Protective role of gap junctions in preconditioning against myocardial infarction

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The aim of the present study was to examine the hypothesis that acceleration of gap junction (GJ) closure during ischemia contributes to anti-infarct tolerance afforded by preconditioning (PC). First, the effects of PC on GJ communication during ischemia were assessed. Isolated buffer-perfused rabbit hearts were subjected to 5-min global ischemia with or without PC with two cycles of 5-min ischemia/5-min reperfusion or a GJ blocker (2 mM heptanol), and then the tissue excised from the ischemic region was incubated in anoxic buffer containing lucifer yellow (LY; 2.5 mg/ml), a tracer of GJ permeability, for 20 min at 37°C. PC and heptanol significantly reduced the area to which LY was transported in the ischemic myocardium by 39% and by 54%, respectively. In the second series of experiments, three GJ blockers (heptanol, 18β-glycyrrhetinic acid, and 2,3-butanedione monoxime) infused after the onset of ischemia reduced infarct size after 30-min ischemia/2-h reperfusion to an extent equivalent to that in the case of PC. In the third series of experiments, Western blotting for connexin43 (Cx43) showed that PC shortened the time to the onset of ischemia-induced Cx43 dephosphorylation but reduced the extent of Cx43 dephosphorylation during a 30-min period of ischemia. Calphostin C, a protein kinase C (PKC) inhibitor, abolished preservation of phosphorylated Cx43 but not the early onset of Cx43 dephosphorylation after ischemia in the preconditioned myocardium. These results suggest that PC-induced reduction of GJ permeability during ischemia, presumably by PKC-mediated Cx43 phosphorylation, contributes to infarct size limitation.

infarct size; connexin43; lucifer yellow

PRECONDITIONING (PC) with brief ischemia delays the progression of myocardial injury during subsequent long-sustained ischemia and significantly limits infarct size (IS) (3, 31, 32). This adaptive response of the myocardium is triggered by activation of adenosine A1/A3 and other G protein-coupled receptors during PC ischemia (3, 43). These receptors have been thought to transmit signals to multiple protein kinase-mediated pathways and to ultimately activate cytoprotective effectors, which have not been clearly identified (3, 17, 34, 35). Several studies, including ours, support the notion that the mitochondrial ATP-sensitive K+ (mitoKATP) channel is an effector of PC downstream of protein kinase C (PKC) (25, 27, 29, 34, 40, 49). A PKC activator has been shown to accelerate opening of the mitoKATP channel (40), and we have demonstrated that a selective inhibitor of this channel [5-hydroxydecanoic acid (5-HD)] applied after PC ischemia abolished the protection of PC in rabbit hearts without inhibiting translocation of PKC-ε (34), a key isoform in PC in this species (26). However, conflicting results have also been reported. Administration of 5-HD after PC failed to eliminate the PC effect in some of the other models of myocardial ischemia (33, 35), and the reasons for the different results remain unclear. One plausible explanation for this inconsistency is the presence of additional or different protective effectors downstream of PKC depending on the experimental models and conditions.

In the present study, we hypothesized that the gap junction may be an effector, downstream of PKC-ε, of cardioprotection afforded by PC. The rationale for this hypothesis is fourfold. First, PKC-ε has been shown to phosphorylate connexin43 (Cx43) (7). Second, phosphorylation of Cx43 by PKC accelerates closure of the gap junction (6, 18, 20, 21). Third, gap junctions remain open for some time after the onset of ischemia (39), and this would allow propagation of Na+ overload from severely ischemic cardiomyocytes to adjacent cells (8, 36, 38). Na+ overload primes the cardiomyocyte for Ca2+ overload via reverse-mode operation of the Na+/Ca2+ exchanger (37), and suppression of Na+ overload in the ischemic myocardium by blocking the Na+ channel or Na+/H+ exchange has been shown to reduce ischemia-reperfusion injury (8, 30). Finally, recent studies (4, 44) suggest that PC modifies the phosphorylation status and protein level of Cx43, a major subunit protein of the gap junction in ventricular cardiomyocytes. To test our hypothesis, we assessed the effects of PC on gap junction permeability and Cx43 dephosphorylation during myocardial ischemia and compared the effects of PC with gap junction blockers in terms of IS-limiting effects. In addition, the contribution of PKC to PC-induced alteration in Cx43 phosphorylation during ischemia was examined using calphostin C, a selective PKC inhibitor.

METHODS

This study was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and was permitted by the Animal Use Committee of Sapporo Medical University.

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GAP JUNCTIONS AND PRECONDITIONING

Experiment 1: Assessment of Gap Junction Permeability in the Myocardium

Preparation of isolated perfused rabbit hearts. Rabbit hearts were prepared as in our previous studies (28, 29, 34). In brief, hearts were isolated from male albino rabbits (Japanese White), weighing 2.0 kg, under pentobarbital anesthesia and mechanical ventilation. Each excised heart was quickly mounted onto a Langendorff apparatus and perfused at 75 mmHg with modified Krebs-Henseleit buffer [containing (in mM) 118.5 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 24.8 NaHCO3, 2.5 CaCl2, and 10 glucose], which was continuously oxygenated with 95% O2-5% CO2 and maintained at 38°C. A fluid-filled latex balloon was placed in the left ventricle (LV), and its volume was adjusted to set LV end-diastolic pressure at <5 mmHg. Hearts were excluded from the study if they showed arrhythmias or failed to develop LV pressure above 70 mmHg after a 20-min stabilization period.

Experimental protocol. Hearts were divided into the control group \( (n = 7) \), PC group \( (n = 7) \), and heptanol group \( (n = 5) \), and hearts in all groups underwent 5-min global ischemia. In the PC group, PC was performed with two cycles of 5-min ischemia/5-min reperfusion, and hearts in the heptanol group received 2 mM heptanol for 5 min before ischemia. At the end of the 5-min period of ischemia, each heart was quickly sliced into four sections parallel to the atrioventricular groove, and lucifer yellow (LY) was loaded for 20 min in anoxic buffer according to the method of Ruiz-Meana et al. (36). In brief, an incision was made on the endocardial surface of the ventricular strip using a sharp surgical blade, and the slices were immediately incubated in anoxic PBS containing 2.5 mg/ml of LY and 2.5 mg/ml of rhodamin-conjugated dextran (RD) at 37°C for 20 min. The anoxic buffer was continuously bubbled with 95% N2-5% CO2 before and during the tissue incubation. The heart slices were then fixed with 1% glutaraldehyde-4% formaldeyde in 0.2 M cacodylate buffer (pH 7.4).

Confocal laser microscopy and image analysis. Histology slides were prepared from the fixed tissues, and transport of LY from the fixed tissues, and transport of LY from the edge of the myocardial incision and staining of mechanically injured myocytes with RD were observed using a confocal laser microscope. The area of gap junction permeation was determined as the area of LY staining without RD staining by using NIH Image. Image analysis was performed in four sampling areas and averaged for each heart.

Experiment 2: Infarct Size Experiments

Surgical preparation. Rabbit hearts were isolated and perfused as in experiment 1, and the same exclusion criteria were applied in this series of experiments. Coronary flow was measured as effluent by a graduated cylinder. When the heart rate was below 210 beats/min, the right atrium was electrically paced at this rate before ischemia. No pacing was performed after the onset of ischemia.

Experimental protocol. Hearts were divided into seven groups, and all of the hearts underwent 30-min global ischemia/2-h reperfusion to induced myocardial infarction as shown in Fig. 1. Hearts in the control group received anoxic vehicle alone. The vehicle was normal Krebs-Henseleit buffer, which was made anoxic by bubbling with 95% N2-5% CO2 for >30 min, and was infused at the rate of 2% of preischemic coronary flow using a syringe pump (model 55–2226, Harvard Apparatus; South Natick, MA). Two vehicle groups in which vehicle infusion was commenced at 3 or 15 min after ischemia and continued for 5 min were prepared. However, data obtained from hearts in these groups were combined as a control group because IS did not differ in these two groups. Hearts in the heptanol group, 2,3-butanedione monoxime (BDM) group, and 18β-glycyrrhetinic acid (18β-GA) group received 2 mM heptanol, 30 mM BDM (48), and 40 μM 18β-GA (5, 11), respectively, during ischemia. Because these gap junction blockers markedly suppress contractile function of the heart, which could indirectly modify anti-infarct tolerance, each agent was infused after ischemic arrest of the heart. To minimize the confounding effects of continuous infusion during ischemia, gap junction blockers and the vehicle were infused for only 5 min, and these agents were allowed to remain in the ischemic myocardium until reperfusion. In the heptanol and BDM groups, each agent was infused for 5 min commencing 15 min after the onset of ischemia. Infusion of 18β-GA was commenced at 3 min after ischemia to have a sufficient incubation period. In the heptanol-reperfusion (heptanol-R) group and BDM-reperfusion (BDM-R) group, heptanol and BDM, respectively, were infused for 10 min from the time of reperfusion. In the PC group, PC was performed with two cycles of 5-min ischemia/5-min reperfusion before sustained ischemia.

Analysis of infarct size. At the end of reperfusion, hearts were frozen and sliced into 2-mm-thick sections from apex to base. The uppermost sections were excluded, and other sections were incubated with 1% triphenyltetrazolium solution (pH 7.4 in 100 mM phosphate buffer) to visualize infarcts. The sizes of infarct and the LV were determined by computer-assisted planimetry as in previous studies (28, 29, 34).

Experiment 3: Western Blotting for Cx43

Surgical preparation. Isolated buffer-perfused rabbit hearts were prepared as in experiments 1 and 2.

![Fig. 1](https://example.com/fig1.png)

Fig. 1. Experimental protocol in infarct size experiments. In the control group, vehicle (anoxic buffer) alone was infused at 2% of preischemic coronary flow for 5 min (striped bars) commencing at 15 or 5 min after the onset of ischemia. In the heptanol (Hept) group and 2,3-butanedione monoxime (BDM) group, heptanol and BDM, respectively, were infused for 5 min starting 15 min after ischemia. In the 18β-glycyrrhetinic acid (18β-GA) group, 18β-GA was administered for 5 min starting 3 min after ischemia. To minimize washout of ischemic catabolites, infusion of each test agent was continued for only 5 min (hatched bars), and the agent was trapped in the ischemic myocardium (open bars) until reperfusion. In the heptanol-reperfusion (heptanol-R) group and BDM-reperfusion (BDM-R) group, heptanol and BDM, respectively, were infused for the first 10 min of reperfusion (hatched bars). Hearts in the preconditioning (PC) group received PC with two cycles of 5-min ischemia separated by 5-min reperfusion.
Experimental protocol. Hearts underwent 30-min global ischemia after one of four pretreatments: no treatment (control, n = 5), PC (n = 5), calphostin C (n = 5), or calphostin C plus PC (n = 5). PC was performed with two cycles of 5-min ischemia/5-min reperfusion. Calphostin C (200 nM) was infused during a 20-min preischemic period and allowed to be retained in the myocardium during ischemia. The dose of calphostin C used was based on its reported IC50 for inhibiting PKC (i.e., 50 nM) and results of our previous studies (29, 47) showing that 200 nM calphostin C abolished PKC-<sup>H9280</sup>translocation and the cardioprotective mechanism of PC in isolated rabbit hearts.

Ventricular tissues were biopsied using precooled ophthalmology scissors under baseline conditions, immediately before ischemia, 10 min after ischemia, and 30 min after ischemia. The tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until Western blotting.

Western blotting. Tissue samples were processed for Western blotting by the method used in our previous studies (28, 34) with slight modifications. Myocardial tissues were homogenized in ice-cold buffer containing 20 mM Tris·HCl (pH 7.4), 1 mM EGTA, 5 mM Na<sub>H18528</sub>H<sub>9252</sub>N<sub>2</sub>, 50 mM NaCl, 10 mM β-mercaptoethanol, 100 mM Na<sub>9252</sub>VO<sub>4</sub>, and a protease inhibitor cocktail (Complete Mini, Roche Diagnostics). The homogenate was centrifuged at 600 g for 10 min, and the supernatant was centrifuged at 10,000 g for 20 min. The 10,000-g pellet was used as the intercalated disk fraction. The 10,000-g supernatant was recentrifuged at 100,000 g for 60 min to obtain a cytosol fraction (supernatant) and a particulate fraction (pellet). Intercalated disk fractions and particulate fractions were solubilized by 0.3% Triton-X-containing buffer and centrifuged at 10,000 g for 10 min to prepare samples for electrophoresis. Protein concentration was determined using a Bio-Rad Protein Assay kit (Bio-Rad; Hercules, CA).

Samples were electrophoresed on a 12.5% polyacrylamide gel and then electoblotted onto polyvinylidene difluoride membranes (Millipore; Bedford, MA). After being blocked with 5% nonfat dry milk in buffer containing 100 mM NaCl, 10 mM Tris·HCl (pH 7.4), and 0.1% Tween 20, the blots were incubated with 1,000-fold-diluted antibodies against Cx43 (Transduction Laboratories; Lexington, KY) or unphosphorylated Cx43 (Zymed Laboratories; South San Francisco, CA). The anti-Cx43 antibodies and anti-unphosphorylated Cx43 antibodies were directed to cytoplasmic sequences, amino acids 252–270, and amino acids 360–376, respectively, of rat Cx43. The signals of Cx43 were visualized using an ECL Western Blotting Detection kit (Amersham; Buckinghamshire, UK) and quantified by using SigmaGel, a gel analysis software (SPSS; Chicago, IL).

Statistics

All data are presented as means ± SE. Intergroup comparisons were performed by one-way ANOVA. Differences within a group during experimental periods were examined by repeated-measures ANOVA. When ANOVA indicated a significant difference, the Student-Newman-Keuls post hoc test was used for multiple comparisons of groups or time points. SigmaStat (SPSS) was used to run these statistical analysis. The difference was considered significant if the P value was <0.05.

RESULTS

Experiment 1

Figure 2A shows representative images of cell-to-cell diffusion of LY and staining of injured cells with RD, and a summary of the area stained with LY without RD is shown for each study group in Fig. 2B. Heptanol and PC significantly reduced the area of LY diffusion by 54% and by 39%, respectively. These results indicate that both PC and heptanol suppressed gap junction communication during myocardial ischemia.
Table 1. Hemodynamic data in infarct size experiments

<table>
<thead>
<tr>
<th></th>
<th>Reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>228 ± 7</td>
</tr>
<tr>
<td>Heptanol</td>
<td>228 ± 9</td>
</tr>
<tr>
<td>BDM</td>
<td>222 ± 2</td>
</tr>
<tr>
<td>18β-GA</td>
<td>229 ± 8</td>
</tr>
<tr>
<td>Heptanol-R</td>
<td>224 ± 5</td>
</tr>
<tr>
<td>BDM-R</td>
<td>223 ± 8</td>
</tr>
<tr>
<td>PC</td>
<td>229 ± 6</td>
</tr>
<tr>
<td>LVDP, mmHg</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>113 ± 8</td>
</tr>
<tr>
<td>Heptanol</td>
<td>114 ± 8</td>
</tr>
<tr>
<td>BDM</td>
<td>108 ± 8</td>
</tr>
<tr>
<td>18β-GA</td>
<td>118 ± 6</td>
</tr>
<tr>
<td>Heptanol-R</td>
<td>113 ± 9</td>
</tr>
<tr>
<td>BDM-R</td>
<td>111 ± 6</td>
</tr>
<tr>
<td>PC</td>
<td>107 ± 5</td>
</tr>
<tr>
<td>Coronary flow, ml/min</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>72 ± 2</td>
</tr>
<tr>
<td>Heptanol</td>
<td>70 ± 4</td>
</tr>
<tr>
<td>BDM</td>
<td>67 ± 4</td>
</tr>
<tr>
<td>18β-GA</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>Heptanol-R</td>
<td>63 ± 5</td>
</tr>
<tr>
<td>BDM-R</td>
<td>69 ± 3</td>
</tr>
<tr>
<td>PC</td>
<td>72 ± 3</td>
</tr>
</tbody>
</table>

Values are means ± SE. BDM, 2,3-butanedione monoxime; 18β-GA, 18β-glycyrrhetinic acid; R, reperfusion; PC, preconditioning; LVDP, left ventricular (LV) developed pressure. *P < 0.05 vs. control.

Experiment 2

Hemodynamic data. As summarized in Table 1, there were no significant differences between baseline heart rates, LV developed pressure (LVDP), or coronary flow in the study groups. Recovery of LVDP was improved by the gap junction blockers and PC. In the heptanol-R and BDM-R groups, hearts did not develop significant LV pressure during the 10-min postreperfusion period when heptanol or BDM was infused.

Infarct size data. There were no significant intergroup differences in heart weight or LV volume at risk (Table 2). Figure 3 shows ES in each rabbit together with its group average. All three gap junction blockers significantly reduced IS as a percentage of the LV (%IS/LV); %IS/LV was 7.3 ± 3.5% in the heptanol group, 18.4 ± 4.2% in the BDM group, and 20.2 ± 2.7% in the 18β-GA group, which were significantly smaller than the control infarct size (%IS/LV = 41.6 ± 4.0%). PC limited infarct size (%IS/LV = 21.9 ± 3.1%), as in our previous studies (28, 34). No significant changes in %IS/LV were observed in the heptanol-R and BDM-R groups.

Table 2. Infarct size data

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>LV, cm³</th>
<th>Infarct, cm³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>2.58 ± 0.14</td>
<td>1.08 ± 0.12</td>
</tr>
<tr>
<td>Heptanol</td>
<td>5</td>
<td>2.90 ± 0.18</td>
<td>0.23 ± 0.12*</td>
</tr>
<tr>
<td>BDM</td>
<td>5</td>
<td>2.32 ± 0.12</td>
<td>0.44 ± 0.11*</td>
</tr>
<tr>
<td>18β-GA</td>
<td>5</td>
<td>2.50 ± 0.13</td>
<td>0.51 ± 0.08*</td>
</tr>
<tr>
<td>Heptanol-R</td>
<td>4</td>
<td>2.72 ± 0.15</td>
<td>1.37 ± 0.16</td>
</tr>
<tr>
<td>BDM-R</td>
<td>4</td>
<td>2.75 ± 0.05</td>
<td>1.25 ± 0.17</td>
</tr>
<tr>
<td>PC</td>
<td>8</td>
<td>2.53 ± 0.10</td>
<td>0.37 ± 0.10*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of hearts. *P < 0.05 vs. control.

Fig. 3. Effects of gap junction blockers and PC on infarct size. Each open circle represents infarct size in an individual heart, and closed circles with error bars are group means ± SE. *P < 0.05 vs. control.

Experiment 3

Cx43 was detected by Western blotting almost exclusively in the intercalated fraction, and a slight signal of Cx43 was detected in some of the particulate fractions but in none of the cytosol fractions (data not shown). Although the total Cx43 level tended to be slightly reduced after ischemia, the difference was not statistically significant (Fig. 4). Most of the Cx43 was phosphorylated under baseline conditions, and nonphosphorylated Cx43 was barely detected. In the control group, the percentage of nonphosphorylated Cx43 was significantly increased after 30 min of ischemia. In the group that received PC, significant dephosphorylation had occurred at 10 min after ischemia (Fig. 4B), but phosphorylated Cx43 was significantly more preserved at 30 min after the onset of ischemia than in controls. Calphostin C did not modify the time course of Cx43 dephosphorylation in non preconditioned hearts. However, this PKC inhibitor tended to increase Cx43 dephosphorylation 10 min after ischemia and had abolished preservation of phosphorylated Cx43 at 30 min after ischemia in the preconditioned myocardium (Fig. 5).

DISCUSSION

Studies using electrophysiological indexes (intracellular resistance and whole tissue resistance in vitro or myocardial impedance in situ) have suggested that gap junctions in the myocardium close ~15 to 20 min after the onset of ischemia (2, 10, 46), electrically segregating injured myocytes from nonischemic myocytes. In earlier studies (10, 46), PC caused no effect or an inhibitory effect on electrical uncoupling of cardiomyocytes during ischemia. However, electrical uncoupling may not necessarily reflect complete interruption of transport through the gap junction. Kwak et al. (19) showed that electrical conductance and gap junction permeability could change in opposite directions in neonatal rat cardiomyocytes. Furthermore, Ruiz-Meana et al. (39) recently demonstrated that cell-to-cell transport of LY, a gap junction tracer, was persistent during a 30-min period of myocardial ischemia in rat hearts. In the present study, we were able to reproduce such a LY transport during 25-min ischemia in rabbit hearts in vitro (Fig. 2). In addition, contribution of the gap junction to that LY transport was confirmed by the finding that heptanol, a gap junction blocker, significantly suppressed LY transport during ischemia. Furthermore, we found for the first time that PC...
inhibited LY transport during ischemia, indicating that gap junction closure after ischemia is accelerated in the preconditioned myocardium.

To gain an insight into the mechanism by which PC inhibits gap junction communication during ischemia, we assessed the effects of PC on the phosphorylation status of Cx43. PC accelerated the appearance of nonphosphorylated Cx43 after the onset of ischemia but reduced the extent of dephosphorylation of Cx43, preserving phosphorylated Cx43, during a 30-min period of ischemia (Fig. 4). This finding of preservation of Cx43 phosphorylation by PC is consistent with immunohistochemical observation in a recent study by Schulz et al. (44). They found that PC markedly suppressed the increase in intensity of nonphosphorylated Cx43 staining in intercalated discs after 85–90 min of ischemia in pig hearts in situ. In the present study, the preservation of phosphorylated Cx43 by PC was abolished by calphostin C (Fig. 5), indicating that activated PKC is responsible for the preservation of Cx43 phosphorylation.

It is notable that dephosphorylation of Cx43 after 10 min of ischemia in the preconditioned myocardium was not attenuated but rather tended to be enhanced by calphostin C. This finding suggests that the earlier onset of Cx43 dephosphorylation after ischemia in the preconditioned myocardium is not due to PKC-mediated activation of phosphatases (15, 24) but possibly due to the effect of PC on the intracellular ATP level. Earlier studies (14, 32) have demonstrated that PC causes a partial loss of the adenine nucleotide pool so that the preconditioned myocardium has less ATP at the onset and an early phase of sustained ischemia compared with nonpreconditioned myocardium, although the rate of decline in ATP level after ischemia is slowed by PC. The lack of a change in Cx43 phosphorylation status immediately after PC presumably indicates that ATP depletion by PC ischemia alone was not large enough to induce overall dephosphorylation of Cx43. Because PKC inhibitors have been shown to abolish the cardioprotection afforded by PC (3), persistence of the early onset of Cx43 dephosphorylation during the early phase of ischemia is insufficient or unnecessary for cardioprotection of PC.

PC activates PKC-ε (26, 34), and this protein kinase is expected to phosphorylate serine residues of Cx43, resulting in closure of gap junctions (7, 18, 20). Thus PKC-mediated preservation of Cx43 (Figs. 4 and 5) probably reflects phosphorylation of Cx43 at PKC phosphorylation sites, which

![Fig. 4. Dephosphorylation of Cx43 during ischemia. Top: representative Western blots; bottom: summary of phosphorylated and nonphosphorylated Cx43 expressed as percentages of the total. Base, baseline; Pre-I, immediately before ischemia; I-10, 10 min after ischemia; I-30, 30 min after ischemia. P1 and P2, phosphorylated Cx43; NP, nonphosphorylated Cx43. *P < 0.05 vs. baseline; #P < 0.05 vs. corresponding data in the control by two-way repeated-measures ANOVA with Student-Newman-Keuls post hoc test.](image)

![Fig. 5. Effects of calphostin C on dephosphorylation of Cx43 during ischemia. Calphostin C (200 nM) was infused during a 20-min preischemic period. Phosphorylated and nonphosphorylated Cx43 are expressed as percentages of the total as in Fig. 4. *P < 0.05 vs. baseline; #P < 0.05 vs. corresponding data in the calphostin C group by two-way repeated-measures ANOVA with Student-Newman-Keuls post hoc test.](image)
could have contributed to suppression of gap junction permeability in the preconditioned myocardium (Fig. 2). However, a study by Schulz et al. (44) demonstrated that PC increased colocalization of not only PKC but also p38 MAPK-β with Cx43 in the intercalated disk. Involvement of PKC and other protein kinases, including p38 MAPK, in the change in gap junction permeability caused by PC warrants further investigation.

Inhibition of gap junction communication by PC could be cardioprotective, because gap junctions provide routes for propagation of Na⁺ overload in ischemic myocytes (8, 36, 38). However, it has not been determined whether the persistence of gap junction patency during ischemia indeed contributes to myocardial necrosis after ischemia-reperfusion. In the present study, we used three structurally different blockers of gap junctions (heptanol, BDM, and 18β-GA). Of these three agents, 18β-GA is thought to be most selective to the gap junction, and BDM inhibits actomyosin ATPase (50) in addition to gap junction function (48). To avoid the confounding effects of these gap junction blockers on residual contraction and the rate of ATP depletion during the first 3–10 min of ischemia (14, 45), we administered each gap junction blocker after 3 or 15 min of ischemia in infarct size experiments. Despite differences in structure and pharmacological features, all of the three gap junction blockers limited IS (Fig. 3), whereas the same blockers administered at the time of reperfusion failed to protect the myocardium from infarction. These findings strongly indicate that early closure of the gap junction during ischemia prevents myocardial necrosis after ischemia-reperfusion, although the possibility of involvement of nonspecific effects of gap junction blockers in the results cannot be excluded.

The IS-limiting effect of PC was comparable with that of gap junction blockers administered during ischemia (Fig. 3). Both PC and the gap junction blocker were shown to suppress gap junction permeability in the ischemic myocardium (Fig. 2), and this change in the gap junction permeability could delay cell-to-cell propagation of Na⁺ and Ca²⁺ overload and thus myocyte necrosis (8, 36–38). Taken together, these findings are consistent with our hypothesis that reduction of gap junction permeability is one of mechanisms of PC-induced protection, i.e., delay of ischemic myocardial necrosis (3, 31). The contribution of gap junction closure to PC would be more easily tested if we could assess the effects of a selective opener of the gap junction on PC. Unfortunately, however, there is no such selective agent available at the present time.

In contrast with the results of the present study, the failure of heptanol pretreatment to reduce IS has been reported by Gysenbergh et al. (12) and Li et al. (23). However, Gysenbergh et al. (12) used a much lower dose of heptanol (0.5 mM) than the dose used in the present experiments, and Li et al. (23) washed out heptanol from the myocardium for 10 min before sustained ischemia. These differences in the protocols may be responsible for the different results of their studies and ours. However, an interesting observation in the study by Li et al. (23) is that heptanol infusion during the trigger phase of PC (i.e., infusion before PC and subsequent washout before sustained ischemia) abolished IS limitation by PC in mice hearts. Furthermore, Schwanke et al. (42) found that PC failed to afford cardioprotection in heterozygous Cx43-deficient mice. Taken together, the results in the two murine studies (23, 42) indicate that gap junction communication during PC ischemia (i.e., trigger phase of PC) is important for PC to be cardioprotective. Nevertheless, these studies do not necessarily argue against the possible importance of accelerated gap junction closure during sustained ischemia in the preconditioned myocardium.

A number of studies have indicated a crucial role of the mitoKATP channel in PC, although it is still controversial whether this channel is an effector, a trigger, or both an effector and a trigger of PC (29, 33–35, 40, 46, 49). The relationship between this channel and the gap junction in the mechanism of PC would be an interesting subject for further investigation. However, because the mitoKATP channel is currently thought to serve as an effector or trigger of PC, two relationships between the mitoKATP channel and the gap junction can be speculated. First, the mitoKATP channel and gap junction may be independent effectors distal to PKC, and the contribution of each effector to the overall PC effect might vary depending on experimental conditions. Such a difference in the contribution of the mitoKATP channel, if indeed exists, might underline the discrepancy in observed effects of mitoKATP channel blockers administered after the trigger phase of PC in earlier studies (33–35, 49). The second possibility is that signal transduction triggered by the mitoKATP channel regulates the gap junction function. In a preliminary study, we found that diazoxide, a mitoKATP channel opener, mimicked the effect of PC on gap junction permeability, which was assessed by LY, in the ischemic myocardium (unpublished observations). In a recent study by Jain et al. (13), a mitoKATP channel blocker inhibited the effect of PC causing delay in electrical uncoupling in the ischemic myocardium. The findings in those two studies are not necessarily contradictory, because gap junction permeability and the electrical conductance of gap junctions can change in opposite directions (19). Nevertheless, both of those studies suggest a possible regulation of the gap junction by KATP channel activity in PC.

One possible argument against the contribution of gap junctions to PC is that PC protects isolated cardiomyocytes that lack gap junctions. However, the role of the mitoKATP channel as an effector of PC (25–27, 40) may be predominant in PC in isolated cardiomyocytes. In addition, there is the possibility that PC modifies gap junction hemichannels in isolated cardiomyocytes (16, 22). Nevertheless, it is notable that the extent of myocardial salvage by PC is modest in isolated cardiomyocyte preparations than in whole hearts (1, 26, 27), suggesting a possible difference between PC mechanisms in these preparations.

Several investigators have shown that administration of heptanol and BDM upon reperfusion attenuated myocardial injury. Garcia-Dorado et al. (9) reported that administration of gap junction blockers at the time of reperfusion suppressed reperfusion injury in rat hearts in vitro and swine hearts in situ. They infused heptanol into the coronary artery during the first 15 min after 30 or 48 min of ischemia. This treatment suppressed contraction band necrosis and reduced IS. In a study by Schlack et al. (41), intracoronary infusion of BDM for 65 min commencing 5 min before reperfusion limited IS in canine hearts. However, such protective effects of heptanol and BDM were not detected in isolated rabbit hearts in the present experiments, and we do not have a clear explanation for the different outcomes. However, differences in duration of treat-
ment and preparation for myocardial ischemia might be involved.

In summary, the present study showed that PC reduced the extent of Cx43 dephosphorylation by a PKC-dependent mechanism and suppressed gap junction permeability during ischemia. Conversely, pharmacological blockade of gap junction communication during ischemia mimicked the IS-limiting effect of PC. These results suggest that reduction of gap junction permeability, presumably by PKC-mediated Cx43 phosphorylation, is one of the mechanisms of cardioprotection afforded by PC.

GRANTS
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REFERENCES


