Role of the gap junction in ischemic preconditioning in the heart

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Miura T, Miki T, Yano T. Role of the gap junction in ischemic preconditioning in the heart. Am J Physiol Heart Circ Physiol 298: H1115–H1125, 2010. First published January 29, 2010; doi:10.1152/ajpheart.00879.2009.—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. The 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 the ischemia-induced suppression of chemical GJC, whereas IPC delays the reduction of electrical GJC after ischemia. The inhibition of GJC during sustained ischemia and reperfusion by GJC blockers mimics the effect of IPC on myocardial necrosis. IPC induces distinct effects on the interaction of connexin-43 with protein kinases, and the phosphorylation of connexin-43 at Ser368 by PKCε is a primary mechanism of inhibition of chemical GJC by IPC. Several lines of evidence support the notion that the 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 the 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. The pathological reduction of gap junction conductance or the reduction of gap junction proteins promotes the development of arrhythmias (111). However, the 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 cytotoxic agents (16). The effects of the inhibition of gap junction communication (GJC) or the downregulation of the gap junction protein expression on cell viability can be just the 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 García-Dorado et al. They reported that the 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+/Ca2+-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), and p38 mitogen-activated protein kinase (p38MAPK)] (3, 100, 117) are known to participate in GJC regulation (36, 102). With this background, the effects of IPC on the gap junction and its functional significance have been investigated by several laboratories. The 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 the roles of the gap junction in IPC and discuss the possibility that gap junction modulation is a part of the mechanisms of IPC protection. The 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, the reduction of the number of gap junctions or the 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 the depletion of ATP and the accumulation of H+/Ca2+ in cytoplasm, which have been shown to decrease gap junction conductance in isolated paired cardiomyocytes (19). In addition, ischemia provokes the redistribution of connxin-43 (Cx43) from the intercalated disk region to the nonintercalated disk region (so-called “lateralization” of Cx43) (7, 58, 113), indicating the reduction of the number of gap junctions.

The mechanism by which ischemia induces the 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. The reduction of cellular ATP and protein phosphorylation 1A (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 the dephosphorylation of Ser325, Ser328, and Ser330 and the 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. The 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 (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 the 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. The regulatory factors of gap junction conductance (for example, intracellular Ca2+, H+, and ATP) follow different time courses after the onset of ischemia (19, 65, 66). Ischemia induces the dephosphorylation of Cx43 at Ser325, Ser328, and Ser330, and Ser368 (52, 102, 103) and increased the 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 the configuration of the Cx43 protein. In fact, ischemia induces a shift of the Cx43 bands by ~2–4 kDa in electrophoresis (7, 43, 62, 91), which cannot be simply explained by the loss of several phosphates from the Cx43 molecule. Furthermore, we (71) recently found that there were marked differences in the time courses of the physical interaction of Cx43 with PKC, Src, and p38MAPK during 35 min ischemia in the rat myocardium. 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 activates multiple signaling pathways, and the “mediator phase” of IPC, when mediators (or effectors) that are activated by triggered signaling suppress the lethal mechanisms during sustained ischemia and/or reperfusion (21). IPC induces the activation of multiple G protein-coupled receptors (adenosine, bradykinin, and δ-opioid receptors) and the trans-activation of EGF receptors and TNF-α receptors, which result in the activation of redundant pro-survival signal pathways, including phosphatidylinositol 3-kinase-Akt-, PKC-, PKG-, and Jak2-STAT3-mediated pathways during the trigger phase of IPC (21, 40, 117). The contribution of each pathway to myocardial protection by IPC has been indicated by findings that the pharmacological inhibition or genetic deletion of relevant kinases significantly attenuated or abolished the IPC-mediated protection. Although cross talk of the pathways has not been clarified, the signal transduction during the trigger phase of IPC is thought to upregulate mechanisms (i.e., actions of mediators of cytoprotection) that afford resistance to ischemia-reperfusion injury. Candidates of such cytoprotective mediators that are phospho-Ser9-glycogen synthase kinase-3 (GSK-3) (48, 61), mitochondrial ATP-sensitive K+ channel (mKATP channel) (1, 2, 42), and gap junctional Cx43 (62, 63, 71).

Several lines of evidence indicate that the gap junction or other connexin complexes which are sensitive to gap junction blockers are involved in the trigger phase of IPC. As summarized Table 1, the 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), the infusion of heptanol, a reversible gap junction blocker, for 5 min before IPC ischemia, which was followed by a 10-min washout period, abolished the 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...
Table 1. Effects of gap junction blockers on IPC and on infarct size in nonpreconditioned hearts

<table>
<thead>
<tr>
<th>Authors (Ref)</th>
<th>Preparation</th>
<th>Gap Junction Blocker</th>
<th>End Point</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<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* (Fig. 1)</td>
<td>Isolated rat hearts</td>
<td>Carbenoxolone</td>
<td>Infarct size</td>
<td>Loss of 65% of protection</td>
</tr>
<tr>
<td>Papp et al. (79)</td>
<td>Dog hearts in situ</td>
<td>Heptanol (1 mM)</td>
<td>Arrhythmia</td>
<td>Loss of &gt;50% of protection</td>
</tr>
</tbody>
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In infusion of a gap junction blocker before or during IPC

<table>
<thead>
<tr>
<th>Authors (Ref)</th>
<th>Preparation</th>
<th>Gap Junction Blocker</th>
<th>End Point</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<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>BDM (10 mM)</td>
<td>Infarct size</td>
<td>No protection</td>
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</table>

In infusion of a gap junction blocker before ischemia

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<thead>
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<th>Authors (Ref)</th>
<th>Preparation</th>
<th>Gap Junction Blocker</th>
<th>End Point</th>
<th>Outcome</th>
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<tbody>
<tr>
<td>Gysembergh 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>BDM (30 mM)</td>
<td>Infarct size</td>
<td>56% reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18β-GA</td>
<td>Heptanol (1 mM)</td>
<td>52% reduction</td>
<td></td>
<td></td>
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<tr>
<td>Rodríguez-Sinovas et al. (83)</td>
<td>Isolated rat hearts</td>
<td>Palmitoleic acid</td>
<td>LDH release</td>
<td>41% reduction</td>
</tr>
<tr>
<td>18α-GA</td>
<td>Heptanol (1 mM)</td>
<td>19% reduction</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Miura et al. (63)</td>
<td>Isolated rabbit hearts</td>
<td>Heptanol (1 mM)</td>
<td>Infarct size</td>
<td>36% reduction</td>
</tr>
</tbody>
</table>

In infusion of a gap junction blocker upon reperfusion

<table>
<thead>
<tr>
<th>Authors (Ref)</th>
<th>Preparation</th>
<th>Gap Junction Blocker</th>
<th>End Point</th>
<th>Outcome</th>
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<tbody>
<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>BDM (30 mM)†</td>
<td>Infarct size</td>
<td>36% reduction</td>
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IPC, ischemic preconditioning; BDM, 2,3-butanedione monoxime; 18β-GA, 18β-glycyrrhetinic acid; 18α-GA, 18α-glycyrrhetinic acid. *Preconditioning mechanism was triggered by activation of the δ-opioid receptor (see text for details). †In this negative study, duration of gap junction 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.

GAP JUNCTION AND ISCHEMIC PRECONDITIONING

The effects of IPC on GJC During Ischemia

The 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 an ∼5–10 min delay in uncoupling of electrical GJC by IPC during sustained ischemia (10, 43, 79, 96). In contrast, the determination of GJC by Lucifer yellow showed that IPC facilitated the reduction of chemical GJC during ischemia (62, 71) (Fig. 2). Furthermore, the activation of two different steps in the signaling mechanisms of IPC, the activation of the δ-opioid receptor (63) (Fig. 2B), and

Protection afforded by an IPC mimic, a δ-opioid receptor agonist, (D-Ala²,D-Leu⁵)-enkephaline acetate (DADLE), in isorereperfusion was shorter than those in the positive studies (10 min vs. 15 min), which might have been responsible for the different results.
the activation of the mKATP 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 GJCs 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, Vetterlein 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 the 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 PKCε is primarily responsible for the IPC-induced suppression of chemical GJC. First, the reduction of GJC permeability to Lucifer yellow in the ischemic myocardium by IPC was abrogated by a pretreatment with PKCε translocation inhibitory peptide (PKCε-TIP), a PKCε-selective inhibitor (71) (Fig. 2). Second, the 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 PKCε coimmunoprecipitated with Cx43 after ischemia and phosphorylation of Cx43 at Ser368, a PKC phosphorylation site (71). These effects of IPC on Cx43-PKCε interaction were mimicked by the preischemic activation of the δ-opioid receptor, which is known to trigger mechanisms of IPC (63). Finally, the phosphorylation of Cx43 at Ser368 has
been shown to alter the 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 an overexpression of Cx43 with S262D (simulating a phosphorylated state) neither suppressed chemical GJC nor attenuated the inhibition of GJC by a PKC-activating phorbol ester. Interestingly, the overexpression of S262A-Cx43 increased chemical GJC and cell necrosis during simulated ischemia in vitro (104). Taken together, these results indicate that the phosphorylation of Cx43 at Ser368 by PKCe is a primary mechanism of IPC-induced suppression of chemical GJC, whereas the phosphorylation of Cx43 at Ser262 may be responsible for baseline resistance of cardiomyocytes to ischemic injury.

PKCe is not the only protein kinase involved in the modulation of gap junction by IPC. p38MAPK is known to be activated by ischemia, and its inhibitory effect on GJC has been observed in noncardiac tissues (74, 119). In our recent study (71), 10–35 min of ischemia-increased p38MAPK coimmunoprecipitated with Cx43 in the rat myocardium, but IPC rather suppressed the p38MAPKα-Cx43 interaction. This effect of IPC was associated with a reduction of p38MAPK activity in the intercalated disk-rich fraction, and SB-203580, a p38MAPK inhibitor, mimicked the effect of IPC on the Cx43-p38MAPK complex level in the ischemic myocardium. Interestingly, chemical GJ at 15 min after ischemia, but not that at 25 min after ischemia, was increased by SB-203580. These results indicate that IPC reduces the p38MAPK-Cx43 interaction via the 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 PKCe during an early phase of ischemia. This p38MAPK-mediated gap junction modulation in rat hearts may be species specific, since an immunohistochemical analysis showed that IPC enhanced the colocalization of p38MAPK and Cx43 in the swine myocardium (91).

Since PKCe-TIP almost completely inhibited the 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 the production of ROS as a signaling molecule (21), we hypothesized that mKATP channel opening induces the activation of MEK1-ERK1/2 signaling via ROS, leading to Cx43 phosphorylation by ERK1/2. Consistent with this hypothesis, the activation of the mKATP channel by diazoxide induced the complex formation of ERK with Cx43 and the 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 PD-98059, a MEK-1 inhibitor. An adjunctive role of GJC inhibition in the protection by mKATP channel activation was suggested by the results showing that PD-98059 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 the prevention of GJC-mediated ischemia-reperfusion injury.

Although reported data obtained from rabbit and rat hearts support the contribution of PKCe to IPC-induced GJC modulation as discussed above (62, 63, 71), this PKCe isoform is unlikely to play the same role in the mouse heart. Opposite to the observation in the rat (63, 71), IPC reduced the myocardial phospho-Ser368-Cx43 level after ischemia in the mouse (39). Furthermore, the phospho-Ser368-Cx43 level after ischemia was twofold higher in the PKCe-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 the delayed loss of electrical GJC (7, 43), but how IPC slows down the overall Cx43 dephosphorylation during ischemia is still unclear. A simple plausible explanation is the preservation of the intracellular ATP level during ischemia by IPC (110) and/or the inactivation of PPs relevant to Cx43 dephosphorylation (36, 37). The IPC-induced preservation of ATP during ischemia has been demonstrated in some but not all preparations (15, 68, 74, 114). On the other hand, the involvement of PPs in IPC-induced modification of phospho-Cx43 levels was not supported by a recent study by Totzek et al. (108). They found that PPIα and PP2Aα (but not PP2Bα) were expressed in the swine myocardium and that only PP2Aα was coimmunoprecipitated with Cx43. However, IPC did not change PP2Aα-Cx43 interaction or PP2A activity.

IPC-Induced Suppression of Chemical GJC During Ischemia and Infarct Size Limitation

The 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 1). 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 the 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, the inhibitors of PKCe, 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 a reverse-mode operation of the Na+/Ca2+ 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. Ca2+ overload via Na+/Ca2+ exchange is suppressed during ischemia by acidosis, but reperfusion eliminates acidosis-induced suppression of Na+/Ca2+ exchange, resulting in massive Ca2+ 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 the contribution of other death factors shown in different types of cells, such as Ca$^{2+}$, inositol 1,4,5-trisphosphate, and cAMP (16), cannot be excluded.

What percentage of IPC protection, if any, is attributable to the 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 the dephosphorylation of Ser297 and Ser368 in Cx43 during ischemia, indicating the activation of a protein kinase and/or the inhibition of a PP. 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 an 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 δ-opioid receptor in the rat. The unique feature of cytoprotective signaling by the δ-opioid receptor in this species is that the δ-isof orm of PKC, but not the ε-isof orm, 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 PKCε inhibitor if only PKCε 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 PKCε (PKCε-TIP) but not by an inhibitor of PKCδ (rottlerine) (Fig. 2B). PKCε-TIP and rottlerine reduced the infarct size-limiting effect of DADLE by 48% and by 65%, respectively. These findings suggest that the contribution of the GJC-mediated mechanism is no more than 35% of the protection afforded by IPC mechanisms triggered by the δ-opioid receptor (Fig. 3). This interpretation, however, clearly has limitations in that we assume the complete selectivity of rottlerine to PKCδ and the independency of the PKCδ-mediated pathway from the PKCε-mediated pathway in cardioprotection, which is perhaps an oversimplification.

Nevertheless, the modulation of GJC could be a part of the mechanisms of IPC. On the other hand, the 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 the opening of the mitochondrial permeability transition pore, the putative final mechanism of reperfusion-induced cell necrosis (21, 32, 33, 109). The mKATP channel and phospho-GSK-3β are putative molecules participating in inhibition of mitochondrial permeability transition pore 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 the 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 the prolongation of transmural conduction time during ischemia and also during the early period of reperfusion in the isolated guinea pig right
ventricle. The 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 the 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 the 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 the 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 the entire protection afforded by IPC. In that series of experiments, we activated the δ-opioid receptor by DADLE before ischemia to provoke the PKCε-mediated suppression of chemical GJC during ischemia and simultaneously blocked PKCδ by rottlerine to eliminate the 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 the preischemic 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 (63). It is also notable that both the binding of PKCε to Cx43 and the augmentation of the PKCε-Cx43 interaction by IPC were increased as ischemia duration was prolonged (71). These results argue for the possibility that the PKCε-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, the 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 the suppression of arrhythmias by IPC. IPC with a single episode of 5 min ischemia and 20 min reperfusion induced a delay in the onset of phase IIb 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 the 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 the animal models of myocardial ischemia.

The intramural reentry in the border zone, the increase in longitudinal resistance, and the 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 the prolongation of transmural conduction time during ischemia (118). In studies that assessed electrical GJC by myocardial tissue resistance, the anti-arrhythmic effects of IPC were associated with the delayed interruption of electrical GJC (10, 79). However, the contribution of the preserved electrical GJC to the 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 nonischemic myocytes in ischemic border zones are thought to be involved in the slow conduction in surviving tissues. Because of the 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 or triggered activity (116), though it prevented reentrant ventricular tachycardia 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 the anti-arrhythmic effects of IPC are not achieved solely by the 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 the survival and death of various types of cells (16, 76). A part of ischemia-reperfusion injury may be attributable to the 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 the opening of the hemichannel (13, 95, 107). A sustained opening of hemichannels can induce a profound derangement of ionic homeostasis and metabolites (such as ATP) during ischemia and lead to cell necrosis. In
fact, the 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 the sustained opening of the hemichannel, its transient opening can be cytoprotective. Schock et al. (88) showed that transient depolarization of primary rat cardiac 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 the deletion of Cx36 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 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), indicating the involvement of the adenosine A$_1$ 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.

An analysis of functions of Cx43 hemichannels and their regulation in intact tissue is a technical challenge. Methodologies that have been used to examine the roles of the gap junction in IPC (i.e., the use of pharmacological blockers of GJC and fluorescent probes for chemical GIC) cannot differentiate the roles of the gap junction from those of the hemichannels. Experiments using isolated cardiomyocytes certainly provide insights into the 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 the 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, the development of an inhibitor specific to hemichannels or methodology that can specifically manipulate hemichannel expression would be necessary.

### Conclusion

Whereas ischemia induces the intracellular redistribution of Cx43 and the interruption of electrical and chemical GIC in the myocardium, chemical GIC is maintained for a considerable time after the loss of electrical GIC, possibly leading to intercellular propagation of ischemic injury. The 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 a reduction in chemical GJC during sustained ischemia, while the inhibition of electrical GJC is delayed. Phosphorylation of Cx43 at multiple kinase target sites and the interactions of Cx43 with protein kinases are modified by IPC, but PKC-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 the modulation of GJC by IPC is a part of the mechanisms leading to IPC-induced tolerance against infarction and arrhythmias during ischemia-reperfusion.

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### DISCLOSURES

No conflicts of interest are declared by the author(s).

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