Contribution of Intracellular Calcium and pH in Ischemic Uncoupling of Cardiac Gap Junction Channels Formed of Connexins 43, 40, and 45: A Critical Function of C-Terminal Domain

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Abstract
Ischemia is known to inhibit gap junction (GJ) mediated intercellular communication. However the detail mechanisms of this inhibition are largely unknown. In the present study, we determined the vulnerability of different cardiac GJ channels formed of connexins (Cxs) 43, 40, and 45 to simulated ischemia, by creating oxygen glucose deprived (OGD) condition. 5 minutes of OGD decreased the junctional conductance (Gj) of Cx43, Cx40 and Cx45 by 53±3%, 64±1% and 85±2% respectively. Reduction of Gj was prevented completely by restricting the change of both intracellular calcium ([Ca2+]i) and pH (pHi) with potassium phosphate buffer. Clamping of either [Ca2+]i or pHi through BAPTA (2 mM) or HEPES (80 mM) respectively, offered partial resistance to ischemic uncoupling. Anti-calmodulin antibody attenuated the uncoupling of Cx43 and Cx45 significantly but not of Cx40. Furthermore, OGD could reduce only 26±2% of Gj in C-terminal (CT) truncated Cx43 (Cx43-Δ257). Tethering CT of Cx43 to the CT-truncated Cx40 (Cx40-Δ249), and Cx45 (Cx45-Δ272) helped to resist OGD mediated uncoupling. Moreover, CT domain played a significant role in determining the junction current density and plaque diameter. Our results suggest; OGD mediated uncoupling of GJ channels is primarily due to elevated [Ca2+]i and acidic pH, though the latter contributes more. Among Cx43, Cx40 and Cx45, Cx43 is the most resistant to OGD while Cx45 is the most sensitive one. CT of Cx43 has major necessary elements for OGD induced uncoupling and it can complement CT of Cx40 and Cx45.

Introduction
Gap junctions (GJs) participate in traffic of signalling molecules and propagation of electrical impulse between adjacent cells by forming intercellular channels. Six connexin (Cx) molecules assemble to form cell surface hemichannel, whereas GJs are formed by the docking of two hemichannels from the adjacent cells [1]. Till date, more than 20 Cxs have been reported in human [2]. Cxs have four transmembrane domains, two extracellular loops, one intracellular loop and cytoplasmic N and C-termini [1]. In mammalian heart, 3 major Cx isoforms i.e. Cx45, Cx43 and Cx40 are expressed. Expression of Cx43 predominates in all parts of the heart, whereas expression of Cx40 and Cx45 are compartmentalized [3,4]. Overlapping expression pattern of Cxs raised the possibility of the formation of homomeric, heteromeric, homotypic and heterotypic junctions in heart [5-8]. Un-apposed hemichannels on the cell surface can function in certain physiological conditions, whereas GJs are constituted active. The length of C-terminus (CT) varies among different Cx isoforms. CT plays a crucial role in the assembly, degradation and function of GJs. CT of Cx43 harbours multiple phosphorylation sites for several protein kinases [9,10].

During ischemia, gap junctional communication is compromised which may cause cardiac arrhythmia [11-13]. Interestingly, hemichannels and GJs behave differently in response to metabolic inhibition or simulated ischemia. Metabolic inhibition induces the opening of hemichannels but reduces the gap junctional coupling [14,15]. Uncoupling of GJs, limit the spread of ischemic damage [16]. The association of Cx43 in both ischemia-reperfusion injury and ischemic preconditioning, a mechanism by which repeated sub-lethal ischemia protects tissue from severe ischemia have been widely studied [17,18]. Ischemia-induced dephosphorylation of Cx43 and its translocation to the intracellular pool plays a major contribution in the inhibition of junctional communication [12]. Ischemia lowers the intracellular pH (pHi) and increase cytoplasmic calcium ([Ca2+]i). Acidic pHi and elevated [Ca2+]i have been shown to disrupt cell-cell communication in many cells including cardiomyocytes [19,20]. The inhibitory effect of [Ca2+]i rise on junctional communication is possibly mediated through calmodulin [21]. Effect of intracellular acidification varies among different Cxs. Among the 3 cardiac Cxs, Cx45 and Cx40 showed highest and lowest pH-sensitivity, with Cx43 being intermediate [22]. CT of many Cxs dictates the pH sensitivity. Removal of CT impairs the pH gating of Cx43 and Cx40, but it had no effect on the pH
sensitivity of Cx45. Further, a chimera of Cx40 having CT domain from Cx43 restored the pH sensitivity and vice versa [22].

Most of the studies relating to the ischemic uncoupling included ‘whole heart’ or ‘isolated cardiomyocytes’ where Cx45 and Cx40 containing GJs do exist, apart from Cx43 [12,13,23]. Therefore, the observed cumulative effect does not throw light on the susceptibility of individual Cxs to ischemia. Also, the molecular mechanism underlying ischemia mediated uncoupling of GJs is poorly understood. To study the effect of intracellular acidification on GJs, 100% CO2 have often been used, which hardly mimics ischemic condition. Most studies, related to the effect of pHi or [Ca^2+]i on GJs studied individually in a non-ischemic milieu.

In the present study, we mimicked ischemia by creating oxygen glucose deprivation (OGD) and studied its effect on homotypic, homotypic GJs constituting of Cx45, Cx43 and Cx40, expressed in Neuro-2a (N2a) cells. The cumulative effect as well as individual contribution of pHi drop and [Ca^2+]i, rise on the uncoupling of different GJs were studied in OGD condition. We observed that Cx45 is more susceptible to OGD, compared to Cx43 and Cx40. OGD caused maximum inhibition of junctional conductance (Gj) in Cx45 followed by Cx40 and Cx43. The role of CT in ischemic uncoupling was also studied by generating CT truncated mutants and swapping the CT between different Cxs.

Materials and Methods

Molecular biology
cDNA of mouse Cx45 and Cx40 was kindly provided by Prof. Klaus Willecke, Germany. Mouse eGFP tagged Cx43 (Cx43-eGFP) was provide by Prof. Feliksas F. Bukauskas, Albert Einstein College of Medicine, New York. Cx43, Cx45 and Cx40 were sub-cloned into pIRESV-DsRed or pIRESV-eGFP expression vector (Clontech, USA) by using standard molecular biology techniques. Cx45 was also sub-cloned into pEGFPN1 vector (Clontech, USA) to express it as a fusion protein with eGFP. All the constructs were sequenced, followed by functional studies using patch clamp.

CT truncated Cx45, Cx43 and Cx40 were generated with the help of PCR based site directed mutagenesis by placing a stop codon at amino acid positions 272, 257 and 249 respectively, to generate Cx45-D272, Cx43-D257 and Cx40-D249. The primers used for generating CT truncated mutants are listed in table S1.

For creating CT-chimeric connexins, two step PCR based site directed mutagenesis was used according to previously published protocol [24]. Briefly, in the first PCR reaction, the Cx’s CT-domain that has to be ligated with other Cx, was used as a cDNA template. Primers having complementary sequences of both Cxs were used in the first PCR reaction. The amplified products of first PCR reaction bearing the complete CT part, was purified with the help of gel extraction kit (Qiagen, Germany) and used as mega-primers for the second PCR cycle. The Cx that contributes the N-terminus (NT) was used as template in the second PCR cycle to generate the CT-chimera. Constructs were confirmed by sequencing. The primer sets used for generating different chimeras are listed in table S2. Cx43-C40 represents chimeric Cx43 with the CT of Cx40; other chimeras are designated similarly.

Cell culture and transfection

N2a cells were obtained from National Centre for Cell Science, India. For routine maintenance, cells were grown in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% heat inactivated foetal bovine serum and 100 units/ml amantycine and antibiotic mixture (Gibco, USA). Cells were maintained at 37°C with 5% CO2 in a humidified incubator (Thermo scientific, USA). For patch clamp recording, cells were grown on size-0 glass cover slips (Himedia Labs, India). Cells were transfected with desired Cx cDNA constructs using Lipofectamine-2000 (Invitrogen, USA) in serum free media. After 4–5 hours of transfection, the serum free media was replaced with normal growth media containing 10% serum. All the experiments were performed after 24–36 hours of transfection.

Oxygen glucose deprivation (OGD)

Ischemia was simulated by exposing the cells to OGD, as described in earlier reports [25–27]. Cells grown on cover slips, were perfused first with bicarbonate external solution (ES; pH 7.4) containing (in mM) 124 NaCl, 4 KCl, 26 NaHCO3, 1.5 NaH2PO4, 1.5 MgSO4, 10 D-Glucose, 2 CaCl2. The solution was continuously bubbled with 5% CO2 and 95% air, at room temperature (22–24°C). OGD was created by replacing the ES with ischemic solution (IS). IS has the same composition as ES with the exception of glucose being replaced with sucrose. IS was degassed for 1 hour followed by continuous bubbling with mixed gas containing 5% CO2 and 95% of Argon. To assure complete removal of dissolved O2, O2 scavenger sodium dithionite (2 mM) was added. Complete removal of dissolved O2 was confirmed by analysing the IS with a dissolved O2 analyser (Mettler Toledo, USA). To study the effect of ischemia, cells were exposed to IS for 5 minutes.

Patch clamp recording

For electrophysiology, N2a cells on cover slips were transferred to a recording chamber (RC-26G, Warner Instruments, USA), mounted on the stage of an Olympus IX71 inverted microscope, attached with EMCCD camera (Andor Technology, UK). The chamber was continuously perfused with ES. For measuring junctional conductance, cell pairs were patched with two Axopatch 200B (Molecular devices, USA) amplifiers. Thin walled glass pipettes of resistances 3–5 MΩ were prepared using pipette puller P-97 (Sutter Instrument Company, USA). The pipette solution contained (in mM) 10 NaCl, 140 KCl, 1 MgCl2, 0.2 CaCl2, 3 Mg-ATP, 5 HEPES (pH 7.2), 2 EGTA, unless otherwise mentioned. After obtaining whole cell, both cells were held at 0 mV. Holding potentials of cell-1 and cell-2 are designated as V1 and V2 respectively. To create junctional voltage gradient (Vj = V1 - V2), Vj was stepped to different voltages levels, keeping the V2 at 0 mV. The current recorded from cell-2, represents the junctional current (Ij = Ij1 - Ij2). The current traces were low pass filtered at 1 kHz and sampled at 10 kHz with the help of Digidata 1440 (Molecular devices, USA).

In OGD experiments, Vj of ±15 mV was applied to cell-1 for 5 second, with 5 second recovery step at 0 mV in between the pulses. The Ij obtained from cell-2, was normalized to 15 mV of Vj at every 10th second to calculate Gj. The maximum conductance, Gjmax of each experiment was used to normalize Gj. The mean±SEM of normalized Gj generated from 5–7 independent experiments were plotted against time, to check the degree of uncoupling due to OGD treatment.

To determine the voltage sensitivity of different Cxs, step protocol with an increment of 20 mV was applied to cell-1 ranging from -120 mV to +120 mV for 20 s. There was a pause of 30 s at 0 mV between two sweeps. Is obtained in response to 10 mV pre pulse were used to normalize the respective current traces. The normalized steady state Gj (Gj0) was plotted with respect to Vj. To calculate voltage dependency, Gjmax-Vj plot was fitted with two-states Boltzmann’s equation that assumes channel gating is a two steps process. The following form of Boltzmann’s equation was used: Gjmax = [Gjmax-Gjmin/{1+exp[(AVj-V0)/V1]}]+Gjmin, where Gjmax is the normalized maximum conductance (equal to

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compared to check the effect of truncation on channel gating. Ij

temperature, respectively.

charge q that acts as voltage sensor for the transition from open to

The value of RpH = 7 was obtained at the end of each experiment,

ment were performed after 10 minutes of washing in BCECF free

ments were performed after 10 minutes of washing in fura-2-

m

M Fura-2-AM (Molecular Probes, USA) for 10 minutes in the same buffer, used

were washed in fura-2 free buffer for 30 minutes. Fura-2 loaded

Intracellular pH (pHi) measurement

Intracellular calcium was measured ratio-metrically with Fura-

Calcium imaging

Intracellular calcium was measured ratio-metrically with Fura-

pHi was normalized the Gj(ss). The normalized Gj(ss) (with respect to

For clamping pHi at a particular value during OGD, pipette

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 20 minutes, washed thoroughly with 1X phosphate buffer saline (PBS), (GIBCO, USA), followed by blocking with 10% FBS and 0.3%

For clamping pHi at a particular value during OGD, pipette solution with increasing concentrations of HEPES (5, 10, 20, 50 & 80 mM) was used. With increasing concentration of HEPES, equimolecular amount of KCl was removed to maintain the osmolarity. The pipette solutions were usually supplemented with 50 μM BCECF free acids (Molecular Probes, USA) to avoid dilution of the BCECF dye inside the cell. At the onset of the experiments, the pipette solutions were adjusted to pH 7.2 with KOH.

Statistical analysis

Data provided here are representative of 3–5 independent experiments. Values are mean±SEM of 5–11 replicates as described in the respective figure legends. Student paired t-test and one way ANOVA were performed for comparison between two and multiple groups respectively.

Results

Biophysical characterization of wild type and eGFP tagged gap junctions

Wild type Cx43, Cx40 and Cx45 readily formed GJs when expressed in N2a cells. Figure 1 shows the representative current traces at different junctional voltages (Vj), recorded from the cell pair containing homotypic junctions of Cx43, Cx40 and Cx45. The average junctional conductance (Gj) for Cx43, Cx40 and Cx45 were 60 ± 6 nS (n = 45), 43 ± 5 nS (n = 46) and 36 ± 4 nS (n = 48) respectively (Table 1). $G_{feas}-V_j$ dependence of Cx40, Cx40 and Cx45 are presented in the lower panel of figure 1. A 10 mV pre-pulse was applied before every voltage step to normalize the $G_{feas}$. The normalized $G_{feas}$ (with respect to 10 mV pre pulse) versus $V_j$ plots were fitted with two state Boltzmann equation to determine the voltage sensitivity. The Boltzmann parameters are presented in table 2. $V_0$ represents the $V_j$ at which $G_{feas}$ is reduced by 50%. In agreement with previous reports, $V_0$ for Cx45 (+11 ± 1.8 and $-11 ± 1.3$) was lowest among all three Cxs (Cx43: +57 ± 0.5 and $-56 ± 0.5$, Cx40: +50 ± 0.7 and
2.49, indicating its highest $V_j$ sensitivity. The $V_j$ sensitivity followed the order Cx45>Cx40>Cx43.

GJs are often studied by attaching different fluorescent proteins at the CT of Cx [31]. We analysed the $V_j$ sensitivity of Cx43-eGFP and Cx45-eGFP as these were used in our study. As shown in figure S1, $G_{j(ss)}-V_j$ plot of Cx45-eGFP is indistinguishable from the wild type Cx45. On the other hand, $G_{j(ss)}-V_j$ plot slightly shifted towards higher voltage upon attachment of eGFP with Cx43 indicating the decrease of voltage sensitivity, which is consistent with earlier reports [31]. $V_0$ (in mV) for Cx43 were +57±0.5 and −56±0.5, whereas for Cx43-eGFP the values were +62±1.4 and −62±1.2 respectively (Table 2). Cx40-eGFP was

Table 1. Average conductance and junctional plaque diameter of different Connexins.

| Connexin       | Average Conductance, nS (n) | Average plaque diameter, μM (n) |
|----------------|-----------------------------|---------------------------------|
| Cx45 (WT)      | 36±3.5 (48)                 | 3.8±0.3 (30)                    |
| Cx45-D272      | 3±0.5 (23)                  | ND (20)                         |
| Cx45-C43       | 49±4.6 (37)                 | 5.8±0.3 (35)                    |
| Cx45-C40       | 37±4.3 (33)                 | 2.3±0.1 (33)                    |
| Cx43 (WT)      | 60±4.4 (45)                 | 7.0±0.4 (32)                    |
| Cx43-D257      | 72±4.6 (39)                 | 9.6±0.5 (35)                    |
| Cx43-C40       | 59±3.2 (33)                 | 6.6±0.4 (30)                    |
| Cx43-C45       | NF                          | NF                              |
| Cx40 (WT)      | 43±4.6 (46)                 | 4.9±0.3 (27)                    |
| Cx40-D249      | 4±0.5 (20)                  | ND (15)                         |
| Cx40-C43       | 40±3.6 (35)                 | 3.4±0.2 (30)                    |
| Cx40-C45       | NF                          | NF                              |

WT: wild type; ND: not detectable; NF: not functional; data presented are mean±SEM.
doi:10.1371/journal.pone.0060506.t001
non-functional for reasons unknown and thus not used in our study.

Ischemia uncoupled Cx43, Cx40 and Cx45 to different extents

Ischemia was simulated by exposing the cells to OGD for 5 minutes. Cell pairs expressing Cx43, Cx40 or Cx45 were voltage clamped with two patch pipettes in whole cell configuration. Junctional current (Ij) from cell-2 was monitored continuously in external solution (ES), bubbled with 5% CO2+95% air, by stepping cell-1 from holding potential, 0 mV to

Table 2. Boltzmann fitting parameters for different Connexins.

| Connexin              | Gmin (mV) | Vj=Vj (mV) | A     | t1 and t2 (ms) |
|-----------------------|-----------|------------|-------|----------------|
| Cx45 (WT)             | 0.10±0.02 | +11±1.8    | 11.3±0.9 | 37±4          |
| (n = 7)               |           |            |       |                |
| Cx45-eGFPN1           | 0.10±0.01 | +12±1.7    | 11.9±0.9 | 40±6          |
| (n = 7)               |           |            |       |                |
| Cx45-A272             | 0.12±0.01 | +12±2.2    | 10.2±1.9 | 31±3          |
| (n = 5)               |           |            |       |                |
| Cx45-C43              | 0.12±0.01 | +11±2.3    | 14.8±0.9 | 50±3          |
| (n = 7)               |           |            |       |                |
| Cx45-C40              | 0.22±0.01 | +18±1.9    | 9.1±1.5 | 144±11        |
| (n = 7)               |           |            |       |                |
| Cx45 (WT)             | 0.23±0.02 | -18±1.7    | 9.4±1.3 | 1895±119      |
| (n = 7)               |           |            |       |                |
| Cx43                  | 0.35±0.01 | +57±0.5    | 7.9±0.7 | 70±5          |
| (n = 7)               |           |            |       |                |
| Cx43-eGFP             | 0.25±0.02 | +62±1.4    | 11.5±1.4 | ...          |
| (n = 5)               |           |            |       |                |
| Cx43-A257             | 0.29±0.01 | +59±0.9    | 8.7±0.8 | ...          |
| (n = 3)               |           |            |       |                |
| Cx43-C40              | 0.32±0.01 | +62±0.7    | 11.3±0.4 | 99±7         |
| (n = 5)               |           |            |       |                |
| Cx40 (WT)             | 0.26±0.02 | +50±0.7    | 13.1±0.7 | 86±12        |
| (n = 6)               |           |            |       |                |
| Cx40-A249             | 0.20±0.01 | +51±0.8    | 14.1±0.7 | 91±15        |
| (n = 3)               |           |            |       |                |
| Cx40-C43              | 0.26±0.02 | +51±2.0    | 14.4±1.9 | 107±5        |
| (n = 7)               |           |            |       |                |
| Gmin: minimum conductance; Vj: junctional voltage; A: slope factor. For calculating time constants t1 and t2 of voltage desensitization, the junctional current decay in response to 100 mV voltage step, was fitted with mono or double exponential function.

doi:10.1371/journal.pone.0060506.t002

Figure 2B, Cx43 uncoupled at a much slower rate in comparison to Cx45 and Cx40. Tagging of GFP to CT of some Cxs has been reported to alter their properties. For example, Cx45-eGFP, failed to rescue the Cx45 knockout mice from embryonic lethality [22]. We compared the susceptibility of Cx43-eGFP and Cx45-eGFP to OGD with their wild type counterpart. Effect of OGD on Cx43-eGFP was same as Cx43 (data not shown). However Cx45-eGFP showed higher sensitivity to OGD. As shown in figure 3, Gj of Cx45-eGFP reduced by 99±0.2% (n = 8) in 5 minutes of OGD treatment.

[Ca2+]i rises and pHj decreases during simulated ischemia

Intracellular acidification and [Ca2+]i, rise are known to be associated with ischemia [26]. Fura-2 loaded N2a cells when exposed to OGD, [Ca2+]i increased immediately and maintained a steady value through the entire duration of OGD (Figure 4A). [Ca2+]i returned to basal level when IS was replaced with ES. F340/F380 increased to 1.6±0.2 from 0.92±0.1 (n = 25). Cx transfected cells also showed [Ca2+]i, rise to the same extent and there was no difference among Cxs (data not shown).

pHj was estimated ratio-metrically with BCECF. pHj dropped from 7.25±0.1 to 6.35±0.4 (n = 20) in 5 minutes of OGD. pHj was restored once IS was replaced with ES (Figure 4B). The degree of intracellular acidification does not depend on the level Cx expression. The pHj of untransfected cells also dropped to the same extent following 5 minutes of OGD (data not shown).
Role of $[Ca^{2+}]_i$ and $pHi$ in uncoupling

The contributions of $[Ca^{2+}]_i$, and $pHi$ in ischemic uncoupling of $GJ$ were evaluated individually as well as in combination, by restricting their change during OGD. To clamp $[Ca^{2+}]_i$, 2 mM BAPTA was included in the pipette solution. After obtaining ‘whole cell’, cells were allowed to equilibrate with BAPTA for 5 minutes. Intracellular administration of BAPTA prevented OGD-induced $[Ca^{2+}]_i$ rise (Figure 5A) and attenuated the reduction of $G_j$.

Figure 2. Effect of OGD on different cardiac gap junctions. A. representative $I_j$ trace of Cx43 gap junction recorded from transfected N2a cells. $I_j$ decreased at the onset of OGD. Solid line above the current trace represents duration of OGD. Voltage protocol is presented on top of the current trace. B. OGD reduced the $G_j$ (normalized) of all gap junctions. Cx45 showed maximum reduction. Values are the mean±SEM. C. quantitative representation of the data obtained from panel B.

doi:10.1371/journal.pone.0060506.g002

Figure 3. OGD inhibited Cx45-eGFP more than Cx45. OGD uncoupled eGFP tagged Cx45 almost completely, compared to 85±2% inhibition in wild type Cx45. The difference is statistically significant ($p<0.001$).

doi:10.1371/journal.pone.0060506.g003
of all Cxs significantly (Figure 6). After 5 minutes of OGD, $G_j$ of BAPTA treated cell pair reduced by: $35 \pm 2\%$ (n = 6) for Cx43, $52 \pm 2\%$ (n = 6) for Cx40 and $59 \pm 4\%$ (n = 7) for Cx45. In control experiment (without BAPTA), $G_j$ of Cx43, Cx40 and Cx45 decreased by $53 \pm 5\%$, $64 \pm 1\%$ and $85 \pm 2\%$ respectively. It is evident that [Ca$^{2+}$]$_i$ has lesser contribution in the uncoupling of Cx40 in comparison to Cx43 and Cx45. Effect of BAPTA is independent of pH$_i$ changes. It did not affect intracellular acidification during OGD (Figure 5B).

To study the role of acidification on the uncoupling of GJ, we restricted the pH$_i$ change by using high concentration of HEPES in pipette solution. As shown in figure 5C, gradual increase of HEPES concentrations from 5 mM to 80 mM, prevented the pH$_i$-drop in a graded fashion. 80 mM of HEPES restricted the pH$_i$ change by using high concentration of HEPES in pipette solution. As shown in figure 5D. Like clamping of [Ca$^{2+}$]$_i$ when pH$_i$ was maintained to 7.2, all Cxs showed lesser vulnerability to ischemic uncoupling. Reduction of $G_j$ was prevented significantly (Figure 7), pH$_i$-clamped cell pair showed the reduction of $G_j$: 26$\pm$1$\%$ (n = 7) for Cx43, 29$\pm$2$\%$ (n = 6) for Cx40 and 27$\pm$1$\%$ (n = 7) for Cx45.

To evaluate the cumulative role of [Ca$^{2+}$]$_i$, and pH$_i$, we clamped both by injecting high concentration of potassium phosphate buffer. Addition of 150 mM KPO$_4$ in pipette solution prevented acidification as well as [Ca$^{2+}$]$_i$ rise during OGD, as shown in figure 5B and 5D. When [Ca$^{2+}$]$_i$, and pH$_i$ were clamped, OGD could not uncouple any of the junctions as observed by the steady $G_j$ throughout (Figure 7). Cx43, Cx40 and Cx45 became completely resistant to OGD.

Calmodulin (CaM) is involved in ischemic uncoupling

CaM has been reported to modulate both voltage and chemical gating of gap junctions [33,34]. CO$_2$ mediated uncoupling of Cx43 was prevented by suppressing the expression CaM with antisense RNA [35]. We studied the role of CaM in ischemic uncoupling. Anti-CaM antibody (Sigma-Aldrich, USA) was injected into the cell pair through patch pipette by supplementing pipette solution with 7 $\mu$M of antibody. Antibody was allowed to diffuse for 5 minutes after obtaining whole cell and $I_j$ was recorded. As shown in figure 6, the antibody attenuated ischemic uncoupling of Cx43 and Cx45, GJs significantly. However, uncoupling of Cx40 by OGD was not affected at all by anti-CaM antibody. $G_j$ of the antibody treated Cx43 expressing cell pair reduced by 26$\pm$2$\%$ (n = 7) in response to 5 minutes OGD, whereas in control (without anti-CaM antibody) the reduction was 53$\pm$3$\%$. In case of Cx45, antibody treated and the control cell pair showed the reduction of $G_j$ by 54$\pm$2$\%$ (n = 8), and 85$\pm$2$\%$ respectively (Figure 6D).

Role of carboxy terminal (CT) tail in ischemic uncoupling

Properties of CT deletion mutants. CT of Cx43 has been implicated in many physiological and pathophysiological processes [22,32,36]. To study the role of CT in ischemic uncoupling, we generated CT-deletion mutants Cx43-A257, Cx40-A249 and Cx43-A272 (see material and methods). Deletion of CT impaired the $G_j$ of Cx40 and Cx45. The average $G_j$ of Cx40 reduced from 43$\pm$4.6 nS (n = 46) to 4$\pm$0.5 nS (n = 20) upon deletion of CT. Similarly, Cx43-A272 showed average $G_j$ of 3$\pm$0.5 nS (n = 23) which is about 12 fold lesser than wild type (36$\pm$3.5 nS, n = 48), (Table 1). Consistence with earlier reports, Cx43-A257 displayed higher average $G_j$ and larger plaque diameter than the wild type (Table 1). Deletion of CT also altered the gating parameters of GJs. In accordance with a previous report, removal of CT from Cx43 and Cx40 did not alter their sensitivity to $V_j$ (Table 2) as $V_0$ remained unaltered. However there was a small change of minimum conductance ($G_{min}$) and the desensitization kinetics altered significantly (Table 2). To study the desensitization kinetics, $V_j$ was stepped to -100 mV. The decay of $I_j$ was fitted with either single or double exponential function as required. The time constants for relaxation, $\tau_1$ and $\tau_2$, are presented in figure S2 and table 2. Most GJs are known to follow two gating processes: slow gating and fast gating [31,37,38]. In accordance with previous reports [37,38], all wild type GJs in our experiment showed double exponential decay of $I_j$ (Figure 2 and Table 2). Fast time constant and slow time constant of voltage relaxation reflect closure of fast gate and slow gate respectively [37]. Cx43-A257 and Cx40-A249 followed monophasic decay compared to biphasic relaxation of the corresponding wild types, suggesting loss of one gate in both cases.

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*Figure 4. OGD increases intracellular calcium ([Ca$^{2+}$]$_i$) and decreases intracellular pH (pH$_i$). OGD induced rise of [Ca$^{2+}$]$_i$, and decrease of pH$_i$ in N2a cells are shown in A and B. [Ca$^{2+}$]$_i$ and pH$_i$ were measured ratio-metrically using Fura-2 and BCECF dye. Fluorescence intensity was calculated from background subtracted images using ANDOR IQ programme. Values are the mean$\pm$SEM of 20–25 cells.*
Interestingly removal of CT did not affect the gating of Cx45 as both slow and fast remained unaltered (Figure S2 and Table 2).

Immunofluorescence analysis revealed the characteristics of junctional plaque formed by different CT-truncated mutants (Figure 8). Both wild type and Cx43-D257 showed bright fluorescent plaque at the cell-cell junction. Average diameter of the Cx43-D257 junctional plaque is significantly bigger than the wild type Cx43 (Table 1), which is in agreement with the observed higher Gj of Cx43-D257. Although clear junctional plaques were detected in cell pairs containing wild type Cx40 or Cx45, it was not visible in corresponding CT truncated mutants (Figure 8); a possible reason for their very low Gj.

Effect of OGD on CT truncated mutants

Cx43-D257 when exposed to OGD, showed significant resistance to uncoupling. 5 minutes of OGD resulted in only 26±2% (n = 5) reduction of Gj, compared to 53±3% in wild type (Figure 9), suggesting the involvement of Cx43-CT in ischemic uncoupling. OGD on deletion mutants of Cx40 and Cx45 could not be performed due to their low average Gj.

We observed that among 3 cardiac Cxs, Cx45 is the most sensitive to OGD, whereas Cx43 is the least one. To check if this variability is conferred by the CT, we generated several Cx chimeras e.g. Cx43-C45, Cx43-C40, Cx40-C45, Cx40-C43, Cx45-C43 and Cx45-C40 by swapping CT. Biophysical properties of different chimeras and their sensitivity to OGD are described below.

Biophysical properties of CT chimeras

Deletion of the CT of Cx40 and Cx45 attenuated their Gj by more than 10 fold (Table 1). CT truncated mutants Cx40-D249 and Cx45-D272 did not assemble properly to form junctional plaque as evident from confocal microscopy. When CT of Cx43 was tethered to truncated Cx40 and Cx45, it rescued their Gj completely (40±3.6 nS, n = 35 for Cx40-C43 and 49±4.6 nS, n = 37 for Cx45-C43), (Table 1). Cx45-C43 even showed higher Gj than wild type Cx45 (36±3.5, n = 48). Also, both the chimeras showed bright junctional plaques with diameters comparable to
their wild type counterpart (Figure 8 and Table 1). Unlike other Cxs, truncation of CT significantly improved the Gj of Cx43 (60.6 ± 4.4 nS, n = 45 for Cx43 and 72 ± 4.6 nS, n = 39 for Cx43-D257, p < 0.05), (Table 1). Tethering of CT of Cx40 to the Cx43-D257 restored its Gj to the wild type value (Table 1). CT of Cx40 also rescued Cx45-D272. Cx45-C40 chimera behaved like wild type Cx45 in terms of Gj (37 ± 4.3 nS, n = 33 for Cx45-C40 and 36 ± 3.5 nS, n = 48 for Cx45), though the plaque diameter was slightly smaller (Figure 8 and Table 1). Interestingly, CT of Cx45 was not compatible with either Cx43 or Cx40. None of the chimeras with CT of Cx45 formed functional channels (Figure 8 and Table 1).

The voltage sensitivity and relaxation kinetics of different CT chimeras was studied by step protocol. The results are shown in Table 2. Except Cx45-C40, voltage sensitivity of all chimeras resembled their respective wild type. V0 of Cx45-C40 shifted towards higher voltage indicating decrease of Vj sensitivity. Like wild type Cx, voltage relaxation of all chimeras showed biphasic decay of Ij. In comparison to wild type, all chimeras except Cx45-C40 showed slower decay as indicated by higher t1 and t2. t1 and t2 of Cx45-C43 are not significantly different from wild type Cx45.

Ischemic uncoupling of CT chimeras

Effect of 5 minutes OGD on different chimeras, are presented in Figure 10. If the CT of a particular Cx is the sensor for ischemia, chimera would respond like the Cx that contributed the CT. Cx43-D257 showed 26 ± 2% inhibition of Gj in response to OGD. Tethering of CT of Cx40 did not improve the sensitivity to OGD significantly. Gj of Cx43-C40 inhibited by 33 ± 2% (n = 7), whereas wild type Cx40 exhibited 64 ± 1% inhibitions. Similarly, CT of Cx40 though rescued Gj of truncated Cx45; the chimera exhibited far lesser sensitivity to OGD than Cx40 and wild type Cx45. Gj of Cx45-C40 reduced by 38 ± 1% (n = 7) as shown in figure 10. Interestingly, CT of Cx43 not only rescued the Gj of truncated Cx45 and Cx40, it brought their OGD sensitivity closer to wild type Cx43. After 5 minutes of OGD, Cx43, Cx40-C43 and Cx45-C43 showed reduction of Gj by 53 ± 3% (n = 7), 44 ± 2% (n = 8) and 45 ± 1% (n = 6) respectively. It suggests that unlike CT of Cx40, CT of Cx43 is partially associated with OGD mediated uncoupling.

Discussion

Ischemia reduces gap junctional communication in many cell types. Intracellular acidification and [Ca2+]i rise, generally observed during ischemia, are known to promote uncoupling of
However, the molecular mechanisms are not clearly understood. In the present study, we simulated ischemia by depriving oxygen and glucose and studied its effect on cardiac GJ channels, formed of Cx43, Cx40 and Cx45. Contribution of \([\text{Ca}^{2+}]_i\) and pHi in uncoupling was studied. Additionally, we demonstrated the role of CT of Cxs in OGD-mediated uncoupling.

We studied the effect of ischemia on different cardiac gap junctions, over-expressed in N2a cells. N2a cells have been used extensively in many earlier reports for studying different connexins as they do not have endogenous gap junctions [39,40]. Also, N2a cells are a good model system for studying ischemia [41,42]. Cardiomyocytes or cell line of cardiac origin would have been an appropriate model for studying cardiac connexins. However, cardiomyocytes express all three connexins (Cx45, Cx43 and Cx40) which may form homomeric and heteromeric channels in different combinations, thereby making it difficult to study the effect of ischemia on individual connexins. Moreover, available gap junction blockers are not very specific. Similarly, all available cell lines of cardiac origin express multiple connexins.

GJs made of Cx45, Cx43 and Cx40 varied remarkably in their biophysical properties as well as sensitivity to OGD. In accordance with earlier report [43], we showed \(V_j\) sensitivity follows the order Cx45>Cx40>Cx43. \(V_j\) sensitivity is an inherent property of GJs and determined by several factors including number of charge residues in the voltage sensor. Voltage sensors in cardiac GJs are ill defined and the underlying mechanism of differential voltage sensitivity is unknown. Voltage sensitivity of Cxs may play an important role during ischemic uncoupling. Ischemia affected cardiomyocytes, depolarize quickly which may cause development of higher \(V_j\) between ischemic cell and healthy cell. GJs that are less sensitive to \(V_j\) will remain open longer and may prevent development of arrhythmia.

OGD reduced gap junctional communication of all cardiac Cxs. However, the degree of uncoupling varied among Cxs. None of the GJs closed completely upon exposure to OGD for 5 minutes. Cx45 showed maximum reduction of \(G_j\), while Cx43 was most resistant to OGD. Once cells were exposed to IS, there was elevation of \([\text{Ca}^{2+}]_i\) and pHi dropped. When \([\text{Ca}^{2+}]_i\) rise was prevented with BAPTA without altering the acidification, all GJs showed partial resistance to ischemic uncoupling. In calcium clamped condition, when pHi was allowed to drop to the same extent, \(G_j\) of Cx40 reduced by 52±2% which is close to control value, suggesting that acidification is the major contributor in uncoupling. In the same condition, \(G_j\) of Cx43 and Cx45 reduced by 35±2% and 59±4% compared to 53±3% and 85±2%.
Figure 8. Carboxy terminal (CT) domain of connexins, regulate junctional plaque formation. A. I, II, III and IV are the representative confocal microscopic images of N2a cells expressing Cx43, Cx43-Δ257, Cx43-C40 and Cx43-C45 respectively. Junctional plaques formed by Cx43-Δ257 were significantly bigger (p<0.05) than that of wild type Cx43. There was no significant difference in plaque diameter between Cx43-C40 and Cx43. Cx43-C45 did not form detectable plaque. Most of the connexins were detected in the perinuclear area (shown with white arrows). B. I, II, III and IV are the images of Cx40, Cx40-Δ249, Cx40-C43 and Cx40-C45 expressing cells respectively. Cx40-Δ249 plaques were not detectable. Plaques formed by Cx40-C43 were thin and punctuated (shown with white arrows). Cx40-C45 did not form detectable plaque. C. I, II, III and IV, images of Cx45, Cx45-Δ272, Cx45-C43 and Cx45-C40 transfected N2a cells respectively. Cx45-Δ272 junctional plaques were not detectable. Cx45-C43 plaques were significantly larger than corresponding Cx45 plaques. Cx45-C40 plaques were punctuated and smaller compared to wild type Cx45.

doi:10.1371/journal.pone.0060506.g008

Figure 9. OGD induced uncoupling of CT truncated Cx43 (Cx43-Δ257). A. Gj of CT truncated Cx43 reduced to a lesser extent compared to wild type Cx43, in response to OGD. B. quantitative representation of data generated from 5–7 independent experiments. Values are the mean±SEM. ***, p<0.001.

doi:10.1371/journal.pone.0060506.g009
reduction in control (Figure 6 and Table 3). When pHi change was restricted without affecting [Ca$^{2+}$]$_i$ rise, all Cxs showed only 26–29% reduction of Gj suggesting that acidosis indeed contributes more than [Ca$^{2+}$]$_i$ elevation in uncoupling. When both pHi and [Ca$^{2+}$]$_i$ were maintained to the normal physiological levels, OGD had no effect on any of the GJs (Figure 7 and Table 3). It confirms that acidosis and [Ca$^{2+}$]$_i$ elevation are the primary causes of ischemic uncoupling of GJs. There are ample evidences suggesting that [Ca$^{2+}$]$_i$ works through CaM [21]. To check the involvement of CaM in ischemic uncoupling of GJs, we administrated anti-CaM antibody intra-cellularly through patch pipette. In case of Cx43, anti-caM antibody prevented uncoupling to a greater extent than BAPTA, suggesting its additional role, independent of calcium (Figure 6 and Table 3). CaM has been reported to bind to lens gap junction protein in calcium independent fashion [44]. In case of Cx45, buffering of [Ca$^{2+}$]$_i$ and inactivating CaM prevented the uncoupling to the similar extent, suggesting that [Ca$^{2+}$]$_i$ primarily worked through CaM in this case. Anti-CaM antibody had no effect on the uncoupling of Cx40. Unlike Cx43 and Cx45, Cx40 does not have putative CaM binding domain [45]. Therefore CaM promotes ischemic uncoupling of Cx43 and Cx45 but not in Cx40. It is not clear how CaM binding induces uncoupling [21,45]. It has been proposed that CaM occludes channel mouth of Cx32 from cytosolic side inducing channel closure [20,33]. This may be true also for Cx43 and Cx45. From the above discussion, it is apparent that lesser sensitivity to acidic pH makes Cx43 more resistant to OGD, compared to Cx45 and Cx40.

To investigate the role of CT in ischemic uncoupling, we generated different CT truncated Cxs and chimeras. CT deletion mutant Cx43-A257 when exposed to OGD, uncoupling was attenuated significantly, suggesting its involvement in uncoupling (Figure 9). Earlier studies demonstrated that the intracellular loop, but not the CT of Cx43, is involved in calcium-CaM mediated reduction of Gj [45,46]. Therefore, observed uncoupling in Cx43-A257 is possibly executed through calcium-CaM pathway. In the same line, OGD reduced the Gj of Cx43-A257 to the same extent of wild type Cx43, when pHi was clamped in the latter. This suggests that CT of Cx43 is involved in acidosis mediated component of the uncoupling. This is consistent with the findings that CT-truncated Cx43 showed lesser sensitivity towards low pH, and resulted in increase of infarct size and arrhythmia due to acute coronary occlusion [36]. Upon tethering of CT of Cx43 to the truncated Cx40 and Cx45, not only did it improve their expression (Figure 8), but also the chimeras showed moderate sensitivity to ischemia (Figure 10). It was not possible to assess directly the contribution of CT of Cx40 and Cx45 in uncoupling, due to the low expression of truncated mutants. When CT of Cx40 was tethered to Cx43-A257, the sensitivity of the chimera to OGD did not improve much, suggesting its minor role in ischemic uncoupling (Figure 10). Cx45 chimera containing CT of Cx40 showed remarkably improved expression (Figure 8). However, OGD mediated uncoupling of Cx40 is mainly due to acidosis and calcium-CaM
had minimum contribution in it. If CT of Cx40 is involved in the reduction of $G_\text{t}$, more robust uncoupling of Cx45-C40 would have been observed. Therefore CT of Cx40 possibly does not participate in ischemic uncoupling. We could not assess the role of CT of Cx45 in ischemic uncoupling as tethering of it with Cx43 or Cx40 did not yield a functional construct.

Apart from their role in ischemic uncoupling, we observed that the CT determines the characteristics of junctional plaque (Figure 8). In concurrence with previous report [47], removal of CT increased the plaque diameter made of Cx43. The $G_\text{j}$ also improved (Table 1). However we could not detect junctional plaque in case of CT truncated Cx45 and Cx40. They also exhibited very little $G_\text{j}$, possibly due to removal of phosphorylation and protein-protein interaction sites. Attachment of CT of Cx43 to truncated Cx40 and Cx43 enabled them to form junctional plaque with some altered properties. Plaque diameter of Cx40-C43 was smaller than Cx40 and appeared punctuated but the $G_\text{j}$ of both were comparable. Intriguingly, tethering CT of Cx43 to truncated Cx43 resulted in bigger plaque than wild type Cx43 and increased $G_\text{j}$. Similarly Cx45, when tethered to CT of Cx40, it formed distinct plaque and functional $G_\text{j}$. It suggests that CT of Cx43 is an essential component required for the expression and formation of junctional plaque and it can complement CT of Cx40 and Cx45. However it is not required for the formation of its own junctional plaque. CT of Cx40 is necessary for its expression and plaque formation; it can also complement CT of Cx45. In contrary, CT of Cx45 does not complement Cx40 or Cx43.

In accordance with previous reports, CT domain showed its involvement in voltage gating [37,38,42]. Truncation of CT reduced $G_\text{min}$ and changed slope factor in case of Cx43 and Cx40. Following voltage step, $I_\text{j}$ of CT-truncated Cx43 and Cx40 decayed mono-exponentially and bi-exponentially for corresponding wild type. Relaxation kinetics became slower for the CT-truncated Cx43 and Cx40 resulted in bigger plaque than wild type Cx43 and increased $G_\text{j}$. Similarly Cx45, when tethered to CT of Cx40, it formed distinct plaque and functional $G_\text{j}$. The $G_\text{j}$ also increased the plaque diameter made of Cx43. The $G_\text{j}$ also decreases its voltage sensitivity. The $G_\text{j}(\text{ss})$-$V_\text{i}$ plot shifted towards higher voltage.

**Conclusions**

This work figures out the molecular mechanisms that uncouple GJ channels formed of Cx43, Cx40 and Cx45 during ischemic condition. Ischemia was simulated by depriving oxygen and glucose. OGD caused the reduction of $G_\text{t}$ of all Cxs significantly. Cx43 was most sensitive, while Cx43 showed maximum resistant to OGD. Elevated [Ca$^{2+}$], and acidic pH$_\text{i}$ were the primary causes, as uncoupling was prevented completely by restricting their change. Acidosis has greater contribution than elevated calcium in reducing the $G_\text{t}$, particularly in the uncoupling of Cx40. Calcium worked through CaM, though calcium independent role of CaM in the uncoupling of Cx43 cannot be ruled out. Further, CT of Cx43 played significant role in ischemic uncoupling. Tethering of it to the CT-truncated Cx45 and Cx40 enabled them to respond to OGD, same like Cx43. CT also played significant role in determining plaque diameter, voltage dependent gating and current relaxation kinetics with the exception of Cx43-CT domain. Taken together, this study provides an explanation for the comprehensive mechanism of the ischemic uncoupling of cardiac gap junctions.

**Supporting Information**

**Figure S1** $V_\text{i}$ sensitivity of wild type and eGFP tagged Cx43 and Cx45. **A**. Tagging of eGFP to the C-terminus of Cx43 decreases its voltage sensitivity. The $G_{\text{max}}$-$V_\text{i}$ plot shifted towards higher voltage. **B**. Voltage sensitivity of Cx43 did not change after tagging eGFP. $G_{\text{max}}$-$V_\text{i}$ plot of Cx45-eGFP is indistinguishable from that of wild type Cx45.

**Figure S2** Desensitization kinetics of wild type and CT-truncated connexin containing gap junctions. A and AII, representative $I_\text{j}$ traces of Cx43 and Cx43-$\Delta$257. $V_\text{i}$ was stepped to 100 mV. $I_\text{j}$ decayed mono-exponentially and bi-exponentially for truncated Cx43 and wild type Cx43 respectively. Predicted best fittings are presented with solid line. AIII, merged fitted curves of A and AII. Corresponding $\tau$ values are indicated with arrows. **B**, relaxation kinetics of Cx40 and Cx40-$\Delta$249. Figures are presented similar to fig. A. Current decay of Cx40 and Cx40-$\Delta$249 are best fitted with double and single exponentially. **C**, voltage relaxation of Cx45 and Cx45-$\Delta$272. Both Cx45 and truncated Cx45 showed double exponential decay of $I_\text{j}$.

**Table S1** Primers used for generating truncated C-terminal domain mutants.

**Table S2** Primers used for generating C-terminal chimeric mutants.

**Acknowledgments**

We thank S. Divya for critical comments on the manuscript. We sincerely acknowledge R. Suryaraja for his help in confocal microscopy.

**Author Contributions**

Conceived and designed the experiments: GS AKB. Performed the experiments: GS. Analyzed the data: GS AKB. Contributed reagents/materials/analysis tools: GS AKB. Wrote the paper: GS AKB.
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