Introduction

Communication between adjacent cells through gap junctions occurs in nearly every tissue and is fundamental to coordinated cell behavior. 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 kD), including nutrients, metabolites, second messengers, and peptides, without transit through the extracellular space (Goodenough et al., 1996; Harris, 2001; Saez et al., 2003). Gap junctions play important roles in normal tissue function and organ development (Reaume et al., 1995; Sohl and Willecke, 2004; Wei et al., 2004) and have been implicated in a great diversity of biological processes, notably, electrical synchronization of excitable cells, energy metabolism, growth control, wound repair, tumor cell invasion, and antigen cross-presentation (Kwak et al., 2001; Qiu et al., 2003; Mesnil et al., 2005; Oliveira et al., 2005; Neijssen et al., 2005; Bernstein and Morley, 2006; Mori et al., 2006). 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 (Gerido and White, 2004; Wei et al., 2004).

Connexin43 (Cx43) is the most abundant and best-studied mammalian connexin. Cx43-based gap junctional communication is of a particular interest because it is regulated by both physiological and pathophysiological stimuli. In particular, Cx43-based...
cell–cell coupling is rapidly disrupted after stimulation of certain G protein–coupled receptors (GPCRs), such as those for endothelin, thrombin, nucleotides, and bioactive lipids (Hill et al., 1994; Venance et al., 1995; Postma et al., 1998; Spinella et al., 2003; Meme et al., 2004; Blomstrand et al., 2004; Rouach et al., 2006). Disruption is transient, as communication is restored after ~20–60 min, depending on the GPCR involved (Postma et al., 1998). GPCR-mediated inhibition of intercellular communication will have broad consequences for long-range signaling 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, PKC, MAP kinase, and c-Src, but the results remain ambiguous (Lampe and Lau, 2004; Warn-Cramer and Lau, 2004; Laird, 2005). One of the difficulties with unraveling the regulation of Cx43 channel function is that Cx43 functions in a multiprotein complex that is currently ill understood (Giepmans, 2004). One established component of this assembly is the scaffold protein zona occludens 1 (ZO-1), which binds directly to the C terminus of Cx43 via its second PDZ domain (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998). 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 (Hunter et al., 2005; Laing et al., 2005).

In the present study, we sought to identify the signaling 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)P₂) 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)P₂-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)P₂.

**Results**

**Regulation of Cx43 gap junctional communication by the Goαᵢ/PLCβ–PtdIns(4,5)P₂ hydrolysis pathway**

Rat-1 fibroblasts are ideally suited for studying Cx43 channel function because they express Cx43 as the only functional connexin (Postma et al., 1998; Ponsioen et al., 2007). Stable knockdown of Cx43 expression (using pSuper short hairpin RNA [shRNA]) resulted in a complete loss of intercellular communication, consistent with Cx43 being the only functional gap junction protein in Rat-1 cells (Fig. 1 A). Fig. 1 B shows that the Cx43 binding partner ZO-1 retains its submembranous localization in Cx43-knockdown cells.

To assess which G proteins mediate inhibition of gap junctional communication, we introduced active versions of Goα₁₂, Goα₁₃, and Goα₁₃ subunits into Rat-1 cells and examined their impact on cell–cell coupling. Expression of active Goα₁ resulted in complete inhibition of intercellular communication, whereas active Goα₁₂, Goα₁₃, and Goα₁₃ left cell–cell coupling unaltered, as indicated by Lucifer yellow (LY) diffusion and electrophysiological assays (Fig. 2 A). Disruption of gap junctional communication induced by active Goα₁ was persistent, as opposed to the transient inhibition observed after GPCR stimulation (Postma et al., 1998). Similarly, treatment of Rat-1 cells with *Pasteurella multocida* toxin, a direct activator of Goα₃ (Orth et al., 2005), caused persistent abrogation of cell–cell coupling (Fig. 2 B). Goα₁ couples to PLCβ to trigger PtdIns(4,5)P₂ hydrolysis, leading to production of the second messengers inositol-1,4,5-trisphosphate (IP₃) and DAG (Rhee, 2001). We monitored PtdIns(4,5)P₂ in living cells by using a GFP fusion protein of the pleckstrin homology (PH) domain of PLCβ1 (GFP-PH) as a probe (Stauffer et al., 1998; Varnai and Balla, 1998; van der Wal et al., 2001). We monitored PtdIns(4,5)P₂ in living cells by using a GFP fusion protein of the pleckstrin homology (PH) domain of PLCβ1 (GFP-PH) as a probe (Stauffer et al., 1998; Varnai and Balla, 1998; van der Wal et al., 2001). In control cells, the PtdIns(4,5)P₂ probe was concentrated at the plasma membrane. In cells expressing active Goα₁, however, the probe was spread diffusely throughout the cytosol, indicative of PtdIns(4,5)P₂ depletion from the plasma membrane (Fig. 2 C). Although these results are consistent with Goα₁ mediating agonist-induced inhibition of intercellular communication, they should be interpreted with caution because...
Figure 2. Activated Gαq disrupts Cx43-based gap junctional communication: correlation with PtdIns(4,5)P2 depletion. (A) Intercellular communication in Rat-1 cells transfected with various activated (GTPase-deficient) Gα subunits. (top) Electrical cell–cell coupling measured by a single patch-clamp electrode (Postma et al., 1998). Whole-cell current responses to 10-mV voltage pulses (duration, 100 ms; holding potential, −70 mV) were recorded from confluent Rat-1 cells. Note the dramatic increase in cellular input resistance (i.e., a decrease in conductance) by activated Gαs but not other Gα subunits. (middle and bottom) Rat-1 cells cotransfected with active Gαs and GFP (10:1 ratio). GFP-positive cells were microinjected with LY, and dye diffusion from the microinjected cells was monitored. Wide-field pictures of GFP and LY diffusion as indicated. Bars, 10 μm. (B) Disruption of gap junctional communication by 1 μg/ml P. multocida endotoxin (PMT; 3-h preincubation), an activator of Gαs, as measured by LY diffusion. Bar, 20 μm. (C) Depletion of PtdIns(4,5)P2 from the plasma membrane by activated Gαq. HEK293T cells were transfected with the PtdIns(4,5)P2 sensor GFP-PH, alone or together with active Gαq (1:10 ratio). GFP-PH localizes to the plasma membrane, where it binds PtdIns(4,5)P2 (left). Coexpression with activated Gαq causes GFP-PH to relocate to the cytosol (right). (D) Monitoring PtdIns(4,5)P2 levels (PIP2; red trace) and intercellular communication (black; n ≥ 20 per time point) in Rat-1 cells after addition of 50 nM endothelin (top) or 50 μM TRP (bottom). Data points show the percentage of injected cells that spread LY to their neighbors. Temporal changes in plasma membrane–bound PtdIns(4,5)P2 were measured by changes in FRET between CFP-PH and YFP-PH. Ionomycin, which evokes an immediate constitutive depletion of PtdIns(4,5)P2 from the plasma membrane promotes apoptosis (Kranenburg et al., 1999; Halstead et al., 2006).

To monitor the kinetics of PtdIns(4,5)P2 hydrolysis and resynthesis with high temporal resolution, we made use of the fluorescence resonance energy transfer (FRET) between the PH domains of PLCδ1 fused to CFP and YFP, respectively (van der Wal et al., 2001). When bound to plasma membrane PtdIns(4,5)P2, CFP-PH and YFP-PH are in close proximity and show FRET. After PtdIns(4,5)P2 breakdown, the probes dilute out into the cytosol, and FRET ceases. The prototypic Gq-coupled receptor agonist endothelin, acting through endogenous endothelin A receptors, induced an acute and substantial decrease in PtdIns(4,5)P2, reaching a maximum after 30–60 s; thereafter, PtdIns(4,5)P2 slowly recovered to near basal levels over a period lasting as long as 45–60 min (Fig. 2 D, top, red trace). Sustained PtdIns(4,5)P2 hydrolysis by endothelin A receptors has been reported previously (Cramer et al., 1997) and may be explained by the fact that activated endothelin A receptors follow a recycling pathway back to the cell surface rather than the lysosomal degradation route (Bremnes et al., 2000). The kinetics of endothelin-induced inhibition and recovery of cell–cell communication followed those of PtdIns(4,5)P2 hydrolysis and resynthesis, respectively, with communication being restored after ~75 min (Fig. 2 D, top, black trace).

More transient PtdIns(4,5)P2 depletion and recovery kinetics were observed with a thrombin receptor (PAR-1 [protease-activated receptor 1]) activating peptide (TRP), which correlated with a more short-lived inhibition of gap junctional communication (Fig. 2 D, bottom). Furthermore, a desensitization-defective mutant NK2 receptor (for neurokinin A) that mediates prolonged PtdIns(4,5)P2 hydrolysis (Alblas et al., 1996) inhibits gap junctional communication for prolonged periods of time when compared with the wild-type NK2 receptor (Postma et al., 1998). Although these results reveal a close correlation between the duration of PtdIns(4,5)P2 depletion and that of communication shutoff, we note that the restoration of cell–cell communication consistently lagged behind the recovery of PtdIns(4,5)P2 levels. Nevertheless, our findings strongly suggest that the Gq/PLCβ-mediated hydrolysis and subsequent resynthesis of PtdIns(4,5)P2 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; Rhee, 2001). 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. 3 A and not depicted). We stably suppressed PLCβ3 expression using the pSuper shRNA expression vector (Brummelkamp et al., 2002). Four different target sequences were selected to correct for clonal variation and off-target effects. Immunoblot analysis shows a marked reduction and complete depletion of PtdIns(4,5)P2 from the plasma membrane when applied at high doses (5 μM), together with 5 mM Ca2+ (van der Wal et al., 2001) was used for calibration.
control cells, agonist-induced PtdIns(4,5)P$_2$ breakdown was
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pared 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. 3 B). PLCβ3-
knockdown cells showed normal basal cell–cell communication
but failed to shut off cell–cell communication after GPCR stim-
ulation (Fig. 3 C). 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 G$_q$/PLCβ3 pathway.

PLC-mediated PtdIns(4,5)P$_2$ hydrolysis generates the sec-
ond messengers IP$_3$ and DAG, leading to Ca$^{2+}$ mobilization
and PKC activation, respectively. Previous pharmacological
studies have already suggested that neither Ca$^{2+}$ nor PKC have
a critical role in GPCR-mediated inhibition of cell–cell coupling
(Postma et al., 1998), a notion supported by additional experi-
ments using “caged” IP$_3$, the cell-permeable Ca$^{2+}$ chelator
BAPTA-AM (1,2-bis[2-aminophenoxy]ethane-N,N,N,N-tetra-
acetic acid), and a PKC-activating bacterial PLC (van Dijk
et al., 1997; Table S1, available at http://www.jcb.org/cgi/content/
full/jcb.200610144/DC1). Whether PtdIns(4,5)P$_2$-derived sec-
ond messengers are dispensable for Cx43 channel closure upon
GPCR activation remains debatable, however, as the supporting
pharmacological evidence is indirect.

**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-phosphatase (5-ptase; Suh et al., 2006; Varnai
et al., 2006). The method is based on the rapamycin-induced
heterodimerization of FRB (fragment of mammalian target of
rapamycin that binds FKBP12) and FKBP12 (FK506 binding pro-
tein 12), as schematically illustrated in Fig. 4 A. In this approach,
a mutant version of 5-ptase with a defective membrane targeting
domain (CAAX box) is fused to FKBP12 and tagged with mono-
meric red fluorescent protein (mRFP), whereas its binding part-
ner FRB (fused to CFP) is tethered to the plasma membrane
through palmitoylation (construct PM-FRB-CFP; Varnai et al.,
2006). In the absence of rapamycin, 5-ptase resides in the cyto-
sol and leaves PtdIns(4,5)P$_2$ levels at the plasma membrane un-
altered (Fig. 4 A, left). Upon addition of 100 nM rapamycin,
FKBP12 and FRB undergo heterodimerization and the 5-ptase is
recruited to the plasma membrane (Fig. 4 A, right).

We expressed the mRFP–FKBP12–5-ptase and PM-FRB-
CFP fusion proteins in Rat-1 cells and confirmed their proper
intracellular localization by confocal microscopy (unpublished
data). Addition of rapamycin caused a rapid and complete deple-
tion 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. 4,
B and C, top trace; n ≥ 25 for each dataset). LY injections were
done at 2 min after addition of agonist.

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. Ex-
pression of PLCβ3 in Rat-1 cells expressing a nonfunctional 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 after thrombin receptor stimulation of normal (red trace) and PLCβ3-
deficient Rat-1 cells (blue trace). TRP was used at 50 μM, and ionomycin
was used at 5 μM, n = 5. (C) Bar graphs showing the percentage of com-
municating cells in control and PLCβ3-knockdown cells (clone 1) treated
with either 50 nM endothelin (Et) or 50 μM TRP, as indicated (n ≥ 25 for
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tion 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. 4,
B and C, top trace; n = 10). As expected, no Ca$^{2+}$ signal was
detected after the 5-ptase–mediated conversion of PtdIns(4,5)P$_2$
in PtdIns(4)P (Fig. 4 C; n = 4). To determine how the 5-ptase–
induced hydrolysis of PtdIns(4,5)P$_2$ affects gap junctional commu-
nication, we measured the intercellular diffusion of calcine
(added as membrane-permeable calcine-AM) using FRAP
(Ponsioen et al., 2007; Fig. 4 D). Rat-1 cells expressing mRFP–
FKBP12–5-ptase and PM-FRB-CFP showed efficient intercellular
transfer of calcine. At 2 min after rapamycin addition, however,
intracellular dye diffusion was inhibited as inferred from a strongly
reduced fluorescence recovery rate (Fig. 4 D; n = 15; P < 0.005;
~0.25× the recovery rate before rapamycin addition). The recovery
of calcine fluorescence could not be decreased any further by addi-
tion of 50 μM of the gap junction blocker 2-aminoethoxy-
diphenylborane (Ponsioen et al., 2007; Fig. 4 D). Rapamycin did
not affect cell–cell communication in nontransfected cells (un-
published data). Thus, PtdIns(4,5)P$_2$ depletion by 5-phosphatase
activation is sufficient to inhibit Cx43 gap junctional communica-
tion, with no need for PtdIns(4,5)P$_2$-derived second messengers.
Overexpression of PtdIns(4,5)P₂ 5-kinase (PIPK5) prevents inhibition of cell-cell communication

PtdIns(4,5)P₂ at the plasma membrane is generated mainly from PtdIns(4)P by PIP5K (Hinchliffe et al., 1998; Anderson et al., 1999). As a further test of the PtdIns(4,5)P₂ hypothesis, we stably overexpressed PIP5K (type Ia, fused to GFP) in Rat-1 cells in an attempt to prevent PtdIns(4,5)P₂ depletion after GPCR stimulation (Fig. 5 A). As shown in Fig. 5 B, transfected GFP-PIP5K localizes to the plasma membrane. In the PIP5K-overexpressing cells, PtdIns(4,5)P₂ levels remain elevated (i.e., above FRET threshold levels) after agonist addition (Fig. 5 C). Nonetheless, GPCR agonists still induced transient rises in IP₃ and Ca²⁺ (Fig. 5 C), indicating that excessive synthesis of PtdIns(4,5)P₂ 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. 5 C). 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)P₂ depletion (Fig. 2 D). Expression of a kinase-dead version of PIP5K had no effect on either PtdIns(4,5)P₂ hydrolysis or inhibition of cell–cell communication (Fig. 5 D). We conclude that Cx43 channel closure is prevented when PtdIns(4,5)P₂ is maintained at adequate levels.

No detectable PtdIns(4,5)P₂ binding to the C-terminal tail of Cx43

PtdIns(4,5)P₂ can modulate the activity of various ion channels and transporters, apparently through direct electrostatic interactions (Hilgemann et al., 2001; Suh and Hille, 2005). By analogy, regulation of Cx43 channels by PtdIns(4,5)P₂ would imply that basic residues in Cx43 bind directly to the negatively charged PtdIns(4,5)P₂. Indeed, the regulatory cytosolic tail of Cx43 (aa 228–382) contains a membrane-proximal stretch of both basic and hydrophobic residues (21VFFKGVKDR/VKR243) that could constitute a potential PtdIns(4,5)P₂ binding site. Local depletion of PtdIns(4,5)P₂ 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)P₂, mutations within this domain might interfere with PtdIns(4,5)P₂-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,R243A; K237A,K241A; R239A,K241A,R243A; K237A,R239A,K241A; and the “4A” mutant, K237A,R239A,K241A,R243A. When expressed in Cx43-deficient cells, however, all these mutants were trapped intracellularly and failed to localize to the plasma membrane (Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200610144/DC1). Although 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)P₂ interaction hypothesis.

We next examined whether PtdIns(4,5)P₂ 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 (PLCβ1) was used as a positive control. In the first approach, agarose beads coated with GST-PH were never observed (n = 15; P < 0.005). The gap junction blocker 2-aminoethoxy-diphenylborane (2-APB) was added at 50 μM.
either PtdIns(4,5)P_2 or PtdIns(4)P were incubated with GST-Cx43CT or GST alone and then pulled down by centrifugation. PtdIns(4,5)P_2 beads readily brought down the GST-PH polypeptide but not GST-Cx43CT (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200610144/DC1). Second, we incubated GST-Cx43CT with ^32P-labeled PtdIns(4,5)P_2 and examined the ability of excess phosphoinositides to displace bound ^32P-PtdIns(4,5)P_2. Although 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, as indicated (Fig. S2 B). Finally, we found that PtdIns(4,5)P_2 (and other phosphoinositides) immobilized on nitrocellulose strips failed to bind either Cx43CT or a 35-amino-acid juxtamembrane domain peptide (Cx43JM; aa 228–263; Giepmans et al., 2001b; unpublished data). Thus, PtdIns(4,5)P_2 does not detectably bind to the juxtamembrane domain of Cx43, or 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. 1 B). Conversely, when ZO-1 expression was knocked down by shRNA, Cx43 levels were unaltered (Fig. 6 A). ZO-1–knockdown Rat-1 cells retained their fibroblastic morphology and showed normal Cx43 punctate staining and cell–cell coupling (Fig. 6, B and 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. 6 C). Importantly, overall PtdIns(4,5)P_2-dependent Ca^{2+} mobilization was not affected in the ZO-1–knockdown cells (Fig. 6 D). We conclude that ZO-1 is essential for the regulation of gap junctional communication by Gs/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.
Acting as a signaling molecule in its own right, PtdIns(4,5)P2 can regulate local cellular activities when its levels rise and fall; in particular, PtdIns(4,5)P2 can modulate the activity of Cx43 binding partner ZO-1 binds to PLCβ3 and is essential for PtdIns(4,5)P2-hydrolyzing receptors to regulate gap junctional communication.

**Discussion**

A critical and long-standing question in gap junction biology is how junctional communication is regulated by physiological and pathophysiological stimuli. Relatively little progress has been made in identifying receptor-induced signaling 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 signaling has not been systematically examined to date. In the present study, we identify the Gq-linked PLCβ–PtdIns(4,5)P2 hydrolysis pathway as a key regulator of Cx43-based gap junctional communication in normal fibroblasts. We demonstrate that loss of PtdIns(4,5)P2 from the plasma membrane is necessary and sufficient to close Cx43 channels, without a role for PtdIns(4,5)P2-derived second messengers. In other words, PtdIns(4,5)P2 itself is the responsible signaling molecule. A second novel finding is that the PtdIns(4,5)P2 as a key regulator

Our conclusion that PtdIns(4,5)P2 at the plasma membrane regulates Cx43 channel function is based on several lines of evidence. First, active Gq (but not Gα13, or Gα12, or Gα11) depletes PtdIns(4,5)P2 from the plasma membrane and abrogates gap junctional communication. Second, knockdown of PLCβ3 inhibits agonist-induced PtdIns(4,5)P2 depletion and prevents disruption of cell–cell communication. Third, conversion of PtdIns(4,5)P2 into PtdIns(4)P by a translocatable 5-phosphatase is sufficient to inhibit intercellular communication. Fourth, maintaining PtdIns(4,5)P2 at adequate levels by overexpression of PIP5K renders Cx43 channels refractory to GPCR stimulation, although second messenger generation still occurs. Acting as a signaling molecule in its own right, PtdIns(4,5)P2 can regulate local cellular activities when its levels rise and fall; in particular, PtdIns(4,5)P2 can modulate the activity of...
Various ion channels and transporters, presumably through electrostatic interactions (Hilgemann et al., 2001; Suh and Hille, 2005). Although the existence of such interactions in living cells remains largely inferential and PtdIns(4,5)P₂ binding consensus sequences have not been clearly defined, the common theme is that the negatively charged PtdIns(4,5)P₂ binds to a motif with multiple positive charges interdispersed with hydrophobic residues (Janmey et al., 1992; McLaughlin and Murray, 2005). The Cx43 C-terminal juxtamembrane domain indeed contains such a putative PtdIns(4,5)P₂ binding motif (aa 231–243), although this stretch also meets the criteria of a tubulin binding domain (Giepmans et al., 2001b). Extension of the above model to Cx43 channel gating would then imply that local loss of PtdIns(4,5)P₂ 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)P₂ 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)P₂ does bind directly to Cx43 in situ.

Aside from modulating ion channel activity, PtdIns(4,5)P₂ has been implicated in cytoskeletal remodeling, vesicular trafficking, and recruitment of cytosolic proteins to specific membranes (Yin and Janmey, 2003; McLaughlin and Murray, 2005). Although Cx43 can interact with cytoskeletal proteins, such as tubulin and drebrin (Giepmans et al., 2001b; Butkevich et al., 2004), cytoskeletal reorganization does not play a significant role in regulating Cx43 junctional communication because cytoskeleton-disrupting agents (cytochalasin D, nocodazole, and Rho-inactivating C3 toxin) have no detectable effect on GPCR regulation of cell–cell coupling (Giepmans et al., 2001b; 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 (Lampe and Lau, 2004; Laird, 2005). 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 (Postma et al., 1998; Giepmans et al., 2001a). 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 (Table S1; Rouach et al., 2006). Therefore, tyrosine phosphorylation of Cx43 leading to loss of cell–cell coupling, as observed with constitutively active c-Src and v-Src (Warn-Cramer and Lau, 2004), 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 (Giepmans and Mooallem, 1998; Toyofuku et al., 1998). Originally identified as a major component of epithelial tight junctions (Stevenson et al., 1986), 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 -2 act redundantly to some extent in the formation of tight junctions (Umeda et al., 2004; Rouach et al., 2006). In nonepithelial cells lacking tight junctions, ZO-1 has been attributed a role in the assembly and stabilization of Cx43 gap junctions (Hunter et al., 2005; Singh et al., 2005), but its precise role has remained elusive. Our knockdown studies herein show that ZO-1 is essential for G_{i/o}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)P₂, as schematically illustrated in Fig. 8. As we found no evidence for direct binding of PtdIns(4,5)P₂ to Cx43 in vitro, PtdIns(4,5)P₂ might regulate junctional communication in an indirect manner, for example, via a Cx43-associated protein that modulates the Cx43 regulatory tail and thereby shuts off channel function. Precisely how PtdIns(4,5)P₂ regulates the Cx43 multiprotein complex remains a challenge for future studies.
Materials and methods

Reagents

Materials were obtained from the following sources: endothelin, TRP (sequence SFLRNL), neurokinin A, Cx43 polyclonal, and α-tubulin monoclonal antibodies from Sigma-Aldrich; P. multocida toxin from Calbiotech-Novabiochem; Cx43 NT monoclonal antibody from the Fred Hutchinson Cancer Research Center; actin monoclonal from Chemicon International; polyclonal PLCβ antibody from Cell Signaling; ZO-1 monoclonal antibody from Zymed Laboratories; HRP-conjugated secondary antibodies from DakoCytomation, and secondary antibodies for immunofluorescence (goat anti-mouse [Alexa488] and goat anti-rabbit [Alexa594]) from Invitrogen. Hα, Myc, and GST monoclonal antibodies were purified from hybridoma cell lines 12CA5, 9E10, and 2F3, respectively. GFP antisera was generated in our institute.

cDNA constructs

Constructs encoding active (GTPase-deficient) Gα subunits, EGFP-PLCβ1, ECFP-PLCβ1, EYFP-PLCβ1, and EFGP-tagged mouse type 1a PIP5K have been described (Kranenburg et al., 1999; van der Wal et al., 2001; van Horck et al., 2002). Mouse PLCβ3 cDNA was obtained from MRC Genescience, cloned into pcDNA3-HA by PCR (see Table S2, available at http://www.jcb.org/cgi/content/full/jcb.200610144/DC1, for primers) and ligated into pcDNA3-HA Xhol–NotI site. HA-PLCβ3ΔN terminus was obtained by restriction of the full-length construct with Eco47II, cleaving off the very C-terminal 14 residues. Human ZO-1 was cloned into Xhol and KpnI sites of pEGFP C2 (CLONTECH Laboratories, Inc.). GFP-based yellow cameleon 2.1 has been described (van der Wal et al., 2001). Constructs encoding cytosolic 5-phosphatase fused to FK12-mRFP and PAW-FRB-CFP have been described (Varnai et al., 2006).

Cell culture and cell-cell communication assays

Cells were cultured in DMEM containing 8% fetal calf serum, glutamine, and antibiotics. For cell-cell communication assays, cells were grown in 3-cm dishes and serum starved for at least 4 h before experimentation. Monitoring the diffusion of LY from single microinjected cells and single-electrode electrophysiological measurements of cell–cell coupling were done as described previously (Postma et al., 1998). Images were acquired on an inverted microscope (Axiovert 135; Carl Zeiss Microlmaging, Inc.), equipped with an Acplan 40× objective (NA 0.60) and a camera (F301; Nikon).

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 (GE Healthcare). For immunoprecipitation, cells were harvested in 1% NP-40 lysis buffer, boiled for 10 min, and subjected to immunoprecipitation using protein A–conjugated antibodies for 4 h at 4°C. Proteins were eluted by boiling for 10 min in LSB and analyzed by immunoblotting.

Immunostaining and fluorescence microscopy

Cells 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 (Calbiotech). Confocal fluorescence images were obtained on a confocal system (TCS NT; Leica), equipped with an Ar/Kr laser. Images were taken using a 63× NA 1.32 oil objective. Standard filter combinations and Kalman averaging were used. Processing of images for presentation was done on a PC using Photoshop (Adobe).

Live imaging

All live imaging experiments were performed in bicarbonate-buffered saline containing 140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM glucose, 23 mM NaHCO3, and 10 mM Hepes, pH 7.2, kept under 5% CO2, at 37°C. Images of live cells expressing GFP-PLCβ and GFP-PIP5K were recorded on a confocal microscope (TCS-SP2; Leica) using a 63× lens (NA 1.4).

PtdIns(4,5)P2, IP3, and Ca2+ imaging by FRET ratiometry

Temporal changes in PtdIns(4,5)P2 levels in living cells were assayed by the FRET-based PtdIns(4,5)P2 sensor, PH-PLC1, as described previously (van der Wal et al., 2001). In brief, Rat-1 cells were transiently transfected with CFP-PLCβ and YFP-PLCβ constructs (1:1 ratio) using Fugene transfection agent and placed on a inverted microscope (Nikon) equipped with an Achromat 63× oil objective (NA 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 baseline. Temporal changes in IP3 levels were monitored using a FRET-based IP3 sensor, in which the IP3 binding domain of the human type 1 IP3 receptor (aa 224–605) is fused between CFP and YFP, essentially analogous to the sensor described previously (Tanigura et al., 2004). In vitro binding studies showed that it bound IP3 with apparent Kd of ∼3 nM. Intracellular Ca2+ mobilization was monitored using the CFP/YFP-based Ca2+ sensor yellow cameleon 2.1 (Miyawaki et al., 1997; Rhee, 2001; van der Wal et al., 2001). Traces were smoothed in Excel (Microsoft) using a moving average function ranging from 3 to 6.

PtdIns(4,5)P2 depletion by rapamycin-induced translocation of phosphoinositide 5-phosphatase

Rat-1 cells were transiently transfected with PM-CFP-FRB and mRFP–FKB12–5-phosphate (Varnai et al., 2006). Cells were selected for experimentation when sufficient protein levels were expressed as judged by CFP and mRFP fluorescence. For PtdIns(4,5)P2 measurements, the YFP-PLCβ construct was cotransfected. For Ca2+ measurements, cells were loaded with Oregon Green-AM. To monitor gap junctional communication, cells were loaded with calcein-AM and analyzed by FRAP (Ponsioen et al., 2007). These experiments were performed on a confocal microscope (TCS-SP2; Leica) using 63× lens (NA 1.4).

Overexpression of PIP5K

To overexpress PIP5K (type la; van Horck et al., 2002), virus containing the LZR5-PIP5K constructs was generated as described in the following paragraph. Rat-1 cells were incubated with 1 ml viral supernatant supplemented with 10 μl Dotap (Roche). 48 h after infection, cells were plated in selection medium. Transfected cells were selected on 200 μg/ml zeocin (Invitrogen) for 2 wk, and colonies were examined for PIP5K expression.

RNAi

To generate Cx43-deficient Rat-1 cells, Cx43 was knocked down by stable expression of retroviral pSuper (pr5; Brummelkamp et al., 2002) containing the RNAi target sequence GGTGTTGCGTGTAGTGCCT. pr5-Cx43 was transfected into Phoenix/Eco package cells, and the supernatant containing viral particles was harvested after 72 h. For infection, cells were incubated with 1 ml viral supernatant supplemented with 10 μl Dotap (1 mg/ml). 48 h after infection, cells were selected on 2 μg/ml puromycin for 2 wk. 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, ACTAGTCTGGCCGTGGAATA, GGATGTCGTTACTGTTGGGC, TTACGTGAAGGGGCATCAAG, and CCCCCCCGTCTCCCAAGG. Nonfunctional 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 GGAGGGCCGAGCTGGAGGAC were ligated into pSuper after oligo annealing. Next, the oligos, together with the H1 RNA promoter, were subcloned into pEn1.1 (BanH-H-Xhol) and recombined into pAd/PLDest 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.

Online supplemental material

Fig. S1 shows intracellular accumulation of the Cx43-4A mutant. Fig. S2 shows lack of detectable binding of phosphoinositides to the Cx43 Cterminal tail. Table S1 summarizes the results of pharmacological experiments. Table S2 shows sequences of the oligos used. Online supplemental material is available at www.jcb.org/cgi/content/full/jcb.200610144/DC1.

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