$^{2+}$ wave was always biased by an extracellular flow of fluid, in contrast to the intercellular Ca$^{2+}$ wave through gap junction in this study and others (55).

[Ca$^{2+}$]$_i$ of the proximal cell often reaches its peak during the lag period of intercellular communication before the initiation of a response by an adjacent cell. This period may represent the time taken for the concentration of messengers to reach the threshold, triggering the initiation of Ca$^{2+}$ release from an intracellular store. Increases in [Ca$^{2+}$]$_i$, have been observed to decrease gap junctional coupling in a number of different cell types (8, 9, 17). If the increase in [Ca$^{2+}$], associated with the Ca$^{2+}$ wave results in the closure of gap junctions, this should interrupt the intercellular propagation of the messenger that mediates the Ca$^{2+}$ wave. Assuming that an adjacent cell responds directly to a threshold concentration of a messenger, a Ca$^{2+}$ response to the messenger in an adjacent cell should not occur after the peak increase in [Ca$^{2+}$], in the proximal cell, in contrast to our observation. Thus, we suggested that increases in [Ca$^{2+}$], of up to 500 nM, which was the peak increase in [Ca$^{2+}$], observed in this study, did not inhibit gap junctional communication.

**Cysteine Residues in the Extracellular Loops Are Crucial for Gap Junctions**—The presence of intramolecular disulphide bonds, including inter-loop ones, in connexin has been proposed...
We observed that Cys 54 in the first loop and Cys 187, Cys 192, and Cys 198 in the second loop of connexin-43 are crucial for the intercellular Ca\(^{2+}\) wave. On \([^{14}C]\)iodoacetamide labeling of free thiols of cysteine residues, two disulfide bonds between Cys 54 and Cys 192 and between Cys 187 and Cys 198 were revealed to form. Thus, the disulfide bonds of the extracellular loops of connexin-43 are necessary for gap junctional communication.

It is unclear at which step cysteine mutants of connexin-43 failed to form functional gap junctions from the results of the functional assay, because it relies solely on open channels. As inferred from the results of structural studies on isolated gap junctions (60, 61), the assembly of a gap junction is a multi-stage process, which comprises the oligomerization of six connexin monomers, integration of oligomerized connexins into the plasma membrane through the endoplasmic reticulum to the Golgi transport system, dense clustering at the cell-cell interface, and docking with an oligomerized connexin in an apposing cell membrane to form an intercellular channel. In this study, we observed the immunological localization of connexin-43 mutants at the interfaces between cells and the disulfide bond formation between Cys 54 to Ser, Cys 187 to Ser, Cys 192 to Ser, and Cys 198 to Ser, the increased [Ca\(^{2+}\)], in several clustered cells in response to caffeine (15 mM) did not propagate across neighboring cells.

Fig. 6. Roles of the cysteine residues in the extracellular loops on the intercellular Ca\(^{2+}\) wave in response to caffeine. [Ca\(^{2+}\)], maps in a field of cells at 0, 10, and 20 s after the onset of the peak increase in [Ca\(^{2+}\)], in caffeine-responsive cells are shown. [Ca\(^{2+}\)], is indicated by a pseudocolor scale bar. A, in the Cys\(^{54}\) to Ser and Cys\(^{68}\) to Ser mutant connexin-43s, the increased [Ca\(^{2+}\)], in several clustered cells in response to caffeine (15 mM) propagated across neighboring cells. The spatial pattern and peak [Ca\(^{2+}\)], in cells expressing each mutant were revealed to be identical to those of the wild-type connexin-43. Octanol (500 \(\mu\)M) was added to the same cells, and 5 min later caffeine stimulation was performed. The intercellular Ca\(^{2+}\) wave across the cells was markedly decreased in magnitude. B, in the connexin-43s mutants containing mutations of Cys\(^{54}\) to Ser, Cys\(^{187}\) to Ser, Cys\(^{192}\) to Ser, and Cys\(^{198}\) to Ser, the increased [Ca\(^{2+}\)], in several clustered cells in response to caffeine (15 mM) did not propagate across neighboring cells.

Fig. 7. Junctional conductance (Gj) in pairs of wild-type and mutant connexin-43-expressing cells. Whole cell voltage-clamp recordings were obtained from homotypic pairing of cells expressing connexin-43 or mutants. Gj was calculated as described under “Experimental Procedures.” The column and bar represent mean ± S.D. of \(n\) experiments (number in parentheses).

(56–59). We observed that Cys\(^{54}\) in the first loop and Cys\(^{187}\), Cys\(^{192}\), and Cys\(^{198}\) in the second loop of connexin-43 are crucial for the intercellular Ca\(^{2+}\) wave. On \([^{14}C]\)iodoacetamide labeling of free thiols of cysteine residues, two disulfide bonds between Cys\(^{54}\) and Cys\(^{192}\) and between Cys\(^{187}\) and Cys\(^{198}\) were revealed to form. Thus, the disulfide bonds of the extracellular loops of connexin-43 are necessary for gap junctional communication.

It is unclear at which step cysteine mutants of connexin-43 failed to form functional gap junctions from the results of the functional assay, because it relies solely on open channels. As inferred from the results of structural studies on isolated gap junctions (60, 61), the assembly of a gap junction is a multi-stage process, which comprises the oligomerization of six connexin monomers, integration of oligomerized connexins into the plasma membrane through the endoplasmic reticulum to the Golgi transport system, dense clustering at the cell-cell interface, and docking with an oligomerized connexin in an apposing cell membrane to form an intercellular channel. In this study, we observed the immunological localization of connexin-43 mutants at the interfaces between cells and the disulfide bond formation between Cys\(^{54}\) and Cys\(^{192}\) and between Cys\(^{187}\) and Cys\(^{198}\) in the extracellular loops, which should occur outside the cell, namely in an oxidative environment. Therefore, connexin-43 mutants should be integrated into the plasma membrane, such that their extracellular loops are positioned outside the cell. Thus, the gap junctional discommunication in the mutants containing mutations of Cys\(^{54}\), Cys\(^{187}\), Cys\(^{192}\), and Cys\(^{198}\) to Ser probably occurred at the steps of hemichannel docking and/or channel opening.

An electrophysiological study involving a paired oocyte system has demonstrated that the mutation of all six cysteine residues to serine abolished the junctional communication (56, 57). On the contrary, our results showed that the Cys\(^{61}\) and Cys\(^{68}\) mutants showed an intercellular Ca\(^{2+}\) wave as well as electrical coupling. Immunohistochemical analysis of these mutants on the surface of oocytes revealed the same distribution.
as in the case of the wild type (57). Therefore, the discrepancy between the two studies may be due to differences in the regulation of channel opening.

The present findings substantiate the hypothesis of Sanderson and colleagues that the intercellular Ca\(^{2+}\) wave results from the flux of Ca\(^{2+}\) through InsP\(_3\) receptor in the Ca\(^{2+}\)-storing structure and that the Ca\(^{2+}\) excitation reflects a regenerative action of [Ca\(^{2+}\)]\(\text{e}\), toward the InsP\(_3\) receptor. Although the Ca\(^{2+}\) wave that reflects the excitability of excitable cells progresses thousands of times more slowly than the electrical counterpart, i.e., changes in membrane potential, it is this formal parallel between electrical excitability and Ca\(^{2+}\)-based excitability that may have major and multiple modulating effects on the long range signaling network in excitable cells.

Acknowledgments—We are indebted to Dr. David H. MacLennan for the ryanodine receptor cDNA and Dr. Noriyuki Yamada for help in the electrophysiological analysis.

REFERENCES

Additions and Corrections


The role of C2 domains in Ca⁡²⁺-activated and Ca⁡²⁺-independent protein kinase Cs in aplysia.

Antonio M. Pepio, Xiaotang Fan, and Wayne S. Sossin

Page 19047, Fig. 10: CBR2 was incorrectly assigned to the region between strand 3 and strand 4. There is, thus, not a large difference between the CBR2 domain of APL1 and those of the other PKCs, contrary to the original statement. The correct Fig. 10 is shown below.

\[\text{FIG. 10}\]


Intercellular calcium signaling via gap junction in connexin-43-transfected cells.

Toshihiko Toyofuku, Masanori Yabuki, Kinya Otsu, Tsuruho Kuzuya, Masatsugu Hori, and Michihiko Tada

Pages 1523 and 1524: The legends to Figs. 3 and 4 were transposed. The legend appearing with Fig. 3 should appear with Fig. 4 and vice versa.

Page 1526, Fig. 7: The junctional conductance (Gj) of C54S and C68S should be exchanged.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Intercellular Calcium Signaling via Gap Junction in Connexin-43-transfected Cells
Toshihiko Toyofuku, Masanori Yabuki, Kinya Otsu, Tsunehiko Kuzuya, Masatsugu Hori
and Michihiko Tada

doi: 10.1074/jbc.273.3.1519

Access the most updated version of this article at http://www.jbc.org/content/273/3/1519

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 65 references, 34 of which can be accessed free at
http://www.jbc.org/content/273/3/1519.full.html#ref-list-1