Role of the gap junction in ischemic preconditioning in the heart

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Abstract

The gap junction plays roles not only in electrical coupling of cardiomyocytes but also in intercellular transport of biologically active substances. Furthermore, the gap junction participates in decision-making on cell survival versus cell death in various types of cells, and a part of reperfusion injury in the heart has been indicated to be gap junction-mediated. Contribution of gap junction communication (GJC) and/or mitochondrial “hemichannels” to protective signaling during the trigger phase of ischemic preconditioning (IPC) is suggested by observations that IPC failed to protect the heart when GJC was blocked during IPC. Although ischemia suppresses both electrical and chemical GJC, chemical GJC persists for a considerable time after electrical GJC is lost. IPC facilitates ischemia-induced suppression of chemical GJC, whereas IPC delays reduction of electrical GJC after ischemia. Inhibition of GJC during sustained ischemia and reperfusion by GJC blockers mimics the effect of IPC on myocardial necrosis. IPC induces distinct effects on interaction of connexin-43 (Cx43) with protein kinases, and phosphorylation of Cx43 at Ser368 by PKC-ε is a primary mechanism of inhibition of chemical GJC by IPC. Several lines of evidence support the notion that modulation of GJC is a part of the mechanism of IPC-induced protection against myocardial necrosis and arrhythmias, though what percentage of IPC protection is attributable to inhibition of GJC during ischemia/reperfusion still remains unclear.
The gap junction consists of two hexameric connexin complexes (connexons) in the opposing membranes of neighboring cells and functions as a conduit for molecules smaller than 1 kDa with regulations on its permeability and selectivity. In the heart, the gap junction plays a crucial role in electrical coupling of cardiomyocytes, which enables physiological synchronized contraction of the atria and ventricles. Pathological reduction of gap junction conductance or reduction of gap junction proteins promotes development of arrhythmias [111]. However, roles of the gap junction are not limited to electrical coupling of adjacent cells. In many types of cells, the gap junction participates in decision-making on cell survival versus cell death under various insult conditions such as hypoxia, ischemia, irradiation and action of cytototxic agents [16]. The effects of inhibition of gap junction communication (GJC) or down-regulation of the gap junction protein expression on cell viability can be just opposite depending on the type of cell and type of insult. In the heart, GJC contributes to necrosis of cardiomyocytes after ischemia/reperfusion. This untoward role of the gap junction in the heart was first indicated by Garcia-Dorado et al. They reported that physical cell-to-cell interaction as a cell death mechanism is necessary to reproduce “confluent” myocardial infarct in a computer simulation [27], that significant limitation of infarct size was achieved by administration of a gap junction blocker upon reperfusion in swine hearts in situ [25] and that Na⁺-induced and Na⁺-Ca²⁺-mediated hypercontracture of a cardiomyocyte is propagated to adjacent cardiomyocytes by transport of overloaded Na⁺ via gap junctions in vitro [84]. However, there is no clinical strategy that specifically targets gap junction-mediated myocardial necrosis after ischemia/reperfusion.

Ischemic preconditioning (IPC), which is a brief episode or episodes of
ischemia before long sustained ischemia, affords protection against myocardial
necrosis and arrhythmias during the sustained ischemia/reperfusion
[67,94,112,117,118]. Since IPC can protect isolated cardiomyocytes from
simulated ischemia/reperfusion injury [57,73], the gap junction is clearly not
requisite for IPC protection, but the possibility that the gap junction plays a role in
IPC mechanisms in whole hearts cannot be excluded. IPC is generally more
potent in whole hearts than in isolated cardiomyocytes, and protein kinases
involved in IPC mechanisms (for example, Src, protein kinase C [PKC], p38
mitogen-activated protein kinase [p38MAPK]) [3,100,117] are known to
participate in GJC regulation [36,102]. With this background, effects of IPC on
the gap junction and its functional significance have been investigated by several
laboratories. Roles of non-gap junctional connexins (i.e., hemichannel and
mitochondrial connexin) in IPC have also been investigated, and intriguing
observations have been reported. In the present article, we summarize recent
observations regarding roles of the gap junction in IPC and discuss the possibility
that gap junction modulation is a part of the mechanisms of IPC protection.
Involvement of non-gap junctional connexins in IPC is mentioned briefly in the
present review, since functions of these connexins have been extensively
discussed in recent reviews [6,14,86,90].

Effects of ischemia on electrical and chemical GJC of cardiomyocytes

Gap junction permeability in the myocardium is reduced by ischemia.
Theoretically, reduction of the number of gap junctions or reduction of the
conductance of each gap junction could suppress overall GJC, and both appear to
be involved in ischemia-induced inhibition of GJC. Ischemia induces depletion of
ATP and accumulation of H\(^+\) and Ca\(^{2+}\) in cytoplasm, which have been shown to
decrease gap junction conductance in isolated paired cardiomyocytes [19]. In
addition, ischemia provokes redistribution of Cx43 from the intercalated disk region to the non-intercalated disk region (so-called “lateralization” of Cx43) [7,58,113], indicating reduction of the number of gap junctions.

The mechanism by which ischemia induces lateralization of Cx43 in the myocardium is not clear. However, Lampe et al. [52] recently showed that Cx43 lateralized after ischemia was dephosphorylated at Ser325, Ser328 and Ser330, though Cx43 remaining in the intercalated disk continued to be phosphorylated at these Ser residues. Reduction of cellular ATP and PP1A-like phosphatase has been suggested to be involved in ischemia-induced Cx43 dephosphorylation [37,46,110]. On the other hand, the level of phosphorylation of Cx43 at Ser368 has been shown to increase after ischemia [23,63], and Ek-Vitorin et al. [23] found that phospho-Ser368-Cx43 locates in the intercalated disk but not in the sarcolemma. These findings suggest that dephosphorylation of Ser325, Ser328 and Ser330 and lack of increased phosphorylation at Ser368 allow traffic of Cx43 from the intercalated disk to the sarcolemma, though their causative relationship remains to be confirmed.

Ischemia depresses both electrical and chemical GJC in the heart. However, there is a significant difference between the time course of electrical GJC and that of chemical GJC after the onset of ischemia. Loss of electrical GJC (i.e., electrical uncoupling of cardiomyocytes), which was determined by extra- and intracellular electrodes in isolated papillary muscles or by the “four electrode method” in ventricular walls of hearts in vivo or ex vivo, occurred at 10~20 min after ischemia, when ischemia-induced Ca$^{2+}$ overload and rigor had developed [5,19,49,99]. On the other hand, chemical GJC, assessed by fluorescent dyes, persisted for up to 30~60 min of no-flow ischemia [62,63,70,71,85]. This persistent GJC during late ischemia has been observed by using anionic and cationic tracers (for example, Lucifer yellow and ethidium bromide, respectively).
The mechanism by which chemical GJC is maintained after interruption of electrical GJC in ischemic myocardium remains unclear. However, a plausible explanation is the difference in ischemia-responsive gating mechanisms between electrical GJC and chemical GJC. Regulatory factors of gap junction conductance (for example, intracellular Ca\(^{2+}\), H\(^{+}\) and ATP) follow different time courses after the onset of ischemia \[19,65,66\]. Ischemia induces dephosphorylation of Cx43 at Ser325, Ser328, Ser330 and Ser365 \[52,102,103\] and increased phosphorylation at Ser368 \[23,63\]. As demonstrated in the case of Ser368 phosphorylation \[5\], changes in phosphorylation at Ser residues (and possibly other residues as well) in Cx43 after ischemia are likely to be accompanied by changes in configuration of Cx43 protein. In fact, ischemia induces a shift of Cx43 bands by 2~4 kDa in electrophoresis \[7,43,62,91\], which cannot be simply explained by loss of several phosphates from the Cx43 molecule. Furthermore, we recently found that there were marked differences in time courses of physical interaction of Cx43 with PKC, Src and p38MAPK during 35-min ischemia in the rat myocardium \[71\]. Taken together, these observations are consistent with the notion that multiple GJC regulatory factors are activated (or inactivated) in sequence after the onset of ischemia and that they differently regulate gating mechanisms for electrical currents and for transport of chemical substances. Inhibitory mechanisms specific for each of the chemical and electrical GJCs remain to be investigated.

Roles of the gap junction in trigger phase of IPC

The mechanism of myocardial protection by IPC can be separated into two phases, the “trigger phase”, when IPC ischemia activates multiple signaling pathways, and the “mediator phase” of IPC, when mediators (or effectors) that are activated by triggered signaling suppress lethal mechanisms during sustained...
ischemia and/or reperfusion [21]. IPC induces activation of multiple G protein-coupled receptors (adenosine, bradykinin and δ-opioid receptors) and transactivation of EGF-receptors and TNF-α receptors, which results in activation of redundant pro-survival signal pathways, including PI3K-Akt-, PKC-, PKG- and Jak2-STAT3-mediated pathways, during the trigger phase of IPC [21,40,117]. Contribution of each pathway to myocardial protection by IPC has been indicated by findings that pharmacological inhibition or genetic deletion of relevant kinases significantly attenuated or abolished IPC-induced protection. Although cross-talk of the pathways has not been clarified, the signal transduction during the trigger phase of IPC is thought to up-regulate mechanisms (i.e., actions of mediators of cytoprotection) that afford resistance to ischemia/reperfusion injury. Candidates of such cytoprotective mediators are phospho-Ser9-glycogen synthase kinase-3β (phospho-GSK-3β) [48,61], mitochondrial ATP-sensitive K⁺ channel (mKATP channel) [1,2,42,61] and gap junctional Cx43 [62,63,71]. Several lines of evidence indicate that the gap junction or other connexin complexes that are sensitive to gap junction blockers is involved in the trigger phase of IPC. As summarized in the table, administration of a gap junction blocker during the trigger phase of IPC inhibited myocardial protection afforded by IPC. In a study by Li et al. [55], infusion of heptanol, a reversible gap junction blocker, for 5 min before IPC ischemia, which was followed by a 10-min washout period, abolished infarct size limitation by IPC in isolated mouse hearts. In our experiments [Yano and Miura, unpublished data], we examined the effect of heptanol on myocardial protection afforded by an IPC mimic, a δ-opioid receptor agonist, [D-Ala²,D-Leu⁵]-enkephaline acetate (DADLE), in isolated rat hearts. This agonist was selected since the δ-opioid receptor is mainly responsible for activation of pro-survival pathways by IPC in rat hearts [24,54,89]. As shown in Figure 1, heptanol infused during δ-opioid receptor activation
eliminated 65% of protection (i.e., infarct size-limiting effect). Similar to the
infarct size-limiting effect of IPC, the anti-arrhythmic effect of IPC was attenuated
by intracoronary administration of a gap junction blocker, carbenoxolone, before
IPC in dogs [79]. Furthermore, IPC failed to protect the myocardium of Cx43
heterozygous knockout mouse from infarction [53,92]. Unfortunately,
pharmacological gap junction blockers used in earlier studies are not specific to
gap junctions or to hemichannels. Taken together, these results suggest that
gap junctions or hemichannels need to be open for protective signal transduction
during the trigger phase of IPC.

No study has examined transport of molecules via the gap junction during
IPC in the myocardium. However, a significant role of GJC in transmitting
secondary signals to adjacent cells has been demonstrated in non-cardiac cells
[16,105]. Thus, there is the possibility that signaling molecules activated by IPC,
such as cyclic GMP and nitric oxide [21], are intercellularly transported via gap
junctions during the trigger phase of IPC.

Accumulating evidence indicates that mitochondrial Cx43 in a
“hemichannel-like” structure plays a significant role in the trigger phase of IPC
[8,34,59,82]. IPC increases protein level of mitochondrial Cx43 [8].
Production of reactive oxygen species (ROS) by activation of the mKATP channel,
an important signal step in the trigger phase of IPC, is attenuated in Cx43
heterozygous knockout mice [34]. A recent study by Miro-Casas et al. [59]
indicated that mitochondrial Cx43 is in a “hemichannel-like” structure and is
sensitive to gap junction blockers. They showed that a gap junction tracer,
Lucifer yellow, was taken up by isolated mitochondria and that the uptake was
inhibited by gap junction blockers. Furthermore, activation of the mKATP channel
increased mitochondrial K⁺ uptake in a Cx43-dependent manner, and a gap
junction blocker, 18α-glycyrrhetinic acid, significantly inhibited the K⁺ uptake.
These results suggest that mitochondrial Cx43 forms a hemichannel-like structure and functions as a positive regulator of the mK$_{ATP}$ channel during the trigger phase of IPC.

Contribution of Cx43 hemichannels to IPC as ATP release channels has been reported for neuronal tissues [56]. However, that is unlikely to be the case in the myocardium. Three to 5 min of ischemia is sufficient to trigger IPC protection in the cardiomyocyte, whereas more than 30 minutes of ischemia is necessary to induce significant opening of the hemichannel [12,50,95]. Furthermore, there is no evidence to indicate that activation of G protein-coupled receptors relevant to IPC in the heart requires opening of the hemichannel.

Effects of IPC on GJC during ischemia

Opposing effects of IPC on electrical GJC and on chemical GJC during sustained ischemia (i.e., mediator phase of IPC) have been reported to date. Studies using a “four electrode method” to determine tissue resistance and phase angle showed no significant change [78] or a 5~10 min delay in uncoupling of electrical GJC by IPC during sustained ischemia [10,43,79,96]. In contrast, determination of GJC by Lucifer yellow showed that IPC facilitated reduction of chemical GJC during ischemia [62,71] (Figure 2AB). Furthermore, activation of two different steps in signaling mechanisms of IPC, activation of the δ-opioid receptor [63] (Figure 2C) and activation of the mK$_{ATP}$ channel [70], mimicked the effect of IPC on chemical GJC in the ischemic myocardium. Taken together, these findings indicate that IPC affords distinct effects on chemical and electrical GJC during myocardial ischemia.

Mechanisms of IPC-induced suppression of chemical GJC

The effect of IPC on Cx43 level in intercalated disk regions in the ischemic
myocardium has been examined in two studies, which showed conflicting results. Jain et al. [43] reported that IPC attenuated the loss of Cx43 protein from the intercalated disk region during 30-min ischemia in isolated rat hearts. In contrast, Vetterline et al. [113] showed that IPC significantly enhanced ischemia-induced Cx43 redistribution from intercalated disks to the free sarcolemma during 15~45-min ischemia in rat hearts in situ. The reason for the discrepancy is unclear. Nevertheless, the change in the number of gap junctions caused by IPC, if any, is unlikely to be mainly responsible for IPC-induced changes in chemical GJC since ample evidence suggests that modulation of gap junction Cx43 by protein kinases underlies the effect of IPC as follows.

There are several lines of evidence indicating that Cx43 phosphorylation by PKC-ε is primarily responsible for IPC-induced suppression of chemical GJC. First, reduction of GJC permeability to Lucifer yellow in the ischemic myocardium by IPC was abrogated by pretreatment with PKC-ε translocation inhibitory peptide (PKC-ε-TIP), a PKC-ε-selective inhibitor [71] (Figure 2). Second, preservation of phospho-Cx43 level by IPC in the ischemic myocardium was abolished by a PKC inhibitor, calphostin-C [62]. Third, a PKC activator, phorbol 12-myristate 13-acetate, significantly reduced chemical coupling of rat insulinoma cells in which transfected Cx43s were only gap junction proteins [38]. Fourth, IPC significantly increased both the level of PKC-ε co-immunoprecipitated with Cx43 after ischemia and phosphorylation of Cx43 at Ser368, a PKC phosphorylation site [71]. These effects of IPC on Cx43-PKC-ε interaction were mimicked by pre-ischemic activation of the δ-opioid receptor, which is known to trigger mechanisms of IPC [63]. Finally, phosphorylation of Cx43 at Ser368 has been shown to alter selectivity of the gap junction [23]. PKC phosphorylates Cx43 not only at Ser368 but also at Ser262 [104]. However, Doble et al. [20] have shown that over-expression of Cx43 with S262D (simulating a
phosphorylated state) neither suppressed chemical GJC nor attenuated inhibition of GJC by a PKC-activating phorbol ester. Interestingly, over-expression of S262A-Cx43 increased chemical GJC and cell necrosis during simulated ischemia in vitro [104]. Taken together, these results indicate that phosphorylation of Cx43 at Ser368 by PKC-ε is a primary mechanism of IPC-induced suppression of chemical GJC, whereas phosphorylation of Cx43 at Ser262 may be responsible for baseline resistance of cardiomyocytes to ischemic injury.

PKC-ε is not the only protein kinase involved in modulation of gap junction by IPC. p38MAPK is known to be activated by ischemia and its inhibitory effect on GJC has been observed in non-cardiac tissues [74,119]. In our recent study [71], 10~35 min of ischemia increased p38MAPK co-immunoprecipitated with Cx43 in the rat myocardium, but IPC rather suppressed the p38MAPKα-Cx43 interaction. This effect of IPC was associated with reduction of p38MAPK activity in the intercalated disk-rich fraction, and SB203580, a p38MAPK inhibitor, mimicked the effect of IPC on Cx43-p38MAPK complex level in the ischemic myocardium. Interestingly, chemical GJC at 15 min after ischemia, but not that at 25 min after ischemia, was increased by SB203580. These results indicate that IPC reduces p38MAPK-Cx43 interaction via suppression of p38MAPK activity during ischemia, and the reduced inhibitory effect of p38MAPK on the gap junction may partially counteract the inhibition of GJC by PKC-ε during an early phase of ischemia. This p38MAPK-mediated gap junction modulation in rat hearts may be species-specific, since immunohistochemical analysis showed that IPC enhanced co-localization of p38MAPK and Cx43 in the swine myocardium [91].

Since PKC-ε-TIP almost completely inhibited IPC-induced suppression of GJC, the contribution of other kinases to the GJC reduction appears negligible. However, there is an additional signal pathway that potentially suppresses GJC
during ischemia. Considering the role of the mKATP channel in production of ROS as a signaling molecule [21], we hypothesized that mKATP channel opening induces activation of MEK1-ERK1/2 signaling via ROS, leading to Cx43 phosphorylation by ERK1/2. Consistent with this hypothesis, activation of the mKATP channel by diazoxide induced complex formation of ERK with Cx43 and phosphorylation of Cx43 at Ser279/282 [70]. Diazoxide reduced chemical GJC during ischemia to a level similar to that by IPC, and this effect of diazoxide was sensitive to a mKATP channel inhibitor, 5-hydroxydecanoate, and to PD98059, a MEK-1 inhibitor. An adjunctive role of GJC inhibition in protection by mKATP channel activation was suggested by results showing that PD98059 prevented the infarct size-limiting effect of a low dose of diazoxide but not that of a high dose of diazoxide. Thus, mKATP channel-MEK1-ERK1/2 signaling is an inhibitory mechanism of chemical GJC, which potentially contributes to prevention of GJC-mediated ischemia/reperfusion injury.

Although reported data obtained from rabbit and rat hearts support the contribution of PKC-ε to IPC-induced GJC modulation as discussed above [62,63,70,71], this PKC isoform is unlikely to play the same role in the mouse heart. Opposite to the observation in the rat [63,71], IPC reduced myocardial phospho-Ser368-Cx43 level after ischemia in the mouse [39]. Furthermore, phospho-Ser368-Cx43 level after ischemia was two-fold higher in the PKC-ε-knockout mouse than in the wild-type mouse. These observations indicate marked differences between the rat and mouse in GJC regulation by Cx43 phosphorylation and possibly also in their functional outcomes.

Besides phosphorylation at specific Ser residues of Cx43, IPC preserves the overall phosphorylation level of Cx43 during ischemia [62,91]. Preserved Cx43 phosphorylation has been proposed to underlie delayed loss of electrical GJC [7,43], but how IPC slows down overall Cx43 dephosphorylation during
ischemia is still unclear. A simple plausible explanation is preservation of intracellular ATP level during ischemia by IPC [110] and/or inactivation of protein phosphatases (PPs) relevant to Cx43 dephosphorylation [36,37]. IPC-induced preservation of ATP during ischemia has been demonstrated in some but not all preparations [15,68,74,114]. On the other hand, involvement of PPs in IPC-induced modification of phospho-Cx43 levels was not supported by a recent study by Totzeck et al. [108]. They found that PP1\(\alpha\) and PP2A\(\alpha\) (but not PP2B\(\alpha\)) were expressed in the swine myocardium and that only PP2A\(\alpha\) was co-immunoprecipitated with Cx43. However, IPC did not change PP2A\(\alpha\)-Cx43 interaction or PP2A activity.

IPC-induced suppression of chemical GJC during ischemia and infarct size limitation

Contribution of suppressed chemical GJC during ischemia to myocardial salvage by IPC has not been conclusively demonstrated. However, several lines of evidence support its contribution. First, except for one study using mice [31], studies to date have shown that blockers of the gap junction significantly limited infarct size when administered before and/or during ischemia, during hypoxia or at the time of reperfusion [25,62,63,83,87] (Table). Second, IPC not only limits infarct size but also changes infarct morphology from confluent infarcts to patchy infarcts (or scattered foci of small infarcts) as described in the original report of IPC [67]. That change in infarct morphology can be explained by interruption of gap junction-mediated propagation of lethal injury [27], and, in fact, myocardial salvage by intracoronary infusion of heptanol upon reperfusion resulted in patchy infarcts [25]. Third, inhibitors of PKC-\(\varepsilon\), which abolish IPC-induced protection [117], abrogated the effect of IPC on chemical GJC [71]. Fourth, ischemia provides a circumstance in which GJC induces myocardial injury via reverse mode
operation of Na\(^+\)-Ca\(^{2+}\) exchange [41,66]. There are transmural gradients in severity of blood flow deficiency [45] and elevation of intracellular Na\(^+\) level [44] within the ischemic region. Thus, theoretically, Na\(^+\) accumulated in cardiomyocytes in a severely ischemic subendocardial zone can be transported via gap junctions to myocytes in the midmyocardial and then subepicardial zones under the condition of sustained ischemia. Ca\(^{2+}\) overload via Na\(^+\)-Ca\(^{2+}\) exchange is suppressed during ischemia by acidosis, but reperfusion eliminates acidosis-induced suppression of Na\(^+\)-Ca\(^{2+}\) exchange, resulting in massive Ca\(^{2+}\) influx in the Na\(^+\)-overloaded cells, which leads to reperfusion injury. In other words, Na\(^+\) possibly functions as a “death factor” transported by the gap junction in ischemic myocardium, whereas contribution of other death factors shown in different types of cells, such as Ca\(^{2+}\), inositol 1,4,5-triphosphate and cyclic AMP [16], cannot be excluded.

What percentage of IPC protection, if any, is attributable to suppression of GJC? It would be easy to determine the extent of myocardial salvage by GJC inhibition if there were a specific and direct opener of closed gap junctions. A new gap junction opener, rotigaptide [4,11,35], is apparently selective. However, it suppresses dephosphorylation of Ser297 and Ser368 in Cx43 during ischemia, indicating activation of a protein kinase and/or inhibition of a protein phosphatase. In addition, rotigaptide has been shown to modify gap junction expression after ischemia/reperfusion [35]. Thus, whatever the effect of rotigaptide on IPC might be, it cannot be attributable solely to increase in chemical GJC.

To assess what percentage of IPC protection is explained by the GJC-mediated mechanism, we took advantage of a feature in signal pathways activated by the cardiac \(\delta\)-opioid receptor in the rat. The unique feature of cytoprotective signaling by the \(\delta\)-opioid receptor in this species is that the
δ-isofom of PKC, but not the ε-isofom, plays a major role in myocardial protection [24]. We postulated that we could assess the contribution of the GJC-mediated mechanism to IPC protection by use of a PKC-ε inhibitor if only PKC-ε is responsible for the effects of IPC on GJC. To test this hypothesis, we activated the δ-opioid receptor by a selective agonist, DADLE, before regional ischemia in isolated rat hearts. Pretreatment with DADLE significantly suppressed chemical GJC during 30-min ischemia, and this effect on GJC was abolished by an inhibitor of PKC-ε (PKC-ε-TIP), but not by an inhibitor of PKC-δ (rottlerine). PKC-ε-TIP and rottlerine reduced the infarct size-limiting effect of DADLE by 48% and by 65%, respectively. These findings suggest that contribution of the GJC-mediated mechanism is no more than 35% of protection afforded by IPC mechanisms triggered by the δ-opioid receptor (Figure 3). This interpretation, however, clearly has limitations in that we assume complete selectivity of rottlerine to PKC-δ and independency of the PKC-δ-mediated pathway from the PKC-ε-mediated pathway in cardioprotection, which is perhaps oversimplification.

Nevertheless, modulation of GJC could be a part of the mechanisms of IPC. On the other hand, reduction of GJC-mediated cell necrosis does not explain all of the protection afforded by IPC. IPC has been shown to protect isolated cardiomyocytes [57,73], and accumulating evidence from recent studies supports the notion that multiple signal pathways activated by trigger mechanisms of IPC converge to steps that inhibit opening of the mitochondrial permeability transition pore (mPTP), the putative final mechanism of reperfusion-induced cell necrosis [21,32,33,109] (Figure 2). The mK_{ATP} channel and phospho-GSK-3β are putative molecules participating in inhibition of mPTP opening during the mediator phase of IPC [1,2,21,42,48,61]. It is notable, however, that the role of phospho-GSK-3β as a mediator of protection may differ depending on animal
species [28,72,98] and that the mKATP channel plays roles in both the trigger phase and in the mediator phase of IPC [1,2,21,42].

Effects of IPC on GJC during reperfusion

The effects of IPC on electrical GJC after reperfusion compared with the effects during sustained ischemia have been examined in a few studies. In a study by Padilla et al. [78], IPC had no effect on recovery of both resistivity and phase angle in tissue impedance recordings during reperfusion in isolated rat hearts. On the other hand, Zhu and Ferrier [118] reported that IPC significantly attenuated prolongation of transmural conduction time during ischemia and also during early period of reperfusion in the isolated guinea pig right ventricle. Preservation of electrical GJC in the transverse direction by IPC is a possible explanation for this finding.

Changes in chemical GJC during reperfusion by IPC have not been examined because of technical difficulties. In assessment of chemical GJC using fluorescent tracers, it takes a long time to load cardiomyocytes with a tracer and to follow the intercellular transport of the tracer via the gap junction. In previous studies, including ours, the myocardium was incubated in a buffer containing tracers for 10~25 min for visualization of chemical GJC [62,63,71,85]. On the other hand, reperfusion induces rapid changes in determinants of gap junction conductance, including intracellular H⁺, Ca²⁺ and ATP [65,66]. Thus, it is very unlikely that current fluorescent dyes can be used to detect rapid change in GJC after reperfusion and its modification by IPC.

Although direct data on chemical GJC upon reperfusion are lacking, there is an observation suggesting that suppression of chemical GJC by IPC might persist after reperfusion. As mentioned in a preceding section, we used a δ-opioid receptor agonist (DADLE) to get an insight into the proportion of
GJC-mediated protection in entire protection afforded by IPC. In that series of experiments, we activated the δ-opioid receptor by DADLE before ischemia to provoke PKC-ε-mediated suppression of chemical GJC during ischemia and simultaneously blocked PKC-δ by rottlerine to eliminate protection by this PKC isoform, which is not GJC-mediated. This combined treatment (DADLE plus rottlerine) reduced infarct size by 25%. If GJC after reperfusion is not modified by pre-ischemic activation of the δ-opioid receptor, additional treatment with a pharmacological gap junction blocker at the time of reperfusion should afford further protection. However, that was not the case. It is also notable that both binding of PKC-ε to Cx43 and augmentation of the PKC-ε-Cx43 interaction by IPC were increased as ischemia duration was prolonged [71]. These results argue for the possibility that PKC-ε-mediated suppression of chemical GJC by IPC persists during an early and critical period of reperfusion injury.

Effects of IPC on arrhythmia and gap junction modification

In contrast to its effect on infarct size, effects of IPC on arrhythmias are not consistent in different animal models of myocardial ischemia/reperfusion [10,30,77,79,94,96,112]. Furthermore, different patterns have been reported for suppression of arrhythmias by IPC. IPC with a single episode of 5-min ischemia and 20-min reperfusion induced delay in onset of phase IB ventricular premature beats (VPBs) in pig hearts in situ [10], and IPC with four cycles of 5-min ischemia/5-min reperfusion similarly delayed the peak of IB VPBs in canine hearts [96]. On the other hand, IPC with a single episode or two episodes of 5-min ischemia/20-min reperfusion suppressed VPBs throughout the ischemic period (i.e., both IA and IB arrhythmias) in canine hearts [79,112]. It is also notable that the infarct size-limiting effect of IPC was not always accompanied by suppression of lethal arrhythmias during ischemia/reperfusion [30,67,77].
These findings suggest that anti-arrhythmic mechanisms of IPC are different depending on the IPC protocol and/or animal models of myocardial ischemia.

Intramural reentry in the border zone, increase in longitudinal resistance and secretion of catecholamine underlie the development of early ischemic arrhythmias [9]. A study using microelectrodes in isolated right ventricle walls showed that IPC significantly attenuates prolongation of transmural conduction time during ischemia [118]. In studies that assessed electrical GJC by myocardial tissue resistance, anti-arrhythmic effects of IPC were associated with delayed interruption of electrical GJC [10,79]. However, the contribution of preserved electrical GJC to anti-arrhythmic effects of IPC is not clear.

Conduction velocity depends not only on gap junction resistance but also on resistance of the cytoplasm. In addition, electrotonic interactions between depolarized myocytes and non-ischemic myocytes in ischemic border zones are thought to be involved in slow conduction in surviving tissues. Because of reduction in such electrotonic interaction, gap junction uncoupling in the viable tissue could improve conduction in the ischemic region of the myocardium [17]. Furthermore, studies using a pharmacological opener of the gap junction, rotigaptide, showed that its effects on arrhythmias were not similar to those of IPC. Rotigaptide did not suppress ischemia-induced focal ventricular tachycardia (VT) or triggered activity [116], though it prevented reentrant VT induced by programmed pacing in the ischemic myocardium [115]. Reperfusion arrhythmias were suppressed by rotigaptide administered before reperfusion, but it required a very high dose of this agent [35]. Taken together, these findings suggest that anti-arrhythmic effects of IPC are not achieved solely by preservation of electrical GJC during ischemia, and alterations in ion channels [22], Ca²⁺ handling by the mitochondria [1,2,42] and norepinephrine release from sympathetic nerves [60,64] may also be involved.
Effects of IPC on functions of hemichannels

Gap junction hemichannels are in a process of migration from their formation in the endoplasmic reticulum to the intercalated disk where they dock with hemichannels in adjacent cells to form gap junctions [47,51,97]. In contrast with the gap junction, Cx43 hemichannels have very low open probability under physiological conditions. However, hemichannels are capable of functioning as channels through which signaling molecules and metabolites (such as ATP, glutamate and glutathione) are released into the extracellular space [81,93,106]. In addition to the open probability under baseline conditions, responses to some modulatory factors differ between the gap junction and hemichannel. De Vuyst et al. [18] showed that lipopolysaccharide and basic fibroblast growth factor suppress GJC but stimulate ATP release via hemichannels in glioma cells. On the other hand, pharmacological blockers of the gap junction and activated PKC similarly suppress conductances of the gap junction and the Cx43 hemichannel.

Accumulating evidence indicates that the hemichannel, like the gap junction, plays important roles in survival and death of various types of cells [16,76]. A part of ischemia/reperfusion injury may be attributable to opening of the Cx43 hemichannel in the heart and brain [13,95,107]. In isolated cardiomyocytes and also in neuronal cells, metabolic inhibition or simulated ischemia induces increases in both inward currents under blockade of multiple specific channels and GJC tracing dye transfer, which were sensitive to gap junction blockers, indicating opening of the hemichannel [13,95,107]. Sustained opening of hemichannels can induce profound derangement of ionic homeostasis and metabolites (such as ATP) during ischemia and lead to cell necrosis. In fact, blockade of hemichannels with a Cx43 mimetic peptide,
Gap26, significantly reduced necrosis of isolated rat cardiomyocytes after simulated ischemia/reperfusion [95]. Similar cytoprotection by structurally different gap junction blockers has been observed in neuronal tissues [75,107].

In contrast to sustained opening of the hemichannel, its transient opening can be cytoprotective. Schock et al. [88] showed that transient depolarization of primary rat cortical cells with KCl induced extracellular release of ATP and that activation of the P2Y receptor by released ATP afforded protection against simulated ischemia by a PKA/phospholipase C-mediated mechanism. This protection was abrogated either by pharmacological blockade of the gap junction or by knocking down Cx36 expression. In a study by Lin et al. [56], ATP release from C6 glioma cells occurred after IPC, and it was abolished either by structurally different gap junction blockers or by deletion of Cx43 expression. The extent of cytoprotection by IPC was in parallel with the level of ATP released into extracellular space, and the protection was abrogated by DPCPX (8-cyclopentyl-1,3-dipropylxanthine), indicating involvement of the adenosine A1 receptor as a mechanism downstream of hydrolysis of extracellular ATP.

Whether these autocrine and paracrine types of signaling via hemichannels are present in cardiomyocytes has not yet been examined.

Analysis of functions of Cx43 hemichannels and their regulation in intact tissue is a technical challenge. Methodologies that have been used to examine roles of the gap junction in IPC (i.e., use of pharmacological blockers of GJC and fluorescent probes for chemical GJC) cannot differentiate roles of the gap junction from those of hemichannels. Experiments using isolated cardiomyocytes certainly provide insights into functions of hemichannels under ischemia, but the function and regulation of hemichannels might be different from those in the intact and contracting myocardium in vivo. Nevertheless, there is the possibility that blockade of hemichannels of cardiomyocytes during
ischemia/reperfusion is a part of the mechanism of IPC protection in the myocardium. To critically address this issue, development of an inhibitor specific to hemichannels or methodology that can specifically manipulate hemichannel expression would be necessary.

Conclusion

Whereas ischemia induces intracellular redistribution of Cx43 and interruption of electrical and chemical GJC in the myocardium, chemical GJC is maintained for a considerable time after loss of electrical GJC, possibly leading to intercellular propagation of ischemic injury. Roles of the gap junction appear to be different in the trigger phase and mediator phase of IPC. GJC and/or opened mitochondrial hemichannel-like structures are necessary for IPC to trigger cytoprotective signaling. On the other hand, IPC facilitates reduction in chemical GJC during sustained ischemia, while inhibition of electrical GJC is delayed. Phosphorylation of Cx43 at multiple kinase target sites and interactions of Cx43 with protein kinases are modified by IPC, but PKC-ε-mediated phosphorylation of Cx43 at Ser368 is a primary mechanism of IPC-induced inhibition of chemical GJC in the rat ventricular myocardium. Evidence to date supports the notion that modulation of GJC by IPC is a part of the mechanisms leading to IPC-induced tolerance against infarction and arrhythmias during ischemia/reperfusion.

Grants

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Figure 1. Effects of heptanol on infarct size limitation by pre-ischemic activation of the δ-opioid receptor.

In isolated Sprague-Dawley rat hearts, the left coronary artery was occluded for 35 min and reperfused for 2 hrs. Upper panel shows experimental protocols. CAO = coronary artery occlusion, DADLE = two episodes of 5-min infusion of 300 nM DADLE (DA) followed by 5-min washout period before ischemia, Hept+DADLE = DADLE infusion as in the DADLE group and infusion of 1 mM heptanol (hatched bar) from 5 min before the first infusion of DADLE to the end of the second infusion of DADLE, Hept = infusion of heptanol for 3 min before coronary occlusion. Infarct size and area at risk were determined as previously reported. N = 5~8. *p<0.05 vs. Control

Figure 2. Suppression of chemical GJC in ischemic myocardium by IPC and an IPC mimetic (δ-opioid agonist).

Size of area where Lucifer yellow (LY) was transported via gap junctions during ischemia ex vivo was used as an index of gap junction permeability. Areas stained with rhodamine-conjugated dextran (RD) indicate those with disruption of sarcolemma, which was made for LY loading. RD-stained areas were subtracted from LY-stained area for determination of gap junction communication (GJC). A and B: Effects of an IPC mimetic, δ-opioid agonist (DADLE) and inhibitors of PKC on GJC determined at 30 min after ischemia. Representative images of control and preconditioned hearts (A) and group mean data (B). Scale bars in panel A indicate 200 μm. C: Effects of IPC, PKC-ε inhibitor (PKC-ε-TIP), negative control of PKC-ε inhibitor (scrambled TIP) and p38MAPK inhibitor (SB203580) on GJC. C: Effects of an IPC mimetic, δ-opioid agonist...
(DADLE) and inhibitors of PKC on GJC. In this series of experiments, GJC was determined at 25 min after ischemia (a) and at 15 min after ischemia (b). *p<0.05 vs. Control. Reproduced from Refs. 63 and 71 with permission.

Figure 3. Proposed roles of the gap junction and Cx43 in infarct size limitation by IPC.

IPC = ischemic preconditioning, GPCRs = G protein-coupled receptors, GJC = gap junction communication, mK_{ATP} ch = mitochondrial K_{ATP} channel, mCx43 = mitochondrial connexin-43, ROS = reactive oxygen species, NCX = Na^{+}-Ca^{2+} exchanger.
Figure 1

The figure illustrates the effects of different treatments on infarct size in a CAO (Catheter Artery Occlusion) model. The treatments include Control, DADLE, Heptanol, and Heptanol + DADLE. The bar graph shows the infarct size (% of area at risk) over time, with bars representing the treatments at specific time points. The x-axis represents time in minutes, ranging from -15 to 155 min, and the y-axis represents infarct size (% of area at risk) from 0 to 35.

- **Control**: The treatment group showing the highest infarct size.
- **DADLE**: Shows a reduced infarct size compared to Control.
- **Heptanol + DADLE**: Demonstrates a further reduction in infarct size.
- **Heptanol**: Exhibits a significant reduction in infarct size, indicated by the asterisk (*), compared to Control.

The asterisk (*) indicates statistical significance, indicating that the treatment has a significant effect on reducing infarct size compared to the control group.
Figure 2

Gap Junction Communication
(arbitrary units)

- Control
- Hepatol
- DADLE
- Rottlerin+DADLE
- Scr-TIP+DADLE
- PKCe-TIP+DADLE
- Rottlerin
- PKC

* indicates a significant difference from the control group.
Figure 3

IPC

↓

GPCRs

↓

GJC-mediated transport of signaling molecules within IPC region?

↓

mK_{ATP} ch under regulation by mCx43

↓

ROS

↓

MEK1

↓

PKC-ε

↓

ERK1/2

↓

Cx43

↓

Inhibition of hemichannel opening?

↓

Prevention of ionic derangements

↓

Protection of myocyte from necrosis

↓

Inhibition of cell-to-cell propagation of Na^+ overload?

Attenuated cell-to-cell propagation of Na^+ overload?

↓

Accelerated loss of chemical GJC

↓

Attenuated Ca^{2+} overload via NCX?

↓

Inhibition of cell-to-cell propagation of injury

Reperfusion

Sustained ischemia

Trigger phase of IPC
Table. Effects of gap junction blockers on ischemic preconditioning (IPC) and on infarct size in non-preconditioned hearts

<table>
<thead>
<tr>
<th>Authors (ref)</th>
<th>Preparation</th>
<th>GJ blocker</th>
<th>End-point</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion of a gap junction blocker before or during IPC</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li et al. (55)</td>
<td>Isolated mouse hearts</td>
<td>Heptanol (0.5 mM)</td>
<td>Infarct size</td>
<td>Complete loss of protection</td>
</tr>
<tr>
<td>Yano and Miura* (figure 1)</td>
<td>Isolated rat hearts</td>
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<td>Loss of 65% of protection</td>
</tr>
<tr>
<td>Papp et al. (79)</td>
<td>Dog hearts in situ</td>
<td>Carbenoxolone</td>
<td>Arrhythmia</td>
<td>Loss of &gt;50% of protection</td>
</tr>
<tr>
<td>Infusion of a gap junction blocker before ischemia</td>
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<td></td>
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<tr>
<td>Saltman et al. (87)</td>
<td>Isolated rabbit heart</td>
<td>Heptanol (1 mM)</td>
<td>Infarct size</td>
<td>52% reduction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BDM (10 mM)</td>
<td></td>
<td>No protection</td>
</tr>
<tr>
<td>Infusion of a gap junction blocker during ischemia</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Gysemberg et al. (31)</td>
<td>Isolated rabbit hearts</td>
<td>Heptanol (0.5 mM)</td>
<td>Infarct size</td>
<td>No protection</td>
</tr>
<tr>
<td>Miura et al. (62)</td>
<td>Isolated rabbit hearts</td>
<td>Heptanol (2 mM)</td>
<td>Infarct size</td>
<td>82% reduction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BDM (30 mM)</td>
<td></td>
<td>56% reduction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18b-GA</td>
<td></td>
<td>52% reduction</td>
</tr>
<tr>
<td>Rodriguez-Sinovas et al. (83)</td>
<td>Isolated rat hearts</td>
<td>Palmitoleic acid</td>
<td>LDH release</td>
<td>41% reduction</td>
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<tr>
<td></td>
<td></td>
<td>18a-GA</td>
<td></td>
<td>19% reduction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Heptanol (1 mM)</td>
<td></td>
<td>55% reduction</td>
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<tr>
<td>Infusion of a gap junction blocker upon reperfusion</td>
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</tr>
<tr>
<td>Garcia-Dorado et al. (25)</td>
<td>Pig hearts in situ</td>
<td>Heptanol (1 mM)</td>
<td>Infarct size</td>
<td>54% reduction</td>
</tr>
<tr>
<td>Miura et al. (62)</td>
<td>Isolated rabbit hearts</td>
<td>Heptanol (2 mM)†</td>
<td>Infarct size</td>
<td>No protection†</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BDM (30 mM)†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miura et al. (63)</td>
<td>Isolated rat hearts</td>
<td>Heptanol (1 mM)</td>
<td>Infarct size</td>
<td>36% reduction</td>
</tr>
</tbody>
</table>

GJ blocker = gap junction blocker, BDM = 2,3-butanedione monoxime, 18b-GA = 18b-glycyrrhetinic acid, 18a-GA = 18a-glycyrrhetinic acid.
*Preconditioning mechanism was triggered by activation of the d-opioid receptor (see text for details).
†In this negative study, duration of GJ blocker infusion after reperfusion was shorter than those in the positive studies (10 min vs. 15 min), which might have been responsible for the different results.