Regulation of Connexin43 Protein Complexes by Intracellular Acidification


Abstract—Ischemia-induced acidification of astrocytes or cardiac myocytes reduces intercellular communication by closing gap junction channels and subsequently internalizing gap junction proteins. To determine whether such coupling changes might be attributable to altered interactions between connexin43 (Cx43) and other proteins, we applied the nigericin/high K+ method to vary intracellular pH (pHi) in cultured cortical astrocytes. Intracellular acidification was accompanied by internalization of Cx43 with retention of Cx43 scaffolding protein Zonula Occludens-1 (ZO-1) at cell surfaces, suggesting that ZO-1 and Cx43 dissociate at low pHi. Coimmunoprecipitation studies revealed decreased binding of ZO-1 and increased binding of c-Src to Cx43 at low pHi. Resonant mirror spectroscopy was used to quantify binding of the SH3 domain of c-Src and the PDZ domains of ZO-1 to the carboxyl terminal domain of Cx43 (Cx43CT). Data indicate that the c-Src/Cx43CT interaction is highly pH dependent whereas the ZO-1/Cx43CT interaction is not. Moreover, binding of c-Src to Cx43CT prevented and reversed ZO-1/Cx43CT binding. We hypothesize that increased affinity of c-Src for Cx43 at low pHi aids in separation of Cx43 from ZO-1 and that this may facilitate internalization of Cx43. These data suggest that protracted acidification may remodel protein-protein interactions involving Cx43 and thus provide an important protective mechanism to limit lesion spread after ischemic injury. (Circ Res. 2004;94:215-222.)

Key Words: gap junctions ■ ischemia ■ protein-protein interactions ■ resonant mirror spectroscopy ■ central nervous system

Connexins are membrane proteins that oligomerize to form gap junction channels, through which ions and small molecules diffuse between cells. The present view is that these channels are not passive pores insulated from the rest of the molecular environment but rather are dynamic protein complexes that can both filter and amplify passage of information between cells. Among proteins known to associate with Connexin43 (Cx43) (the most abundant gap junction in heart and in astrocytes) are the scaffolding protein Zonula Occludens-1 (ZO-1) and the tyrosine kinase c-Src. Recent studies show that this association is potentially interlinked, because binding of Cx43 to constitutively active c-Src is correlated with a decrease in the Cx43/ZO-1 interaction. Still unclear are whether there is a direct interdependence to these interactions and whether these associations are modified by factors that close gap junctions. The present study centers on the pH dependence of interactions of Cx43 with ZO-1 and c-Src. Intracellular acidification leads to closure of gap junction channels, which is relevant in the context of ischemia, where a drop in intracellular pH (pHi) has been demonstrated both in heart and in brain. Cardiac ischemia leads to electrical uncoupling as well as to dephosphorylation and internalization of Cx43. Changes in the composition of gap junction complexes attributable to sustained intracellular acidification may therefore be fundamental in determining the extent of ischemic damage and, in the case of heart, development of life-threatening ventricular arrhythmias. In this study, we assess whether changes in function and cellular distribution of gap junctions are concurrent with modifications in the composition of the c-Src/Cx43 molecular complex. Our data indicate that intracellular acidification-induced internalization of Cx43 coincides with an enhanced association of Cx43 to c-Src and a loss of association of Cx43 to ZO-1. Spectroscopic analysis of protein binding using relevant domains additionally revealed that the dissociation of Cx43 from ZO-1 is a consequence of enhanced Cx43/c-Src association at low pH. Such changes in intermolecular affinity may be critical steps in gap junction remodeling after infarct in brain and in heart.

Materials and Methods
Composition of all primers and solutions used that have not been previously published is provided in the online data supplement, available at http://circres.ahajournals.org.

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Production of Purified Protein

Full-length rat Cx43 carboxyl terminal domain was cloned, expressed, and purified as previously described. Recombinant Cx43CT after cleavage from GST contained the sequence 255-382 of rCx43 preceded by four additional amino acids (GPLG). PDZ domains of ZO-1 were cloned into GEX-6P-2 plasmids, and the SH3 domain of c-Src was cloned as previously described, transformed into BL-21 competent bacterial cells, and expressed as previously described.

Cell Culture

Pregnant C57Bl6 mice from Charles River distributors (Wilmington, Mass) were maintained in AALVC-accredited facilities, and all procedures were approved by the Albert Einstein College of Medicine Animal Use Committee. Astrocyte cultures were prepared as described.

pH Treatments

Intracellular pH was clamped to that of the extracellular solution using high K+ nigericin solutions at different pH values. Cultured astrocytes were incubated in K+/nigericin at pH 7.4 or 6.5 at 37°C in a room-air isolation chamber.

Scrape-Loading

Astrocytes were grown to confluence and then incubated for 30 minutes in high K+/nigericin solution at either pH 7.4 or 6.5. Incisions through monolayers were made in the presence of 0.5% Lucifer Yellow (Sigma). Data were processed and analyzed as previously described.

Lucifer Yellow Microinjection

Confluent cultures were rinsed in K+/nigericin buffer (as above) at pH 7.4 or pH 6.5 and then incubated in matching K+/nigericin buffer for 15 minutes at 37°C. Dye injections were as previously described. To determine extent of reversibility of acidification-induced uncoupling, cells were injected at 3- to 4-minute intervals and photographed 1 to 2 minutes after injection using film or digital media on a Nikon Diaphot microscope equipped with FITC illumination and excitation filters.

Immunostaining

Astrocytes were incubated in K+/nigericin at pH 7.4 or 6.5 for 30 minutes, stained for either Cx43 or ZO-1, as previously described. Cells were examined and photographed using an Olympus FV500 confocal fluorescence microscope. Images were analyzed using MetaMorph image analysis software.

Comunoprecipitation

Confluent 100-mm dishes of cortical astrocytes were lysed on ice, scrape-harvested, and sonicated for 10 seconds and then incubated for 30 minutes on ice and spun at 4°C for 10 minutes at 12 000 rpm. Supernatant was precleared with 50 μL CL-4B buffer for 60 minutes and spun for 3 minutes at 4°C, 5000 rpm, and supernatant was retained. Primary antibody (4 μL) was added and rotated for 60 minutes, and then 50 μL CL-4B was added and rotated at 4°C overnight. After spin, supernatant was removed and pellet-washed three times with lysis buffer and spun at 4°C at 5000 rpm for 2 minutes between washes, and then supernatant was removed and 60 μL loading buffer was added. Samples were incubated at 50°C for 5 minutes, immediately placed on ice for 1 minute, and spun for 3 minutes at 4°C and 10 000 rpm. Supernatant was retained for loading into 7.5% (ZO-1) or 10% (Cx43 and c-Src) SDS gels for Western blots.

Resonant Mirror Spectroscopy

Resonant mirror spectroscopy (RMS) used purified recombinant Cx43CT protein bound covalently (EDC/NHS coupling) to a carboxymethyl dextran matrix linked to a loading cuvette (Applied Sensor Technology), as previously described, and data were acquired using the IASYS software package.

Results

Acidification-Induced Uncoupling in Cultured Astrocytes

The purpose of this study was to assess changes in the Cx43/ZO-1/c-Src complex consequent to low pH and correlate them temporally with cell-cell uncoupling and internalization of Cx43. For these studies, we used primary cultures of mouse cortical astrocytes, which are highly coupled primarily by gap junctions formed of Cx43 and are particularly amenable to the various procedures used in this study. Intracellular coupling among astrocytes is important in maintaining ionoic and metabolic homeostasis in brain, and decreased coupling after an ischemic event may limit infarct size in brain.

Intracellular Acidification Limits Dye Spread in Cultured Astrocytes

Astrocytes have been shown to uncouple in response to intracellular acidification achieved by CO2 exposure. To determine the effect on intercellular coupling of longer-term exposure to reduced pH, cells were incubated for 30 minutes in K+/nigericin at pH 7.4 or 6.5, under which conditions extracellular and intracellular pH are very nearly identical. Intercellular coupling was assessed both by scrape-loading, and by intracellular Lucifer Yellow injections.

Scrape-Loading

When cells were maintained in K+/nigericin for 30 minutes at a pH 7.4, dye spread extended well beyond the line of the scrape (black arrow), encompassing large numbers of cells (Figure 1Ai, pH 7.4). However, in a similarly confluent culture at pH 6.5 for 30 minutes, dye coupling was decreased (Fig 1Ai, pH 6.5). We quantified the distance of dye spread using conventional image analysis (representative quantifications illustrated in Figure 1Aii). Statistical analysis revealed that average dye spread at pH 7.4 for 30 minutes was significantly greater (P<0.01) than at pH 6.5 (Figure 1B).

Dye Injections

Spread of dye injected into individual cells within clusters was quantified after 30 minutes of exposure to high K+/nigericin at pH 7.4 or 6.5. As shown in Figure 1C, at pH 7.4, dye spread extensively throughout the cluster (coupled cells marked by asterisks). Under these conditions, dye spread to an average of 4 ± 0.5 cells (n = 38 injections). The number of coupled cells visualized after 30 minutes at pH 6.5 significantly decreased (Figure 1C), on average, to 1.6 ± 0.4 cells per cluster (n = 46, P<0.01). After return to pH 7.4 for 30 minutes, coupling increased to 3.8 ± 0.5 cells per cluster (n = 33, P<0.001). Alterations in coupling are quantified in Figure 1D.

Changes in the Colocalization of Cx43 and ZO-1 in Response to Intracellular Acidification

Previous studies have shown that global ischemia leads to decreased pH and subsequent internalization of Cx43. It has also been shown that Cx43 colocalizes with the scaffolding
protein ZO-1 under normal conditions, although not completely. In this study we asked whether acidification-induced internalization of Cx43 was accompanied by disruption of the Cx43/ZO-1 complex. As a first approach to this question, we examined the cellular localization of Cx43 and ZO-1 in cultured cortical astrocytes after 30 minutes of incubation in K+/H11001/nigericin solutions at pH 7.4 or 6.5. At pH 7.4, astrocytes showed normal morphology with both Cx43 (Figure 2, pH 7.4, red) and ZO-1 (Figure 2, pH 7.4, green), largely localized to plasma membranes between contiguous cells. Under conditions of low pH, membrane localization of ZO-1 persisted (Figure 2, pH 6.5, green), but most of the Cx43 signal was detected in the cytoplasm (Figure 2, pH 6.5, red). Overlay of stacked confocal images showed that the previously reported partial colocalization of the two proteins was most noticeable when cells were maintained at pH 7.4 (right panels). These data suggest that in cells maintaining cell-cell contact, intracellular acidification may lead to the dissociation of the Cx43/ZO-1 complex. In contrast, astrocytes stained for Cx43 and ZO-1 after acute enzymatic dissociation but maintained at normal pH showed that both proteins localized to the cytoplasm (data not shown), consistent with previous findings.

**Coimmunoprecipitation of Cx43 and ZO-1: Effect of Low pH**

To examine the effect of low pH on the Cx43/ZO-1 interaction, either Cx43 or ZO-1 was immunoprecipitated from cortical astrocytes maintained for 30 minutes at pH 7.4 or 6.5. Coimmunoprecipitation of Cx43 and ZO-1: Effect of Low pH

Two proteins were most noticeable when cells were maintained at pH 7.4 (right panels). These data suggest that in cells maintaining cell-cell contact, intracellular acidification may lead to the dissociation of the Cx43/ZO-1 complex. In contrast, astrocytes stained for Cx43 and ZO-1 after acute enzymatic dissociation but maintained at normal pH showed that both proteins localized to the cytoplasm (data not shown), consistent with previous findings.

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Inhibition of ZO-1 Binding to Cx43CT by c-Src
Our confocal microscopy and co-IP studies from cultured astrocytes showed decreased Cx43/ZO-1 association at low pH; however, RMS experiments on isolated proteins revealed no pH dependence to the PDZ-2/Cx43CT association. One simple explanation for this apparent discrepancy could be...
pH-dependent modulation of the ZO-1/Cx43 interaction by a separate molecule, present in astrocytes but absent in the in vitro RMS studies. One possible candidate is c-Src, which shows pH-dependent association with Cx43 (Figure 5) and has been reported to disrupt binding of ZO-1 to Cx43.4,5 To test this hypothesis, we used RMS to evaluate whether c-Src binding to Cx43CT interferes with binding of ZO-1 (Figure 6). In one series of experiments, binding of Cx43CT to PDZ-2 was determined in the presence of varying concentrations of the SH3 domain of c-Src at pH 6.5 (Figure 6A). PDZ-2 (50 μmol/L) was added to the cuvette at the time indicated by the upward closed arrow. After removal of PDZ-2 (downward closed arrow) and reconstitution of the cuvette, 2.4 mmol/L SH3 domain of c-Src was added to the cuvette (2.5 μmol/L; upward open arrow). After 10 minutes, while still in the presence of the SH3 domain, PDZ-2 (50 μmol/L) was added (downward open arrow). Clearly, even though the concentration of PDZ-2 was the same as that tested in the absence of SH3, the response was significantly diminished. Hence, presence of the SH3 domain of c-Src decreased the Cx43CT–PDZ-2 interaction. As shown in
Figure 6B, the decrease in ZO-1 binding was strongly dose-dependent (Figure 6B). Confirmation of the disruption of PDZ-2 binding by the SH3 domain of c-Src was obtained when the SH3 domain was added to the Cx43CT/ZO-1 complex (Figure 6C). Because both peptides bind, it might be expected that binding of these two domains would be additive, in which case an increase in resonance signal would be expected. Instead, a decrease in resonance signal was observed, suggesting that as the SH3 domain of c-Src binds to the Cx43CT, the PDZ-2 domain begins to dissociate, leading to an overall decrease in the resonance signal. These findings indicate that binding of c-Src to Cx43CT interferes, in a concentration-dependent manner, with the association of Cx43CT with ZO-1. We propose that decreased ZO-1 binding to Cx43, as seen by immunofluorescence and co-IP at low pH, may be, in part, a consequence of increased Cx43-c-Src interaction.

Discussion

Recent studies have demonstrated that connexin molecules bind noncovalently to a variety of scaffolding as well as regulatory proteins.24 These interactions are not expected to be static; rather, modifications in protein-protein interactions are known to be fundamental to regulation of cell function.25 This study focuses on the effects of intracellular pH changes on interactions between Cx43 and two of its partners, the scaffolding protein ZO-1 and the tyrosine kinase c-Src. Although these interactions have been demonstrated in the past, their dynamic regulation consequent to changes in intracellular environment has not been previously investigated. Our data show for the first time that intracellular acidification leads to an enhanced association between Cx43 and c-Src and consequently reduces the association of Cx43CT to ZO-1.

Ischemia produces decreases in pHi in brain9 and heart8 and decreases cell-cell coupling.6,7 Whereas rapid uncoupling in response to low pHi apparently involves conformational changes in the connexin molecule itself,12 prolonged intracellular acidification induces relocalization of Cx43 protein from the plasma membrane to the cytoplasm.11,26,27 The present studies indicate that decreased pHi alters Cx43-protein interactions in cultured murine astrocytes, leading to the hypothesis that changes in affinities to binding partners may contribute to translocation of Cx43 in ischemic cells.

Studies of protein complexes in neuronal synapses have shown that interactions of membrane proteins, such as the N-methyl-D-aspartate (NMDA) receptor and the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor with PDZ-containing scaffolding proteins (PSD-95 for the NMDA receptor, PICK and GRIP for the AMPA receptor), play a major role in channel number, localization at synapses, and regulation.28 The data presented here suggest that the interaction of Cx43 with the PDZ domain–containing protein ZO-1 may also play such a role in gap junctions. It has been suggested that interactions between Cx43 and ZO-1 may be important for trafficking of gap junction proteins to appositional membranes.3 Examination of exogenously expressed Cx43 truncated at amino acid 257, thus removing the PDZ-binding domain that interacts with ZO-1, shows that this
construct readily forms functional channels, although the gating properties of these channels differ from those formed of full-length Cx43. Although detailed trafficking studies of truncated mutants have not appeared, these observations suggest that Cx43/ZO-1 interactions are not required for localization of Cx43 at junctional membranes. This hypothesis is supported by the apparently normal trafficking of C-terminal EGFP-tagged or aequorin-tagged Cx43, which presumably disrupts the Cx43/ZO-1 interaction. Alternatively, ZO-1 may act as a scaffold by which Cx43CT is held in close contact with protein partners that are important in normal function of Cx43 gap junction channels. Regulation of this interaction by intracellular acidification would therefore alter connexin function.

Alterations in binding of protein partners under conditions of intracellular acidification may have functional implications for regulation of the Cx43 channel. It has been known for some time that interactions of Cx43 with the tyrosine kinase c-Src can close gap junction channels. c-Src becomes activated on acidification, and constitutively active Src has been shown to bind to Cx43 and disrupt the Cx43/ZO-1 interaction. Toyofuku et al argued that dissociation of Cx43 from ZO-1 was secondary to tyrosine phosphorylation of Cx43CT by binding of the SH2 domain of c-Src. In contrast, our data show that disruption of binding of Cx43 to ZO-1 may occur consequent to interactions with the SH3 domain of c-Src, independent of changes in phosphorylation state of Cx43. Indeed, using RMS, we show that decreased pH favored binding of the SH3 domain to Cx43CT, which reduces ZO-1 binding to Cx43CT, even in the absence of phosphorylation of Cx43 and in the absence of the SH2 domain of c-Src. This suggests that loss of interaction of Cx43 with its scaffolding protein might allow it to be targeted for internalization and subsequent degradation. Rather than be a primary mechanism in rapid closure of the channel, this would remold the junctional contacts over a longer time period. This hypothesis would be consistent with the finding that a dominant-negative form of ZO-1 causes a loss of Cx43 at cell membranes, which was originally interpreted as a trafficking defect, although our data suggest this may be attributable to accelerated internalization of Cx43 rather than a deficit in delivery to the surface membranes.

Studies of gap junctions in adult heart in response to ischemia and in astrocytes in response to intracellular acidification (present study) reveal internalization of Cx43. Our studies show that Cx43 internalization is not accompanied by the internalization of ZO-1 at junctional membranes, in contrast to what is seen after dissociation of cells where cell-cell contact is lost. When cells are dissociated, Cx43 is internalized along with other junctional types, and ZO-1 increases its association with Cx43. Although the increased Cx43/ZO-1 association seems to contrast with results presented in this study, it is important to note that procedures leading to the uncoupling of cells are drastically different. Particularly relevant is that enzymatic dissociation leads to the physical breakdown of the entire intercalated disk (the cells are no longer in contact) and, at least in rat cardiac myocytes, eventual disappearance of cell adhesion proteins. In contrast, intracellular acidification causes electrical uncoupling and closure of gap junctions, but the physical integrity of cell-cell contacts is preserved. Moreover, the studies of Matsushita et al show that at early times after myocardial infarction (which leads to a drop in intracellular pH), gap junctions almost disappear from the intercalated disk, whereas desmosomes and adherens junctions remain. Because ZO-1 associates with other non–gap junction proteins at the intercalated disk (eg, cadherin), it is tempting to speculate that physical disruption of the whole intercalated disk may lead to internalization of the scaffolding protein (perhaps because little is left in need of a scaffold). Yet, under conditions of low pH (perhaps under conditions of acute myocardial infarction), ZO-1 may stay in contact with other molecular partners, which themselves remain at the intercalated disk. In this regard, we have found that dissociation of astrocytes also leads to internalization of both ZO-1 and Cx43 and colocalization of both proteins in the cytoplasm (data not shown).

Our studies allow us to propose that under conditions of cellular stress, such as intracellular acidification, c-Src, through its SH3 domain, increases its affinity for Cx43CT. Binding of these domains would lead to a conformational change in the Cx43CT structure and disruption of the Cx43CT/ZO-1 interaction. It is tempting to speculate that free Cx43CT would then be able to interact with other binding partners. Either the interaction of Cx43CT with other molecules or the conformational change itself may mark the protein for internalization. Intracellular acidification is expected to reduce intercellular coupling through both rapid gating effects and the slower internalization shown here. Whereas rapid uncoupling may be neuroprotective or cardioprotective, preventing passage of toxins or apoptotic signals between cells that have undergone an ischemic insult, it seems likely that acidification-induced removal of Cx43 from junctional domains may set the stage for subsequent remodeling of junctional connections in postinfarct brain and heart.

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References


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Primers Used:
(Primers were: **PDZ-1** forward GAC GGA TCC ATG GAG GAA ACA GCT ATA TG, reverse CTG GAA TTC TCA ATC TTC TTC ATT ATC AGA TA C; **PDZ-2** forward GAC GGA TCC AGT GCA AGT AGA GAG AGG AGC, reverse GTC GAA TTC TCA TGA AAT GTC GTC TCT CTC AGA G; **PDZ-3** forward GAC GGA TCC CCT GTC AGT CCA TCT GAT G, reverse GTC GAA TTC TCA ATA GAA AGA ATC TCC TAC ATC

Solutions Used:
For acidification of the intracellular space we used a K⁺/nigericin (145mM KCl, 10 mM glucose, 1mM CaCl₂, 1 mM MOPS, 0.5 ?M nigericin) solution, pH 7.4 or 6.5. For co-immunoprecipitation studies we used a standard lysis buffer (150mM NaCl, 50 mM Tris, 1% Tween-20, 0.2% sodium dodecyl sulfate, 1 tablet “Complete” protease inhibitor cocktail (Roche Laboratories, Palo Alto, CA). Sepharous A beads for immunoprecipitation were prepared from 0.5 g of Protein Sepharose A powder (Pharmacia Biotech AB, Uppsala Sweden) with 4 ml lysis buffer for 15 min on ice, rinsed in lysis buffer then diluted to 1:1 (Protein Sepharose A:Lysis buffer). The loading buffer for Western blots was a standard 2X Laemmli + DTT at 10:1 ratio.