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

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Close the Gap

A study on the regulation of Connexin43 gap junctional communication

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Cover: Co-staining of microtubules and Cx43 in migrating Rat-1 fibroblasts visualised by immunofluorescence
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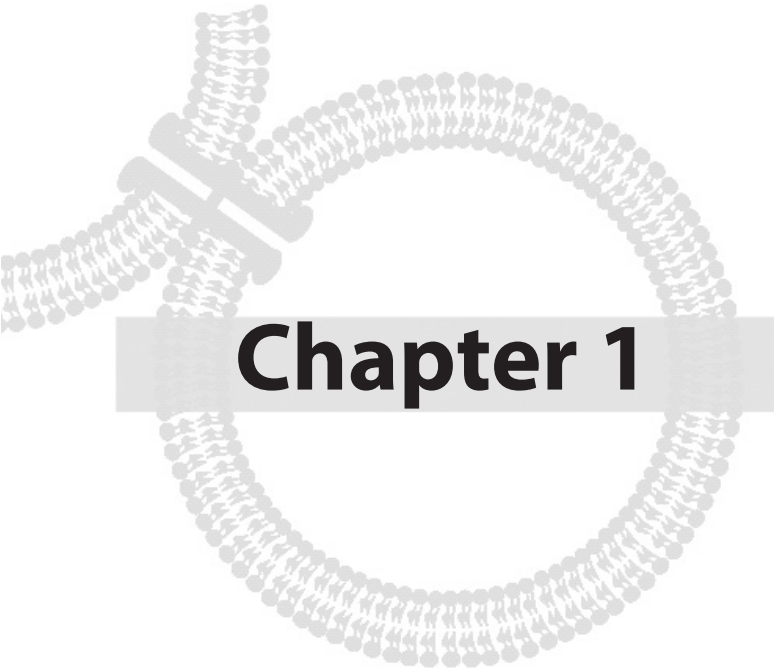
Contents



Chapter 1

General introduction

Gap junctions

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, connecting the cytoplasms of adjacent cells, that mediate the diffusion of small molecules ($\leq$ 2kDa, depending on stoichiometry and polarity), such as ions, metabolites, second messengers and even small peptides[1-4]. Through gap junctional communication (GJC), the behaviour of a cell can be influenced by its neighbours; Gap junctional communication is essential for tissue homeostasis and plays and important role in the control of processes like proliferation, migration and differentiation[5-13].
Gap junctional communication is an evolutionary conserved property of nearly all multi-cellular organisms. Throughout evolution, two families of gap junction proteins have evolved: The invertebrate innexins and the vertebrate connexins. Recent evidence suggests that innexins are conserved even in humans, the so called pannexins, but to date it is unclear whether these proteins are capable of forming functional gap junctions[14-17].

Connexins

The building blocks of gap junctions are connexin proteins. Connexins are transmembrane proteins, that span the membrane four times, with an intracellular C-terminal tail, that varies in length and composition between the different connexins. In the endoplasmatic reticulum (ER) and Golgi, six connexin proteins aggregate to form a connexon, or hemichannel[18]. Although intact microtubules are not necessary for the formation of gap junctions, it is thought that microtubules facilitate the transport of vesicles containing the connexons to the cell surface, where they merge with the plasma membrane[19,20]. Once inserted into the membrane, connexons diffuse freely until they encounter a connexon from an apposing cell, with which they can form a functional cell-to-cell channel. The interaction between the apposing connexons is established by the formation of disulfide bridges between cysteine residues, three of which are present in both extracellular loops of each connexin (Fig.1D)[18,21].
Alternatively, connexons may function as hemichannels, which only open under certain (pathophysiological) conditions and are permeable to small molecules, such as ATP, NAD^+ and glutamate. Misregulation of hemichannel closure may lead to the loss of chemical gradients across the plasma membrane and the induction

of cell death[23-30].

Twenty different connexins have been identified in mice and twenty-one in humans. The names that are currently in use refer to their predicted molecular weight.

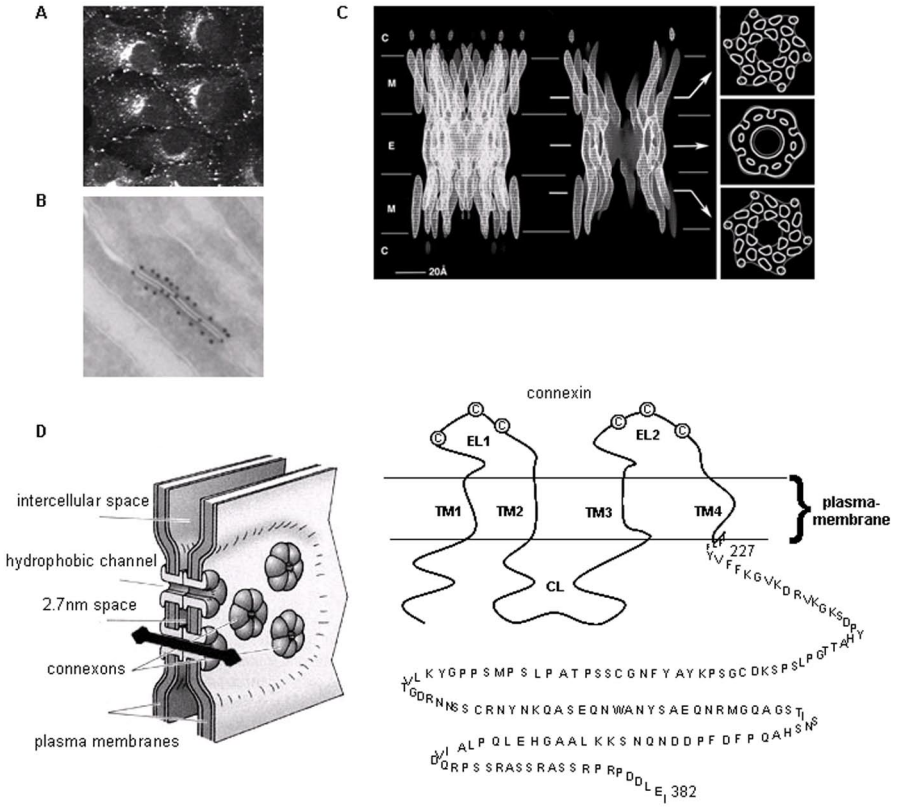


Figure 1: Gap junction

A: Confocal picture of immunostained Cx43 in Rat-1 fibroblasts. The outline of the cells is marked by punctate Cx43 staining. In addition, a bulk of Cx43 is localised to the perinuclear region.

B: Electron microscopic image of a Cx43 gap junction in Rat-1 cells. Cx43 is labelled with immunogold. Clearly visible are the membranes of two adjacent cells being very close to each other at the site of the gap junction, surrounded by immunogold labelling of Cx43 C-terminal tails in the cytoplasm of both cells.

C: Reconstruction of a recombinant gap junction channel. left: side view, middle: the channel interior. M: membrane, E: extracellular gap, C: cytoplasm. Right, top: top view, showing the 24 transmembrane α–helices per connexon. Right, middle: the channel in the extracellular space, right, bottom: bottom view, like the top view, but tilted ~30 degrees. Reproduced from [22].

D: Drawing of a gap junction. (adapted from academic.brooklyn.cuny.edu/biology) Right: schematic drawing of Cx43. TM: transmembrane domain, EL: extracellular loop, CL: cytoplasmic loop.

All connexins share the same topology, with a short intercellular N-terminus, four transmembrane domains and an intercellular C-terminal tail[6] (Fig.1D). The C-terminal tail varies in length and composition between connexins and contains putative regulatory and protein-protein interaction sites. Connexins have a distinct and partially overlapping tissue distribution and one cell type may express several connexins[1,6,31].

Different connexins can selectively form channels composed of different combinations of connexins[32] (Fig. 2).This not only raises the possibility to form gap junctions between cells from different tissues, but also provides an ingenious way to differentially regulate GJC within a tissue or with cells from surrounding tissues[33,34]. In addition, it gives a cell the opportunity to compensate for loss or mutation of one of its connexins. That this is not an airtight mechanism is shown by a range of mutations in different connexins that lead to a disease phenotype. Loss and misregulation of GJC have been implicated in several human diseases[35,36] and in many tumours GJC is defective[37,38]. An overview of connexin linked diseases is given in table 1.

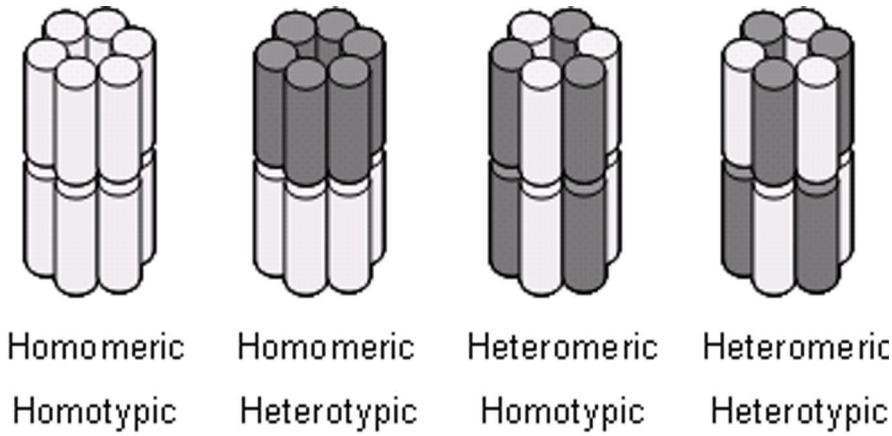


Figure 2: Composition of a gap junction channel
Six connexins aggregate to form a connexon, while two connexons form a gap junction channel. A connexon may be composed of one or more connexin species, resulting in several different possible compositions of a gap junction channel.

Gap junctions and cancer

Ever since the discovery of gap junctions, it has been hypothesised that GJC is involved in growth control and may play a role in cancer. Tumour cells and oncogene transformed cells often show reduced or even complete inhibition of GJC, which may be caused by loss of connexin expression or because of mislocalisation of connexins. In many cases, transformed cells have completely lost the ability

Table 1: Overview of connexin related diseases

Connexin	phenotype	mutation	references
Cx26	Skin disease with or without deafness	>100 mutations, most loss of function mutations >12 additional mutations lead to both skin disease and deafness	35 ,36, 48-59
	for example:		
	• Bart-Pumphrey syndrome		60,61
	• Vohwinkel's syndrome		62
	• Palmoplantar keratoderma with deafness		63-66
	• Keratitis-ichthyosis-deafness (KID) syndrome • Hystrix-like ichthyosis-deafnesss (HID) syndrome		56,67-69
	Non-syndromic deafness		58,70,71
Cx30	Clouston's syndrome (hidrotic ectodermal dysplasia)	G11R, A88V, V37E	72, 73
	Non-syndromic deafness		
Cx30.3	Erythro-keratoderma variablis	F137L, G12D, R22H, T85P, F189Y	74, 75
Cx31	Erythro-keratoderma variablis	G12R/D, C86S, R42P, F137L, L209F, L34P, E100K	76-82
	Peripheral neuropathy and hearing impairment Non-syndromic deafness	?	83-85
Cx32	X-linked Charcot-Marie Tooth disease	> 270 mutations, most point mutations interfering with trafficking, missassembly of gap junctions or abnormal gating	35,86- 88
Cx40	Atrial fibrilation	M163V, A96S, P88S, G38D	89
Cx43	ODDD	28 mutations, as far as known loss of function	35,90-100
	Non syndromic deafness		101
Cx46	Zonular pulverulent cataract-3	D3Y, N63S, FS at codon 380	102,103
Cx50	Zonular pulverulent cataract-1	P88Q, E48K	104-106

communicate via gap junctions, either within the tumour or with surrounding tissue. It has also been reported that communication within a tumour is still intact, while communication with normal tissue is inhibited[10,37-41].
Furthermore, in studies using cell lines, a strong correlation was found between treatment with transforming agents or mitogens, or transfection with oncogenes, and the inhibition of GJC[38,42-46]. It should be kept in mind, however, that most of these treatments have a marked effect on cell-cell contacts, and the observed inhibition of GJC may be caused by disruption of cell-cell contacts, rather then by modification of gap junctions.
It is unlikely that loss of GJC directly leads to tumourigenesis, but it has been demonstrated that reconstitution of GJC in communication deficient tumours has a strong effect on tumour growth and invasiveness. This suggests that, even though defective GJC is not sufficient for loss of contact inhibition, restoration of GJC may contribute to contact inhibition. Alternatively, healthy surrounding tissue may control the behaviour of deranged cells, or even induce apoptosis. The transfer of apoptotic signals through gap junctions is known as the "bystander" killing effect[47].

Connexin43

The most ubiquitous and best studied connexin is connexin43 (Cx43) (Fig.1D shows a schematic drawing of Cx43). Although it is also known as the heart connexin, it is expressed in many other tissues[35].

Regulation of Cx43 based gap junctional communication
Cx43, like all connexins, has a very short half life, varying from ~1.5 hours in the heart to ~ 5 hours in other tissues[35,107-110]. For a transmembrane protein, this is a very fast turnover, suggesting the need for a cell to be able to rapidly adjust its GJC to changing circumstances. In addition, several post translational modifications, like serine and tyrosine phosphorylation and ubiquitination, have been described, that provide the possibility to rapidly regulate the level of GJC.

Serine phosphorylation
Immunoblots from total cell lysates almost always show 3 bands for Cx43 (Fig. 3), named P0, P1 and P2, with P0 being the fastest running isoform, and P1 and P2 running slightly slower as a result of serine phosphorylation[111].
The P1 isoform is thought to be phosphorylated on serines 364 and/or 365, and is localised mainly to the cell-cell contacts[112,113]. The P2 isoform, on the other hand, is

phosphorylated on serines 325, 328 and/or 330, and is exclusively localized to the gap junctions. Moreover, it was found that the Triton x-100 insoluble fraction consists predominantly of P2 Cx43[113,114]. Thus, it appears that the P1 and P2 isoforms are both associated with functional gap junctions. however, it remains to be investigated whether phosphorylation of these residues is essential for the accumulation of gap junctions. In addition, MAPK is reported to phosphorylate Cx43 on serines 279, 282 and 255, which is associated with a migration shift comparable to P2, but no direct relation to functionality has been found[113,115-120].

In contrast, phosphorylation on S368 by PKC, downstream from TPA, is associated with decreased GJC and increased internalisation and turnover of Cx43. Phosphorylation of S368 may occur on all three Cx43 isoforms and does not induce a migration shift of Cx43 on SDS-page[26,121-126]

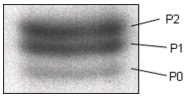


Figure 3: Cx43 isoforms
On SDS-PAGE, Cx43 appears as three different isoforms, named P0 for the non-phosphorylated form and the slower running P1 and P2 serine phosphorylated isoforms (See text for details).

Tyrosine phosphorylation

Tyrosine phosphorylation on residues Y265 and, to a lesser extent, Y247, is associated with inhibition of GJC. The only tyrosine kinase that phosphorylates Cx43 that has been identified so far is Src. The correlation between tyrosine phosphorylation and downregulation of GJC has been observed mainly in systems overexpressing active Src[113,127-133]. Because of the association between Src induced cell transformation and loss of GJC, it was even proposed that loss of Cx43 function contributes to cell transformation[39,44,134]. The role of Src in gap junction function is discussed in more detail below.

Ubiquitination

Ubiquitination of Cx43 is linked to gap junction turnover. Cx43 is thought to be monoubiquinated on multiple lysines[35,110,135]. Mono-ubiquitination is usually a trigger for internalisation, whereas poly-ubiquitination is a precursor for proteasomal degradation[173]. Internalisation of gap junctions occurs via so called annular junctions; part of the gap junction plaque, including the membranes and connexins from both cells, invaginates into one cell, forming a vesicular structure, which is called annular junction, or connexosome (Fig. 4). These annular structures are rapidly taken up by lysosomes, where they are degraded[35,136-138]. Leithe and Rivedal nicely demonstrated that internalisation of ubiquitinated Cx43 is a clathrin dependent process. The same authors suggest that ubiquitination may be regulated

by MAPK mediated phosphorylation of Cx43[135,137]. Recently, E3 ubiquitin ligase Nedd4 was found to interact with Cx43[139]. More details on the role of Nedd4 in gap junction turnover are discussed below.

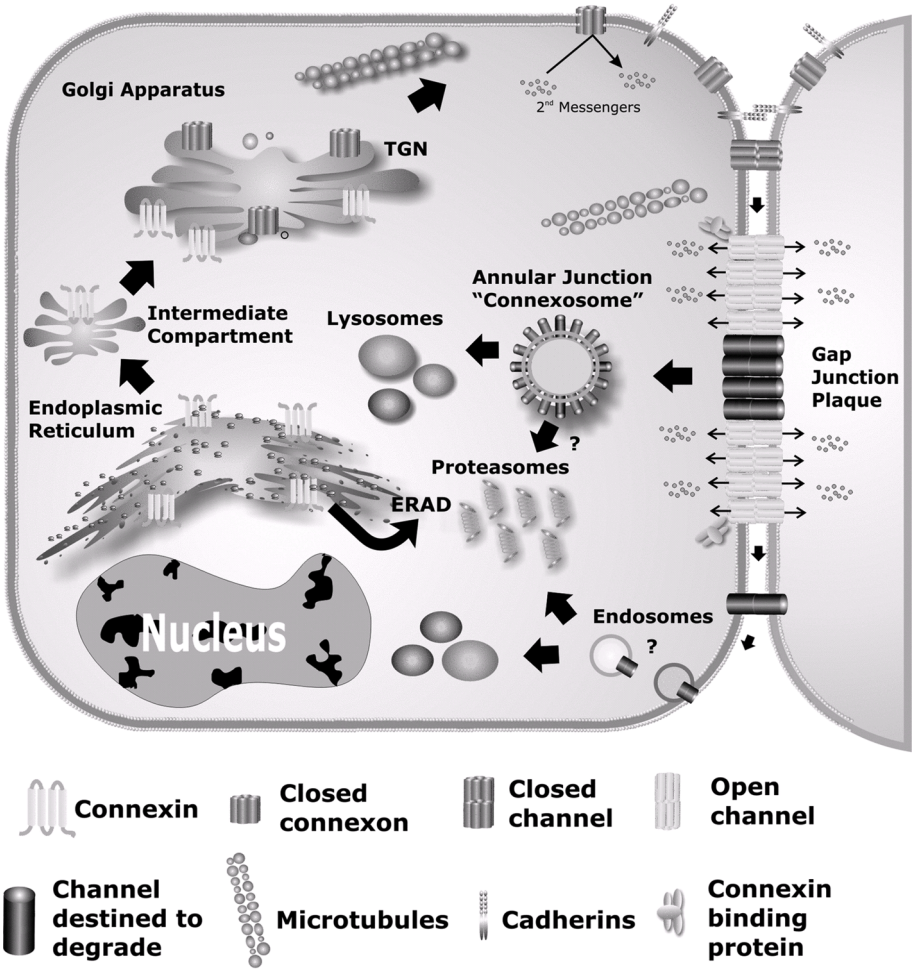


Figure 4: The Cx43 life cycle
Upon synthesis, connexins are inserted into the ER. Improperly folded connexons are subject to ER associated degradation (ERAD). While going through the ER and Golgi, connexins are assembled into connexons. Pleiomorphic vesicles and transport intermediates are thought to deliver closed connexons to the cell surface, a process that is facilitated by microtubules. New gap junction channels are recruited to the margins of gap junction plaques and older channels are found in the centre of the plaques. Gap junction plaques and fragments of gap-junction plaques are internalised into one of two adjacent cells as a double-membrane structure commonly referred to as an annular junction. Internalised gap junctions are targeted for degradation in lysosomes, although some evidence suggests a role in proteasomal degradation. (Reproduced from [35])

Regulation by G-protein coupled receptor signalling

Modulation of gap junctional communication by physiological stimuli can occur through activation of G-protein coupled receptors (GPCR). For example lysophosphatidic acid (LPA), thrombin, neurokinin A, endothelins and angiotensin all rapidly and transiently inhibit Cx43 based GJC[133,140-144], the kinetics of which is agonist and cell type dependent. An overview of GPCR signalling is shown in Fig. 5.

Postma *et al.* and Giepmans *et al*. showed that this inhibition is independent of PKC, MAPK, Ca^{2+} and membrane potential and does not require Rho or Ras activation. Instead, they attribute an essential role to tyrosine phosphorylation by c-Src[132,133], which was later confirmed by Spinella *et al*.[143]. It has been suggested that the intercellular C-terminal tail of Cx43 is essential for regulation of Cx43 gap junctions. We showed that GPCR induced inhibition of GJC is dependent on depletion of $PI(4,5)P_2$ by PLCβ3, downstream of Gαq activation. In addition, we proposed that ZO-1, which interacts with the very C-terminus of Cx43, facilitates local regulation of $PI(4,5)P_2$ levels by recruiting PLCβ3 to the Cx43 gap junctions[145].

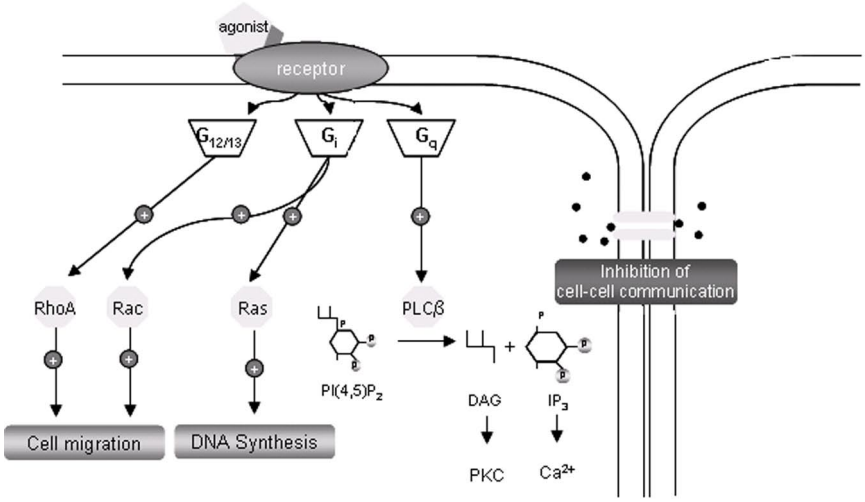


Figure 5: Overview of G protein-coupled receptor signalling
G protein-coupled receptors couple to distinct G proteins, all linked to their specific pathways. The balance between the different pathways determines the behaviour of a cell in response to the different GPCR agonists.

Cx43 interacting proteins

During the past decade, increasing interest has been developed in Cx43 interacting proteins. So far, however, most protein interactions remain without function (table 2). An overview of Cx43 interacting proteins and their putative function is given in table 2. The interaction partners that are of interest for the work described in this thesis, namely ZO- 1, Src and Nedd4, are discussed in more detail below.

Zona Occludens 1

The first Cx43 interacting protein that was identified is Zona Occludens 1 (ZO-1)[146,147]. ZO-1 was originally identified in the zona occludens, or tight junction, and is also known as tight junction protein 1 (TJP1)[148]. ZO-1 is a 220 kDa scaffold protein, consisting mainly of protein-protein interaction motifs, including three PDZ domains, the second of which interacts with Cx43 (Fig. 5A). In general, PDZ domains bind the very C-terminus of other proteins. The last four amino acids of a protein are essential for interaction with a PDZ domain. The following amino acid sequences form classic putative PDZ interaction sites: x-S/T-x-V/I/L and x-V/I/L-x-V/I/L[149]. In addition, PDZ domains can bind other PDZ domains. The last four amino acids of Cx43 are DLEI. Other connexins which have been reported to interact with ZO-1 are Cx45 (KSSI), Cx47 (TVWI), Cx36 (SAYV), Cx46 (DLAI), Cx50 (DLTI) and Cx31.9 (DLAI)[150-157]. Based on sequence analysis, also Cx40 (DLSV), Cx31 (LTPI) and Cx31.1 (KTIL) may interact with ZO-1. Other ZO-1 interacting proteins include ZO-2, tight junction proteins occludin and claudins[158,159] , cytoskeletal components actin[160-163], α-actinin[164], α and β-catenin[159,161,165], transcription factor ZONAB[166,167] and signalling protein PLCβ3[145].

Several reports address the function of ZO-1 in Cx43 gap junctions, mostly by interfering with the Cx43-ZO-1 interaction through a C-terminal (GFP-) tag on Cx43. The drawback of most studies is that the observations are made in cells without endogenous gap junctions, thus, the observed effects may be due to an overexpression artefact or because of the absence of one or more proteins of the complex that is involved in regulation of gap junctions. For example, our group published that Cx43 gap junctions fail to close in response to exogenous stimuli, when expressed in communication deficient cells. Furthermore, GFP-tagging of Cx43 interferes with much more than just the interaction with ZO-1, like, for example, phosphorylation by PKC.

The most convincing study was carried out by Hunter *et al,* who showed that the distribution of Cx43-GFP, which appears as large plaques at the cell-cell contacts, is normalised by co-expression with untagged Cx43, to the typical punctate pattern

Table 2: Cx43 kinases and Cx43 interacting proteins

protein	interaction domain	Cx43 site	function	references
α-catenin	putative, localisation only			193
β-catenin	putative, localisation only		Link between Wnt signalling and gap junctions.	194
P120 catenin	putative, localisation only			195
Cadherin	putative, localisation only	cytoplasmic loop		193,196
α–/β tubulin, microtubules	Distal ends of microtubules	234-262	anchorache of micro-tubules to the membrane/microtubule stability. regulation of Cx43 expression and distribution (all putative)	19,174,197-199
Caveolin 1, -2	82-101 (caveolin-scaffolding domain) and/or 135-178 (C-terminal domain)	C-terminal tail	enhancement of GJC	200,201
Cdc2	kinase	255	serine phosphorylation	202,203
CK1	kinase	325, 328 and/or 330	serine phosphorylation, regulation of gap junction assembly (putative)	204
CIP75	UBA domain	264-302	promotes turnover and degradation of Cx43	205
CIP85	SH3 domain	P(253) LSP(256) motif	induction of Cx43 turnover through lysosomal degradation (putative)	206
Drebrin	nd	C-terminal tail	maintaining functional Cx43 gap junctions at the plasma membrane	207
Akt		369, 373	serine phosphorylation, induces the interaction with 14-3-3 (putative)	208
14-3-3	nd	373		208,209
NOV/CCN3	nd	C-terminal tail	growth suppression (putative) through upregulation of NOV expression by Cx43	210-212
MAPK	kinase	255, 279, 282	serine phosphorylation	120, 213
PKA	kinase	364, 365, 368, 369, 373	serine phosphorylation, upregulation of GJC	214-216
PKC	kinase	368, 372	serine phosphorylation, disruption of GJC	116,117,121,217
PKG	kinase	257 (rat/ mouse)	serine phosphorylation, inhibition of GJC	217,218
RPTPμ	phosphatase	265 (putative)	tyrosine dephosphorylation (putative)	219

that is usually observed for Cx43. In addition, they show that peptides that mimic the PDZ binding site of Cx43, and thus compete with the interaction between Cx43 and ZO-1, induce a change in distribution of Cx43 that is similar to that of Cx43-GFP, while control peptides have no effect on the Cx43 spreading pattern[168]. Thus, it appears that the interaction between ZO-1 and Cx43 regulates the turnover, size and distribution of gap junction plaques via a yet unknown mechanism.

Src

Both v-Src and activated c-Src have been reported to phosphorylate Cx43[126,129,132,134,169,170], which is associated with reduced cell-cell communication[113,130-133,171]. In the most likely model, the Src SH3 domain binds to a proline rich area in the C-terminal tail of Cx43. Subsequently, upon phosphorylation of Cx43, the SH2 domain of Src binds to the phosphorylated residue, thereby stabilising the interaction between Src and Cx43 (Fig. 5B). Expression of v-Src or constitutively active c-Src transforms cells[172], which is accompanied by massive phosphorylation of many proteins, including Cx43, and a strong reduction of gap junctional communication. It should be taken into account that transformation by Src also causes the cells to loose their cell-cell contacts, which automatically makes them poor communicators (see also chapter 3 of this thesis), so the effect of Src on Cx43 based GJC may be indirect, rather then a direct effect of Cx43 phosphorylation. The major target in Cx43 for phosphorylation by Src is residue Y265, while Y247 is a secondary target[129,132,169]. Overexpression studies show that communication of v-Src expressing cells can be rescued by mutation of Y265[132], suggesting that phosphorylation of this residue negatively regulates GJC. Src is the only tyrosine kinase that has been found to phosphorylate Cx43, so far.

Nedd4

Increasing evidence suggests that ubiquitination plays a vital role in the internalisation and turnover of Cx43[35,109,110,135,139]. No E3 ubiquitin ligase had been associated with Cx43, until Leykauf *et al.* showed that Nedd4 interacts with Cx43. They found that knockdown of Nedd4 increases Cx43 plaque size, without effecting Cx43 protein level, suggesting that Nedd4 is essential for Cx43 internalisation[139].

The Nedd4 family of E3 ubiquitin ligases is evolutionary conserved and has eight members in mice and nine members in human (review[173]). All Nedd4-like proteins consist of two or more WW domains, which are protein-protein interaction motifs that bind primarily to PPxY motifs of target proteins, although interactions with T/SPxY and other motifs have also been reported. It has been suggested that the WW2 domain of Nedd4 binds a PY motif in the C-terminal tail of Cx43[139] (Fig. 6C).

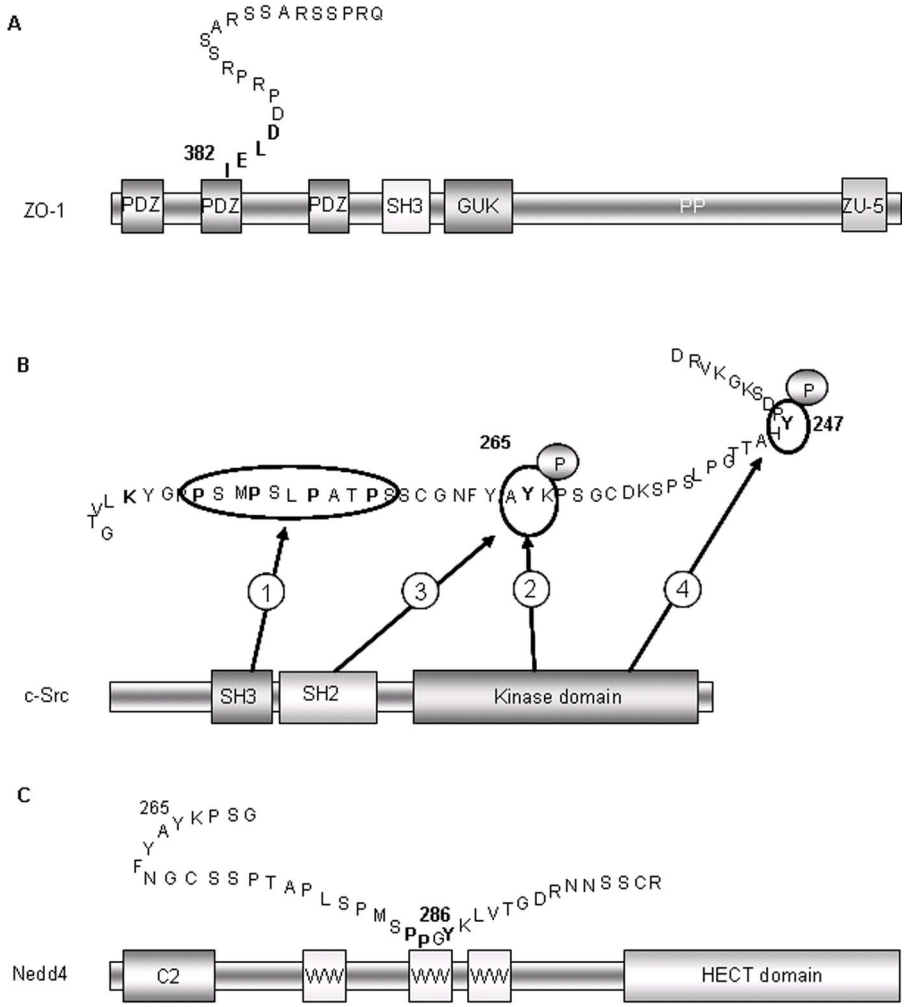


Figure 6: Interaction of Cx43 with ZO-1, c-Src and Nedd4
A: The second PDZ domain of multi protein-protein interaction domain protein ZO-1 interacts with the four most C-terminal residues of Cx43[146,147,174] B: The first step in the interaction between Src and Cx43 is binding of the SH2 domain of Src to the proline rich area of the C-terminus of Cx43 (1). Next, the kinase domain of Src phosphorylates Y265 of Cx43 (2), which facilitates binding of the Src SH3 domain to phospho Y265 (3). Subsequently, Y247 of Cx43 may be phosphorylated by Src (4)[126,127,129,132,134,169]. C: According to Leykauf *et al*, binding of Nedd4 to Cx43 occurs through binding of the second WW domain of Nedd4 to the PPxY motif at Y286[139].

Furthermore, Nedd4 family members consist of an N-terminal C2 domain, which can bind phospolipids and is essential for membrane localisation, and a C-terminal HECT domain, which is responsible for the ligation of ubiquitin to the target protein. Ubiquitination by a Nedd4 family member is responsible for the internalisation and/or targeted degradation of a number of proteins, including sodium channel ENaC and other ion channels, as well as components of the TGFβ signalling pathway[175].

Cx43 linked diseases

Heart disease

Connexin43 (Cx43) is the most abundant gap junction protein in the heart, particularly in the contractile ventricles, and is essential for electrical coupling and efficient propagation of the action potential throughout the heart[5,12,107,176]. Cardiac ischemia may be caused by GPCR agonists angiotensin and endothelins, which are not only very potent vasoconstrictors, but also inhibitors of Cx43 based gap junctional communication[140-142,144,177,178]. Inhibition of GJC protects the heart during pathological conditions by limiting the spreading of damage[179,180]. On the downside, however, closure of Cx43 gap junctions may be the cause of (re-entry) arrhythmia and it has been suggested that genetic defects in Cx43 may underlie a predisposition for arrhythmia[181-186].

Oculodentodigital Dysplasia (ODDD)

A few hundred cases have been described of a hereditary disease called oculodentodigital dysplasia (ODDD), which is associated with a range of loss of function Cx43 mutations[35,90,107,187-189]. Patients suffer from syndactyly (webbed fingers), craniofacial abnormalities, dry and slow growing hair, brittle nails, conductive hearing loss, cataracts, glaucoma, keratoderma, cornea defects, abnormally small teeth and sometimes neurological and heart problems. Considering that Cx43 is the most universal connexin, and that mutant Cx43 impairs the function of co-expressed wildtype Cx43, it is surprising to see how long most patients live, and in relatively good health. This suggests that the mechanism of redundancy by other connexins works very effectively, or that mere expression of Cx43, without the formation of functional channels, is sufficient to partially rescue a knockout phenotype.

Cx43 mouse models

ODDD models

Cx43 loss of function mutations (Cx43 mutants G138R, G60S, I130T) in mice cause a phenotype similar to human ODDD patients[93,190-192]. At a molecular level, mice bearing loss of function mutations of Cx43 show strongly reduced levels of Cx43 protein. This is accompanied by a loss of serine phosphorylated Cx43 species that are usually associated with functional gap junctions, resulting in a reduced Cx43 functional ability to <20%. In contrast, it appears that such mutations increase the formation of hemichannels, leading to increased ATP release from the cells, which may reduce GJC even further[188]. Even though there are no obvious morphological abnormalities of the heart, these mice show a tendency toward spontaneous arrhythmia[190].

Cx43 knockout mice

Cx43 knockout mice are considerably less fortunate than the ODDD models, since they die immediately after birth due to a non functional, malformed heart, of which the right ventricular outflow tract is obstructed[220]. So, apparently, the presence of Cx43 protein and/or the remaining 20% functionality is sufficient to overcome the lethal phenotype.

Attempting to gain more insight into the role of the Cx43 C-terminus, which contains all the regulatory and protein-protein interaction sites, in mouse development, Maass *et al.* replaced endogenous Cx43 by Cx43K258stop in mice. Mice bearing the Cx43K258stop were born at the expected frequency and viable, indicating that deletion of the Cx43 C-terminus does not impair embryonic development. However, the Cx43K258stop homozygotes rarely make it to adulthood because of a defective epidermal barrier, due to an incomplete differentiation of the keratinocytes, which, together with lipids, form the permeability barrier. The epidermal permeability barrier protects from dehydration and against bacterial infection, therefore (partial) absence of this barrier is lethal *ex utero*[221,222].

Normal development of the epidermal barrier depends on the establishment of a fine tuned calcium gradient across the epidermis, which is essential for terminal differentiation of the keratinocytes and epidermal homeostasis. Cx43K258stop has a prolonged half life, compared to wild type Cx43 and Cx43K258stop/Cx43 knockout heterozygotes do not suffer from the epidermal barrier defect and 50% of these mice reach adulthood[221]. Apparently it is not so much the absence of the C-terminal tail but rather the regulation of Cx43 protein expression which causes the skin defects in the K258stop homozygote mice. Cx43 gap junctions in the hearts of adult Cx43 K258stop/knockout mice are functional, but have an altered

morphology: plaques are increased in size and decreased in number compared to wildtype mice[222].

Tissue specific Cx43 knockout mice

To further elucidate the importance of Cx43 for development and organ function, a number of tissue specific Cx43 knockout mice have been generated.

The role of Cx43 in heart development was further investigated by the creation of neural crest cell (NCC) and cardiomyocyte specific Cx43 knockout mice. The hearts of NCC Cx43 knockout mice are morphologically indistinguishable from control animals. The hearts of cardiomyocyte specific knockout mice, however, are predisposed to postnatal morphological abnormalities. Furthermore, Cx43 knockout correlates with slower ventricular activation and a reduced viability during development. All cardiomyocyte Cx43 knockout mice die within 16 days after birth. Notably, neither NCC, nor cardiomyocyte specific Cx43 ablation resembles the heart phenotype of the complete Cx43 knockout mice[223]. Furthermore, smooth muscle cell (SMC) specific knockout mice have been studied in detail for effects on intestine and uterine function. In the intestine, Cx43 SMC knockout induces morphological changes of the intestinal tunica muscularis, and functional impairments, like gastrointestinal motor dysfunction and altered visceral sensory function[224]. In the uterus, SMC specific Cx43 knockout causes a change in uterine contractility, leading to problems with partition[225]. In addition, Sertoli cell specific Cx43 knockout causes male infertility due to abnormal testicular development and arrested spermatogenesis[226,227]. Finally, osteoblast specific Cx43 knockout results in bone malformation and increases vulnerability of the bones to mechanical stress[228].

In summary, Cx43 is essential for normal development and function of all tissues tested. Notably, the knockout phenotypes only partially overlap with the loss of function mutant phenotypes. This suggest either that the ~20% remaining Cx43 channel function in ODDD model mice is sufficient to partially rescue a knockout phenotype, or that Cx43 also exerts channel independent functions.

Channel independent functions of Cx43

In addition to being a channel-forming protein, Cx43 was shown to influence the migratory and adhesive properties of cells. Expression of Cx43 was reported to either inhibit or stimulate migration in diverse cell lines and mouse models[229-233]. In addition, in a Cx43 knockout mouse model, wound healing is increased compared to wild type mice[9].

In neuronal cells, that lack adherens junctions, it has been reported that Cx43 protein increases the adhesive properties of cells, both within one celltype and to neighbouring tissue. In these cells, migration in the brain is mediated by Cx43 (or Cx26), which is independent of gap junctional communication.
In contrast to the advantage that tumour cells have in early stages of tumourigenesis from shutting down gap junction mediated communication, for escaping growth control and cell detachment, in later stages, tumours may also benefit from expression of Cx43. For example for malignant glioma and breastcancer cells, it has been shown that Cx43 increases the adhesiveness of the tumour cells. Increased adhesion enhances invasive properties of these tumours, by promoting angiogenesis and facilitating metastatic homing of tumour cells. In all cases, there are strong indications that the adhesive effect of Cx43 is independent of gap junctional communication and it is suggested that Cx43 may act as an adhesion molecule itself[234-237].

Outline of this thesis

This thesis reports on the mechanism behind regulation of Cx43 based GJC by GPCR signalling. In *chapter 2*, it is described that depletion of phosphatidylinositol 4,5 bisphosphate ($PI(4,5)P_2$) from the plasma membrane, downstream of Gαq, is both necessary and sufficient for inhibition of GJC. Furthermore, it is shown that ZO-1 plays an essential role, possibly by recruiting $PI(4,5)P_2$ hydrolysing enzyme PLCβ3 to the site of the gap junction. *Chapter 3* focuses on the importance of Cx43 residue Y265 on regulation of cell-cell communication. Mutation of this residue prevents GPCR induced inhibition of GJC. However, no increase in tyrosine phosphorylation of Cx43 was observed by GPCR activation, and inhibition of c-Src, and other tyrosine kinases, does not prevent inhibition of GJC. In *chapter 4*, the role of ubiquitination of Cx43 by Nedd4 is addressed. We show that Cx43 is internalised in response to GPCR signalling. This process depends on $PI(4,5)P_2$ hydrolysis and is accompanied by Cx43 ubiquitination, which is dependent on Nedd4. Furthermore, we show that mutant Y265F no longer binds Nedd4, explaining the importance of this residue in inhibition of GJC. Finally, *chapter 5* describes the importance of Cx43 on cell behaviour. It is shown that knockdown of Cx43 in Rat-1 fibroblasts inhibits the migration of contacted cells. Our results suggest that this effect is independent of cell-cell communication, and is attributed to the downregulation of N-cadherin expression in Cx43 knockdown cells.

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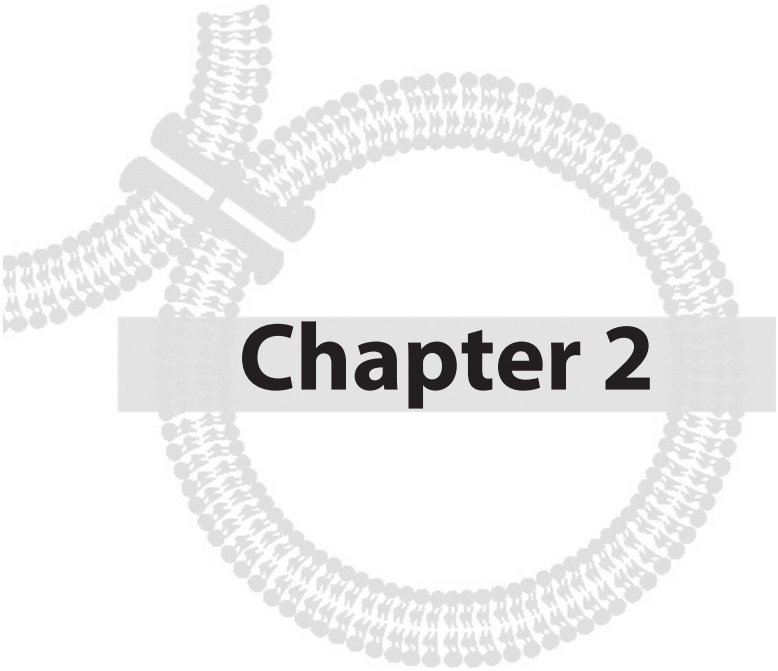

Chapter 2

Regulation of Connexin43 gap junctional communication by phosphatidylinositol 4,5-bisphosphate

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Abstract

Cell-cell communication through connexin43 (Cx43)-based gap junction channels is rapidly inhibited upon activation of various G protein-coupled receptors; however, the mechanism is unknown. Here, we show that Cx43-based cell-cell communication is inhibited by depletion of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) from the plasma membrane. Knockdown of phospholipase Cβ3 (PLCβ3) inhibits PtdIns(4,5)P_2 hydrolysis and keeps Cx43 channels open after receptor activation. Using a translocatable 5-phosphatase, we show that PtdIns(4,5)P_2 depletion is sufficient to close Cx43 channels. When PtdIns(4,5)P_2 is overproduced by PtdIns(4)P 5-kinase, Cx43 channel closure is impaired. We find that the Cx43-binding partner ZO-1 interacts with PLCβ3 via its third PDZ domain. ZO-1 is essential for PtdIns(4,5)P_2-hydrolyzing receptors to inhibit cell-cell communication, but not for receptor-PLC coupling. Our results show that PtdIns(4,5)P_2 is a key regulator of Cx43 channel function, with no role for other second messengers, and suggest that ZO-1 assembles PLCβ3 and Cx43 into a signalling complex to allow regulation of cell-cell communication by localized changes in PtdIns(4,5)P_2.

Introduction

Communication between adjacent cells through gap junctions occurs in nearly every tissue and is fundamental to coordinated cell behaviour. Gap junctions are composed of connexins, consisting of an intracellular N-terminus, four transmembrane domains and a cytosolic C-terminal tail. Six connexins oligomerize into a pore-forming connexon, and alignment of two connexons in apposing cell membranes forms a gap junction channel. These channels allow direct cell-to-cell diffusion of ions and small molecules (<1-2 kDa), including nutrients, metabolites, second messengers and peptides, without transit through the extracellular space[1-3]. Gap junctions play important roles in normal tissue function and organ development[4-6] and have been implicated in a great diversity of biological processes, notably electrical synchronization of excitable cells, energy metabolism, growth control, wound repair, tumour cell invasion and antigen cross-presentation[7-13]. The importance of gap junctions is highlighted by the discovery that mutations in connexins underlie a variety of genetic diseases, including peripheral neuropathy, skin disorders and deafness[5,14].

Connexin43 (Cx43) is the most abundant and best studied mammalian connexin. Cx43-based gap junctional communication is of a particular interest since it is regulated by both physiological and pathophysiological stimuli. In particular, Cx43-based cell-cell coupling is rapidly disrupted following stimulation of certain

G protein-coupled receptors (GPCRs), such as those for endothelin, thrombin, nucleotides and bioactive lipids[15-21]. Disruption is transient as communication is restored after about 20-60 min., depending on the GPCR involved[18]. GPCR-mediated inhibition of intercellular communication will have broad consequences for long-range signalling in cells and tissues where Cx43 is vital, such as dermal fibroblasts, glial cells and heart. However, the link between receptor stimulation and Cx43 channel closure has remained elusive to date. Numerous studies on the 'gating' of Cx43 channels have focused on a possible role for phosphorylation of Cx43 by various protein kinases, in particular protein kinase C (PKC), mitogen-activated protein (MAP) kinase and c-Src, but the results remain ambiguous[22-24]. One of the difficulties with unravelling the regulation of Cx43 channel function is that Cx43 functions in a multiprotein complex that is currently ill understood[25]. One established component of this assembly is the scaffold protein ZO-1, which binds directly to the C-terminus of Cx43 via its second PDZ domain[26,27]. ZO-1 has been suggested to participate in the assembly and proper distribution of gap junctions, but its precise role in the Cx43 complex remains unclear[28,29].

In the present study, we sought to identify the signalling pathway that leads to inhibition of Cx43 gap junctional communication in fibroblasts. Using a variety of experimental approaches, we show that the levels of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) at the plasma membrane dictate the inhibition (and restoration) of Cx43 gap junctional communication in response to GPCR stimulation, with no role for PtdIns(4,5)P_2-derived second messengers. We further show that ZO-1, via its third PDZ domain, interacts with phospholipase Cβ3 (PLCβ3) and is essential for Gq/PLC-coupled receptors to abrogate Cx43-based cell-cell communication. Our results suggest a model in which ZO-1 serves to organize Cx43 and PLCβ3 into a complex to allow exquisite regulation of Cx43 channel function by localized changes in PtdIns(4,5)P_2.

Results

Regulation of Cx43 gap junctional communication by the Gαq-PLCβ-PtdIns(4,5)P_2 hydrolysis pathway

Rat-1 fibroblasts are ideally suited for studying Cx43 channel function since they express Cx43 as the only functional connexin[18,30]. Stable knockdown of Cx43 expression (using pSuper shRNA) resulted in a complete loss of intercellular communication, consistent with Cx43 being the only functional gap junction protein in Rat-1 cells (Fig. 1A). Fig. 1B shows that the Cx43-binding partner ZO-1 retains its submembranous localization in Cx43 knockdown cells.

To assess which G protein(s) mediate(s) inhibition of gap junctional communication, we introduced active versions of Gαq, Gαi, Gα12 and Gα13 subunits into Rat-1

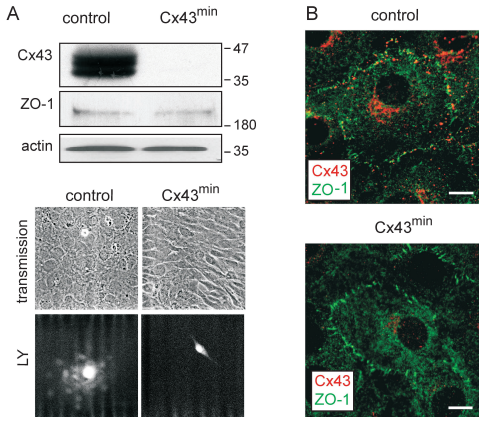

Figure 1. Cx43 is the only functional connexin in Rat-1 cells
A: Rat-1 cells were transduced with Cx43 shRNA (Cx43min) or with a non-functional shRNA (control). Top panel: Immunoblot showing that stable expression of Cx43 shRNA leads to disappearance of Cx43 (Cx43min cells), while leaving ZO-1 expression unaltered. Actin served as loading control. Bottom panel: wide-field images of control and Cx43min Rat-1 cells. Cx43min cells lack cell-cell communication as determined by LY diffusion.
B: Confocal images of control and Cx43-min cells immunostained for Cx43 (red) and ZO-1 (green) (scale bars, 5 μm).

cells and examined their impact on cell-cell coupling. Expression of active Gaq resulted in complete inhibition of intercellular communication, whereas active Gαi, Gα12 and Gα13 left cell-cell coupling unaltered, as evidenced by Lucifer Yellow (LY) diffusion and electrophysiological assays (Fig. 2A). Disruption of gap junctional communication induced by active Gaq was persistent, as opposed to the transient inhibition observed after GPCR stimulatio[18]. Similarly, treatment of Rat-1 cells with Pasteurella multocida toxin, a direct activator of Gαq[31], caused persistent abrogation of cell-cell coupling (Fig. 2B). Gαq couples to PLCβ to trigger PtdIns(4,5)P_2 hydrolysis leading to production of the second messengers inositol-(1,4,5)-trisphosphate (IP_3) and diacylgycerol (DAG)[32]. We monitored PtdIns(4,5)P_2 in living cells by using a GFP fusion protein of the PH domain of PLCδ1 (GFP-PH) as a probe [33-35]. In control cells, the PtdIns(4,5)P_2 probe was concentrated at the plasma membrane. In cells expressing active Gαq, however, the probe was spread diffusely throughout the cytosol, indicative of PtdIns(4,5)P_2 depletion from the plasma membrane (Fig. 2C). While these results are consistent with Gαq mediating agonist-induced inhibition of intercellular communication, they should be interpreted with caution since constitutive depletion of PtdIns(4,5)P_2 from the plasma membrane promotes apoptosis[36,37].

To monitor the kinetics of PtdIns(4,5)P_2 hydrolysis and resynthesis with high temporal resolution, we made use of the fluorescence resonance energy transfer (FRET) between the PH domains of PLCβ1 fused to CFP and YFP, respectively[34]. When bound to plasma membrane PtdIns(4,5)P_2, CFP-PH and YFP-PH are in close proximity and show FRET. Following PtdIns(4,5)P_2 breakdown, the probes dilute out into the cytosol and FRET ceases. The prototypic Gq-coupled receptor agonist endothelin, acting through endogenous ET(A) receptors, induced an acute and substantial decrease in PtdIns(4,5)P_2, reaching a maximum after 30-60 sec.; thereafter, PtdIns(4,5)P_2 slowly recovered to near basal levels over a period lasting

as long as 45-60 min. (Fig. 2D, upper panel; red trace). Sustained PtdIns(4,5)P_2 hydrolysis by ET(A) receptors has been reported previously[38] and may be explained by the fact that activated ET(A) receptors follow a recycling pathway back to the cell surface rather than the lysosomal degradation route[39]. The kinetics of endothelin-induced inhibition and recovery of cell-cell communication followed those of PtdIns(4,5)P_2 hydrolysis and resynthesis, respectively, with communication being restored after about 75 min. (Fig. 2D, upper panel; black trace).

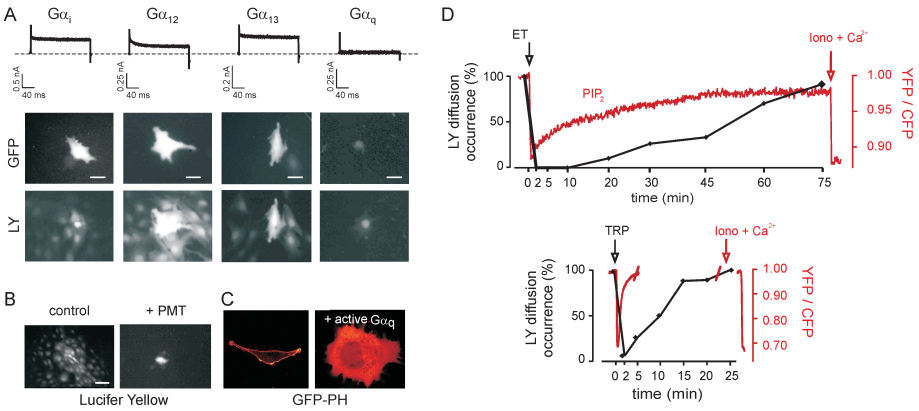

Figure 2. Activated Gαq disrupts Cx43-based gap junctional communication: correlation with PtdIns(4,5)P_2 depletion.

A: Intercellular communication in Rat-1 cells transfected with various activated (GTPase-deficient) Ga subunits. Upper panel: Electrical cell-cell coupling measured by a single patch-clamp electrode[18]. Whole-cell current responses to 10-mV voltage pulses (duration 100 ms; holding potential -70 mV) were recorded from confluent Rat-1 cells. Note dramatic increase in cellular input resistance (i.e. a decrease in conductance) by activated Gαq but not other Gα subunits. Middle and bottom panels: Rat-1 cells cotransfected with active Gαsubunits and GFP (10:1 ratio). GFP-positive cells were microinjected with Lucifer Yellow (LY) and dye diffusion from the microinjected cells was monitored. Wide field pictures of GFP and LY diffusion as indicated (scale bars, 10 μm).

B: Disruption of gap junctional communication by Pasteurella multocida toxin (PMT; 1 μg/ml; 3 hrs preincubation), an activator of Gαq, as measured by LY diffusion (scale bars, 20 μm).

C: Depletion of PtdIns(4,5)P_2 from the plasma membrane by activated Gαq. HEK293T cells were transfected with the PtdIns(4,5)P_2 sensor GFP-PH, alone or together with active Gaq (1:10 ratio). GFP-PH localizes to the plasma membrane where it binds PtdIns(4,5)P_2 (left panel). Co-expression with activated Gaq causes GFP-PH to relocalize to the cytosol (right panel).

D: Monitoring PtdIns(4,5)P_2 levels (PIP_2; red trace) and intercellular communication (black; n>20 per time point) in Rat-1 cells following addition of endothelin (50 nM) (upper panel) or TRP (50 μM) (lower panel). Data points show the percentage of injected cells that spread LY to their neighbors. Temporal changes in plasma membrane-bound PtdIns(4,5)P_2 were measured by changes in FRET between CFP-PH and YFP-PH. Ionomycin, which evokes an immediate and complete depletion of PtdIns(4,5)P_2 from the plasma membrane when applied at high doses (5 μM) together with 5 mM Ca^{2+} (van der Wal *et al*., 2001) was used for calibration.

More transient PtdIns(4,5)P_2 depletion and recovery kinetics were observed with a thrombin receptor (PAR-1) activating peptide (TRP), which correlated with a more short-lived inhibition of gap junctional communication (Fig. 2D, lower panel). Furthermore, a desensitization-defective mutant NK2 receptor (for neurokinin A) that mediates prolonged PtdIns(4,5)P_2 hydrolysis[40] inhibits gap junctional communication for prolonged periods of time when compared to the wild-type NK2 receptor[18]. While these results reveal a close correlation between the duration of PtdIns(4,5)P_2 depletion and that of communication shutoff, we note that the restoration of cell-cell communication consistently lagged behind the recovery of PtdIns(4,5)P_2 levels. Nevertheless, our findings strongly suggest that the Gq/PLCβ-mediated hydrolysis and subsequent resynthesis of PtdIns(4,5)P_2 dictate the inhibition and restoration of Cx43 gap junctional communication, respectively.

Knockdown of PLCβ3 prevents cell-cell uncoupling

The Gαq-activated PLCβ enzymes comprise four members (β1-4)[32]. PLCβ1 and β3 are ubiquitously expressed, whereas PLCβ2 and β4 expression is restricted to hematopoietic cells and neurons, respectively. Rat-1 cells express PLCβ3, but no detectable PLCβ1 (Fig. 3A and results not shown). We stably suppressed PLCβ3 expression using the pSuper short hairpin RNA (shRNA) expression vector[41]. Four different target sequences were selected to correct for clonal variation and off-target effects. Immunoblot analysis shows a marked reduction in PLCβ3 expression in different clones (Fig. 3A). When comparing PtdIns(4,5)P_2 dynamics in PLCβ3 knockdown versus control cells, agonist-induced PtdIns(4,5)P_2 breakdown was strongly reduced in the PLCβ3-deficient cells (Fig. 3B). PLCβ3 knockdown cells showed normal basal cell-cell communication but failed to shut off cell-cell communication following GPCR stimulation (Fig. 3C). These results indicate that PLCβ3 is a key player in the control of intercellular communication, supporting the view that GPCRs inhibit gap junctional communication through the Gq/PLCβ3-PtdIns(4,5)P_2 hydrolysis pathway.

PLC-mediated PtdIns(4,5)P_2 hydrolysis generates the second messengers IP_3 and DAG, leading to Ca^{2+} mobilization and protein kinase C (PKC) activation, respectively. Previous pharmacological studies already suggested that neither Ca^{2+} nor PKC have a critical role in GPCR-mediated inhibition of cell-cell coupling[18], a notion supported by additional experiments using 'caged' IP_3, the cell-permeable Ca^{2+} chelator BAPTA-AM and a PKC-activating bacterial PLC[42] (data summarized in Table S1). Whether PtdIns(4,5)P_2-derived second messengers are dispensable for Cx43 channel closure upon GPCR activation remains debatable, however, since the supporting pharmacological evidence is indirect.

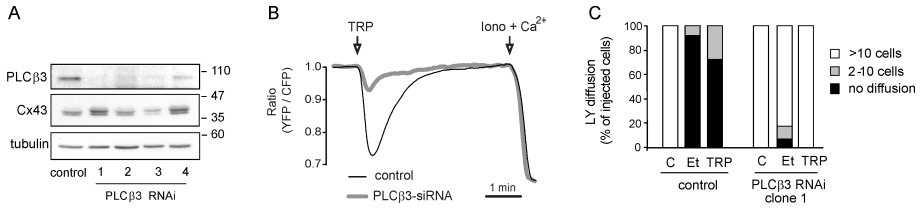

Figure 3. Targeted knockdown of PLCβ3 prevents receptor-mediated PtdIns(4,5)P_2 hydrolysis and inhibition of junctional communication.
A: PLCβ3 knockdown in Rat-1 cells as detected by immunoblotting. Expression of PLCβ3 in Rat-1 cells expressing a non-functional shRNA (control) and in four subclones (1-4) stably expressing different PLCβ3 shRNA constructs. Total cell lysates were immunoblotted for PLCβ3, Cx43 and α-tubulin as indicated.
B: Temporal changes in plasma-membrane PtdIns(4,5)P_2 levels following thrombin receptor stimulation of normal (red trace) and PLCβ3-deficient Rat-1 cells (blue trace). TRP, 50 μM; Ionomycin, 5 μM.
C: Bar graphs showing the percentage of communicating cells in control and v PLCβ3 knockdown cells (clone1) treated with either endothelin (Et, 50 nM) or TRP (50 μM), as indicated (n >25 for each dataset). LY injections were done at 2 min. after addition of agonist.

Conversion of PtdIns(4,5)P_2 into PtdIns(4)P by phosphoinositide 5-phosphatase is sufficient to inhibit cell-cell communication

To examine whether the depletion of PtdIns(4,5)P_2 suffices to inhibit Cx43 gap junctional communication, we used a newly developed method to rapidly deplete PtdIns(4,5)P_2 without activating PLC. In this approach, PtdIns(4,5)P_2 at the plasma membrane is converted into PtdIns(4)P and free phosphate by rapamycin-inducible membrane targeting of the human type IV phosphoinositide 5-phoshatase (5-ptase)[43,44]. The method is based on the rapamycin-induced heterodimerzation of FRB (fragment of mammalian target of rapamycin [mTOR] that binds FKB12) and FKB12 (FK506-binding protein 12), as schematically illustrated in Fig. 4A. In this approach, a mutant version of 5-ptase with a defective membrane targeting domain (CAAX box) is fused to FKBP12 and tagged with monomeric red fluorescent protein (mRFP), while its binding partner FRB (fused to CFP) is tethered to the plasma membrane through palmitoylation (construct PM-FRB-CFP)[43]. In the absence of rapamycin, 5-ptase resides in the cytosol and leaves PtdIns(4,5)P_2 levels at the plasma membrane unaltered (Fig. 4A, left panel). Upon addition of rapamycin (100 nM), FKBP and FRB undergo heterodimerization and the 5-ptase is recruited to the plasma membrane (Fig. 4A, right panel).

We expressed the mRFP-FKBP-5-ptase and PM-FRB-CFP fusion proteins in Rat-1 cells and confirmed their proper intracellular localization by confocal microscopy (not shown). Addition of rapamycin caused a rapid and complete depletion of PtdIns(4,5)P_2, as shown by the disappearance of the PtdIns(4,5)P_2 sensor YFP-PH from the plasma membrane (Fig. 4B and 4C, upper trace n=10). As expected, no Ca^{2+} signal was detected following the 5-ptase-mediated conversion of PtdIns(4,5)P_2

into PtdIns(4)P (n=4; Fig. 4C). To determine how the 5-ptase-induced hydrolysis of PtdIns(4,5)P_2 affects gap junctional communication, we measured the intercellular diffusion of calcein (added as membrane-permeable calcein-AM) using FRAP (Fluorescence Recovery After Photobleaching)[45] (Fig. 4D). Rat-1 cells expressing mRFP-FKBP-5-ptase and PM-FRB-CFP showed efficient intercellular transfer of calcein. At 2 minutes after rapamycin addition, however, intercellular dye diffusion was inhibited as inferred from a strongly reduced fluorescence recovery rate (Fig. 4D; n=15, p<0.005; about 0.25 x the recovery rate before rapamycin addition). The recovery of calcein fluorescence could not be decreased any further by addition of the gap junction blocker 2-APB (2-aminoethoxy-diphenylborane; 50 μM; Fig. 4D)[46].

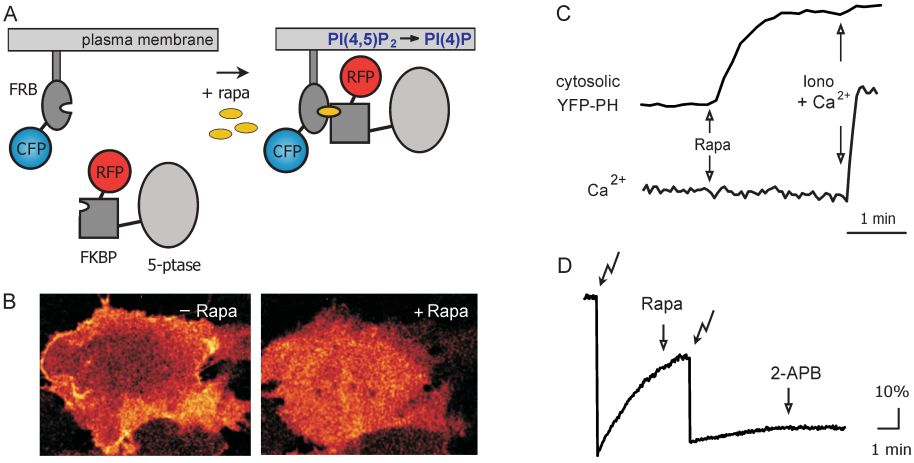

Figure 4. PtdIns(4,5)P_2 depletion by 5-phosphatase inhibits gap junctional communication.
A: Schematic representation of rapamycin-induced PtdIns(4,5)P_2 degradation at the plasma membrane. Rapamycin induces dimerization of FKBP domains to FRB domains. Rapamycin recruits the phosphoinositide-5-phosphatase-FKBP fusion protein (mRFP-FKBP-5-ptase) to FRB tethered to the plasma membrane (PM-FRB-CFP), resulting in the rapid conversion of PtdIns(4,5)P_2 into PtdIns(4)P. B: Confocal images of YFP-PH in Rat-1 cells before (left) and after (right) addition of rapamycin (100nM). In addition to YFP-PH, PM-FRB-CFP and mRFP-FKBP-5-ptase were also correctly expressed (images not shown). Note that the translocation of YFP-PH into the cytoplasm is complete, indicative of massive PtdIns(4,5)P_2 hydrolysis. C: Representative responses to rapamycin and ionomycin. Top, cytosolic levels of YFP-PH; bottom, Ca^{2+} dye Oregon Green. Ionomycin treatment could not induce further translocation of YFP-PH, indicating that rapamycin-induced PtdIns(4,5)P_2 degradation was complete (n=10). Rises in cytosolic Ca^{2+} were never observed (n=4), confirming that PtdIns(4,5)P_2 hydrolysis did not generate second messengers. D; Gap junctional communication in Rat-1 cells transfected with PM-FRB-CFP and mRFP-FKBP-5-ptase, assayed by fluorescence recovery after photobleaching (FRAP) of calcein. While cells showed efficient communication before rapamycin-treatment, gap junctional exchange was significantly decreased at 2 min after addition of rapamycin (0.25 x recovery rate before rapamycin, n=15, p<0.005). The gap junction blocker 2-APB was added at 50 μM.

Rapamycin did not affect cell-cell communication in non-transfected cells (data not shown). Thus, PtdIns(4,5)P_2 depletion by 5-phosphatase activation is sufficient to inhibit Cx43 gap junctional communication, with no need for PtdIns(4,5)P_2-derived second messengers.

Overexpression of PtdIns(4)P 5-kinase prevents inhibition of cell-cell communication

PtdIns(4,5)P_2 at the plasma membrane is generated mainly from PtdIns(4)P by PtdIns(4)P 5-kinase (PIP5K)[47,48]. As a further test of the PtdIns(4,5)P_2 hypothesis, we stably overexpressed PIP5K (type Iα, fused to GFP) in Rat-1 cells in an attempt to prevent PtdIns(4,5)P_2 depletion following GPCR stimulation (Fig. 5A). As shown in Fig. 5B, transfected GFP-PIP5K localizes to the plasma membrane. In the PIP5K-overexpressing cells, PtdIns(4,5)P_2 levels remain elevated (i.e. above FRET threshold levels) after agonist addition (Fig. 5C). Nonetheless, GPCR agonists still induced transient rises in IP_3 and Ca^{2+} (Fig. 5C), indicating that excessive synthesis of PtdIns(4,5)P_2 does not interfere with its hydrolysis. Basal cell-cell communication in PIP5K-overexpressing cells was not significantly different from that in control cells. However, the PIP5K-overexpressing cells failed to close their gap junction channels upon addition of TRP and, to a lesser extent, endothelin (Fig. 5C). That endothelin is still capable of evoking a residual response in PIP5K-overexpressing cells may be explained by the fact that endothelin is by far the strongest inducer of PtdIns(4,5)P_2 depletion (Fig. 2D).

Expression of a 'kinase-dead' version of PIP5K had no effect on either PtdIns(4,5)P_2 hydrolysis or inhibition of cell-cell communication (Fig. 5D). We conclude that Cx43 channel closure is prevented when PtdIns(4,5)P_2 is maintained at adequate levels.

No detectable PtdIns(4,5)P_2 binding to the C-terminal tail of Cx43

PtdIns(4,5)P_2 can modulate the activity of various ion channels and transporters, apparently through direct electrostatic interactions[49,50]. By analogy, regulation of Cx43 channels by PtdIns(4,5)P_2 would imply that basic residues in Cx43 bind directly to the negatively charged PtdIns(4,5)P_2. Indeed, the regulatory cytosolic tail of Cx43 (aa 228-382) contains a membrane-proximal stretch of both basic and hydrophobic residues (231VFFKGVKDRVKGK/R243) that could constitute a potential PtdIns(4,5)P_2 binding site. Local depletion of PtdIns(4,5)P_2 might then dissociate the juxtamembrane region of the Cx43 tail from the plasma membrane leading to channel closure. We reasoned that if the Cx43 juxtamembrane domain binds PtdIns(4,5)P_2 *in situ*, mutations within this domain might interfere with PtdIns(4,5)P_2-regulated channel closure. We therefore neutralized the membrane-proximal Arg and Lys residues by mutation to alanine resulting in eight distinct Cx43 mutants, notably K237A,K241A; R239A,R243A; K241A,R243A; R239A,K241A; K237A,R239A; R239A,K241A,R243A; K237A,R239A,K241A and the '4A' mutant,

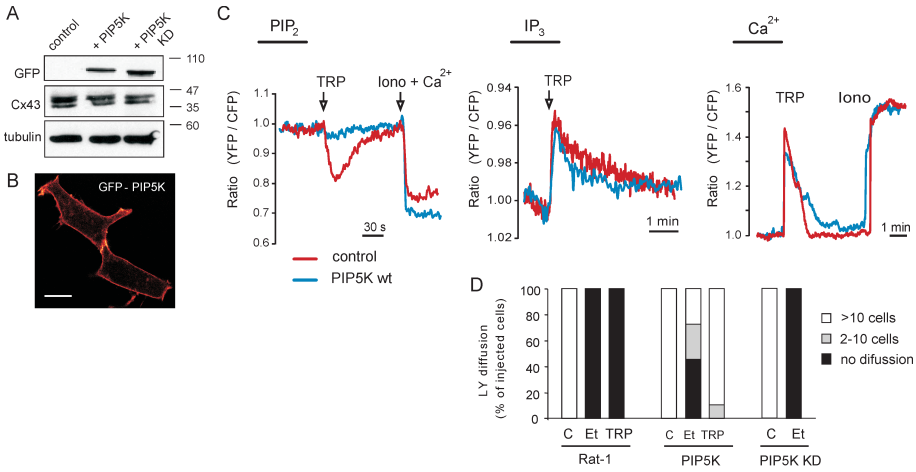

Figure 5. Overexpression of PtdIns(4)P 5-kinase attenuates agonist-induced PtdIns(4,5)P_2 depletion and keeps junctional communication largely intact.
(A) Stable expression of GFP-PIP5K (wild-type, WT, and 'kinase-dead', KD) in Rat-1 cells. Total cell lysates were immunoblotted for GFP, Cx43 and α-tubulin as indicated.
(B) Localization of GFP-PIP5K in Rat-1 cells (scale bar, 10 μm).
(C) Temporal changes in the levels of PtdIns(4,5)P_2, IP_3 and Ca^{2+} measured by the respective FRET-based sensors, as detailed in the Methods section.
Control and PIP5K-overexpressing Rat-1 cells were stimulated with TRP (50 μM). In control cells (red trace), PtdIns(4,5)P_2 levels rapidly fall after TRP stimulation, whereas PIP5K overexpression (blue trace) largely prevents the drop in FRET indicating that PtdIns(4,5)P_2 levels remain high (i.e. above FRET threshold). Ionomycin, 5 μM.
(D) Bar graphs showing the percentage of communicating cells (LY diffusion) in control Rat-1 cells and cells expressing either wild-type (wt) or kinase-dead (KD) PIP5 kinase. Cells were left untreated (C) or stimulated with GPCR agonists (endothelin, 50 nM; TRP, 50 μM) as indicated (n >20 for each dataset). Residual response to endothelin is explained by excessive depletion of PtdIns(4,5)P_2 (Fig. 2D). LY injections were done at 2 min. after addition of agonist

K237A,R239A,K241A,K243A. When expressed in Cx43-deficient cells, however, all these mutants were trapped intracellularly and failed to localize to the plasma membrane (Fig. S1A). While this result reveals a previously unknown role for the membrane-proximal Arg/Lys residues in Cx43 trafficking, it precludes a test of the Cx43-PtdIns(4,5)P_2 interaction hypothesis.

We next examined whether PtdIns(4,5)P_2 can specifically bind to either the Cx43 C-terminal tail (Cx43CT; aa 228-382) or a Cx43CT-derived juxtamembrane peptide (Cx43JM; aa 228-263) in vitro. We generated a GST-Cx43CT fusion protein and determined its ability to bind phosphoinositides in vitro using three distinct protocols. GST-PH(PLCδ1) was used as a positive control. In the first approach, agarose beads coated with either PtdIns(4,5)P_2 or PtdIns(4)P were incubated with GST-Cx43CT or GST-PH and then pulled down by centrifugation. PtdIns(4,5)P_2 beads readily brought down the GST-PH polypeptide but not GST-Cx43CT (Fig. S2A). Second, we incubated GST-Cx43CT with ^{32}P-labeled PtdIns(4,5)P_2 and examined the ability of excess phosphoinositides to displace bound ^{32}P-PtdIns(4,5)P_2.

While GST-PH showed again strong PtdIns(4,5)P_2 binding that could readily be displaced by excess PtdIns(4,5)P_2, there was no detectable binding of PtdIns(4,5)P_2 to Cx43CT above that observed with GST alone (Fig. S2B). Finally, we found that PtdIns(4,5)P_2 (and other phosphoinositides) immobilized on nitrocellulose strips failed to bind either Cx43CT or a 35-aa juxtamembrane domain peptide (Cx43JM; aa 228-263[55]) (results not shown). Thus, PtdIns(4,5)P_2 does not detectably bind to the juxtamembrane domain of Cx43, nor to the full-length regulatory tail (aa 228-362), at least *in vitro*.

ZO-1 is required for GPCRs to inhibit junctional communication

The very C-terminus of Cx43 binds directly to the second PDZ domain of ZO-1, but the functional significance of the Cx43-ZO-1 interaction is not understood. We asked if ZO-1 has a role in modulating gap junctional communication in response to GPCR stimulation. We already showed that RNAi-mediated depletion of Cx43 does not significantly affect the levels and localization of ZO-1 (Fig. 1B). Conversely, when ZO-1 expression was knocked down by shRNA, Cx43 levels were unaltered (Fig. 6A). ZO-1 knockdown Rat-1 cells retained their fibroblastic morphology and showed normal Cx43 punctate staining and cell-cell coupling (Fig. 6B,C), showing that ZO-1 is dispensable for the formation of functional gap junctions. When ZO-1 knockdown cells were stimulated with endothelin, however, the inhibition of cell-cell communication was severely impaired (Fig. 6C). Importantly, overall PtdIns(4,5)P_2-dependent Ca^{2+} mobilization was not affected in the ZO-1 knockdown cells (Fig. 6D). We conclude that ZO-1 is essential for the regulation of gap junctional communication by Gq/PLC-coupled receptors, but not for linking those receptors to PLC activation. A plausible explanation for these findings is that ZO-1 serves to bring the PtdIns(4,5)P_2-metabolizing machinery into proximity of Cx43 gap junctions.

Direct interaction between ZO-1 and PLCβ3

As a test of the above hypothesis, we examined if ZO-1 can interact with PLCβ3. PLCβ3 can associate with at least two scaffold proteins, NHERF2 (in epithelial cells) and Shank2 (in brain), via a C-terminal PDZ domain-binding motif[51,52]. We co-expressed HA-PLCβ3 and GFP-ZO-1 in HEK293 cells and performed immunoprecipitations using anti-GFP antibody (Fig. 7A). Cell lysates and immunoprecipitates were blotted for GFP and HA. As shown in Fig. 7B, PLCβ3 and ZO-1 can indeed be coprecipitated. Next, we co-expressed ZO-1 and a PLCβ3 truncation mutant that lacks the C-terminal 14 residues (HA-PLCβ3-ΔPBD; Fig. 7A), and performed anti-GFP immunoprecipitations. Fig. 7B shows that truncated PLCβ3 fails to interact with ZO-1, indicating that PLCβ3 interacts with ZO-1 through its very C-terminus, containing the PDZ domain-binding motif. Considering that ZO-1 has three distinct

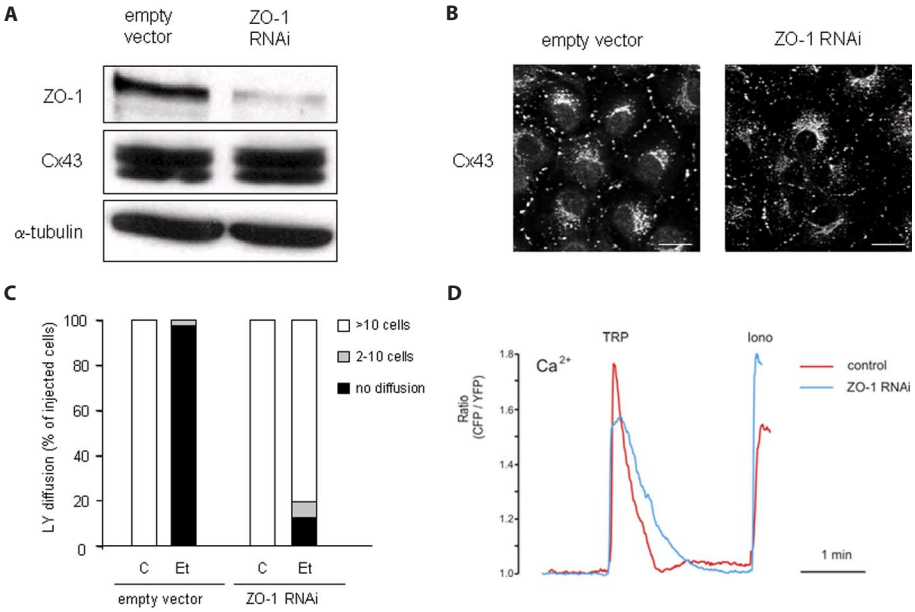

Figure 6. Knockdown of ZO-1 largely prevents agonist-induced disruption of junctional communication, while leaving Ca^{2+} mobilization intact.
A: Immunoblots showing strongly reduced ZO-1 expression by adenoviral ZO-1 RNAi compared to control virus ('empty vector'). ZO-1 knockdown did not affect Cx43 expression, as indicated.
B: Immunostaining of Cx43 in control and ZO-1 knockdown Rat-1 cells. Note that ZO-1 knockdown does not affect Cx43 punctate staining patterns.
C: Bar graphs showing communication in control ('empty vector') and ZO-1 knockdown cells (ZO-1 RNAi) before and after addition of endothelin (Et, 50 nM) (data sets represent totals of at least two independent experiments; number (n) of injected cells: empty vector -/+ Et, n=53/85; ZO-1 RNAi -/+ Et, n=43/56). LY injections were done at 2 min. after addition of agonist.
D: GPCR-mediated Ca^{2+} mobilization in control cells (red trace) and ZO-1 knockdown cells (blue trace). Ca^{2+} was measured using the FRET-based Yellow Cameleon probe. TRP, 50 μM; Ionomycin, 5 μM.

PDZ domains, we examined which (if any) PDZ domain binds PLCβ3. We expressed GFP-tagged versions of the three individual PDZ domains in HEK293 cells, either alone or together with HA-PLCβ3. We immunoprecipitated PLCβ3 using anti-HA antibody and blotted total cell lysates and precipitates for both HA and GFP. As shown in Fig. 7C, we find that PLCβ3 binds to PDZ3 but not to PDZ1 or PDZ2.
To verify that the ZO-1-PLCβ3 interaction exists endogenously, we precipitated ZO-1 from Rat-1 cells and blotted for both ZO-1 and PLCβ3. Fig. 7D shows that PLCβ3 co-precipitates with ZO-1. The reverse co-precipitation could not be done, since precipitating antibodies against PLCβ3 are presently not available. Nonetheless, these results suggest that ZO-1, through its respective PDZ2 and PDZ3 domains,

assembles Cx43 and PLCβ3 into a signalling complex and thereby facilitates regulation of gap junctional communication by PLC-coupled receptors.

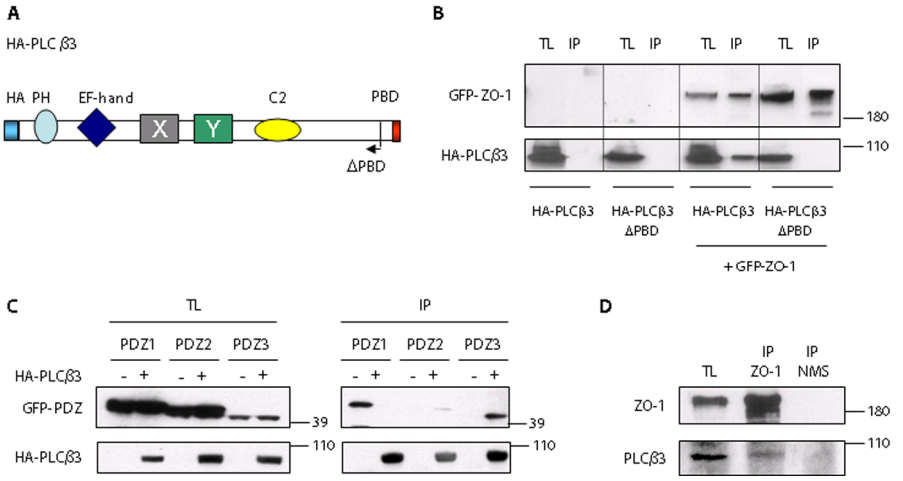

Figure 7. Association of ZO-1 with PLCβ3
A: Schematic representation of HA-PLCβ3. X, Y represent the catalytic domains; the C2 domain interacts with activated $G\alpha q$[32]; ΔPBD: mutant PLCβ3 lacking the C-terminal residues 1220-1234 (comprising the PDZ domain binding motif).
B: Co-immunoprecipitation of GFP-ZO-1 and HA-PLCβ3 expressed in HEK293 cells. TL: total cell lysates; IP, denotes immunoprecipitation using anti-GFP antiserum. Samples were immunoblotted for GFP (top) and HA (bottom).
C: Co-immunoprecipitation of HA-PLCβ3 and GFP-tagged individual PDZ domains of ZO-1 expressed in HEK293 cells. TL: total cell lysates; IP, denotes immunoprecipitation using anti-HA antibody. Samples were immunoblotted for GFP (top) and HA (bottom).
D: Endogenous ZO-1 immunoprecipitated (IP) from Rat-1 cells. Total cell lysates (TL) and samples from ZO-1 immunoprecipitates (IP) were blotted for both ZO-1 and PLCβ3 as indicated. NMS: normal mouse serum.

Discussion

A critical and long-standing question in gap junction biology is how junctional communication is regulated by physiological and pathophysiological stimuli. Relatively little progess has been made in identifying receptor-induced signalling events that modulate the channel function of Cx43, the best studied and most abundant mammalian connexin. In particular, regulation of Cx43 channel activity via G-protein signalling has not been systematically examined to date. In the present study, we identify the Gq-linked PLCβ-PtdIns(4,5)P_2 hydrolysis pathway as a key regulator of Cx43-based gap junctional communication in normal fibroblasts. We demonstrate that loss of PtdIns(4,5)P_2 from the plasma membrane is necessary and sufficient to close Cx43 channels, without a role for PtdIns(4,5)P_2-derived second messengers.

In other words, PtdIns(4,5)P_2 itself is the responsible signalling molecule. A second novel finding is that the Cx43-binding partner ZO-1 binds to PLCβ3 and is essential for PtdIns(4,5)P_2-hydrolyzing receptors to regulate gap junctional communication.

PtdIns(4,5)P_2 as a key regulator

Our conclusion that PtdIns(4,5)P_2 at the plasma membrane regulates Cx43 channel function is based on several lines of evidence. First, active Gαq (but not Gαi, Gα12 or Gα13) depletes PtdIns(4,5)P_2 from the plasma membrane and abrogates gap junctional communication. Second, knockdown of PLCβ3 inhibits agonist-induced PtdIns(4,5)P_2 depletion and prevents disruption of cell-cell communication. Third, conversion of PtdIns(4,5)P_2 into PtdIns(4)P by a translocatable 5-phosphatase is sufficient to inhibit intercellular communication. Fourth, maintaining PtdIns(4,5)P_2 at adequate levels by overexpression of PIP5K renders Cx43 channels refractory to GPCR stimulation, although second messenger generation still occurs.

Acting as a signalling molecule in its own right, PtdIns(4,5)P_2 can regulate local cellular activities when its levels rise and fall; in particular, PtdIns(4,5)P_2 can modulate the activity of various ion channels and transporters, presumably through electrostatic interactions[49,50]. Although the existence of such interactions in living cells remains largely inferential and PtdIns(4,5)P_2-binding consensus sequences have not been clearly defined, the common theme is that the negatively charged PtdIns(4,5)P_2 binds to a motif with multiple positive charges interdispersed with hydrophobic residues[53,54]. The Cx43 C-terminal juxtamembrane domain indeed contains such a putative PtdIns(4,5)P_2-binding motif (aa 231-243), although this stretch also meets the criteria of α tubulin-binding domain[55]. Extension of the above model to Cx43 channel gating would then imply that local loss of PtdIns(4,5)P_2 could release the Cx43 regulatory tail from the plasma membrane to render it susceptible to a modification leading to channel closure. However, our investigations to detect specific binding of PtdIns(4,5)P_2 to the Cx43 C-terminal tail or its juxtamembrane domain in vitro yielded negative results. Rather, mutational analysis revealed that those basic residues in the juxtamembrane domain have a hitherto unrecognized role in the trafficking of Cx43 to the plasma membrane. These findings do not, of course, rule out the possibility that PtdIns(4,5)P_2 does bind directly to Cx43 *in situ*.

Aside from modulating ion channel activity, PtdIns(4,5)P_2 has been implicated in cytoskeletal remodeling, vesicular trafficking and recruitment of cytosolic proteins to specific membranes[54,56]. Although Cx43 can interact with cytoskeletal proteins, such as tubulin and drebrin[55,57], cytoskeletal reorganization does not play a significant role in regulating Cx43 junctional communication because cytoskeleton-disrupting agents (cytochalasin D, nocodazole, Rho-inactivating C3 toxin) have no detectable effect on GPCR regulation of cell-cell coupling[55] (see also Table S1). Furthermore, we found that GPCR-induced inhibition and recovery of gap

junctional communication are insensitive to agents known to interfere with Cx43 trafficking and internalization, including cycloheximide, brefeldin A, monensin, ammonium chloride and hypertonic sucrose (Table S1).

Numerous studies have suggested that closure of Cx43 channels in response to divergent stimuli somehow results from Cx43 phosphorylation[22,23]. Several protein kinases, including PKC, MAP kinase, casein kinase-1 and Src, are capable of phosphorylating Cx43 at multiple sites in the C-terminal tail. These phosphorylations have been implicated not only in Cx43 channel gating but also in Cx43 trafficking, assembly and degradation. The link between Cx43 phosphorylation and altered cell-cell coupling is largely correlative, however, as the functional significance of most of these phosphorylations has not been elucidated. Our previous studies suggested that c-Src-mediated tyrosine phosphorylation of Cx43 underlies disruption of gap junctional communication, as inferred from experiments using both constitutively active and dominant-negative versions of c-Src[18,58]. To date, however, we have been unable to detect GPCR-induced tyrosine phosphorylation of Cx43 in a physiological context. Furthermore, the Src inhibitor PP2 does not prevent GPCR agonists from inhibiting Cx43-based gap junctional communication in either Rat-1 cells or primary astrocytes[17] (Table S1). Therefore, tyrosine phosphorylation of Cx43 leading to loss of cell-cell coupling, as observed with constitutively active c-Src and v-Src 24, may not actually occur under physiological conditions; an issue that warrants further investigation.

Essential role for ZO-1

Another novel finding of the present study concerns the role of ZO-1, an established binding partner of Cx43[26,27]. Originally identified as a major component of epithelial tight junctions[59], ZO-1 is thought to serve as a platform to scaffold various transmembrane and cytoplasmic proteins. ZO-1 and its close relative ZO-2 have several protein-interaction domains, including three PDZ domains, one SH3 domain and one GUK domain. In epithelial cells, ZO-1 and ZO-2 act redundantly to some extent in the formation of tight junctions[17,60]. In non-epithelial cells lacking tight junctions, ZO-1 has been attributed a role in the assembly and stabilization of Cx43 gap junctions[29,61], but its precise role has remained elusive. Our knockdown studies herein show that ZO-1 is essential for Gq/PLC-coupled receptors to inhibit intercellular communication, but not for coupling those receptors to PLC activation, as inferred from Ca^{2+} mobilization experiments; this result suggests that loss of ZO-1 at Cx43 gap junctions is not compensated for by ZO-2. We find that ZO-1 binds directly to the very C-terminus of PLCβ3 via its third PDZ domain. In the simplest model compatible with our findings, ZO-1 serves to assemble Cx43 and PLCβ3 into a complex to permit regulation of gap junctional communication by localized changes in PtdIns(4,5)P_2, as schematically illustrated in Fig. 8. Since we

found no evidence for direct binding of PtdIns(4,5)P_2 to Cx43 *in vitro*, PtdIns(4,5)P_2 might regulate junctional communication in an indirect manner, for example via a Cx43-associated protein that modifies the Cx43 regulatory tail and thereby shuts off channel function. Precisely how PtdIns(4,5)P_2 regulates the Cx43 multiprotein complex remains a challenge for future studies.

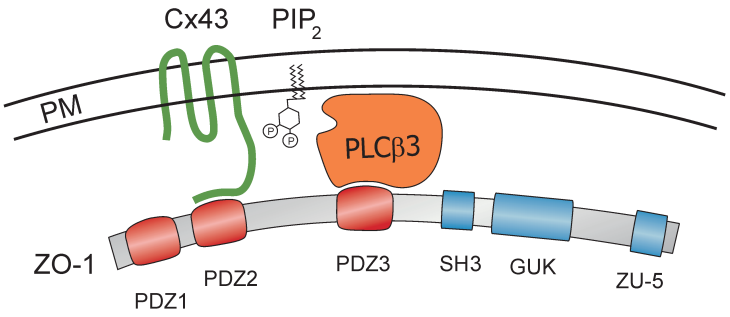

Figure 8. Schematic drawing of the proposed model
ZO-1 is proposed to assemble Cx43 and PLCβ3 into a complex, thereby facilitating regulation of Cx43 channel function by localized changes in PtdIns(4,5)P_2 upon receptor activation. Since we found no evidence for direct binding of PtdIns(4,5)P_2 to Cx43, PtdIns(4,5)P_2 might regulate junctional communication in an indirect manner, for example via a Cx43-associated protein that modifies the Cx43 regulatory tail and thereby shuts off channel function. PM, plasma membrane. See text for details.

Materials and methods

Reagents

Materials were obtained from the following sources: endothelin, thrombin receptor-activating peptide (TRP; sequence SFLLRN), neurokinin A, Cx43 polyclonal and α-tubulin monoclonal antibodies from Sigma (St. Louis, MO); Pasteurella multocida toxin from Calbiochem-Novabiochem (La Jolla, CA); Cx43 NT monoclonal antibody from Fred Hutchinson Cancer Research Center (Seattle WA); actin monoclonal from Chemicon International (Temecula, CA); polyclonal PLCβ3 antibody from Cell Signalling; ZO-1 monoclonal antibody from Zymed; HRP-conjugated secondary antibodies from DAKO and secondary antibodies for immunofluorescence (goat-anti-mouse, Alexa488 and goat-anti-rabbit, Alexa594) from Molecular Probes. HA, Myc and GST monoclonal antibodies were purified from hybridoma cell lines 12CA5, 9E10 and 2F3, respectively. GFP antiserum was generated in our institute.

cDNA constructs

Constructs encoding active (GTPase-deficient) Gα subunits, eGFP-PHPLCδ1, eCFP-PHPLCδ1, eYFP-PHPLCδ1, eGFP-tagged mouse type-Iα PI(4)P 5-kinase have been described[34,37,62]. Mouse PLCβ3 cDNA was obtained from MRC gene service, cloned into pcDNA3-HA by PCR (primers listed in Table S2) and ligated into pcDNA3-HA XhoI/NotI sites. HA-PLCβ3-ΔPBD was obtained by restriction of the full-length construct with Eco47III, cleaving off the very C-terminal 14 residues. Human ZO-1 was cloned into XhoI and KpnI sites of peGFP C2 (Clontech). GFP-based Yellow Cameleon 2.1 has been described[34]. Constructs encoding cytosolic 5-phosphatase fused to FKB12-mRFP and PM-FRB-CFP have been described[43].

Cell culture and cell-cell communication assays

Cells were cultured in DMEM containing 8% fetal calf serum, L-glutamine and antibiotics. For cell-cell communication assays, cells were grown in 3-cm dishes and serum starved for at least 4 hrs prior to experimentation. Monitoring the diffusion of Lucifer Yellow (LY) from single microinjected cells and single-electrode electrophysiological measurements of cell-cell coupling were done as described 18. Images were acquired on a Zeiss Axiovert 135 inverted microscope, equipped with an Achroplan × 40 objective (N.A. 0.60) and a Nikon F301 camera.

SDS-PAGE, immunoblotting and immunoprecipitation

Cells were harvested in Laemmli sample buffer (LSB), boiled for 10 min. and subjected to immunoblot analysis according to standard procedures. Filters were blocked in TBST/5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemoluminescence (Amersham Pharmacia). For immunoprecipitation, cells were harvested in 1% NP-40, 0.25% sodium desoxycholate lysis buffer. Lysates were spun down and the supernatants were subjected to immunoprecipitation using protein A-conjugated

antibodies for 4 hrs at 4°C. Proteins were eluted by boiling for 10 min. in LSB and analyzed by immunoblotting.

Immunostaining and fluorescence microscopy

Cells grown on coverslips were fixed in 3.7% formaldehyde in PBS for 15 min. Samples were blocked and permeabilized in PBS containing 1% BSA and 0.1% Triton X-100 for 30 min. Subsequently, samples were incubated with primary and secondary antibodies for 30 min. each in PBS/1% BSA, washed five times with PBS and mounted in MOWIOL (Calbiochem). Confocal fluorescence images were obtained on a Leica TCS NT (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr laser. Images were taken using a 63x NA 1.32 oil objective. Standard filter combinations and Kalman averaging were used. Processing of images for presentation was done on a PC using the software package Photoshop (Adobe Systems Incorporated Mountain View, California, USA).

Live-cell imaging

All live imaging and time-lapse experiments were performed in bicarbonate-buffered saline containing (in mM) 140 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose, 23 $NaHCO_3$, 10 HEPES (pH 7.2), kept under 5% CO_2, at 37°C. Images of live cells expressing GFP-PH and GFP-PIP5K were recorded on a Leica TCS-SP2 confocal microscope (Mannheim, Germany), using a 63x lens, N.A. 1.4.

PtdIns(4,5)P_2, IP_3 and Ca^{2+} imaging by FRET ratiometry

Temporal changes in PtdIns(4,5)P_2 levels in living cells were assayed by the FRET-based PtdIns(4,5)P_2 sensor, PH-PLCδ1, as described[34]. In brief, Rat-1 cells were transiently transfected with CFP-PH and YFP-PH constructs (1:1 ratio) using Fugene transfection agent and placed on a NIKON inverted microscope equipped with an Achroplan × 63 (oil) objective (N.A. 1.4). Excitation was at 425±5 nm. CFP and YFP emissions were detected simultaneously at 475±15 and 540±20 nm, respectively and recorded with PicoLog Data Acquisition Software (Pico Technology). FRET is expressed as the ratio of acceptor to donor fluorescence. At the onset of the experiment, the ratio was adjusted to 1.0, and FRET changes were expressed as relative deviations from base line. Temporal changes in IP_3 levels were monitored using a FRET-based IP_3 sensor, in which the IP_3-binding domain of the human type-I IP_3 receptor (aa 224 to 605) is fused between CFP and YFP, essentially analogous to the sensor described previously[63]. In vitro binding studies showed that it bound IP_3 with an apparent Kd of approx. 5 nM. Intracellular Ca^{2+} mobilization was monitored using the CFP/YFP-based Ca^{2+} sensor Yellow Cameleon 2.1[32,34,64]. Traces were smoothened in Microsoft Excel using a moving average function ranging from 3 to 6.

PtdIns(4,5)P_2 depletion by rapamycin-induced translocation of phosphoinositide 5-phosphatase

Rat-1 cells were transiently transfected with PM-CFP-FRB and mRFP-tagged FKBP-phosphoinositide 5-phosphatase (mRFP-FKBP-5-ptase)[43]. Cells were selected for experimentation when sufficient protein levels were expressed as judged by CFP and mRFP fluorescence. For PtdIns(4,5)P_2 measurements, the YFP-PH construct was cotransfected. For Ca^{2+} measurements, cells were loaded with Oregon-Green-AM. To monitor gap junctional communication cells were loaded with calcein-AM and analyzed by Fluorescence Recovery After Photobleaching (FRAP)[65]. These experiments were performed on a Leica TCS-SP2 confocal microscope (Mannheim, Germany), using 63x lens, N.A. 1.4.

Overexpression of PtdIns(4)P 5-kinase

To overexpress PtdIns(4)P 5-kinase (PIP5K; type Iα)[62], virus containing the LZRS-PIP5K constructs was generated as described below. Rat-1 cells were incubated with 1 ml of viral supernatant supplemented with 10 µl Dotap. 48 hrs after infection, cells were plated in selection medium. Transfected cells were selected on zeocin (200µg/ml, InVitrogen) for 2 weeks and colonies were examined for PIP5K expression.

RNA interference

To generate Cx43-deficient Rat-1 cells, Cx43 was knocked down by stable expression of retroviral pSuper (pRS)[41] containing the RNAi target sequence GGTGTGGCTGTCAGTGCTC. pRS-Cx43 was transfected into Phoenix-Eco package cells and the supernatant containing viral particles was harvested after 72 hrs. For infection, cells were incubated with 1 ml of viral supernatant supplemented with 10 µl Dotap (Roche; 1 mg/ml). 48 hrs after infection, cells were selected on puromycin (2µg/ml) for 2 weeks. Single cell-derived colonies were tested for Cx43 expression and communication. PLCβ3 was stably knocked down by retroviral expression of PLCβ3 shRNA. Four different target sequences were selected, namely ACTACGTCTGCCTGCGAAATT, GATTCGAGAGGTACTGGGC, TTACGTTGAGCCCGTCAAG, CCCTTTGACTTCCCCAAGG). Non-functional shRNA was used as a control. ZO-1 was transiently knocked down by adenoviral expression of ZO-1 RNAi. First, ZO-1 RNAi oligos containing the ZO-1 target sequence GGAGGGCCAGCTGAAGGAC were ligated into pSuper after oligo annealing. Next, the oligos together with the H1 RNA promotor were subcloned into pEntr1A (BamHI/XhoI) and recombinated into pAd/PL-Dest according to protocol (Virapower Adenoviral Expression System; InVitrogen). Virus was produced in 293A packaging cells according to standard procedures. Supernatant containing virus particles was titrated on Rat-1 cells to determine the amount needed for ZO-1 knockdown.

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Supplemental material

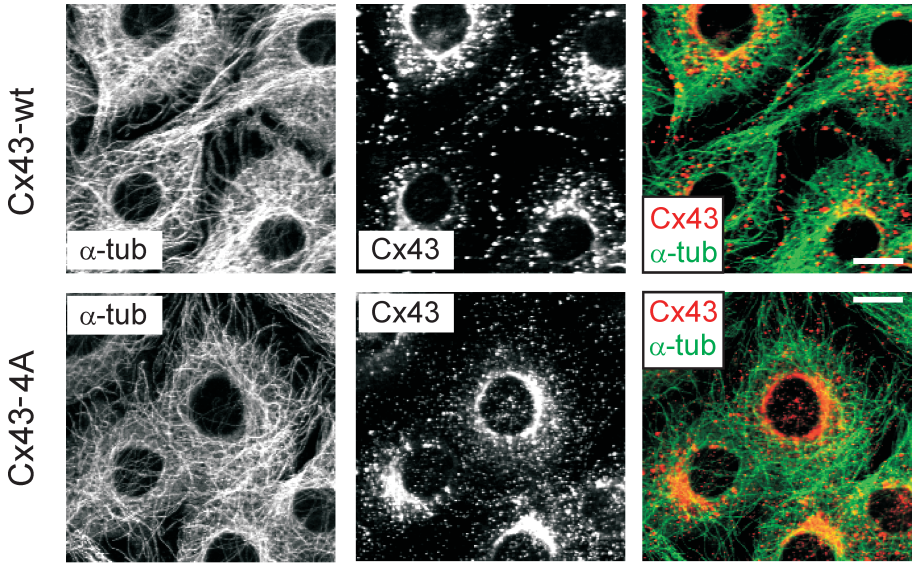

Figure S1. Cx43-4A mutant accumulates intracellularly.
Confocal images of Cx43-deficient Rat-1 cells expressing wild-type Cx43(wt) and Cx43-4A, in which basic Arg/Lys residues in the C-terminal juxtamembrane domain were neutralized by mutation into Ala. Cells were immunostained for Cx43 (red) and α-tubulin (green). Note intracellular accumulation of Cx43-4A and lack of detectable plasma membrane staining. Similar intracellular accumulation was observed with seven other Cx43(K/R->A) mutants, notably K237A,K241A; R239A,R243A; K241A,R243A; R239A,K241A; K237A,R239A; R239A,K241A,R243A; and K237A,R239A,K241A. Scale bars: 5 μm.

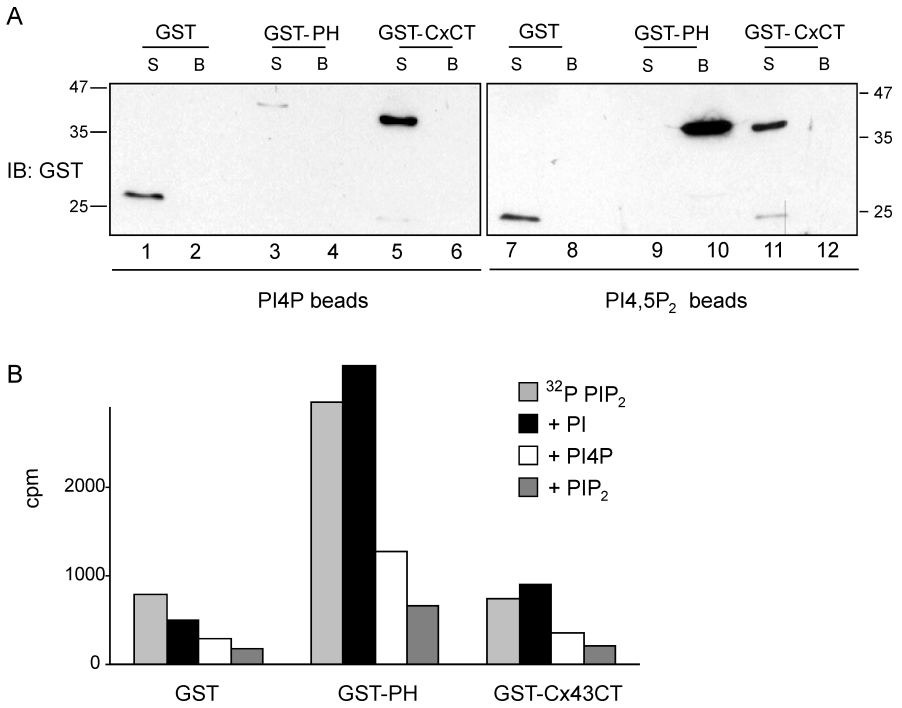

Figure S2. No detectable phosphoinositide binding to the Cx43 C-terminal tail (CT).
A: Pull-down of GST alone, GST-PH and GST-Cx43CT (aa 227-382) fusion proteins using PtdIns(4)P- and PtdIns(4,5)P_2 -coated agarose beads (Molecular Probes), as indicated. GST fusion proteins were purified from DH5α bacteria following standard procedures. Anti-GST monoclonal antibody was purified from hybridoma cell line 2F3. 10 mg of GST or fusion protein was incubated with 7 ml of beads (~70 pmol) in 300 ml Triton-X100 buffer for 4 hrs at 4°C; beads were spun down by centrifugation. 20 μl of the supernatant (S) was used as input control. Protein was eluded from the beads (B) by boiling for 10 min. in Laemmli sample buffer and analyzed by SDS-PAGE and immunoblotting for the presence of GST or GST-fusion protein.
B: Binding of ^{32}P-labeled PtdIns(4,5)P_2 to GST fusion proteins. GST, GST-PH and GST-Cx43CT were coupled to glutathione beads and incubated with [^{32}P]-PtdIns(4,5)P_2 alone or together with excess unlabeled PtdIns, PtdIns(4)P or PtdIns(4,5)P_2 for 1 hr at 4°C, as indicated. Beads were washed extensively (after centrifugation) and bound ^{32}P activity was measured.

Table S1. Pharmacological agents showing no effect on either basal or GPCR-regulated cell-cell communication.

Cell-cell communication in Rat-1 cells was determined by LY diffusion. See also [18]

Agent	Concentration	Remarks
Phorbol ester: TPA (long-term) TPA (acute)	 200 nM 100 nM	 6- 24 hrs preincubation to downregulate PKC 0-15 min.
PC-PLC (*B. cereus*)	1 U/ml	Generates diacylglycerol and activates PKC[42]
Pertussis toxin	200 ng/ml	6-16 hrs preincub.
Caged IP_3 Caged Ca^{2+}		Transient rises in cytosolic Ca^{2+} upon UV-induced uncaging
BAPTA-AM	5 mM	Ca^{2+} signaling blunted
Nocodazole Cytochalasin D Rho-inactivating C3 toxin	10 µM 10 µM 100 ng/ml	Cell contraction[58]
Staurosporine K252a PD598059 Ro-31-8220 PP2	100 µM 5 µM 10 µM 10 µM 50 µM	General protein kinase inhibitor (10 min. preincub.) General protein kinase inhibitor MAP kinase inhibitor PKC inhibitor Src family kinase inhibitor (20 min. preincub.)
Cycloheximide Brefeldin A Monensin NH_4Cl Hyperosmotic sucrose	200 µM 5 µg/ml 5 µM 20 mM 0.5 M	60 min. preincub. 30 min. preincub.

Table S2. Oligos used for cloning, mutagenesis and RNAi constructs.
F, forward; R, reverse. All sequences 5'-> 3'.

name	sequence
PLCβ3 F	GATCCTCGAGTGGTAATCGATGGCGGGCGCGAGGCCCGGCG
PLCβ3 R	GATCTCTAGAGCGGCCGCTCAAAGCTGGGTGTTTTCCTCCTGGCTC
Cx43 RNAi F	GATCCCGGTGTGGCTGTCAGTGCTCTTCAAGAGAGAGCACTGACAGCCACAC-CTTTTTGGAAA
Cx43 RNAi R	AGCTTTTCCAAAAAGGTGTGGCTGTCAGTGCTCTCTCTTGAAGAGCACT-GACAGCCACACCGGG
PLCβ3 RNAi 1 F	GATCCCCACTACGTCTGCCTGCGAAATTTCAAGAGAATTTCGCAGGCAGACG-TAGTGTTTTTGGAAA
PLCβ3 RNAi 1 R	AGCTTTTCCAAAAACACTACGTCTGCCTGCGAAATTCTCTTGAAATTTCGCAG-GCAGACGTAGTGGGG
PLCβ3 RNAi 2 F	GATCCCGATTCGAGAGGTACTGGGCTTCAAGAGAGCCCAGTACCTCTC-GAATCTTTTTGGAAA
PLCβ3 RNAi 2 R	AGCTTTTCCAAAAAGATTCGAGAGGTACTGGGCTCTCTTGAAGCCCAGTAC-CTCTCGAATCGGG
PLCβ3 RNAi 3 F	GATCCCTTACGTTGAGCCCGTCAAGTTCAAGAGACTTGACGGGCTCAACG-TAATTTTTGGAAA
PLCβ3 RNAi 3 R	AGCTTTTCCAAAAATTACGTTGAGCCCGTCAAGTCTCTTGAACTTGACGGGCT-CAACGTAAGGG
PLCβ3 RNAi 4 F	GATCCCCCCTTTGACTTCCCCAAGGTTCAAGAGACCTTGGGGAAGTCAAAG-GGTTTTTGGAAA
PLCβ3 RNAi 4 R	AGCTTTTCCAAAAACCCTTTGACTTCCCCAAGGTCTCTTGAACCTT-GGGGAAGTCAAAGGGGGG
ZO-1 RNAi F	GATCCCGGAGGGCCAGCTGAAGGACTTCAAGAGAGTCCTTCAGCTGGCCCTC-CTTTTTGGAAA
ZO-1 RNAi R	AGCTTTTCCAAAAAGGAGGGCCAGCTGAAGGACTCTCTTGAAGTCCT-TCAGCTGGCCCTCCGGG
Cx43 4A F	ACGGAGAAAACCATCTTCATCATCTTCATGCTGGTGGTGTCCTTGGT-GTCTCTCGCTTTGAACATCATTGAGCTCTTCTACGTCTTCTTCAAAGGCGTT-GCGGATGCCGTGGCGGGAGCAAGCGATCCTTACCACGCCA
Cx43 4A R	GGCGTGGTAAGGATCGCTTGCTCCCGCCACGGCATCCGCAACGCCTTTGAA-GAAGACGTAGAAGAGCTCAATGATGTTCAAAGCGAGAGACACCAAGGACAC-CACCAGCATGAAGATGATGAAGATGGTTTTCTC

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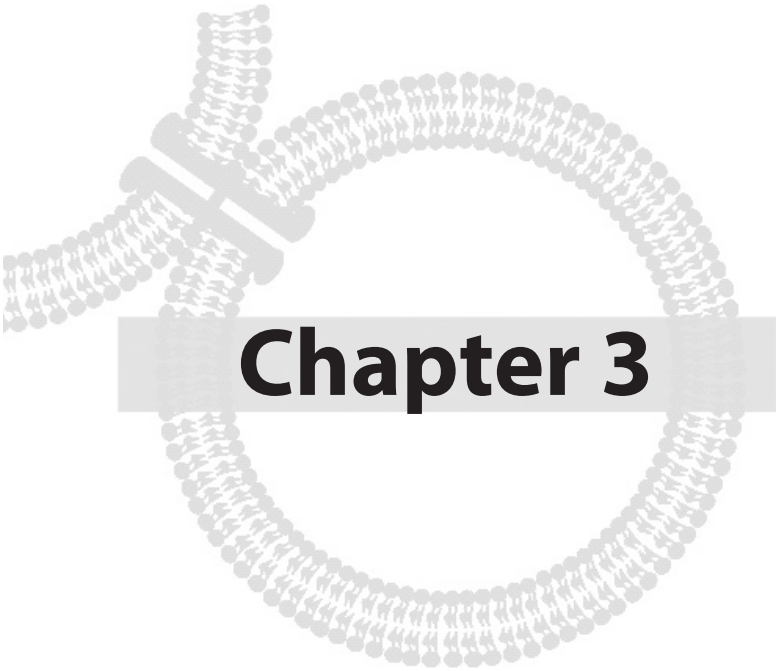

Chapter 3

Inhibition of Connexin43 gap junctional communication by G(q)-coupled receptors: a critical role for residue Tyr265

Leonie van Zeijl, Aafke Ariaens, Wouter H. Moolenaar

Abstract

Cx43-based gap junctional communication (GJC) is rapidly but transiently inhibited by G(q)-coupled receptor agonists, such as endothelin, thrombin and angiotensin. Previously, we showed that inhibition of GJC depends on depletion of phosphatidylinositol 4,5-bisphosphate. However, the molecular details of Cx43 channel regulation remain poorly understood. Previous studies have implicated phosphorylation of the Cx43 C-terminal tail on residues S368, Y247 and Y265, and/or Cx43 internalisation via an Y286-based sorting motif, as key regulatory events. Here, we have analysed the importance of the Cx43 C-terminal domain in channel regulation by studying Cx43 mutants, expressed in connexin-depleted Rat-1 fibroblasts. We find that the Cx43 C-terminal tail (aa 263-382) is essential for channel regulation by GPCR agonists. Furthermore, we find that Cx43 point mutants Y247F, Y265F, Y267F, Y286F and S368A all mediate normal cell-cell communication. After stimulation with GPCR agonist endothelin for 8 minutes, however, the Cx43-Y265F mutant gap junctions are open, in contrast to endogenous Cx43 and the other Cx43 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 broad-spectrum tyrosine kinase and Src inhibitors had no effect on Cx43 channel closure. 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.

Introduction

Gap junctions are groups of intercellular channels that mediate the diffusion of small molecules. The building blocks of gap junctions are proteins called connexins. Six connexins together form a hemichannel, or connexon, that docks with a connexon from the adjacent cell to form a functional channel[1-3]. Gap junctional communication (GJC) is essential for tissue homeostasis and loss or misregulation of GJC is associated with various human pathologies, including cancer[4-14].

In the heart, gap junctional communication between cardiomyocytes ensures efficient electrical coupling and hence the synchronous propagation of action potentials. Connexin43 (Cx43) is the major connexin family member in the myocardium. Misregulation of Cx43 expression, localisation and channel gating may lead to severe cardiac dysfunction[15-19]. In mice, loss of Cx43 in the heart results in lethal ventricular arrhythmias[15].

Previously, our group and others showed that Cx43-based GJC can be inhibited by certain G protein coupled receptor (GPCR) agonists and bioactive lipids[20-27]. Cardiac ischemia may be caused by GPCR agonists angiotensin and endothelins, which are very potent vasoconstrictors[28,29]. Inhibition of gap junctional communication may protect the heart during pathological conditions by limiting the spreading of damage[30,31]. On the downside, however, closure of Cx43 gap junctions may be the cause of arrhythmia and it has been suggested that genetic defects in Cx43 may underlie a predisposition to cardiac arrhythmia[17,19,32,33].

Connexin43 (Cx43) is the most abundant and best studied connexin. Previously, we reported that Cx43 based gap junctional communication can be inhibited by certain G protein coupled receptor (GPCR) agonists[23]. We recently reported that depletion of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) from the plasma membrane is necessary and sufficient for inhibition of communication by GPCRs[20]. However, many details of how GJC is regulated are still unclear.

Various studies suggest that the intracellular C-terminal tail of Cx43 is essential for regulation of Cx43 gap junctions. Many studies have addressed the role of phosphorylation in Cx43 channel regulation; all known phosphorylation sites of Cx43 are located in the C-terminal tail[34-37]. Especially residues Y247 and Y265 as putative targets for tyrosine kinase Src have received their share of attention in the field. In summary, cells expressing v-Src or constitutively active c-Src have been reported to be poor communicators, which is associated with massive tyrosine phosphorylation of Cx43. Mutational analysis has shown that Y265 and, to a lesser extent, Y247 are the Src target residues. Tyrosine phosphorylation may affect GJC by modification of the channel properties and/or by influencing Cx43 turnover and localisation[23,34,38-47]. To date, no other tyrosine kinases that may phosphorylate Cx43 have been identified. It is unclear whether tyrosine phosphorylation of Cx43 plays a role in the inhibition of GJC by GPCRs.

In addition, phosphorylation of residue S368 by protein kinase C (PKC) has been associated with inhibition of communication, possibly by triggering the internalisation and/or promoting the ubiquitination of Cx43[48-54].

Here, we focus on the role of the C-terminal tail of Cx43 in the inhibition of GJC by Gq coupled receptor signalling. We developed a novel system to study Cx43 mutants in their native environment. We knocked down Cx43 in Rat-1 fibroblasts by stable expression of Cx43 shRNA constructs. The Cx43 knockdown cells were used as a reconstitution system for both wildtype and mutant versions of Cx43. Cx43 mutants were compared with wildtype Cx43 for functionality, localisation and response to GPCR agonists.

We show that residue Y265 is essential for regulation of Cx43 based GJC by GPCR signaling. However, tyrosine phosphorylation of Cx43 is not involved in inhibition of GJC. Instead, we propose that Y265 plays a structural role in the multi-protein

complex that regulates Cx43 based gap junctional communication downstream of GPCRs.

Results

Mutational analysis of Cx43 channels in their native cellular context

In our initial attempts to analyze Cx43 mutants, we stably expressed Cx43 wildtype and mutant versions in communication-deficient A431 and HeLa carcinoma cells, and subsequently examined cell-cell communication in response to GPCR stimulation. While stable expression of Cx43 did result in efficient cell-cell coupling, the regulation of Cx43 channel function by receptor agonists was impaired[20,43], suggesting that one or more essential components of the Cx43 gap junction complex, such as for example ZO-1, is lacking or mislocalised.

We therefore sought to analyze Cx43 mutants in their native cellular context, namely Rat-1 fibroblasts, which express Cx43 as the only functional connexin. To this end, we stably knocked down endogenous Cx43 using shRNA, and subsequently introduced shRNA-resistant mutant versions of Cx43 containing two silent mutations in the shRNA target sequence (Fig. 1A). Knockdown of Cx43 reduced its expression by >95% and resulted in a complete loss of intercellular communication[20,56] (Fig. 1B,C), while the Cx43-binding partner ZO-1 retained its submembranous localisation[20]. Expression of shRNA-resistant Cx43 in Cx43 knockdown cells fully restored not only basal cell-cell communication but also its regulation by Gq-coupled receptor agonists, such as endothelin and thrombin (Fig. 1C). It is further seen that reconstituted Cx43 shows an immunostaining pattern similar to endogenous Cx43 (Fig. 1D). Thus, reconstitution of Cx43 in Cx43 knockdown cells provides a suitable system for mutational analysis of Cx43 channel function and regulation.

Critical role for the C-terminal tail of Cx43

The C-terminal tail of Cx43 is thought to act as a regulatory domain. Its integrity is required for disruption of communication induced by non-physiological stimuli such as intracellular acidification and the (v-)Src tyrosine kinase. According to the 'ball-and-chain' model of Cx43 channel regulation, the cytosolic tail of Cx43 is modified to block the channel and thereby inhibit cell-cell communication[40,46]. We expressed a truncated version of Cx43 that lacks the C-terminal 120 residues (aa 263-382; Cx43-Δ263). When expressed in Cx43-knockdown cells, Cx43-Δ263 forms functional channels. However, endothelin stimulation failed to inhibit cell-cell coupling (Fig. 1C), implying that the Cx43 C-terminal tail is essential for GPCR agonists to disrupt cell-cell communication. We also find that this Cx43 mutant is not distributed in the characteristic punctate fashion, but rather forms larger plaques (Fig. 1D), similar to what has been reported for GFP-tagged Cx43[60]. These results

confirm that the Cx43 tail is essential for the regulation of channel function and contributes to the proper formation of junctional plaques.

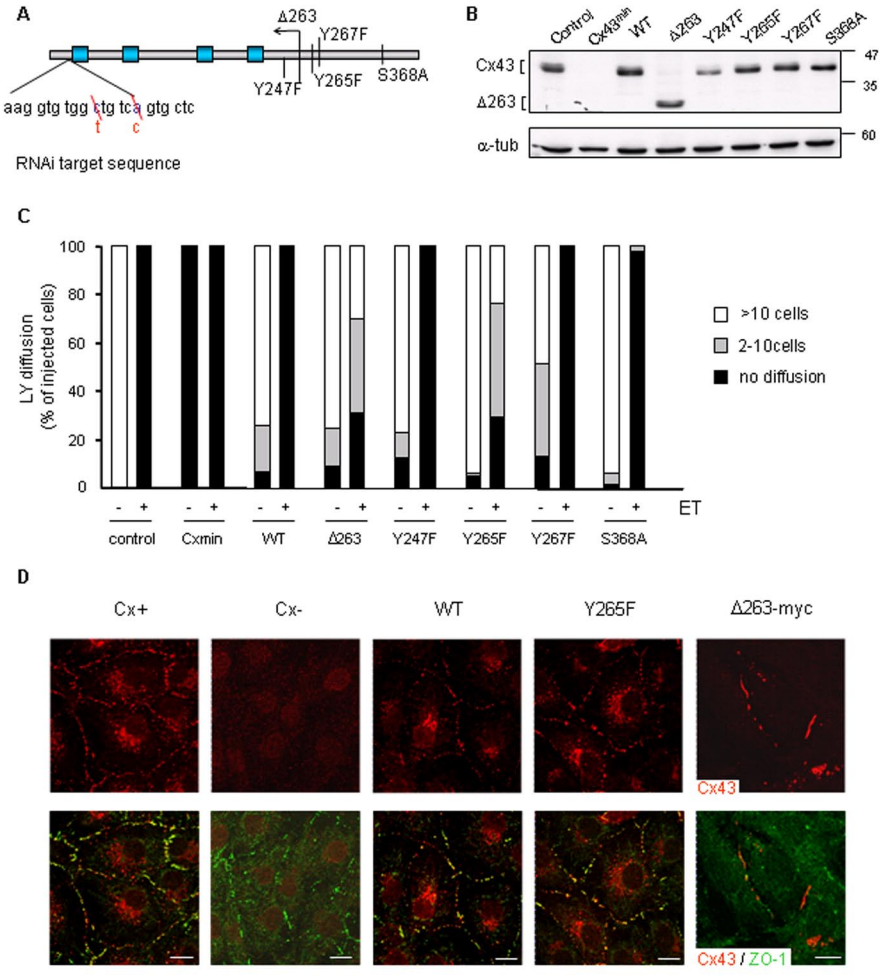

Figure 1: Expression and communication of Cx43 mutants in Cx43 knockdown cells
A: Schematic representation of the Cx43 mutants. Two silent mutations were introduced in the shRNA target sequence to prevent targeting by the Cx43 shRNA construct.
B: Adenoviral expression of Cx43 mutants in Cxmin cells compared to Cx43 expression in control and Cxmin cells. Immunoblot of Cx43 expression (top), α-tubulin is used as loading control (bottom)
C: Bar diagram showing the percentage of cells from which micro-injected Lucifer Yellow spreads to neighbouring cells 8 minutes after endothelin stimulation (n > 80 for each data point).
D: Confocal pictures of Cx43 (red), co-stained with ZO-1 (green) in control cells, Cxmin cells and Cxmin cells reconstituted with Cx43 wild type (WT), Y265F or Δ263-myc. top: Cx43/myc staining, bottom: merge of Cx43/myc and ZO-1 staining. Scale bars: 5 μm.

Residue Ser368 is not involved in inhibition of GJC

Having shown the importance of the Cx43 C-terminal tail, we next created various point mutations in that domain. Phorbol ester-activated PKC phosphorylates Cx43 on residue S368 and thereby decreases gap junctional communication in various cell types[49,51]. We therefore expressed Cx43 mutant S368A in our Cx43 knockdown Rat-1 cells. Cx43(S368A)-based gap junctions show normal intercellular coupling and behave like wild-type junctions in that communication was rapidly and almost completely abolished by endothelin (Fig. 1C). We conclude that PKC target residue S368 is dispensable for the inhibition of communication induced by Gq-coupled receptor agonists. This is consistent with our previous observation that the PKC pathway plays no role in GPCR-induced Cx43 channel inhibition.

Tyrosine265 is essential for GJC inhibition

Earlier studies have shown that Cx43 is tyrosine phosphorylated by oncogenic v-Src, which correlates with permanent inhibition of GJC in v-Src-transformed cells[38,39,44], while activation of c-Src has been implicated in transient inhibition of Cx43-based GJC in normal cells[23,43]. In transfected COS7 cells, activated c-Src phosphorylates Cx43 on residue Y265, but not on Y267. Residue Y247 may serve as a secondary Src target, albeit to a lesser extent than Y265[43,44,47].

We expressed Cx43 mutants Y247F, Y265F and Y267F in the Cx43-depleted Rat-1 cells. All mutants formed functional gap junctions (Fig. 1C), showing an immunostaining pattern similar to that of wt Cx43 (Fig. 1D and data not shown). When the cells were exposed to endothelin, Y247F and Y267F behaved like wildtype Cx43 in that channel function was disrupted upon receptor stimulation. In contrast, in cells expressing mutant Cx43 Y265F GJC was intact when monitored 8 minutes after endothelin stimulation (Fig. 1C). This implicates an essential role for residue Y265.

No tyrosine phosphorylation of Cx43

Since Y265 has been identified as a phosphate acceptor[44], tyrosine phosphorylation of Cx43 in response to GPCR agonists is an obvious explanation for our results. To investigate this, we immunoprecipitated Cx43 from Rat-1 cells after endothelin stimulation, and immunoblotted for phosphotyrosine. As a positive control, we included Cx43 from v-Src-expressing Rat-1 cells, which show massive tyrosine phosphorylation of Cx43. Equal amounts of Cx43 were immunoprecipitated from normal and v-Src-transformed cells. We observed a low level of basal tyrosine phosphorylation of Cx43. However, there was no increase in tyrosine phosphorylated Cx43 in response to endothelin stimulation, while Cx43 from v-Src-transformed cells was strongly phosphorylated (Fig. 2A). As shown in figure 2B, basal tyrosine phosphorylation of Cx43 decreases upon serum starvation. Treatment of the cells with general kinase inhibitor K252a (50 μM) for 30 minutes completely

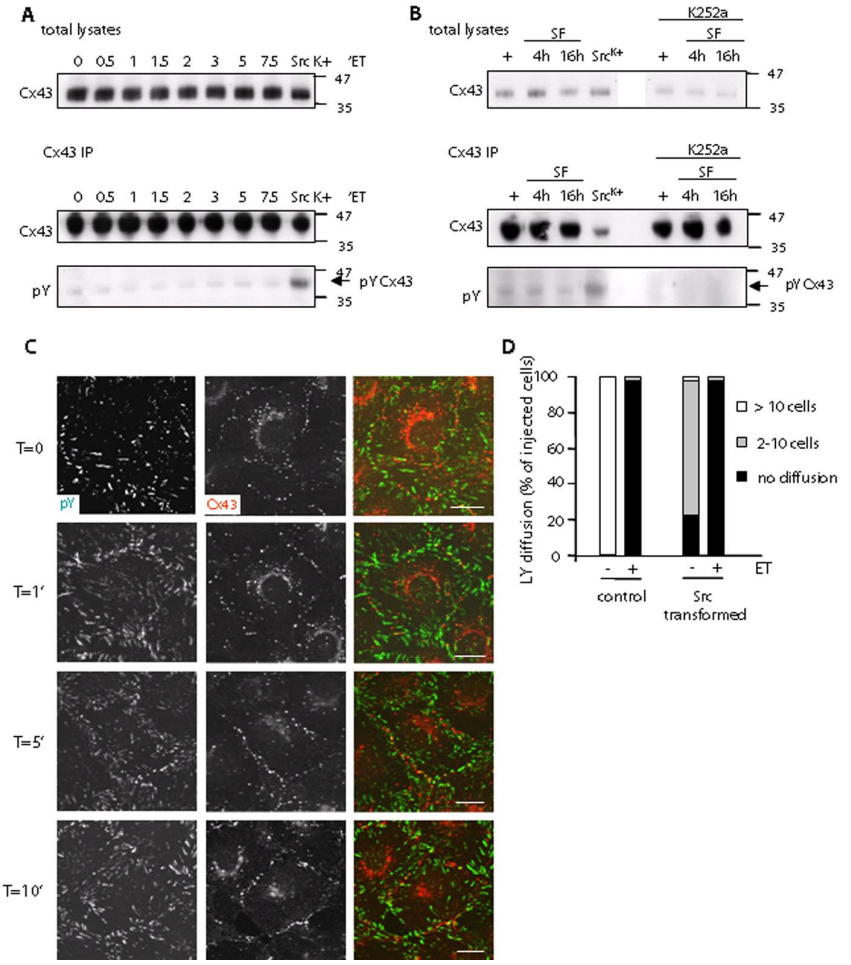

Figure 2: No tyrosine phosphorylation of Cx43 in response to GPCR agonist endothelin

A: Time course of endothelin stimulation, after which Rat-1 cell lysates are subjected to Cx43 immunoprecipitation. Top panel: total lysates immunoblotted for Cx43, Bottom panels: Cx43 immunopricipitates immunoblotted for Cx43 (top) and phosphotyrosine (bottom). V-Src transformed Rat-1 cells were used as a positive control for tyrosine phosphorylation, Cxmin cells were used as a negative control.

B: Left: Rat-1 cells were serum starved for 0, 4 and 16 hours. Cells were lysed and subjected to Cx43 immunoprecipitation. Top panel: Total lysates immunoblotted for Cx43 (top). Cx43 immunopricipitates immunoblotted for Cx43 (top) and phosphotyrosine (bottom) V-Src transformed Rat-1 cells were used as a positive control for tyrosine phosphorylation. Right: Rat-1 cells were serum starved for 0, 4 and 16 hours, and subsequently incubated with K252a (50 μM) for 30 minutes. Cells were lysed and subjected to Cx43 immunoprecipitation. Top panel: Total lysates immunoblotted for Cx43 (top). Cx43 immunoprecipitates, immunoblotted for Cx43 (top) and phosphotyrosine (bottom).

C: Time course of endothelin stimulation, after which Rat-1 cells were fixed and stained for Cx43 (red, middle) and phosphotyrosine (green, left). Scale bars: 5 μm.

D: Bar diagram showing the percentage of cells from which micro-injected Lucifer Yellow spreads to neighbouring cells of control and v-Src transformed cells before and after endothelin stimulation.

inhibited tyrosine phosphorylation of Cx43. Inhibition of GJC by GPCR signalling was not affected by either treatment (Fig. 3A and data not shown). Co-staining the cells for Cx43 and phosphotyrosine revealed no (increase in) co-localization upon agonist stimulation (Fig. 2C).

Additionally, expression of v-Src caused cells to form poor cell-cell contacts, which automatically makes them poor communicators. Cells that do make intercellular contacts showed punctate Cx43 staining at cell-cell contacts and communicated, and communication was inhibited by endothelin (Fig 2D). This suggests that there is no causal relationship between tyrosine phosphorylation of Cx43 and inhibition of communication by GPCR agonists.

Src family kinases do not play a role in inhibition of GJC

When we immunoblotted total lysates of endothelin-stimulated Rat-1 cells with an antibody recognizing Y416 phosphorylated c-Src, we observed a transient increase, indicating activation of c-Src[57] (Fig. 3A). Src activation is at its maximum between 1 and 2 minutes. This is inconsistent with the kinetics of GJC inhibition, which is

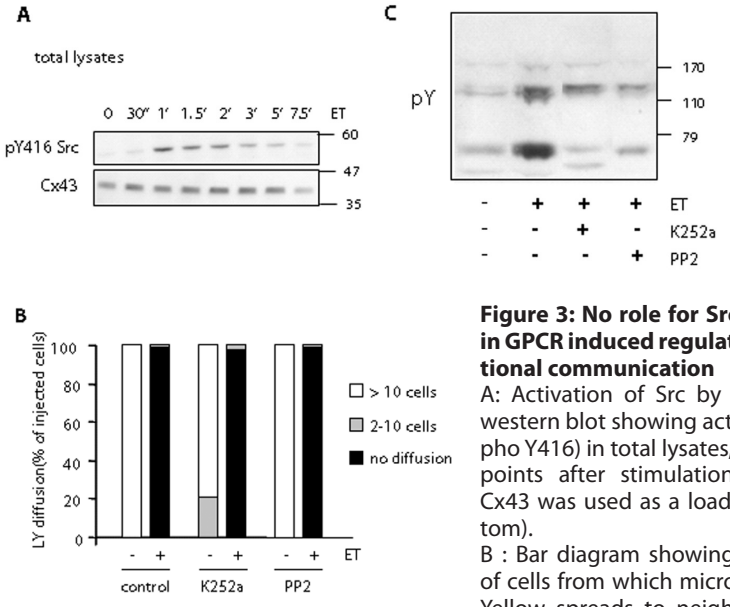

Figure 3: No role for Src family kinases in GPCR induced regulation of gap junctional communication

A: Activation of Src by endothelin. Top: western blot showing activated Src (phospho Y416) in total lysates, at different time points after stimulation of endothelin. Cx43 was used as a loading control (bottom).

B : Bar diagram showing the percentage of cells from which micro-injected Lucifer Yellow spreads to neighbouring cells of control cells and cells preincubated with either Src family kinase inhibitor PP2 (50 μM, 30') or general kinase inhibitor K252a (50 μM, 30') and before and after endothelin (50 nM) stimulation for 8 minutes (n>30 for each data point).

C: Western blot showing tyrosine phosphorylation of total lysates from Rat-1 cells, before and after stimulation with endothelin for 8 minutes (lysates were harvested immediately after microinjection of the cells (Fig. 3B), in control cells and in cells preincubated with either K252a or PP2.

complete within 1 minute after agonist stimulation. Nevertheless, to confirm that phosphorylation of Cx43 by Src is dispensable for GPCR induced inhibition of cell-cell communication, we used PP2, a pharmacological inhibitor of Src family kinases and studied the effect of general kinase inhibitor K252a on GJC regulation. Neither inhibitor had an effect on gap junction closure (Fig. 3B), while showing an almost complete inhibition of general tyrosine phosphorylation (Fig. 3C). Taken together, our results suggest that tyrosine phosphorylation of Cx43 is not required for the GPCR agonist-induced inhibition of cell-cell communication.

Sequence motifs surrounding residue Y265

Aside from being a potential phosphate acceptor, Y265 is part of a YxxΦ motif (where x is any amino acid and Φ is a hydrophobic residue) that may be important for clathrin-mediated endocytosis of Cx43. As described by Piehl *et al.*, a second YxxΦ motif, Y286-F289, is an established Dab2 mediated internalisation

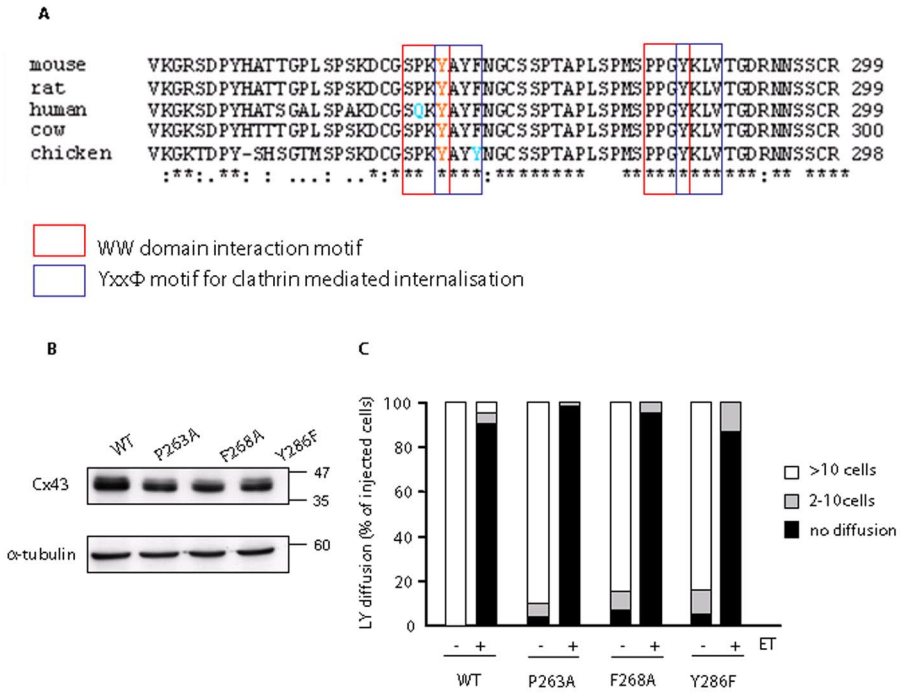

Figure 4: Mutation of putative motifs including Y265

A: Alignment of the residues surrounding Y265 of mouse, rat, human, cow and chicken Cx43. Red and blue rectangles indicate WW domain interaction and YxxΦ motifs, respectively. Y265 is marked in red. Blue residues indicate non-conserved residues that do not fit the indicated motifs.

B: Western blot showing expression of mutants P263A F268A and Y286F (top), α-tubulin is used as loading control (bottom)

C: Bar diagram showing the percentage of cells from which micro-injected Lucifer Yellow spreads to neighbouring cells before and after stimulation of the cells with endothelin (n>30 for each data point).

motif[58] (Fig. 4A). Therefore, we mutated F268 into an alanine to examine the possible involvement of this motif in the GPCR induced block of communication. Mutation of F268 had no effect on inhibition of GJC (Fig. 4B, C), arguing against the hypothesis that Y265 participates in a functional internalisation motif. In addition, we made the Y286F mutation. Also this mutation did not affect inhibition of GJC, excluding the involvement of Y286 and putative surrounding sequence motifs in regulation of GJC by GPCRs.
Finally, we note that rodent, but not human, Y265 is part of a WW binding domain[59] (rodent sequence SPKY versus human: SQKY) (Fig 4A). Mutation of P263 into a glutamine, to resemble human Cx43 or into an alanine had no effect on inhibition of GJC (Fig. 4B,C and data not shown). Thus, known putative sequence motifs surrounding Y265 are seemingly not involved in the regulation of Cx43-based GJC.

Concluding remarks

In this study, we set up a novel system to study Cx43 regulation by expressing shRNA resistant Cx43 mutants in their native cellular environment, Rat-1 Cx43 knockdown cells. This provides Cx43 with an environment in which all components involved in regulation of GJC are present. We found that residue Y265 is essential for inhibition of GJC downstream of GPCR stimulation. Y265 is a known phosphate acceptor and has been reported to be a target for phosphorylation by Src[44]. However, we found no increase in phosphorylation and excluded the involvement of Src family and other kinases. We conclude that tyrosine phosphorylation of Cx43 is not one of the regulating steps in the inhibition of GJC downstream of GPCR's.
Furthermore, we investigated a potential role for Y265 as part of a putative protein motif. The most obvious motifs that include Y265 are a YxxΦ motif and a PxY motif. YxxΦ motifs are involved in clathrin mediated endocytosis[58], and PxY motifs have been reported to mediate binding to WW domains[59]. Notably, Y286 is part of similar motifs (Fig. 4A), and has been implicated in Dab2 mediated, clathrin dependent internalisation, as well as in binding of Cx43 to one of the WW domains of E3 ubiquitin ligase Nedd4. After mutational analysis, we conclude that known protein motifs surrounding Y265 can not explain its importance for regulation of GJC.
We propose that residue Y265 plays an important structural role in the C-terminal tail of Cx43. In spite of the many players that have been identified in the Cx43 protein complex, much remains to be investigated concerning their function in Cx43 gap junctions.

Materials and Methods

Reagents
Materials were obtained from the following sources: PP2 and K252a from Calbiochem; endothelin, thrombin receptor-activating peptide (TRP; sequence SFLLRN), Cx43 polyclonal and α-tubulin monoclonal antibodies from Sigma (St. Louis, MO); Cx43 NT monoclonal antibody from Fred Hutchinson Cancer Research Center (Seattle WA); pY416 Src antibody from Cell Signalling, ZO-1 monoclonal and polyclonal antibodies from Zymed; 4G10 phospho-tyrosine monoclonal antibody from Upstate; HRP-conjugated secondary antibodies from Cell Signalling and secondary antibodies for immunofluorescence (goat-anti-mouse, Alexa488 and goat-anti-rabbit, Alexa594) from Molecular Probes. Myc monoclonal antibody was purified from hybridoma cell line 9E10.

Cell culture and cell-cell communication assays
Cells were cultured in DMEM containing 8% fetal calf serum, L-glutamine and antibiotics. For cell-cell communication assays, cells were grown in 3-cm dishes and serum starved for at least 4 hrs prior to experimentation. Monitoring diffusion of Lucifer Yellow (LY) from single microinjected cells was done as described. Typically, microinjections were started at 3 minutes after addition of endothelin, and monitored at ~8 minutes after endothelin stimulation.

RNA interference
To generate Cx43-deficient Rat-1 cells, Cx43 was knocked down by stable expression of retroviral pSuper (pRS) [55] containing the shRNA target sequence GGTGTGGCTGTCAGTGCTC. pRS-Cx43 was transfected into Phoenix-Eco package cells and the supernatant containing viral particles was harvested after 72 hrs. For infection, cells were incubated with 1 ml of viral supernatant supplemented with 10 µl Dotap (Roche; 1 mg/ml). 48 hrs after infection, cells were selected on puromycin (2 µg/ml) for 2 weeks. Single cell-derived colonies were tested for Cx43 expression and communication. Non-functional shRNA was used as a control.

Construction and expression of cDNA constructs
Mutants Δ263, Y265F and Y267F were described before . All other mutations were created by PCR based site directed mutagenesis (PCR-SDM)
(Primers:
Y247F F: GGGAAGAAGCGATCCTTTCCACGCCACTGG,
R: CCAGTGGCGTGGAAAGGATCGCTTCTTCCC;
P263A F: GCCCATCAAAAGACTGCGGATCTGCAAAATACGCCTACTTCAATGGC,
R: GCCATTGAAGTAGGCGTATTTTGCAGATCCGCAGTCTTTTGATGGGC ;
P263Q: F: GCCCATCAAAAGACTGCGGATCTCAAAAATACGCCTACTTCAATGGC,
R: GCCATTGAAGTAGGCGTATTTTTGAGATCCGCAGTCTTTTGATGGGC;
F268A F: GCGGATCTCCAAAATACGCCTACGCCAATGGCTGCTCCTCACCAACGGC,
R: GCCGTTGGTGAGGAGCAGCCATTGGCGTAGGCGTATTTTGGAGATCCGC;
Y286F F: CGCCTATGTCTCCTCCTGGGTTCAAGCTGGTTACTGGTGACAG,
R: CTGTCACCAGTAACCAGCTTGAACCCAGGAGGAGACATAGGCG;
S368A F: CCTTCCAGCAGAGCCGCCAGCCGCGCCAGCAGCAGGCC,
R: GGCCTGCTGCTGGCGCGGCTGGCGGCTCTGCTGGAAGG).
To prevent targetting of these constructs by Cx43 directed shRNA, we made two silent muta-

tions in the shRNA target site, using PCR-SDM
(primers: F: CCGCTGGAGGGAAGGTGTGGTTGTCCGTGCTCTTCATATTC ,
R: GAATATGAAGAGCACGGACAACCACACCTTCCCTCCAGCGG).
Cx43 mutant cDNA was cloned into pEntr 1A (Invitrogen) by BamHI/Xho restriction and subsequently cloned into pAd/Dest/CMV adenoviral expression vector (Gateway system, Invitrogen) by homologous recombination. Virus was produced in 293A packaging cells according to standard procedures. Supernatant containing virus particles was titrated on Rat-1 Cx43 knockdown cells to determine the amount required for Cx43 expression at levels comparable to endogenous Cx43 expression in Rat-1 cells.

SDS-PAGE, immunoblotting and immunoprecipitation
Cells were harvested in Laemmli sample buffer (LSB), boiled for 10 minutes and subjected to immunoblot analysis according to standard procedures. Filters were blocked in TBST/5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemoluminescence (Amersham Pharmacia).
For immunoprecipitation, cells were harvested in lysis buffer (10mM NaH_2PO_4, pH 7.8, 150mM NaCL, 0.5% NaDoc, 0.5% SDS, 1% NP40), supplemented with protease inhibitor cocktail (Roche) and phosphatase inhibitors (2mM $NaVO_3$, 5 mM NaF, 1mM PMSF). Lysates were spun down and the supernatants were subjected to immunoprecipitation using protein A-conjugated Cx43 antibody for 4 hours at 4°C. Proteins were eluted by boiling for 10 minutes in LSB and analyzed by immunoblotting.

Immunostaining and fluorescence microscopy
Cells grown on coverslips were fixed in methanol for 15 minutes. Samples were blocked in PBS containing 1.5% BSA for 30 min. Subsequently, samples were incubated with primary and secondary antibodies for 30 minutes each in PBS/1.5% BSA, washed five times with PBS and mounted in Immumount (Thermo Scientific). Confocal fluorescence images were obtained on a Leica TCS NT (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr laser. Images were taken using a 63x NA 1.32 oil objective. Standard filter combinations and Kalman averaging were used. Processing of images for presentation was done on a PC using the software package Photoshop (Adobe Systems Incorporated Mountain View, California, USA).

Sequence alignment
Sequences of mouse, rat, human, cow and chicken Cx43 were retrieved from NCBI Gene, and aligned using multiple sequence alignment program Clustal W2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html)

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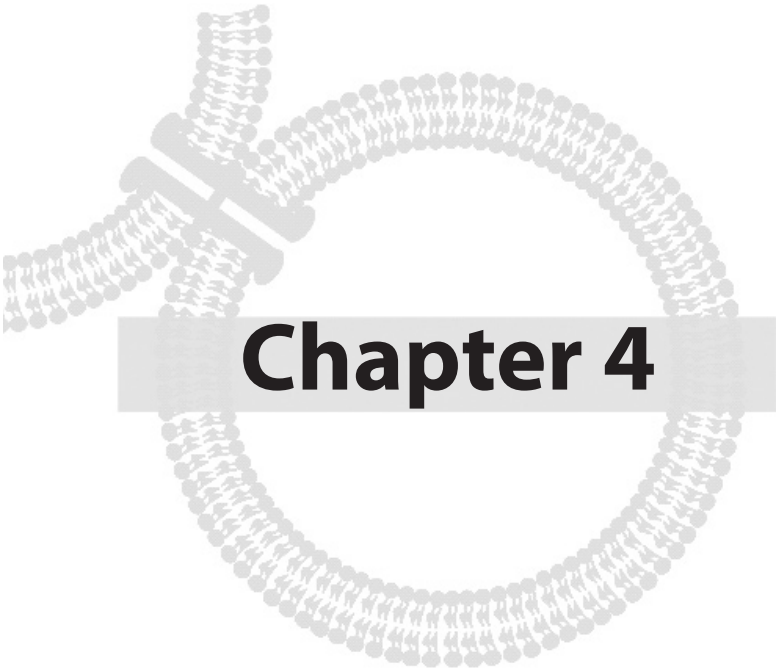

Chapter 4

Dual regulation of Connexin43 gap junctional communication by GPCRs: A key role for ubiquitin ligase Nedd4

Leonie van Zeijl, Bas Ponsioen, Hans Janssen, Kees Jalink, Wouter Moolenaar

Abstract

Connexin43 (Cx43) based gap junctional communication (GJC) is transiently inhibited by several G protein coupled receptor (GPCR) agonists. Recently, we showed that phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) depletion is a key step in inhibition of GJC and that Cx43 residue Y265 is essential. However, it is still not known how Cx43 is modified to cause inhibition of GJC. Mono-ubiquitination at multiple residues of Cx43, followed by internalisation and lysosomal degradation, has been implicated in Cx43 turnover, and the E3 ubiquitin ligase Nedd4 has been shown to interact with Cx43. Here, we examine the possible role of ubiquitination in the regulation of Cx43 based GJC. We find that the interaction of Cx43 with Nedd4 and Cx43 ubiquitination are induced by GPCR activation. In Nedd4 knockdown cells, Cx43 is not ubiquitinated. We show that Cx43 residue Y265 is essential for the interaction with Nedd4 and for ubiquitination. The onset of ubiquitination lagged behind the kinetics of GJC inhibition. Using live cell imaging, we show that inhibition of GJC occurs in two phases. The second phase is absent in Nedd4 knockdown cells and in cells expressing Cx43 mutant Y65F, implicating that ubiquitination of Cx43 by Nedd4 is essential for the second phase. Using electron microscopy, we find a shift in Cx43 localisation away from gap junctional plaques and into lysosomes, consistent with Cx43 internalisation upon GPCR activation. In PLCβ3 knockdown cells we find no shift in Cx43 localisation, which is consistent with earlier observations that these cells continue to communicate after receptor stimulation. Together, our results suggest a model in which Gq-coupled receptor agonists induce inhibition of Cx43 based GJC occurs in two phases. First, closure of the channels mediated by $PI(4,5)P_2$ depletion and second, internalisation of the gap junctions following Cx43 ubiquitination.

Introduction

Gap junctions are groups of intercellular channels that mediate the diffusion of small molecules. The building blocks of gap junctions are proteins called connexins. Six connexins together form a hemi channel, or connexon, which docks with a connexon from the adjacent cell to form a functional channel. Gap junction channels allow passive diffusion of small molecules, up to ~2kDa, such as metabolites, ions and second messengers[1-3]. Gap junctional communication is crucial for tissue homeostasis and is essential for normal development, transportation of action potential in excitable tissues, growth control and metabolic coupling[4-13].

The most abundant and best studied connexin is connexin43 (Cx43). Loss of Cx43 expression or function is associated with severe skin defects, deafness and heart

failure[14]. Furthermore, Cx43 is of interest since Cx43 based gap junctional communication is regulated by (patho)physiological external stimuli, in particular by G-protein coupled receptor (GPCR) agonists, such as endothelin, thrombin and angiotensin[15-20].

Previously, we reported that hydrolysis of $PI(4,5)P_2$ is essential for inhibition of communication[16]. In addition, we showed that residue Y265 in de C-terminal tail is essential, apparently independent of tyrosine phosphorylation and most likely in a structural role (chapter 3 of this thesis). However, exactly how Cx43 is modified downstream of GPCR signalling and whether inhibition of GJC is caused by internalisation or closure of the gap junctions is still an open question.

There is growing evidence that ubiquitination of Cx43 is a crucial step in regulation of GJC and Cx43 turnover[21,22] and E3 ubiquitin ligase Nedd4 was identified as Cx43 binding partner[23]. Nedd4 function is associated with Cx43 turnover and knockdown of Nedd4 was reported to increase Cx43 plaque size, suggesting an important role for Nedd4 in organisation of Cx43 gap junctions.

In this study, we investigated what role ubiquitination plays in the regulation of Cx43 gap junctions in response to GPCR activating agonists. Our results indicate that ubiquitination of Cx43 by Nedd4 is an essential step in inhibition of GJC. Cx43 ubiquitination is independent of $PI(4,5)P_2$ depletion. Mutation of Cx43 residue Y265 abolished the interaction between Cx43 and Nedd4 and Cx43 ubiquitination. We find that GJC inhibition occurs in two temporal phases. The first phase is initiated by $PI(4,5)P_2$ depletion, while the second phase depends on the interaction between Cx43 and Nedd4 and subsequent ubiquitination of Cx43.

Results

Cx43 ubiquitination follows the interaction with E3 ubiquitin ligase Nedd4

The E3 ubiquitin ligase Nedd4 was recently identified as a binding partner of Cx43[23]. Mono-ubiquitination on multiple residues of Cx43 has been suggested to trigger Cx43 internalisation[22,24,25]. We studied the interaction between Cx43 and Nedd4 in our system and how this interaction is regulated. Nedd4 was co-immunoprecipitated with Cx43 from Rat-1 cells at several time points after stimulation with endothelin. In unstimulated cells, we already detect an interaction between Cx43 and Nedd4. However after stimulation with endothelin, there is a marked increase in Nedd4 associated with Cx43. The interaction between Cx43 and Nedd4 increases after 1.5 minutes, with a maximum between 2 and 5 minutes, and is back to basal levels at 7.5 minutes (Fig. 1). Cx43 ubiquitination in response to GPCR activation closely follows the kinetics of the interaction with Nedd4. Cx43 ubiquitination increases after endothelin stimulation, with a maximum at 2-3 minutes, and is back to basal levels after 7.5 minutes of endothelin stimulation. We conclude that Cx43

is ubiquitinated in response to endothelin stimulation and that the extent of ubiquitination correlates with the interaction of Cx43 with Nedd4.

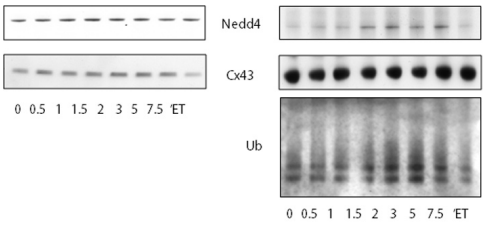

Figure 1: Ubiquitination of Cx43 follows the interaction with Nedd4
Rat-1 cells were stimulated with endothelin and harvested at several different time points in, as indicated. Subsequently, lysates were subjected to Cx43 immunoprecipitation (IP). Total lysates (left) were immunoblotted for Cx43 (bottom) and Nedd4 (top), IPs (right) were immunoblotted for Cx43 (middle), Nedd4 (top) and ubiquitin (bottom).

Nedd4 ubiquitinates Cx43 in response to GPCR activation

Next, we investigated whether Nedd4 is responsible for Cx43 ubiquitination. We knocked down Nedd4 in Rat-1 cells using Nedd4 shRNA. Stable expression of three different shRNA constructs in Rat-1 cells resulted in three different pools with reduced levels of Nedd4 protein. As shown in Figure 2A, pool1 shows a reduction of Nedd4 expression of ~ 60%, whereas pools 2 and 3 have no detectable Nedd4 expression left. Immunofluorescent staining of Cx43 in combination with ZO-1 in Nedd4 knockdown cells reveals no obvious change in Cx43 distribution or cell morphology compared to control cells (Fig. 2C). We immunoprecipitated Cx43 from control cells and from Nedd4 knockdown cells at 3 minutes after endothelin stimulation. Cx43min cells served as a negative control. Control cells showed an increase in ubiquitinated Cx43 after 3 minutes exposure to endothelin, whereas Nedd4 knockdown cells failed to ubiquitinate Cx43 (Fig. 2D). From these results we conclude that Nedd4 is responsible for Cx43 ubiquitination.

Nedd4 is essential for inhibition of GJC

To study the effect of Nedd4 knockdown on (inhibition of) GJC, we monitored Lucifer yellow diffusion after microinjection in control and Nedd4 knockdown cells. All the Nedd4 knockdown cell lines communicate at the same rate as control cells under control conditions. When stimulated with endothelin and TRP (8 minutes), most cells of pool 1 have closed their gap junctions. In pools 2 and 3, however, GJC is largely intact after 8 minutes of stimulation (Fig. 2E). To check whether Nedd4 knockdown cells still respond properly to the stimulus, we measured PI(4,5)P_2 hydrolysis after TRP, and find that knockdown of Nedd4 has no effect on the hydrolysis of PI(4,5)P_2 (Fig. 2B). Thus, Nedd4 expression is required for inhibition of Cx43-based GJC.

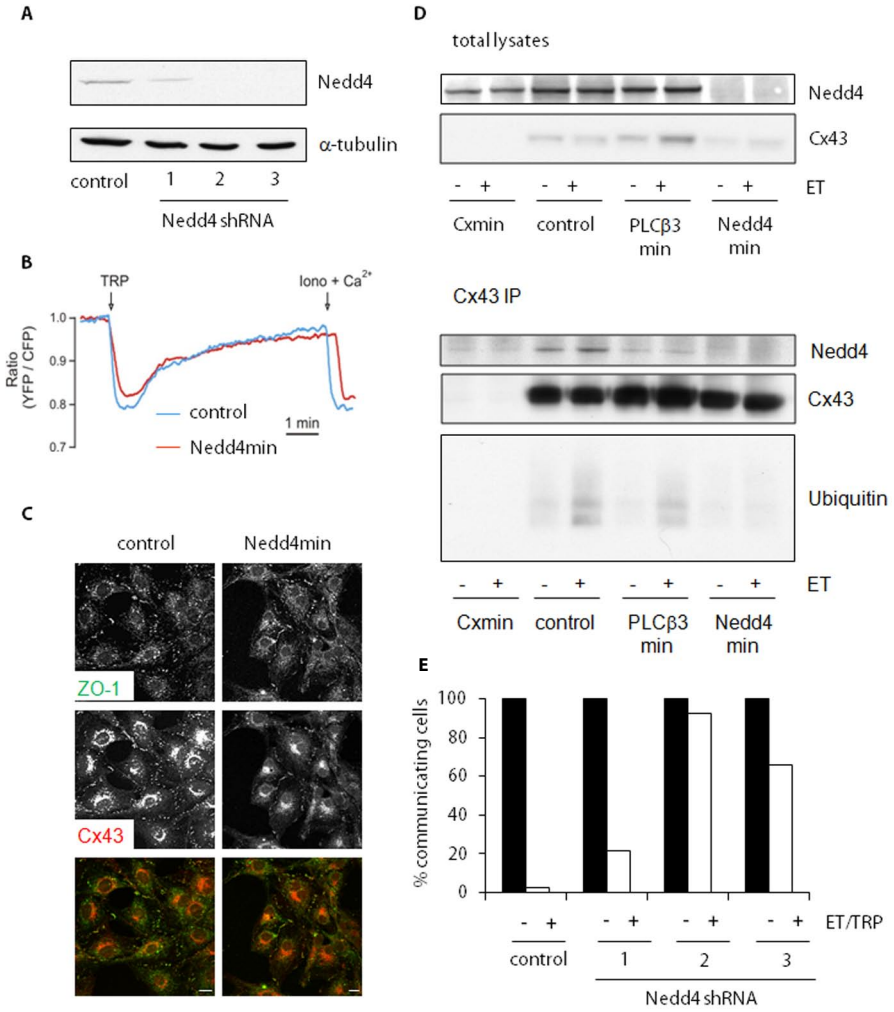

Figure 2: Ubiquitination of Cx43 by Nedd4 is essential for inhibition of gap junctional communication. Cx43 ubiquitination does not require PLCβ3 function

A: Immunoblots of Nedd4 in total lysates from Rat-1 control cells and of pools of cells stably expressing different Nedd4 shRNA constructs. Top: Nedd4, control: control cells, 1-3: pools expressing shRNA constructs 1-3 respectively (see materials and methods for sequences)

B: Temporal changes in plasma membrane $PI(4,5)P_2$ levels after TRP receptor stimulation of normal (blue trace) and Nedd4 min (pool2) (red trace) cells, as determined by changes in YFP-PH/CFP-PH FRET.

C: Confocal images of control and Nedd4min cells immunostained for Cx43 (red) and ZO-1 (green) (scale bars, 5 μm).

D: Rat-1 control cells, Cxmin, PLCβ3min and Nedd4min cells were stimulated with endothelin (50nM) for 3 minutes and harvested. Subsequently, lysates were subjected to Cx43 IP. Total lysates (top) were immunoblotted for Nedd4 and Cx43 as indicated. IPs (bottom) were immunoblotted for Cx43, Nedd4 and ubiquitin as indicated.

E: Bar diagram showing the percentage of communicating cells in control and Nedd4 knockdown cells (pools 1-3) treated with endothelin (Et, 50 nM) and TRP (50 μM), (n >25 for each dataset). Diffusion of Lucifer Yellow after microinjection into a single cell was monitored at 8 minutes after addition of agonists.

GPCR induced Cx43 ubiquitination is independent of prior PI(4,5)P_2 hydrolysis

Since PI(4,5)P_2 hydrolysis is essential for the inhibition of Cx43 based GJC[16], we asked whether there is a relationship between PI(4,5)P_2 hydrolysis and the Cx43-Nedd4 interaction or Cx43 ubiquitination. Therefore, we compared Cx43 ubiquitination and Cx43 interaction with Nedd4 between control and PLCβ3 knockdown cells. PLCβ3 knockdown cells display reduced PI(4,5)P_2 hydrolysis in response to GPCR activation and fail to inhibit GJC[16]. We find that Cx43 ubiquitination is independent of PLCβ3 levels, even though less Nedd4 is co-IPed with Cx43 in PLCβ3 knockdown cells than in control cells (Fig. 2B). We took a second approach to investigate a possible relationship between PI(4,5)P_2 depletion and Cx43 ubiquitination, by precipitating Cx43 from cells overexpressing PIP5-kinase at several time points after endothelin addition (Fig 4A). PI(4,5)P_2 levels in these cells remain high following Gq activation, while second messengers are still being formed, thereby keeping GJC intact[16]. Also in this cell line, Cx43 is ubiquitinated, even more so then in control cells (Fig 4A,B). Strikingly, we see that in PIP5-kinase overexpressing cells, Cx43 ubiquitination is not transient, but rather increases further at later time points through a yet unknown mechanism. We conclude that Cx43 ubiquitination by Nedd4 is independent of prior PI(4,5)P_2 depletion or the formation of PI(4,5)P_2 derived second messengers. .

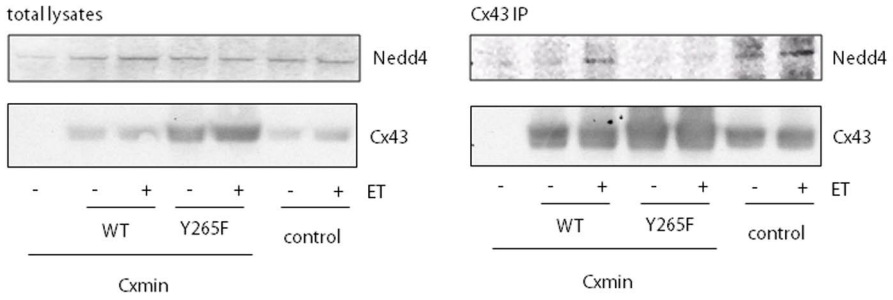

Figure 3: Binding of Nedd4 to Cx43 depends on Cx43Y265
Rat-1 control cells, Cxmin, and Cxmin cells reconstituted with Cx43, either wild type or Y265F mutant, were stimulated with endothelin for 3 minutes and harvested in lysis bufffer. Subsequently, lysates were subjected to Cx43 IP. Total lysates (left) were immunoblotted for Nedd4 and Cx43 as indicated. immunoprecipitates (right) were immunoblotted for Cx43 and ubiquitin as indicated.

Residue Y265 of Cx43 is essential for the interaction with Nedd4

The binding of Nedd4 to Cx43 was suggested to be mediated by one of the WW domains of Nedd4 and a WW binding motif surrounding Y286 in the Cx43 C-terminal tail[23]. In our system, mutation of Y286 did not affect the inhibition of GJC (see chapter 3 of this thesis). Y265 is part of a second putative WW binding domain (SPKY), but further mutation of this motif did not affect GJC inhibition either. Nevertheless, given the importance of residue Y265 for GJC regulation, we

investigated whether mutation of Y265 into a phenylalanine (Y265F) affects the interaction between Cx43 and Nedd4. We reconstituted Cx43 expression in Cxmin cells with both wildtype and Cx43Y265F and co-immunoprecipitated Nedd4 with Cx43 before and after stimulation with endothelin. Again, Cxmin cells served as a

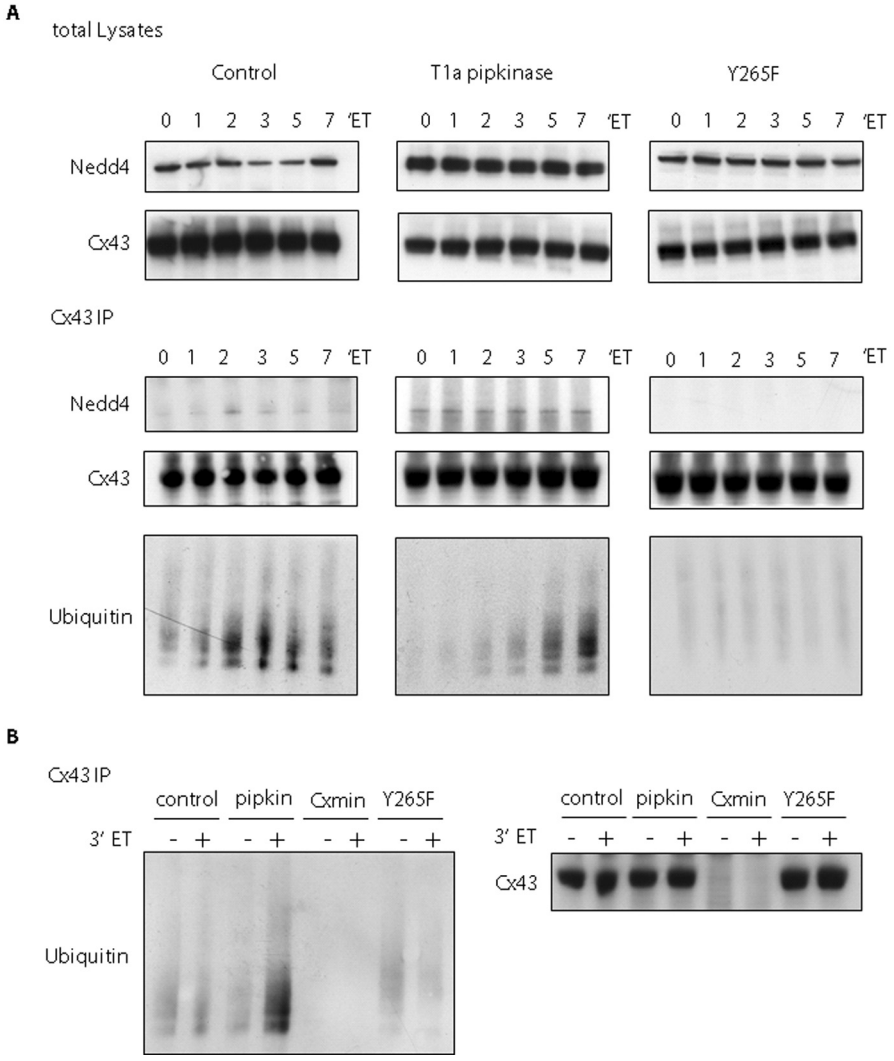

Figure 4: Cx43 ubiquitination of Cx43: Y265 is essential, accumulation in the continuing presence of PI(4,5)P_2
A: Control cells, PIP5kinase expressing cells and cells expressing Cx43Y265F were stimulated with endothelin (50 nM) and harvested at the indicated time points. Lysates were subjected to Cx43 immunoprecipitation. Total lysates were immunoblotted for Nedd4 and Cx43 (top). Precipitates were immunoblotted for Nedd4, Cx43 and ubiquitin.
B: For comparison, equal amounts of the IPs from the 0 and 3 minute time points from A of all the used cell lines were immunoblotted for Cx43 and ubiquitin on the same blot. Precipitates from Cxmin cells were used as a negative control.

negative control. Wildtype Cx43 was found to bind Nedd4 in unstimulated cells, and this interaction was enhanced by endothelin, similar to what is observed with endogenous Cx43. The Cx43Y265F mutant, however, did not interact with Nedd4 in either unstimulated or stimulated cells (Fig. 3). Also, when studied at various time points, the Y265F mutant did not interact with Nedd4, and was not ubiquitinated (Fig. 4A,B).

So, residue Y265 is essential for the interaction between Cx43 and Nedd4 and for Cx43 ubiquitination. This is consistent with our earlier observation that Nedd4 interaction with Cx43 is necessary for ubiquitination of Cx43 and for inhibition of Cx43-based GJC.

We note that the expression of Nedd4 protein is lower in Cxmin cells, and that this is reversed when Cx43 is reintroduced in these cells. We did not find a difference in Nedd4 expression at the mRNA level (RT-QPCR, data not shown), suggesting that Cx43 expression somehow stabilizes the Nedd4 protein through a yet unknown mechanism.

Inhibition of GJC is a two-step process

Since the kinetics of GJC inhibition are faster than those of Cx43 ubiquitination, we examined the kinetics of GJC in Nedd4 knockdown cells in response to endothelin stimulation in more detail. Monitoring Lucifer yellow diffusion after microinjection does not offer the desired temporal resolution, therefore we used a live cell imaging FRAP (fluorescence recovery after photobleaching) assay. To confirm the ability of the cells to communicate, one cell of an unstimulated monolayer, loaded with

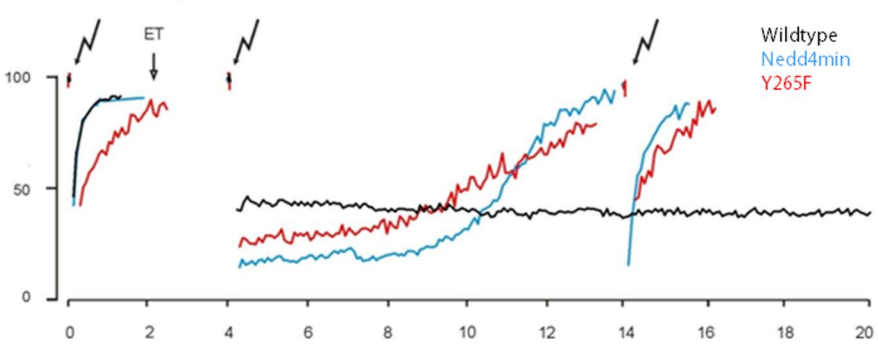

Figure 5: Transient inhibition of GJC in Nedd4 knockdown and Cx43Y265F expressing cells

Gap junctional communication in Rat-1 cells (black trace), Nedd4 knockdown cells (blue trace) and Cxmin cells expressing Cx43Y265F (red trace), assayed by fluorescence recovery after photo bleaching (FRAP) of calcein. The rate of FRAP was determined in control conditions and again starting 2 minutes after addition of endothelin (50nM). While all cells showed efficient communication before endothelin-treatment, gap junctional exchange was significantly decreased at 2 min after endothelin stimulation in all cell lines. However, while GJC remains inhibited for at least twenty minutes in control cells, GJC in both Nedd4min and Cx43Y265F expressing cells is restored after 7 minutes. Presented are representative traces from 5 independent experiments for each cell line.

fluorescent calcein, is bleached and fluorescence recovery is monitored. After full recovery of the fluorescence, endothelin is added and two minutes later the same cell is bleached again, and fluorescence recovery is monitored. As shown in Figure 5, fluorescence rapidly recovered in unstimulated control cells, whereas no recovery is observed for more than twenty minutes in endothelin-stimulated cells, indicating a complete lack of GJC. In Nedd4 knockdown cells (Fig. 5, blue line), we did initially observe inhibition of GJC. However, already 7 minutes after endothelin addition, fluorescence started to recover, implicating recovery of GJC. After full recovery of the fluorescence, the same cell was bleached a third time (at 12 minutes after endothelin stimulation). Now, the rate of recovery resembles that of unstimulated cells, indicating that GJC is fully restored. Cx43Y265F gap junctions were regulated in a similar way to those in Nedd4 knockdown cells (Fig. 5, red line), again stressing the importance of residue Y265 in regulation of GJC by Nedd4. Together, these data suggest that GJC inhibition by of endothelin stimulation occurs in two phases, in which the second, delayed, phase is mediated via Cx43 ubiquitination by Nedd4.

Internalisation of Cx43 upon endothelin stimulation

Mono-ubiquitination of Cx43 has been reported to trigger Cx43 plaque internalisation, followed by lysosomal degradation[22]. Cx43 internalisation is known to occur in so-called annular gap junctions, or connexosomes. An annular junction is a double membrane circular structure that is formed when the entire gap junction or a fragment of it is internalised into one of the two contacting cells[21,22,24,25].

We investigated whether Cx43 is internalised after endothelin stimulation using electron microscopy. We labeled coupes of unstimulated Rat-1 cells and cells stimulated with endothelin with immunogold and quantified the amount of Cx43 labeling at different locations in the cell, i.e. in gap junctions (Fig. 6A, left), in annular gap junctions (Fig. 6A, middle) and in lysosomes (Fig. 6A, right), where degradation takes place. Five or more gold particles in one of these structures is defined as a hit. Since GJC remains intact upon stimulation in PLCβ3 knockdown cells, we used this cell line as negative control. The amount of cell-cell contact localised Cx43 decreased from 36 to 10% of total hits (Fig. 6B), while lysosome localised hits increased from 58 to 82 %, suggesting that endothelin causes massive internalisation and increased turnover of Cx43. The PLCβ3 knockdown cells hardly showed an increase in lysosomal Cx43 and still showed 45% of Cx43 containing structures at cell-cell contacts (n >250 for each condition). This is consistent with our observations that GJC is intact in PLCβ3 knockdown cells. Both cell lines showed only a small difference in relative amount of annular gap junctions, suggesting that these are rapidly taken up by lysosomes after formation (Fig. 6B).

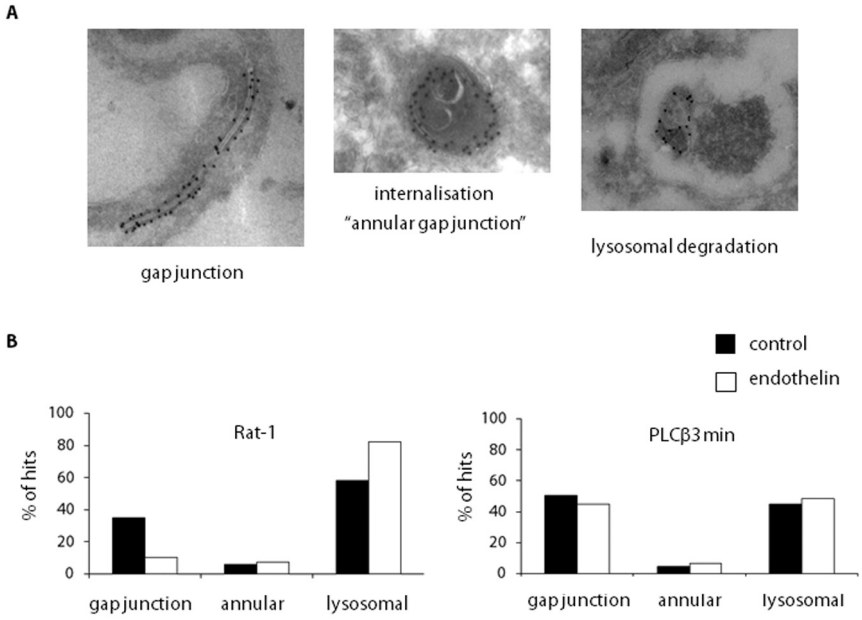

Figure 6: Internalisation of Cx43 upon GPCR stimulation
Rat-1 cells were serum starved overnight and stimulated for 8 minutes with endothelin (50 nM).
A: Electron microscopy images of the different appearances of Cx43 gold labeling. Left: Cx43 labeling in gap junctions. Notice the tight association of the two opposing membranes flanked by Cx43-immunogold labeling (C-terminal tail) at the site of the gap junction. The middle panel represents a so called annular junction: an entire gap junction internalises into one cell, creating a circular structure with a double membrane covered with Cx43 both inside and outside. The right panel shows the remains of an annular gap junction in a lysosome, were Cx43 is degraded.
B: Bar diagrams representing de appearance of Cx43 labeling in the different structures. Under control conditions, in normal Rat-1 cells, 35% of Cx43 labeling events is a gap junction, 6% is present in annular junctions and 58% in lysosomes (n=250). When stimulated with endothelin, 10.1% of Cx43 labeling events is a gap junction, 7.4% is present in annular junctions and 82.4% in lysosomes (n=256). In PLCβ3 knockdown cells, 50.3% of Cx43 labeling events is a gap junction, 4.7% is present in annular junctions and 45% in lysosomes (n=338). After exposure to endothelin, 45.1% of Cx43 labeling events is a gap junction, 6.5% is present in annular junctions and 48.4% in lysosomes (n=277).

Discussion

Cx43-based GJC is transiently inhibited by GPCR signalling. In our quest to unravel the underlying mechanism, we found that closure of Cx43 gap junctions depends on $PI(4,5)P_2$ hydrolysis[16]. In addition, we showed that residue tyrosine 265 of Cx43 is essential for inhibition of GJC. Even though Y265 is an established target for phosphorylation by Src, we excluded the involvement of tyrosine phosphorylation and Src family kinases, implicating that Y265 plays a structural role in the multi-protein

complex that regulates Cx43 based GJC (chapter 3 of this thesis). Despite our previous efforts, it is still unclear what happens to Cx43 at the gap junction after GPCR activation and whether inhibition of communication occurs through a "ball and chain" mechanism, where one or more connexin tails undergo a conformational change to physically block the gap, or rather in a more rigorous manner, through internalisation of the gap junctions.

The past few years, there is a growing awareness of a relationship between Cx43 ubiquitination and internalisation. Several reports suggest that Cx43 is mono-ubiquitinated on several lysines and that ubiquitination correlates with inhibition of GJC and gap junction internalisation[21,22]. In general, mono-ubiquitination is thought to be a trigger for internalisation, followed by lysosomal degradation, in contrast to polyubiquitination, which is often a precursor for proteasomal degradation[25,26]. In addition, E3 ubiquitin ligase Nedd4 was identified as a Cx43 interaction partner and knockdown of Nedd4 was reported to increase Cx43 gap junction plaque size, again providing a link between ubiquitination and gap junction turnover[23]. Other proteins regulated through ubiquitination by Nedd4 family members include TGFβ signalling intermediates and ENaC epithelial sodium channels. Ubiquitination by Nedd4 family members usually results in internalisation followed by either (lysosomal) degradation or recycling[26]. Interestingly, just like Cx43 based GJC, ENaC channel function is inhibited by $PI(4,5)P_2$ depletion. However, no connection has been made between both modes of regulation.

We investigated whether there is a link between GPCR induced inhibition of GJC, Cx43 ubiquitination and the interaction between Cx43 and Nedd4. First, we showed that Cx43 is transiently ubiquitinated in response to endothelin stimulation and that, following similar kinetics, the interaction between Cx43 and Nedd4 is induced by GPCR stimulation.

In the absence of Nedd4, Cx43 is not ubiquitinated and GJC is not inhibited at the 8 minute time point after stimulation of the cells with endothelin. We reported previously that Cx43 mutant Y265F forms gap junctions that are not inhibited in response to endothelin. We now find that Cx43Y265F does not bind to Nedd4 and is not ubiquitinated upon receptor stimulation. This confirms our hypothesis that Y265 plays a structural role in the protein complex that regulates Cx43 based GJC. We conclude that ubiquitination of Cx43 by Nedd4 is an essential step in shutting down communication in response to $PI(4,5)P_2$ hydrolysing agonists.

When first describing the interaction between Cx43 and Nedd4, Leykauf *et al*.[23] suggested that the binding of both proteins is mediated by the WW domain binding motif surrounding Y286 and the WW2 domain of Nedd4. However, they ignored other putative WW binding or protein-protein interaction motifs in Cx43, including a motif surrounding Y265. The details of the interaction between Cx43 and Nedd4 are currently under investigation.

The time frame of Cx43 ubiquitination, which is at its maximum at three minutes,

does not fit the time frame of gap junction shut off, which is apparent already one minute after addition with endothelin and lasts for ~30 minutes. To get a better view on the kinetics of inhibition of GJC, we performed FRAP experiments, which have a much higher temporal resolution than microinjection experiments. With this approach, we were able to determine the rate of communication at any desired time point. In both Nedd4 knockdown cells and cells expressing Cx43 mutant Y265F, GJC is initially inhibited by stimulation of the cells with endothelin. In contrast to control cells, however, GJC starts to recover already within 7 minutes after endothelin addition, implicating that GJC inhibition downstream of Gq coupled receptors is regulated in two phases.

Additionally, we studied the localisation of Cx43 by EM and found that, in response to endothelin, a rather large portion of Cx43 relocalises from the gap junctions to the lysosomes, where degradation takes place. Furthermore, our data suggest that in the continuing presence of normal $PI(4,5)P_2$ levels, ubiquitinated Cx43 is not degraded, but rather accumulates. Together with our EM data, which imply that Cx43 internalisation depends on $PI(4,5)P_2$ depletion since it does not occur in PLCβ3 knockdown cells, this provides a direct link between $PI(4,5)P_2$ hydrolysis and Cx43 internalisation and degradation.

Our results suggest a model in which GPCR agonist induced inhibition of Cx43-based GJC occurs in two phases. First, initial closure of the channels is mediated by $PI(4,5)P_2$ depletion. The 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. It appears that Cx43 ubiquitination is independent of $PI(4,5)P_2$ depletion, but that $PI(4,5)P_2$ depletion and Cx43 ubiquitination are required for internalisation. This suggests that, either directly or indirectly, $PI(4,5)P_2$ depletion is essential for the formation of annular gap junctions. Alternatively, it is possible that $PI(4,5)P_2$ provides Cx43 with a membrane anchor and the presence of $PI(4,5)P_2$ at the gap junction prevents internalisation. However, since Cx43 internalisation occurs in a gap junction-derived membrane integrated structure, dislodging Cx43 from the membrane is no requirement for internalisation, and therefore the second option is unlikely.

All together, this study provides substantial new insights into how Cx43 gap junctions are regulated by external stimuli, particularly GPCR receptor agonists. Whether $PI(4,5)P_2$ hydrolysis is indeed (solely) responsible for the initial inhibition of GJC, why $PI(4,5)P_2$ depletion is necessary for gap junction internalisation and how recovery of GJC is established are subjects for further investigation.

Materials and methods

Reagents

Materials were obtained from the following sources: endothelin, thrombin receptor-activating peptide (TRP; sequence SFLLRN), Cx43 polyclonal and α-tubulin monoclonal antibodies from Sigma (St. Louis, MO); Cx43 polyclonal antibody for electron microscopy from Zymed; ZO-1 monoclonal antibody from Zymed; Nedd4 polyclonal antibody from Upstate; Ubiquitin monoclonal (P4D1) form Covance; HRP-conjugated secondary antibodies from Cell Signalling and secondary antibodies for immunofluorescence (goat-anti-mouse, Alexa488 and goat-anti-rabbit, Alexa594) from Molecular Probes. Phusion High-Fidelity DNA Polymerase was purchased from Finnzymes.

Cell culture and cell-cell communication assays

Cells were cultured in DMEM containing 8% fetal calf serum, L-glutamine and antibiotics. For cell-cell communication assays, cells were grown in 35 mm dishes and serum starved for at least 4 hours prior to experimentation. Monitoring the diffusion of Lucifer Yellow (LY) from single microinjected cells was done as described[15]. Typically, microinjections were started at 3 minutes after addition of endothelin, and monitored at ~8 minutes after endothelin stimulation.

GJC assays using calcein-FRAP: monolayers of cells were loaded with the GJ-permeable dye calcein -AM (10', 5 μM) and washed with DMEM to de-esterify the AM-moiety for 15'. Cytosolic calcein was bleached by high-intensity laser illumination directed at a single cell (~10 s, 50-fold scanning power) and the subsequent GJ exchange of calcein was monitored by confocal time-lapse imaging and normalised to calcein signal from remote, non-bleached cells.

Construction and expression of cDNA constructs

shRNA resistant wildtype Cx43 and mutant Y265F were described before (chapter 3 of this thesis) Cx43 mutant cDNA was cloned into pEntr 1A (invitrogen) by BamHI/Xho restriction and subsequently cloned into pAd/Dest/CMV adenoviral expression vector (gateway system, invitrogen) by homologous recombination. Virus was produced in 293A packaging cells according to standard procedures. Supernatant containing virus particles was titrated on Rat-1 cells to determine the amount needed for Cx43 expression at levels comparable to endogenous Cx43 expression in Rat-1 cells.

Stable cell lines

Cx43 and PLCβ3 knockdown cell lines and GFP-T1α pipkinase expressing cells were described before[16]. To generate Nedd4 knock down Rat-1 cells, Nedd4 was knocked down by stable expression of retroviral pSuper (pRS)[27] containing one of the shRNA target sequences (1: GCATAGGTCTGGCCAAGAA; 2: ATACCAGACTCACCATGTA; 3: AGACTGACATTCCAAACAA). pRS-Nedd4 was transfected into Phoenix-Eco package cells and the supernatant contain-

ing viral particles was harvested after 48 hrs. For infection, cells were incubated with viral supernatant supplemented with polybrene (Sigma; 5ug/ml). 48 hrs after infection, cells were selected on puromycin (2 μg/ml) for 1 week. Pools were tested for Nedd4 expression.

SDS-PAGE, immunoblotting and immunoprecipitation

Cells were harvested in Laemmli sample buffer (LSB), boiled for 10 min. and subjected to immunoblot analysis according to standard procedures. Filters were blocked in TBST/5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemoluminescence (Amersham Pharmacia). For immunoprecipitation, cells were harvested in lysis buffer (containing 1% sodium deoxycholate, 0.5% SDS, 1% NP40) supplemented with protease inhibitor cocktail (Roche) and, in case of ubiquitination experiments, with 10 mM N-Ethylmaleimide. Lysates were spun down and the supernatants were subjected to immunoprecipitation using protein A-conjugated antibodies for 4 hrs at 4°C. Proteins were eluted by boiling for 10 minutes in LSB and analyzed by immunoblotting.

Immunostaining and fluorescence microscopy

Cells grown on coverslips were fixed in methanol for 15 min. Samples were blocked in PBS containing 1.5% BSA for 30 min. Subsequently, samples were incubated with primary and secondary antibodies for 30 min. each in PBS/1.5% BSA, washed five times with PBS and mounted in Immumount (Thermo Shandon). Confocal fluorescence images were obtained on a Leica TCS NT (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr laser. Images were taken using a 63x NA 1.32 oil objective. Standard filter combinations and Kalman averaging were used. Processing of images for presentation was done on a PC using the software package Photoshop (Adobe Systems Incorporated Mountain View, California, USA).

PtdIns(4,5)P_2 imaging by FRET ratiometry

Temporal changes in PtdIns(4,5)P_2 levels in living cells were assayed by the FRET-based PtdIns(4,5)P_2 sensor, PH-PLCδ1, as described[28]. In brief, Rat-1 cells were transiently transfected with CFP-PH and YFP-PH constructs (1:1 ratio) using Fugene transfection reagent and placed on a NIKON inverted microscope equipped with an Achroplan × 63 (oil) objective (N.A. 1.4). Excitation was at 425±5 nm. CFP and YFP emissions were detected simultaneously at 475±15 and 540±20 nm, respectively and recorded with PicoLog Data Acquisition Software (Pico Technology). FRET is expressed as the ratio of acceptor to donor fluorescence. At the onset of the experiment, the ratio was adjusted to 1.0, and FRET changes were expressed as relative deviations from base line. Traces were smoothened in Microsoft Excel using a moving average function ranging from 3 to 6.

Immunoelectron microscopy

Rat-1 cells were grown to confluency and serum starved for 4 hours. Untreated cells and cells stimulated with endothelin for 8 minutes, were fixed for 2 hours in 2% paraformal-

dehyde + 0,2% glutaraldehyde in 0.1 M PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM $MgCl_2$, 10 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning as previously described[29]. Briefly, 50-nm cryosections were cut at -120º C using diamond knives in a cryoultramicrotome (Leica Aktiengesellschaft, Vienna, Austria) and transferred with a mixture of sucrose and methylcellulose onto formvar-coated copper grids. The grids were placed on 35-mm petri dishes containing 2% gelatine. Ultrathin frozen sections were incubated at room temperature with rabbit anti-human connexin-43 and then incubated with 10-nm protein A-conjugated colloidal gold (EM Lab, Utrecht University, Netherlands) as described. After immunolabeling, the sections were embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM10 electron microscope (FEI Company, Eindhoven, The Netherlands).

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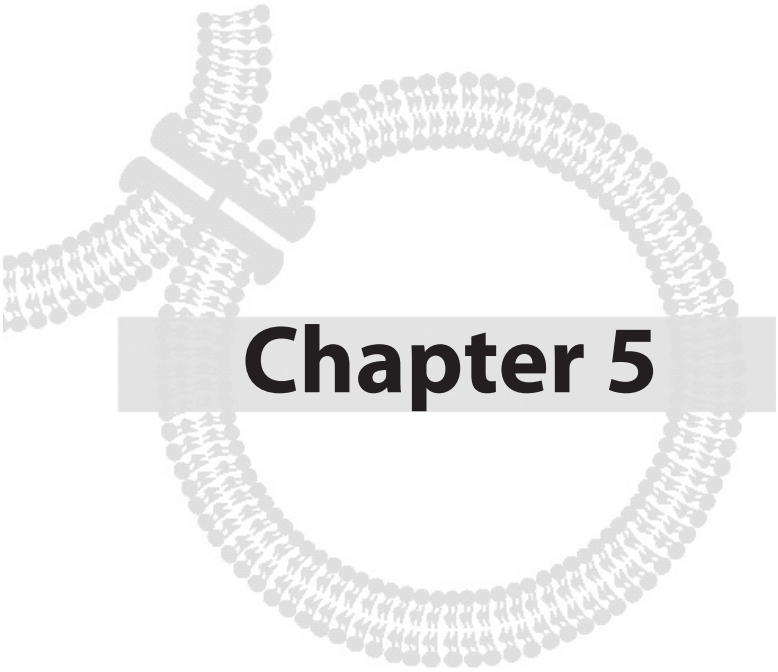

Chapter 5

Dependence of cell migration on Connexin43: modulation of N-cadherin expression

Leonie van Zeijl, Trudi Hengeveld , Wouter Moolenaar

Abstract

Direct cell-cell communication through Cx43-based gap junctions is essential for normal cell behaviour. Increasing evidence suggests, however, that Cx43 influences cell proliferation, cell migration and contact inhibition in a communication-independent manner. To investigate the importance of Cx43 for the behaviour of Rat-1 fibroblasts, we knocked down Cx43 by stable expression of Cx43 shRNA. Cx43 knockdown abolished gap junctional communication (GJC), without affecting cell morphology, cell proliferation, contact inhibition or anchorage-independent growth. However, Cx43 knockdown cells showed a ~50% reduced migration speed in an in vitro wound healing assay. Comparing expression levels of various cell-cell contact proteins revealed a strong reduction in N-cadherin expression, both at the mRNA and protein level. However, reconstitution of Cx43 expression and function, did not rescue N-cadherin expression, nor cell migration, indicating that the effect of Cx43 knockdown on migration is GJC independent. In addition, we show that knockdown of N-cadherin alone, is sufficient to reduce the rate of cell migration similar to what is observed in Cx43 knockdown cells. In summary, knockdown of Cx43 inhibits in vitro fibroblast migration, apparently as a result of reduced N-cadherin expression rather than of reduced GJC.

Introduction

The main function of connexin proteins is to form small intercellular channels, called gap junctions. Gap junctions directly connect the cytoplasms of adjacent cells and mediate the diffusion of small molecules, such as second messengers, ions and metabolites[1-3]. Gap junctions are essential for tissue homeostasis and coordinated cell behaviour and loss of gap junctional communication (GJC) is associated with several diseases[4-6]. Most tumours lack functional gap junctions and reconstitution of connexin expression may (partially) invert the tumourigenic phenotype[7-10]. This suggests that restoration of GJC may contribute to contact inhibition. Alternatively, healthy surrounding tissue may control the behaviour of deranged cells, or even induce apoptosis. The transfer of apoptotic signals through gap junctions is known as the "bystander" killing effect[11].

The most abundant and best studied connexin is connexin43 (Cx43). Cx43 is the main connexin in heart cells and Cx43 knockout mice die immediately after birth due to a malformed heart, which is the result of impaired migration of the neural crest cells[12-14]. Cx43 knockout cells from neural crest cell explants show a reduction in polarised cell movement that is comparable to the effect of N-cadherin knockdown on neural crest cell migration[15]. The same authors reported that over-

expression of Cx43 has a similar effect on cell migration, suggesting that a balanced expression of Cx43 is crucial to cell migration[15,16]. Several studies show that Cx43 expression stimulates cell migration, whereas Cx43 knockdown reduces it. In the brain, Cx43 in migrating neurons, which lack adherens junctions, has been reported to increase the adhesive properties of cells, both within one cell type and to neighbouring tissue. In these cells, migration is mediated by Cx43 (or Cx26), in a manner independent of gap junctional communication[17]. It has been suggested that Cx43 may act as an adhesion molecule itself[18-22]. In a mouse model for wound healing, Cx43 knockdown reduced inflammation, seen both macroscopically, as a reduction in swelling, redness and wound gape, and microscopically, as a significant decrease in neutrophil numbers in the tissue around the wound. In addition, Cx43 knockdown resulted in an increased rate of migration of cells into the wound area, leading to increased wound healing capacity compared to wild type mice[23]. However, given the diverse effects of Cx43 knockdown on wound pathology, this may not be a direct effect of Cx43 knockdown on the migration machinery.
In this study, we set out to determine the importance of Cx43 for diverse aspects of cell behaviour. To this end, we stably knocked down Cx43 in Rat-1 fibroblasts. These cells express Cx43 as the only connexin and form functional and regulatable gap junctions, which makes them a perfect model system. Under control conditions, these cells are contact-inhibited. We found that Cx43 knockdown reduced the rate of cell migration in an *in vitro* wound healing assay. This effect was independent of Cx43 expression or function, but appeared to be an indirect effect of Cx43 knockdown on N-cadherin expression.

Results

Knockdown of Cx43

To investigate the importance of Cx43 expression for cell behaviour, we knocked down Cx43 in Rat-1 fibroblasts. We previously reported that stable knockdown of Cx43 results in Cx43 depletion, and cell-cell communication is completely downregulated. Actin staining revealed no changes in gross cytoskeletal arrangement or cell morphology following Cx43 knockdown (chapters 2 and 3 of this thesis and [20]).

Cx43 knockdown does not affect cell proliferation or contact inhibition

To study whether Cx43 knockdown affects cell growth or contact inhibition, we measured the rate of cell proliferation (Fig. 1A). There was no significant difference in the growth rate of control and Cxmin cells; the number of cells for both cell lines reached a plateau, indicative of contact inhibition.
It was previously reported that addition of endothelin to Rat-1 cells stimulates anchorage-independent growth in the presence of epidermal growth factor (EGF)[24].

We tested whether Cx43 knockdown affects the ability of Rat-1 cells to form colonies in soft agar. Control cells and Cxmin cells were plated in soft agar in 8% FCS alone or in the presence of endothelin (ET), or EGF, or both stimuli. After two weeks, colony outgrowth was monitored (for images, see Fig. 1B). Consistent with previous findings, both endothelin and EGF induced colony formation, while the combination of both stimuli enhanced colony outgrowth even more. In Cxmin cells, we observed enhanced colony outgrowth similar to that in control cells (Fig. 1B). We conclude that Cx43 knockdown does not affect ET/EGF induced anchorage-independent growth of Rat-1 cells.

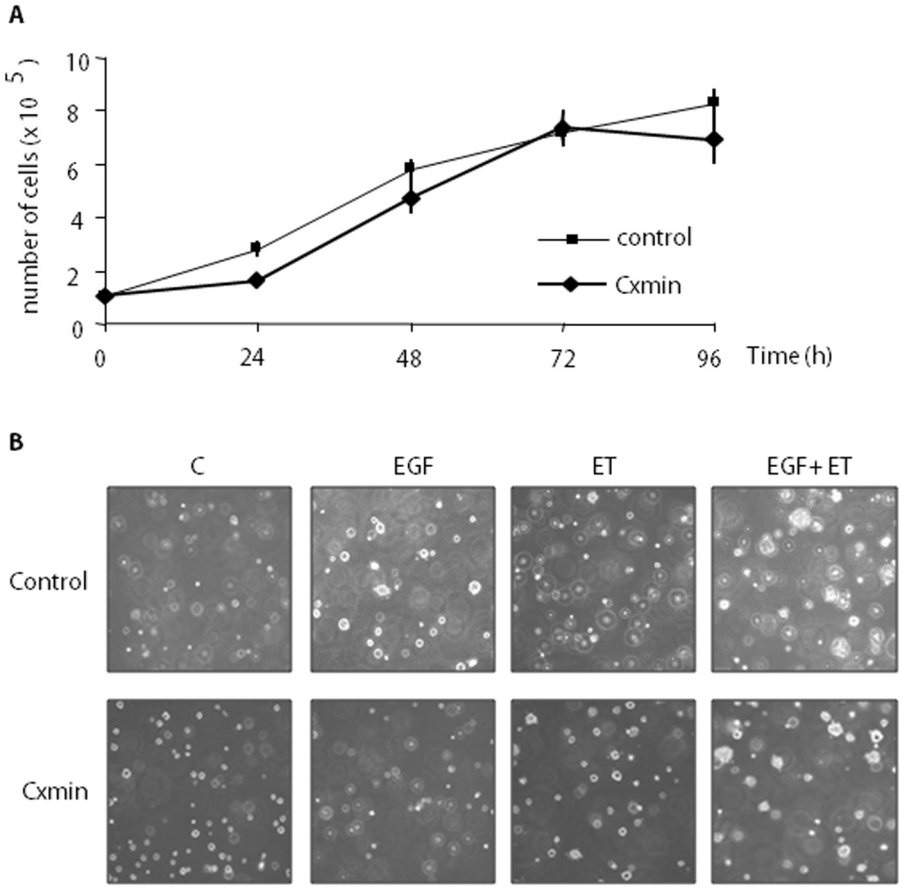

Figure 1. Cx43 knockdown does not affect cell proliferation or contact inhibition.
A: Graph showing the proliferation of both control and Cxmin cells. y-axis: number of cells ($x10^5$), x-axis: time (h)
B: Wide field images of Rat-1 cells growing in soft agar in the presence of 8% FCS. Pictures were taken two weeks plating Left: control cells, right: Cxmin cells. C: control, EGF: incubation with EGF (5ng/ml), ET: incubation with endothelin (50 nM), EGF + ET: incubation with EGF (5 ng/ml) and endothelin (50 nM).

Cx43 knockdown reduces the rate of cell migration

We performed an *in vitro* wound healing assay to investigate the effect of Cx43 knockdown on cell migration. For this assay, cells were grown to confluency and then serum starved for 8 hrs. Subsequently, the monolayer was scratched with a pipette tip. The cells were allowed to close the wound through migration during 16 hours in the presence of 4% FCS. Cells at the edge of the wound and cells within the monolayer communicated normally with their neighbours (as measured by Lucifer yellow diffusion following microinjection[25,26], data not shown). Cx43 was localized to cell-cell contacts and to the perinuclaer region, similar to the distribution of Cx43 in confluent cells (Fig. 3A). At later time points, when the intracellular structure at the rim of the wound became looser, Cx43 was still detected at sites of cell-cell contact (Fig. 3A). So, during migration, cells are contacted and communicate continuously, which is consistent with a previous study[27].

The surface of the wound before and after migration was measured, and the difference was used as a measure for migration. Figure 2A shows pictures of the scratches before (left) and after (right) migration. Quantification of the relative migration of both control and Cxmin cells shows that knockdown of Cx43 leads to a reduction of migration speed to 52% of that in control cells (Fig. 2B). We conclude that knockdown of Cx43 reduces migration of contacted Rat-1 fibroblasts.

Cx43 knockdown affects the formation of stress fibres and focal adhesions.

Directional cell migration requires polarization of the cells toward the desired direction of migration[28]. Therefore, decreased migration ability may be explained by

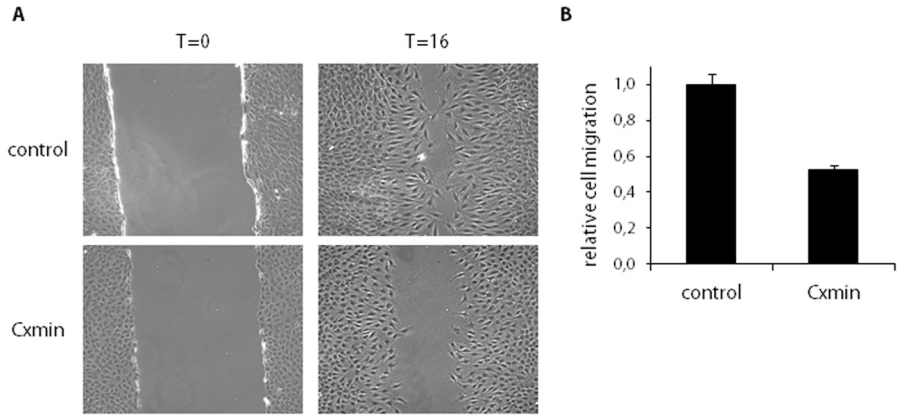

Figure 2. Knockdown of Cx43 inhibits cell migration.
Cells were grown to confluency, serum starved for 8 hours and then scratched with a yellow pipette tip. Cells were left to fill the scratch for 16 hours, images were taken at time point 0 and after 16 h. The reduction of the scratch surface is a measure for migration.
A: Wide-field images of control (top) and Cxmin (bottom) cells at T=0 (left) and T=16 (right)
B: Bar diagram showing relative rate of migration. Migration of the control cells was set to 1.

a defect in cell polarisation. We investigated whether knockdown of Cx43 affects the ability of Rat-1 cells to polarise. Control and Cxmin cells were fixed at various time points after the start of the wound healing assay. As a marker for polarisation, we visualized the orientation of the microtubule organizing centre (MTOC) by staining for α-tubulin. All cells which have their MTOC oriented within 45° from the direction of migration were marked (green dots, Fig. 3B). In both control and Cxmin cells at all time points, all cells at the wound edge and most cells in the second row were polarised, while the MTOC orientation in the cells further from the scratch appeared to be random.

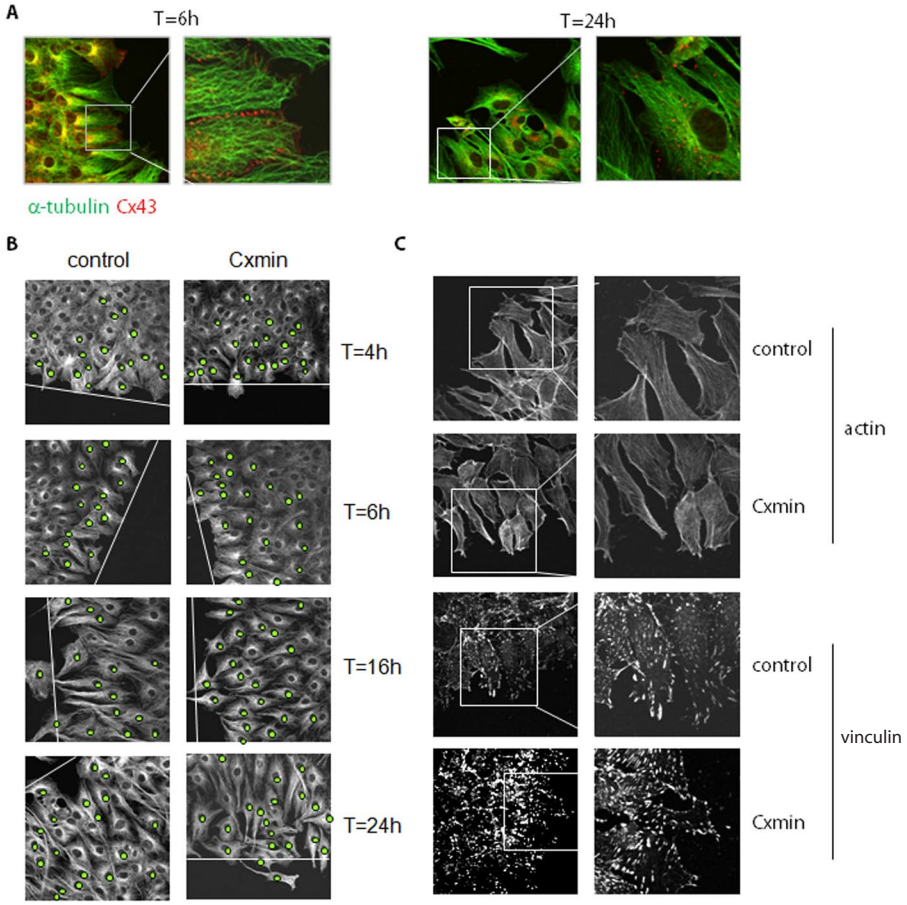

Figure 3. Cx43 knockdown affects polarised cell movement.
A: Confocal images of Rat-1 cells 6 and 24 hours after the start of a scratch assay. Cells on coverslips were fixed and stained for Cx43 (red) and α–tubulin (green)
B: Confocal images of control (left) or Cxmin (right) cells, stained for α-tubulin, fixed at several time points after start of the scratch assay. Cells with their MTOC oriented in the direction of migration are marked with a green dot.
C: Confocal images of cells fixed 8 hours after start of the scratch assay, stained for either actin (top) or vinculin (bottom).

Next, we compared actin stress fibre formation and organisation during the migration of control and Cxmin cells (Fig. 3C, top). We found that control cells were more elongated than Cxmin cells, especially after 16h of migration, and that more rows of cells were elongated. Furthermore, stress fibres in control cells were long and oriented in the direction of migration, whereas in Cxmin cells the stress fibres were shorter and more randomly oriented.
The ability to form focal adhesions was studied by staining for vinculin. Cxmin cells appeared to form more focal adhesions than control cells (Fig. 3C, bottom), suggesting that Cxmin cells are better attached, and thereby may hamper migration. Thus, Cxmin cells are still able to polarize, but not to same extent as control cells.

Knockdown of Cx43 reduces N-cadherin expression

N-cadherin is the only cadherin that is expressed by Rat-1 fibroblasts. Western blots of total cell lysates show that N-cadherin was strongly downregulated in Cxmin cells (Fig. 4A). This was confirmed by RT-PCR (Fig. 4B), indicating that regulation of N-cadherin expression by Cx43 knockdown takes place at the transcriptional level. N-cadherin is known to be essential for migration of neuronal cells and fibroblasts, and is associated with tumour aggressiveness and metastatic potential[29].
Expression levels of the Cx43-interaction partner ZO-1, focal adhesion marker vinculin, and cell-cell contact protein β-catenin were not affected by Cx43 knockdown (Fig. 4A).

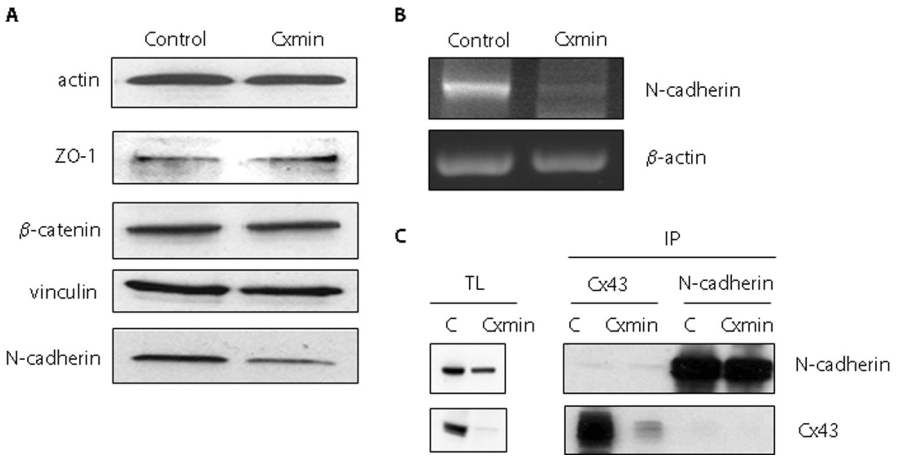

Figure 4. Knockdown of Cx43 inhibits N-cadherin expression.
A: Western blots of total lysates from control (left) and Cxmin (right) cells, showing downregulation of N-cadherin protein, but not of ZO-1, β-catenin and vinculin. Actin was used as loading control.
B: RT-PCR showing downregulation of N-cadherin on mRNA level (top) left: control cells, right: Cxmin cells, RT-PCR of β-actin is used as input control (bottom)
C: No direct interaction between Cx43 and N-cadherin. TL: total lysates blotted for N-cadherin (top) or Cx43 (bottom). IP: Either Cx43 (left 2 lanes) or N-cadherin (right 2 lanes) was immunoprecipitated from control and Cxmin cells. Immunoprecipitates were blotted for Cx43 (bottom) and N-cadherin (top).

To ensure that the effect of Cx43 knockdown on N-cadherin expression is not an off-target effect, we designed two additional shRNA constructs (B and C) against Cx43. We stably knocked down Cx43 in Rat-1 cells, using these constructs and blotted total lysates for Cx43 and N-cadherin (Fig. 5A). In these cell lines, Cx43 expression was knocked down below detection level and resulted in a complete lack of GJC. We found that N-cadherin was downregulated to the same extent as in the Cxmin cells, ruling out an off-target effect of the Cx43 shRNA construct. In the wound healing assay, we found that the Cxmin B and Cxmin C cell lines were impaired in their ability to migrate to the same extent as the original Cxmin cells (Fig. 5B). Thus, the effect of Cx43 knockdown on migration coincides with downregulation of N-cadherin.

Cx43 has been reported to colocalise with N- and E-cadherin and suggested to interact with cadherins, but the evidence is primarily based on colocalisation[30-33]. In Rat-1 cells, Cx43 co-localised with N-cadherin (Fig. 6C). We investigated if Cx43 and N-cadherin are part of the same protein complex by performing co-immunoprecipitations. We precipitated Cx43 or N-cadherin from both control and Cxmin cells and immunoblotted for Cx43 and N-cadherin (Fig. 4C). N-cadherin did not co-IP with Cx43 (Fig. 4C, left two lanes), nor did Cx43 with N-cadherin (right two lanes). So, we found no evidence for a direct interaction between Cx43 and N-cadherin.

Reconstitution of Cx43 expression does not rescue N-cadherin expression or migration.

We asked whether the effect of Cx43 knockdown on migration and N-cadherin expression is communication-dependent. We reconstituted Cx43 expression in Cxmin cells with RNAi- resistant Cx43. We reported previously that GJC in these cells was fully restored and Cx43 was distributed in the same pattern as in the parental Rat-1 cells[26]. Cxmin cells re-expressing Cx43 migrated at the same rate as Cxmin cells (Fig. 5B). Western blot analysis of total lysates showed that N-cadherin expression was not restored in these cells (Fig. 5A). This suggests that Cx43 expression and cell-cell communication are not the key factors in inhibition of migration by knockdown of Cx43 and suggests a correlation between N-cadherin expression and migration speed.

N-cadherin knockdown mimics the Cx43 knockdown cell migration phenotype

We studied the effect of N-cadherin knockdown on Cx43 expression, Cx43-based GJC and cell migration. We stably knocked down N-cadherin in Rat-1 cells using two different shRNA constructs. N-cadherin expression in these cells was reduced to ~80% of that in control cells, while Cx43 was still expressed at normal levels (Fig. 6A). To investigate whether N-cadherin knockdown impaired Cx43 function, we measured gap junctional communication by monitoring the diffusion of Lucifer yellow after microinjection. We found that N-cadherin knockdown cells

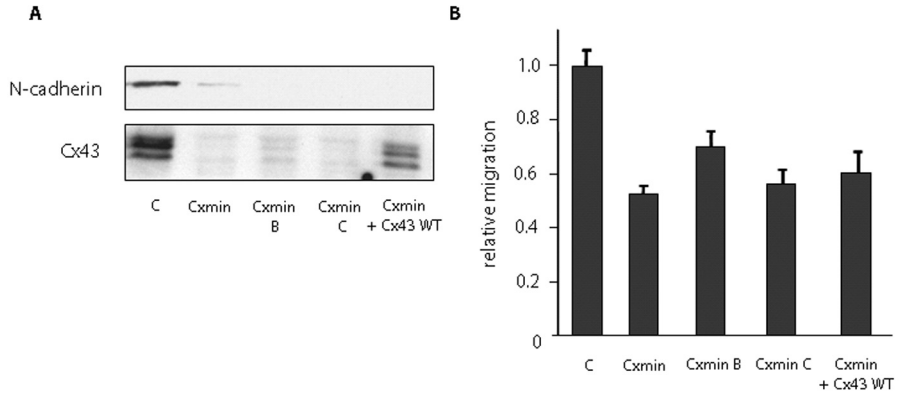

Figure 5. Rescue of Cx43 expression affects neither N-cadherin expression, nor cell migration.
A: Western blot showing downregulation of Cx43 by two additional shRNA constructs (3rd and 4th lanes) and reconstitution of Cx43 expression in Cxmin cells (5th lane) (bottom) and N-cadherin expression in these cells (top), compared to control (1st lane) and Cxmin (2nd lane) cells.
B: Bar diagram showing the relative migration of the diverse cell lines from Fig.A. Migration of the control cells was set to 1.

communicated to the same extent as control cells and that GJC could still be inhibited by endothelin and TRP (Fig. 6B and [20]). We immunostained the cells for Cx43 and N-cadherin and found that, while N-cadherin knockdown cells are flatter and form looser contacts, Cx43 accumulated in the perinuclear region and at cell-cell contacts in a punctate fashion similar to control cells (Fig. 6C). Finally, we studied the effect of N-cadherin knockdown on migration in the in vitro wound healing assay (Fig. 6D). In both N-cadherin knockdown cell lines, migration was reduced to ~50% of that in control cells, similar to the effect of Cx43 knockdown (Fig. 6E). We conclude that reduced N-cadherin expression is sufficient to decrease the migration rate to that of Cx43 knockdown cells.

Discussion

Gap junctional communication (GJC) is essential for tissue homeostasis, growth control and coordinated cell behaviour[1,12,34,35]. Connexins, the building blocks of gap junctions, have been suggested to be tumour suppressors [9,10,34,36]. Loss of communication is associated with loss of growth control, contact inhibition and cell transformation. In this study, we investigated the importance of Cx43 expression on diverse aspects of cell behaviour. We found that knockdown of Cx43 and GJC did not affect cell growth, contact inhibition, cell morphology or cell transformation.
However, in three independent Cxmin cell lines, we observed a ~50% decrease in migration speed of contacted cells. The decrease in migration rate of Cxmin cells

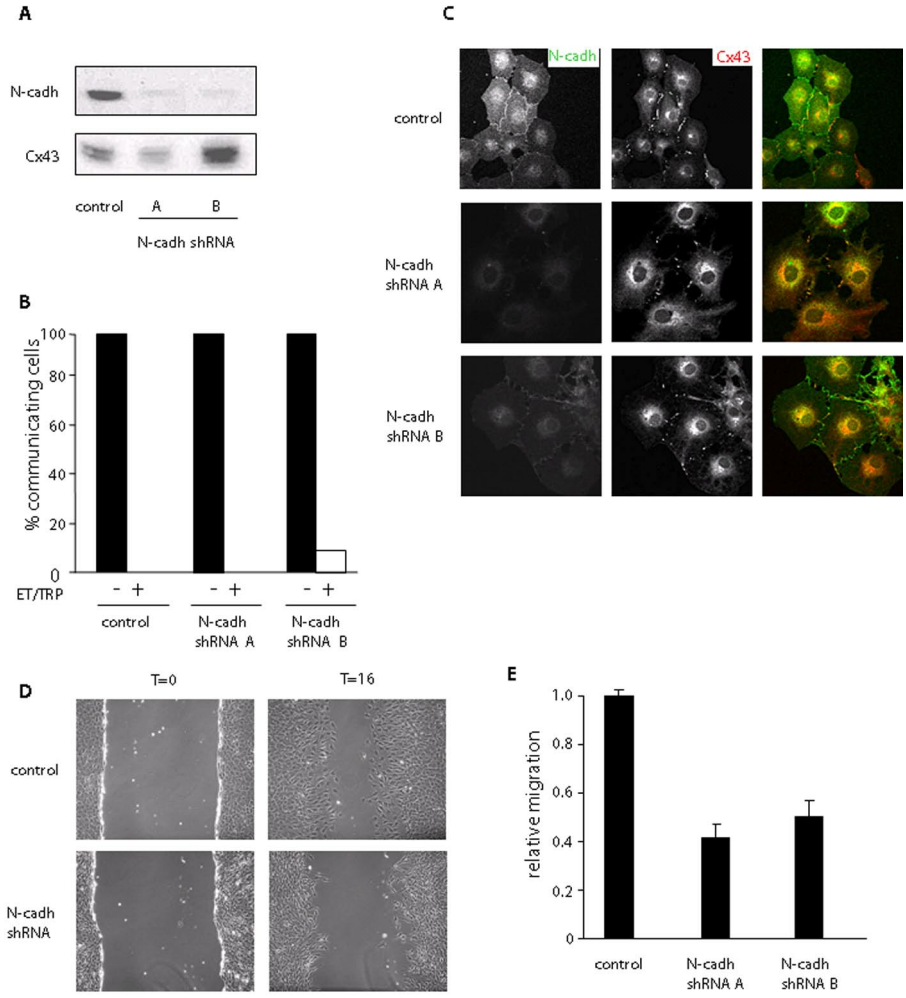

Figure 6. Knockdown of N-cadherin mimics the effect of Cx43 knockdown on cell migration.

A: Western blot showing knockdown of N-cadherin by two different shRNA constructs (top) and the expression of Cx43 in these cells.

B: Bar diagram showing the gap junctional communication of N-cadherin knockdown and control cells, before and after stimulation with endothelin (50 nM).

C: Confocal images of control and N-cadherin knockdown cells. Left (green): N-cadherin, Middle (red): Cx43.

For the migration assay, cells were grown to confluency, serum starved for 8 hours and then scratched with a yellow pipette tip. Cells were left to fill the scratch for 16 hours, images were taken at time point 0 and after 16 h. The reduction of the scratch surface is a measure for migration.

D: Wide-field images of control (top) and N-cadherin knockdown (bottom) cells at T=0 (left) and T=16 (right).

E: Bar diagram showing relative migration of control and N-cadherin knockdown cells. Migration of the control cells was set to 1.

was accompanied by reduced cell elongation and increased focal adhesion formation during migration. In all three Cxmin cell lines, we observed a strong downregulation of N-cadherin expression at the transcriptional level. Rat-1 fibroblasts express N-cadherin as only cadherin. N-cadherin is associated with numerous processes, such as cell-cell adhesion, differentiation, embryonic development, invasion and migration[37]. Although the exact mechanism is unknown, it is generally accepted that N-cadherin is essential for migration of neuronal cell and fibroblasts[37]. Given the less elongated shape of Cxmin cells in the wound healing assay and the disturbance in stress fibre alignment, it seems likely that there is a defect in Rho activation. However, it is unknown how N-cadherin expression is linked to stress fibre organisation, focal adhesion formation or activation of Rho family GTPases.

Reconstitution of Cx43-based GJC did not rescue the migration phenotype, suggesting that that the decrease in migration is independent of GJC and Cx43 expression. However, N-cadherin expression was not rescued by expression of Cx43. We hypothesize that the Cxmin cells had reached a new steady state, in which N-cadherin expression was lower than in control cells. Re-introduction of Cx43 expression did not increase the need for the cells to increase N-cadherin levels. Our results fit the growing notion that Cx43 not only functions as a channel protein, but also is an essential player in a multi-protein complex. This is not only important for regulation of GJC, but may also have a broader cellular effect, through signals emanating from (proteins in) this complex[17,19,38,39]

Furthermore, in N-cadherin knockdown cell lines, Cx43 levels and GJC were not affected. In the wound healing assay, N-cadherin knockdown cells showed a decrease in migration rate, comparable to that in Cxmin cells. Thus, downregulation of N-cadherin expression can explain the decrease in migration speed in Cxmin cells.

In summary, Cx43 influences cell migration through a mechanism that is independent of gap junctional communication, but rather acts by influencing the expression of N-cadherin at the transcriptional level through an as-yet-unknown mechanism.

Materials and methods

Reagents

Materials were obtained from the following sources: endothelin, thrombin receptor-activating peptide (TRP; sequence SFLLRN), actin monoclonal, Cx43 polyclonal, vinculin polyclonal and α-tubulin monoclonal antibodies from Sigma (St. Louis, MO); HRP-conjugated secondary antibodies from Cell Signalling and secondary antibodies for immunofluorescence (goat-anti-mouse, Alexa488 and goat-anti-rabbit, Alexa594) from Molecular Probes. N-cadherin and β-catenin monoclonal antibodies from BD Transduction Laboratories. ZO-1 monoclonal antibody from Zymed.

Cell culture, cell proliferation and cell-cell communication assays

Cells were cultured in DMEM containing 8% fetal calf serum, L-glutamine and antibiotics. Proliferation rate of the cells is determined by plating $1x10^5$ cells in a 35mm dish in triplo for each time point. Cells are counted on 4 consecutive days with an automatic cell counter. From these data, the growth curve is calculated using Excell. For cell-cell communication assays, cells were grown in 35mm dishes and serum starved for at least 4 hrs prior to experimentation. Monitoring the diffusion of Lucifer Yellow (LY) from single microinjected cells was done as described before[25,26] .

RNA Interference

To generate Cx43-deficient Rat-1 cells, Cx43 was knocked down by stable expression of retroviral pSuper[40] (pRS) containing the shRNA target sequence GAAGCAGATTGAAATCAAGAA (Cxmin B) or TCTCGCTTTGAACATCATTGA (Cxmin C). pRS-Cx43 was transfected into Phoenix-Eco package cells and the supernatant containing viral particles was harvested after 72 hrs. For infection, cells were incubated with 1 ml of viral supernatant supplemented with 10 µl Dotap (Roche; 1 mg/ml). 48 hrs after infection, cells were selected on puromycin (2 µg/ml, Sigma) for 1 week. Single cell-derived colonies were tested for Cx43 expression and communication. A clone expressing <5% of residual Cx43 and completely lacking cell-cell coupling was used for further experiments. N-cadherin was stably knocked down by retroviral expression of N-cadherin shRNA. Two different target sequences were selected (CCGATCAACTTGCCAGAAA and TGACTGAGGAGCCGATGAA). Stable knockdown clones were produced as described above. In all cases, a non-functional shRNA was used as control.

Construction and expression of shRNA resistant Cx43 cDNA

Cx43 cDNA was cloned into pEntr 1A (Invitrogen) by BamHI/Xho restriction and subsequently cloned into pAd/Dest/CMV adenoviral expression vector (gateway system, invitrogen) by homologues recombination. To prevent targetting of this construct by Cx43 directed shRNA, we made two silent mutations in the RNAi target site, using PCR-SDM (primers: F: CCGCTGGAGGGAAGGTGTGGTTGTCCGTGCTCTTCATATTC, R: GAATATGAAGAGCACGGACAACCACACCTTCCCTCCAGCGG). Virus was produced in 293A packaging cells according

to standard procedures. Supernatant containing virus particles was titrated on Rat-1 cells to determine the amount needed for Cx43 expression at levels comparable to endogenous Cx43 expression in Rat-1 cells.

Soft agar assay

$1x10^5$ cells were plated in 2 ml of growth medium, containing 0.4% low melting point agar in a 35mm dish. Two weeks after plating, digital images were taken on a Zeiss Axiovert 200M microscope using a 10x NA 0.25 objective. Axiovision software was used to capture the images.

In vitro wound healing assay

To ability of contacted Rat-1 fibroblasts to migrate is investigated with an *in vitro* wound healing assay: cells are grown to confluency in 35mm dishes in triplo. Before starting the assay, cells are serum starved for 8 hours. The monolayer is then scratched with a yellow pipette tip and cells are left to migrate into the created gap for 16 hours in the presence of 4% FCS. At T=0 and T=16h, digital pictures were taken on a Zeiss Axiovert 200M microscope using a 10x NA 0.25 objective. Axiovision software was used to capture the images. The surface of the scratch area on each picture is measured using Image J. The difference in scratch surface between T=16 and T=0 (as calculated using Excell) is a measure for migration.

SDS-PAGE, immunoblotting and immunoprecipitation

Cells were harvested in Laemmli sample buffer (LSB), boiled for 10 min. and subjected to immunoblot analysis according to standard procedures. Filters were blocked in TBST/5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemoluminescence (Amersham Pharmacia). For immunoprecipitation, cells were harvested in lysis buffer containing1% NP-40, 0.25% sodium desoxycholate, supplemented with protease inhibitor cocktail (Roche). Lysates were spun down and the supernatants were subjected to immunoprecipitation using protein A-conjugated antibodies for 4 hrs at 4°C. Proteins were eluted by boiling for 10 min. in LSB and analyzed by SDS page and immunoblotting according to standard protocols.

Immunostaining and fluorescence microscopy

Cells grown on coverslips were fixed in methanol for 15 min. Samples were blocked and in PBS containing 1.5% BSA for 20 min. Subsequently, samples were incubated with primary and secondary antibodies for 30 min. each in PBS/1.5% BSA, washed five times with PBS and mounted in Immumount (Thermo Scientific). Confocal fluorescence images were obtained on a Leica TCS NT (Leica Microsystems, Heidelberg, Germany) confocal system, equipped with an Ar/Kr laser. Images were taken using a 63x NA 1.32 oil objective. Standard filter combinations and Kalman averaging were used. Processing of images for presentation was done on a PC using the software package Photoshop (Adobe Systems Incorporated Mountain View, California, USA).

RT-PCR

Total RNA is isolated from subconfluent control and Cxmin cells using the RNeasy kit (Qiagen). mRNA is converted to cDNA according to standard procedures. The primers that were used for PCR of N-cadherin are: Forward: GATCGAATTCCTCTAGAATGTGCCGGATAGCGGGAGCGC and Reverse: CACAAGTCTCGGCCTCTTG

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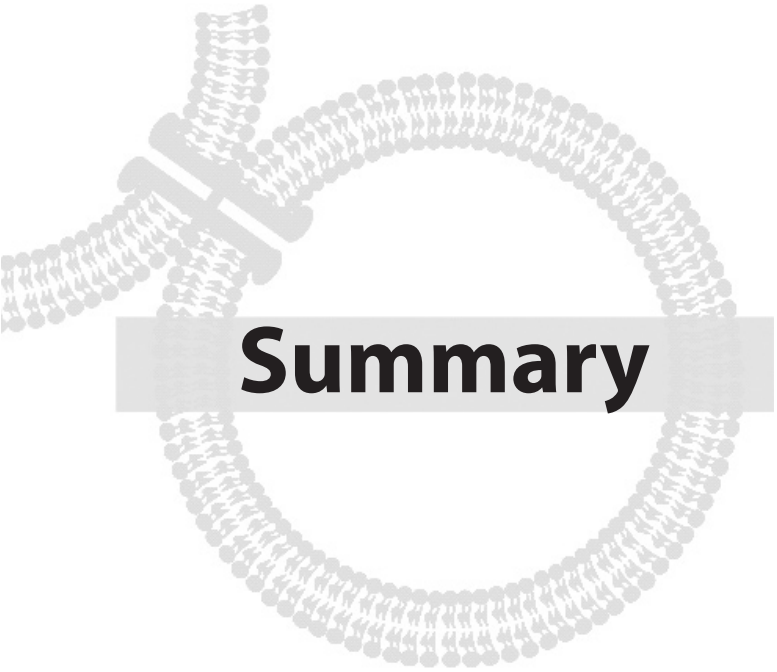


Summary

Summary

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.

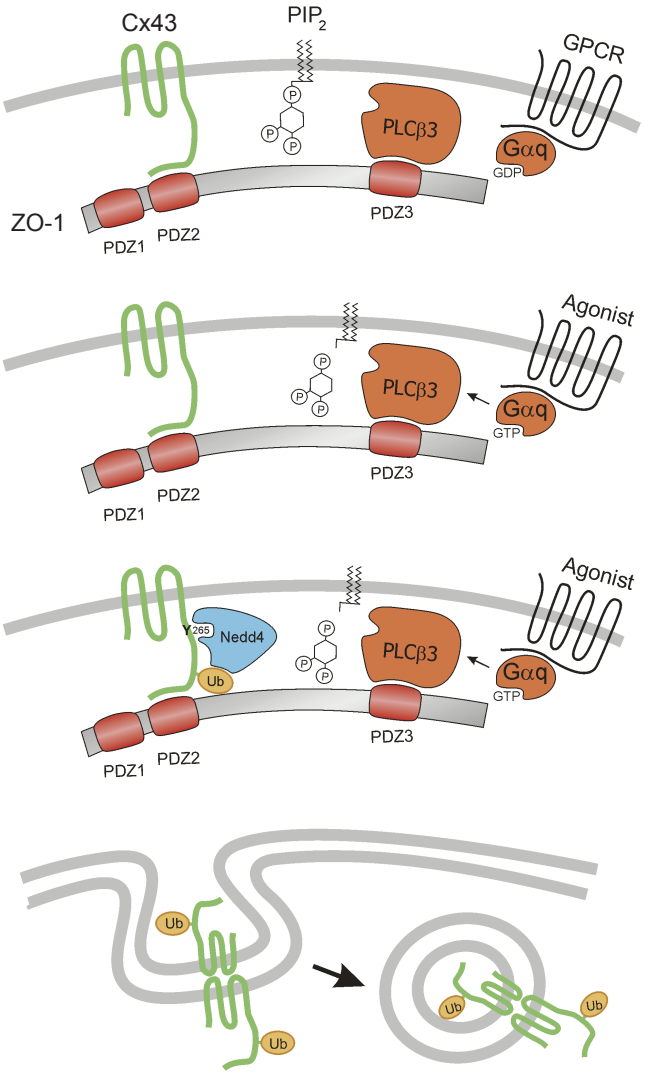


Figure 1. Proposed model: Cx43-based GJC is inhibited by GPCR signalling in two steps.
In this model, ZO-1 recruits PLCβ3 to the Cx43 gap junction plaque. Upon activation of a Gαq coupled receptor, PLCβ3 becomes activated and hydrolyses $PI(4,5)P_2$, followed by disruption of GJC. Next, Nedd4 binds to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

Samenvatting

Samenvatting

Om een meercellig organisme goed te kunnen laten functioneren is het essentieel dat cellen met elkaar communiceren. Intercellulaire communicatie kan plaatsvinden via de uitscheiding van hormonen en groeifactoren of rechtstreeks via cel-cel contacten, bijvoorbeeld via zogenaamde "gap junctions". Gap junctions zijn groepen kanaaltjes in de plasmamembraan die een directe verbinding vormen tussen het cytoplasma van aangrenzende cellen. Door deze kanalen kunnen kleine moleculen zich van de ene naar de andere cel verspreiden. Op deze manier blijven cellen op de hoogte van het doen en laten van hun directe buren en kunnen ze zelfs elkaars gedrag beïnvloeden. Ontregeling van gap junction communicatie (GJC) wordt geassocieerd met verschillende ziektes, met name kanker maar bijvoorbeeld ook hartritmestoornissen.

De bouwstenen van gap juction kanalen zijn de connexine eiwitten. Zes connexines vormen een ring die de helft van het uiteindelijke kanaal vormt. Zo'n half kanaal wordt ook wel connexon genoemd. Om een kanaal te kunnen vormen met een buurcel, wordt een connexon eerst naar de plasmamembraan getransporteerd. Wanneer een connexon in de ruimte tussen twee cellen een connexon van de buurcel tegenkomt, wordt er een chemische verbinding gevormd tussen de connexons, hetgeen resulteert in een functioneel kanaal. Er zijn 21 verschillende connexines in de mens. De meest voorkomende en best onderzochte is connexine43 (Cx43).

De mate van GJC kan op verschillende manieren gereguleerd worden. Bijvoorbeeld door het reguleren van de hoeveelheid connexine eiwit, maar ook door bestaande connexines te modificeren.
Het onderzoek dat beschreven wordt in dit proefschrift richt zich hoofdzakelijk op de regulatie van Cx43 GJC door externe signalen die aangrijpen op zogenaamde G eiwit-gekoppelde receptoren (GPCRs).

Cx43 is het belangrijkste connexine in het hart en communicatie via Cx43 gap junctions is essentieel voor het doorgeven van de actiepotentiaal, waardoor het hele hart in hetzelfde ritme klopt. Een hartaanval kan veroorzaakt worden door vaatvernauwende stoffen zoals angiotensine of endothelines, die werken op GPCRs. Activatie van bepaalde GPCRs leidt tot een tijdelijke remming van GJC.
De tijdelijke sluiting van Cx43 gap junctions speelt een belangrijke rol in het beperken van de schade aan het hart tijdens een hartaanval. Het sluiten van de gap junctions beperkt de verspreiding van moleculen die aanzetten tot celdood door het hartweefsel. Het nadeel van het remmen van Cx43 GJC is dat er hartritmestoornis-

sen kunnen ontstaan. Het is dus van groot belang dat een cel de communicatie via Cx43 gap junctions zeer nauwkeurig kan reguleren.De belangrijkste vraag die aan de basis van dit proefschrift ligt is hoe receptorstimulatie leidt tot remming van Cx43-gebaseerde GJC en welke factoren de signaleringsroute vormen tussen GPCR en Cx43.

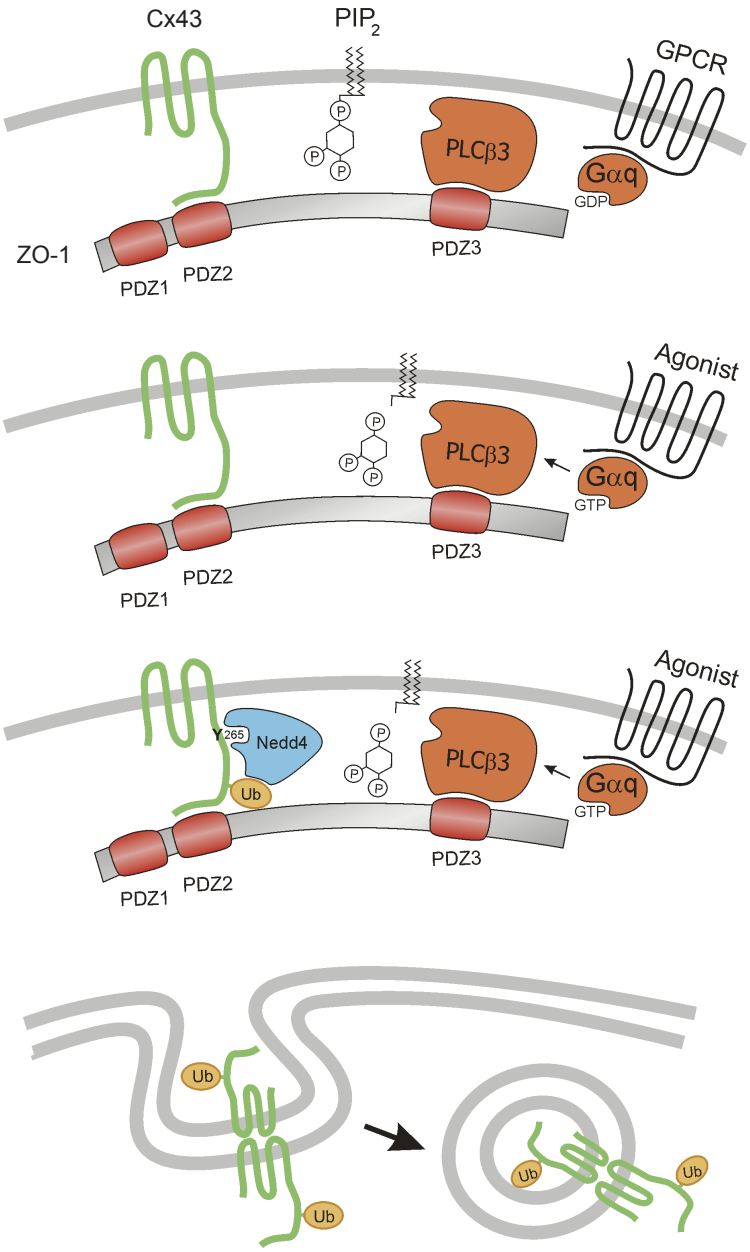
Hoofdstuk 1 geeft een algemene introductie over gap junctions en wat er bekend is van de regulatie van communicatie via Cx43 gap junctions.

In *hoofdstuk 2* tonen we aan dat de verwijdering van fosfolipide PIP_2 van de plasmamembraan noodzakelijk en voldoende is om Cx43 gebaseerde cel-cel communicatie te remmen. Verder hebben we vastgesteld dat de Cx43 bindingspartner ZO-1 essentieel is voor regulatie van GJC. ZO-1 is een eiwit dat voornamelijk bestaat uit eiwit-eiwit interactie domeinen. Naast Cx43 bindt ZO-1 ook aan PLCβ3, een eiwit dat PIP_2 kan afbreken. ZO-1 functioneert dus als een zogenaamde "scaffold" die Cx43 en PLCβ3 bij elkaar brengt, waardoor PIP_2 afbraak locaal geregeld kan worden (Fig.1).

Nadat een eiwit gevormd is kan het, wanneer de cel daar behoefte aan heeft, gemodificeerd worden door een verandering aan een of meer aminozuren. Een manier om de functie van een eiwit te modificeren is fosforylering van een aminozuur. Een kinase bindt een fosfaat groep aan het aminozuur, waardoor het betreffende eiwit andere eigenschappen krijgt. Hierdoor verandert bijvoorbeeld de vorm van het eiwit, of het verandert de herkenning door andere eiwitten. Op deze manier kan een eiwit anders gaan functioneren.

In *hoofdstuk 3* hebben we het belang van de Cx43 C-terminale staart in de regulatie van GJC onderzocht. Eerdere studies hebben aangetoond dat modificatie van Cx43 op bepaalde aminozuren kan leiden tot het remmen van communicatie. Fosforylering van de Cx43 C-terminale staart op aminozuren S368, Y247 en Y265, en/of Cx43 internalisering via een internalisatiemotief rond residu Y286 worden gezien als belangrijkste regulerende gebeurtenissen. Door deze aminozuren afzonderlijk te muteren kunnen we vaststellen hoe belangrijk ze zijn.

Wij bestudeerden Cx43 mutanten door ze tot expressie te brengen in cellen waarin de expressie van endogeen Cx43 geblokkeerd is. We vinden dat na receptorstimulatie de kanalen die gevormd worden door Cx43 mutant Y265F open zijn, dit in tegenstelling tot endogeen Cx43 en de andere mutanten. Hoewel residu Y265 gefosforyleerd kan worden, vinden we geen toename in de fosforylering na receptor stimulatie. Bovendien wordt de remming van de communicatie niet beïnvloed



Figuur 1. Voorgesteld model: Communicatie via Cx43 kanalen wordt geremd door GPCR agonisten in twee stappen.
ZO-1 bindt PLCβ3 via PDZ domein interacties. Na activatie van Gαq door een receptor agonist wordt PLCβ3 geactiveerd en PIP_2 afgebroken. Dit leidt tot remming van GJC. In de tweede stap bindt Cx43 aan Nedd4 en wordt geubiquitineerd. Cx43 residu Y265 is onmisbaar voor de binding van Nedd4 aan Cx43 en voor Cx43 ubiquitinering. Cx43 ubiquitinering leidt tot de internalisatie van de gap junction en langdurige remming van GJC.

door kinase remmers. Dit samen maakt het onwaarschijnlijk dat fosforylering van Cx43 op Y265 belangrijk is voor het remmen van communicatie.
Behalve dat Y265 gefosforyleerd kan worden, maakt het ook deel uit van een motief dat internalisatie kan uit de membraan bewerkstelligen. Verdere mutaties in dit motief hadden echter geen invloed op de sluiting van de Cx43 kanalen. We concluderen dat aminozuur Y265 essentieel is voor remming van cel-cel communicatie en dat Y265 daarbij een structurele rol speelt.

Een andere belangrijke vorm van eiwitmodificatie is ubiquitinering. In dit geval wordt er een klein eiwit, ubiquitin, aan het eiwit geplakt (geligeerd). De enzymen die hiervoor verantwoordelijk zijn heten ubiquitin ligases. Door ubiquitinering kan de bestemming van een eiwit veranderd worden. Ubiquitinering kan bijvoorbeeld een signaal zijn om membraaneiwitten te internaliseren en vervolgens af te breken.

In *hoofdstuk 4* hebben we de mogelijke rol van ubiquitinering bij de regulatie van communicatie via Cx43 gap junctions onderzocht.
Wij hebben gevonden dat Cx43 geubiquitineerd wordt door ubiquitin ligase Nedd4 na stimulatie van GPCRs. Het blijkt dat Cx43 residu Y265 van essentieel belang is voor de interactie van Cx43 met Nedd4 en voor ubiquitinering. We vinden dat remming van GJC in twee fasen gebeurt. Zoals beschreven in hoofdstuk 2, gaan tijdens de eerste fase de gap junctions dicht als gevolg van de afbraak van PIP_2. De tweede fase wordt verzorgd door Nedd4 en ubiquitinering van Cx43 wat leidt tot internalisatie van Cx43 vanaf de plasmamembraan en interruptie van cel-cel commicatie.

Bovengenoemde hoofdstukken geven nieuwe inzichten in de remming van cel-cel communicatie door GPCR agonisten. Onze resultaten suggereren een model waarin GJC in twee fasen geremd wordt. Allereerst de sluiting van de Cx43 kanalen door afbraak van PIP_2. Vervolgens, in de tweede fase, worden de Cx43 eiwitten geïnternaliseerd als gevolg van Cx43 ubiquitinering. Deze tweede fase verlengt de remming van GJC tot ongeveer 30 minuten. Wij stellen een model voor waarin zowel afbraak van PIP_2 als Cx43 ubiquitinering noodzakelijk zijn voor internalisering en dus remming van GJC (Fig. 1).

Ten slotte, zoals beschreven in h*oofdstuk 5*, onderzochten we de invloed van Cx43 op het vermogen van cellen om in een bepaalde richting te bewegen, ofwel migreren. Cellen zonder Cx43 migreren langzamer dan cellen met Cx43. We vonden echter dat het effect van Cx43 op celmigratie onafhankelijk is van GJC. Cellen zonder Cx43 hebben tevens een lagere expressie van cel-cel contact eiwit N-cadherin. Het is bekend dat N-cadherin expressie belangrijk is voor celmigratie. We tonen

aan dat gebrek aan N-cadherin expressie alleen, zonder Cx43 expressie en GJC te beïnvloeden, voldoende is om de cellen net zo langzaam te laten migreren als de cellen zonder Cx43. Kortom, het effect van Cx43 op celmigratie is waarschijnlijk een indirect effect door regulatie van de N-cadherin expressie. Deze studie ondersteunt de hypothese dat Cx43 meer functies heeft dan alleen die van een kanaalvormend eiwit.

List of abbreviations

Cx43	connexin43
ET	endothelin
GJC	gap junctional communication
GPCR	G protein-coupled receptor
$PI(4,5)P_2$	phosphatidylinositol 4,5-bisphosphate
PLC	phospholipase C
RNAi	RNA interference
shRNA	short hairpin RNA
TRP	thrombin receptor activating peptide

Curriculum vitae

Leonie van Zeijl werd geboren op 6 februari 1975 te 's Hertogenbosch. in 1993 behaalde zij haar Gymnasium diploma aan de RSG Magister Alvinus te Sneek. Vervolgens begon zij met de studie Biologie aan de Universiteit Utrecht. Tijdens haar eerste stage bij de faculteit diergeneeskunde (vakgroep Veterinaire Farmacologie) in Utrecht onder begeleiding van Dr. Els de Groene deed zij onderzoek aan het project "Detectie van mutaties in tumor suppressor gen p53". Voor haar tweede stage reisde zij af naar Stockholm om daar bij het Karolinska Instituut (Division of Biochemical Toxicology) onder leiding van Dr. Ian Cotgreave onderzoek te doen aan het project "Differential regulation of two polymorphic variants of Cx37". Het doctoraal diploma Biologie werd behaald in juni 1999. Tot het eind van dat jaar bleef zij verbonden aan haar tweede stage plek en heeft nog 2 maanden aan de Universiteit van Chicago (Department of Pediatrics, Section of Hematology/Oncology) doorgebracht in de groep van Dr. Eric Beyer. In maart 2000 begon zij als AIO aan de VU, op de afdeling Angiogenese. In november van hetzelfde jaar is zij begonnen aan het promotieonderzoek dat in dit proefschrift staat beschreven op het Nederlands Kanker Instituut, afdeling Cellulaire Biochemie, in de groep van Prof. Dr. Wouter Moolenaar. Momenteel is zij in dezelfde groep werkzaam als postdoc op de afdeling Celbiologie I van het Nederlands Kanker Instituut.

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Leonie