Close the Gap : a study on the regulation of Connexin43 gap junctional communication

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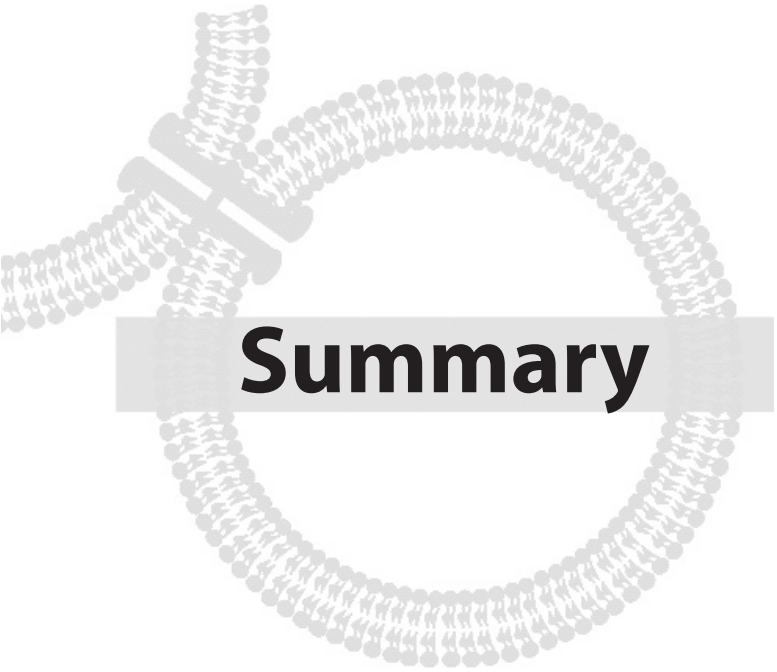

Summary

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For a muliticellular organism to be able to function properly, it is essential that cells communicate with each other. Cell-cell communication can occur either indirectly, via secretion of hormones and growth factors, acting on extracellular receptors, or directly via cell-cell contacts, including adherens junctions, tight junctions and gap junctions. Gap junctions are groups of transmembrane channels, that connect the cytoplasms of adjacent cells, and mediate the diffusion of small molecules, such as ions, metabolites, second messengers and even small peptides (<1-2 kDa).
The building blocks of gap junctions are connexin proteins. Twenty different connexins have been identified in mice and twenty-one in humans. The connexins all share the same topology, with a short intercellular N-terminus, four transmembrane domains and an intercellular C-terminal tail. The C-terminal tail varies in length and composition between connexins and contains putative regulatory and protein-protein interaction sites. The most ubiquitous and best studied connexin is connexin43 (Cx43). Cx43 is also known as the heart connexin. In the heart, gap junctional communication between cardiomyocytes ensures efficient electrical coupling and hence the synchronous propagation of action potentials. Cx43 is the major connexin family member in the myocardium. Misregulation of Cx43 expression, localisation and channel gating may lead to severe cardiac dysfunction.
Cardiac ischemia may be caused by GPCR agonists angiotensin and endothelins, which are very potent vasoconstrictors. Inhibition of gap junctional communication may protect the heart during pathological conditions by limiting the spreading of damage. Cx43-based cell-cell coupling is rapidly disrupted following stimulation of certain G protein-coupled receptors (GPCRs). On the downside, however, closure of Cx43 gap junctions may be the cause of arrhythmia.
The studies presented in this thesis focus on the quest to unravel the signalling pathways leading to the transient inhibition of Cx43-based by GPCR activation.

In *chapter 2*, we show that depletion of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) from the plasma membrane is necessary and sufficient to inhibit Cx43-based cell-cell communication. Furthermore, we found that the third PDZ domain of Cx43-binding partner ZO-1 interacts with PLCβ3. Both ZO-1 and PLCβ3 are essential to inhibit cell-cell communication. Our results show that $PI(4,5)P_2$ is a key regulator of Cx43 channel function, and suggest that ZO-1 recruits PLCβ3 to Cx43 gap junctions to allow regulation of cell-cell communication by localised regulation of $PI(4,5)P_2$ levels.

In *chapter 3*, we have analysed the importance of the Cx43 C-terminal domain in channel regulation. Previous studies have implicated phosphorylation of the

Cx43 C-terminal tail on S368, Y247 and Y265, and/or Cx43 internalisation via an Y286-based sorting motif, as key regulatory events. We studied Cx43 mutants by expressing them in connexin-depleted Rat-1 fibroblasts. We find that, after stimulation with GPCR agonist endothelin for 8 minutes, Cx43 point mutant Y265F mutant gap junctions are open, in contrast to endogenous Cx43 and the other mutants. Although residue Y265 is an established substrate for activated Src, we find that Cx43 tyrosine phosphorylation was not increased in agonist-stimulated cells, while general tyrosine kinase and Src inhibitors had no effect on Cx43 channel closure. In addition, Y265 is part of a putative sorting motif, but further mutation of this motif did not affect Cx43 channel closure. Our results indicate that residue Y265 is essential for Cx43 channel disruption by G(q)-coupled receptors, and suggest that Y265 may play a structural role rather than serving as a phosphate acceptor.

In *chapter 4*, we have examined the possible role of ubiquitination in the regulation of Cx43 based GJC. Mono-ubiquitination at multiple residues of Cx43, followed by internalisation and lysosomal degradation, has been implicated in Cx43 turnover. The E3 ubiquitin ligase Nedd4 has been shown to interact with Cx43. We find that ubiquitination of Cx43 by Nedd4 is induced by GPCR activation. Cx43 residue Y265 is essential for the interaction with Nedd4 and ubiquitination. Inhibition of GJC occurs in two phases. The second phase is absent in Nedd4 knockdown cells and in cells expressing Cx43 mutant Y265F. Furthermore, our results suggest that Cx43 is internalised upon GPCR stimulation. We conclude that inhibition of Cx43-based GJC by GPCR signalling occurs in two phases. The second phase is initiated by Nedd4 mediated Cx43 ubiquitination and results in prolonged inhibition of GJC through internalisation of Cx43.

Together, chapters 2-4, provide new insights into the regulation of Cx43-based GJC, in particular its inhibition by GPCR signalling. Our results suggest a model in which GPCR agonists inhibit Cx43-based GJC in two phases. First, initial closure of the Cx43 channels is mediated by $PI(4,5)P_2$ depletion. This first phase lasts up to ~7 minutes after agonist stimulation. The second phase reflects internalisation of the gap junctions following Cx43 ubiquitination. Cx43 ubiquitination is initiated at approximately 2 minutes after agonist stimulation. The second phase prolongs the inhibition of GJC up to ~30 minutes. Our results suggest that, although Cx43 ubiquitination is seemingly independent of prior $PI(4,5)P_2$ depletion, $PI(4,5)P_2$ depletion and Cx43 ubiquitination are both required for internalisation. (Fig.1)

Finally, in *chapter 5*, we investigated the influence of Cx43 knockdown cell migration. Knockdown of Cx43 strongly reduced migration in an *in vitro* woundhealing assay. Knockdown of Cx43 was accompanied by a decrease in N-cadherin expression. We found that the effect of Cx43 knockdown on migration is independent of GJC. We

show that knockdown of N-cadherin alone, without affecting Cx43 expression and GJC, is sufficient to slow down cell migration as shown for Cx43 knockdown cells. In summary, the effect of Cx43 knockdown on cell migration is independent of a reduction in GJC, and is probably an indirect effect through regulation of N-cadherin expression. This study supports the notion that there is more to connexins than just the channel.

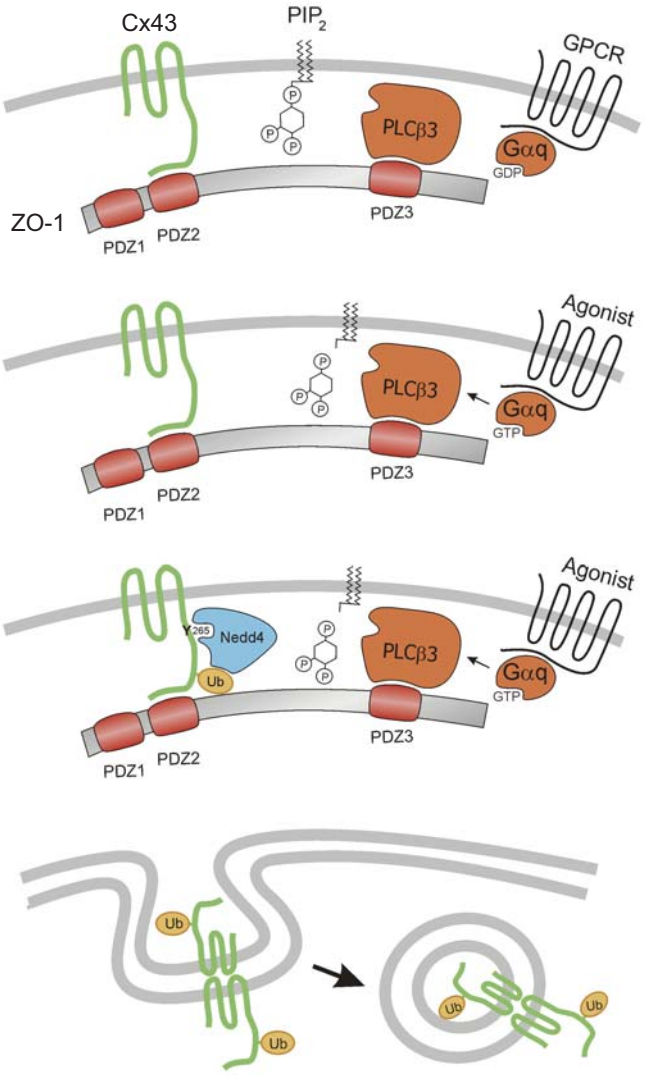

Figure 1. Proposed model: Cx43-based GJC is inhibited by GPCR signalling in two steps.
In this model, ZO-1 recruits PLCβ3 to the Cx43 gap junction plaque. Upon activation of a Gαq coupled receptor, PLCβ3 becomes activated and hydrolyses $PI(4,5)P_2$, followed by disruption of GJC. Next, Nedd4 binds to and ubiquitinates Cx43. Cx43 residue Y265 is indispensible for binding of Nedd4 and Cx43 ubiquitination. Cx43 ubiquitination is followed by internalisation of the gap junction plaque, leading to prolonged inhibition of GJC.