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Roles of Cx43-associated protein kinases in suppression of gap junction-mediated chemical coupling by ischemic preconditioning

Kazuyuki Naitoh,* Toshiyuki Yano,* Tetsuji Miura, Takahito Itoh, Takayuki Miki, Masaya Tanno, Takahiro Sato, Hiroyuki Hotta, Yoshiaki Terashima, and Kazuaki Shimamoto

Second Department of Internal Medicine, Sapporo Medical University School of Medicine

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Naitoh K, Yano T, Miura T, Itoh T, Miki T, Tanno M, Sato T, Hotta H, Terashima Y, Shimamoto K. Roles of Cx43-associated protein kinases in suppression of gap junction-mediated chemical coupling by ischemic preconditioning. *Am J Physiol Heart Circ Physiol* 296: H396–H403, 2009. First published December 19, 2008; doi:10.1152/ajpheart.00448.2008.—Ischemic preconditioning (PC) suppresses chemical coupling of cardiomyocytes via gap junctions (GJs) during ischemia, which is an adjunct mechanism of protection. The aim of this study was to characterize roles of protein kinases in PC-induced GJ modulation. In isolated rat hearts, ventricular tissues were sampled before and after ischemia with or without PC, and intercalated disc-rich fractions were separated for immunoprecipitation and immunoblotting. Levels of protein kinase C (PKC)-ε, p38mitogen-activated protein kinase (MAPK)-α, and Src coimmunoprecipitated with connexin-43 (Cx43) were increased after ischemia, whereas p38MAPKβ was not detected in the Cx43 immunoprecipitates. PC did not modify the level of Cx43-Src complex after ischemia. However, PC enhanced Cx43-PKCε complex formation, which was abolished by PKCε translocation inhibitory peptide (TIP). In contrast, PC reduced Cx43-p38MAPKα complex level and p38MAPK activity in the Cx43 immunoprecipitates after ischemia. The effect of PC on Cx43-p38MAPKα interaction was mimicked by SB-203580, a p38MAPK inhibitor. PC reduced permeability of GJs to Lucifer yellow in the myocardium at 25 min after ischemia, and this effect was abolished by PKCε-TIP. SB-203580 increased the GJ permeability at 15 min after ischemia compared with that in untreated controls, but the difference became insignificant 25 min after ischemia. In conclusion, PC has distinct effects on interaction of GJ Cx43 with PKCε, p38MAPKα, and Src during ischemia. Suppression of GJ permeability during ischemia by PC is primarily achieved by enhanced interaction of Cx43 with PKCε, which overwhelms the counterbalancing effect of reduced Cx43-p38MAPKα interaction.

connexin-43; protein kinase C; p38 mitogen-activated protein kinase; Src

ISCHEMIC PRECONDITIONING (PC) is a potent intervention for protecting the myocardium from infarction and arrhythmias (14, 34). We found that PC significantly suppresses chemical coupling of gap junctions in the ischemic myocardium (19). The contribution of PC-induced gap junction blockade to myocardial protection is difficult to assess since there is no selective and direct opener of closed gap junctions. However, there are several lines of circumstantial evidence suggesting that chemical uncoupling of the gap junction during ischemia-reperfusion is a part of the mechanism of infarct size limitation afforded by PC: infarct size-limiting effects of structurally different gap junction blockers (7, 19, 20, 26), suppression of gap junction permeability by PC mimetics (20, 21), and partial loss of protection afforded by a δ-opioid receptor agonist when suppression of gap junction permeability was abrogated (20). In addition to myocardial necrosis, arrhythmias during ischemia-reperfusion are suppressed by PC (6, 24, 25), which is possibly associated with alteration in electrical coupling of cardiomyocytes (6, 13, 24). However, the molecular mechanisms by which PC modifies gap junction permeability remain unclear.

We hypothesized that direct interaction of connexin-43 (Cx43), a major gap junction protein, with protein kinases activated by PC is responsible for PC-induced chemical uncoupling of the gap junction during ischemia. As candidates of such protein kinases, we selected protein kinase C (PKC)-ε, Src, and p38mitogen-activated protein kinase (p38MAPK). These kinases are involved in myocardial protection by PC (2, 22, 31, 34) and have been shown to inhibit gap junction-mediated cell-to-cell communication in cardiac and noncardiac tissues (8, 9, 12, 18, 23, 32, 35). Interaction of the protein kinases with Cx43 was assessed by using immunoprecipitation (IP) and immunoblotting, and gap junction-mediated chemical coupling was determined by the use of a tracer, Lucifer yellow. The results showed that PC has opposite effects on Cx43-PKCε interaction and Cx43-p38MAPK interaction during ischemia and suggested that enhanced binding of PKCε to Cx43 is a primary mechanism of chemical uncoupling of cardiomyocytes by PC.

METHODS

This study was approved by the Committee for Animal Research, Sapporo Medical University, and was conducted in strict accordance with the Guidelines of Sapporo Medical University for Animal Use in Research and the *Guide for the Care and Use of Laboratory Animals* published by the United States National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Experiment I: IP-Immunoblotting Experiments

Surgical preparation. Sprague-Dawley rats of 8~10 wk of age were anesthetized by pentobarbital sodium (40 mg/kg ip injection), intubated, and mechanically ventilated with a Harvard rodent ventilator using oxygen supplement. Hearts were excised, quickly mounted on a Langendorff apparatus, and perfused with modified Krebs-Henseleit buffer as previously reported (20, 21). Perfusion pressure and temperature of the perfusate were maintained at 75 mmHg and 38°C, respectively.

Experimental protocols. In *protocol 1*, ventricular tissues for IP and immunoblotting were sampled before and at 10 and 35 min into global

* K. Naitoh and T. Yano contributed equally to this work.

Address for reprint requests and other correspondence: T. Miura, Second Dept. of Internal Medicine, Sapporo Medical Univ. School of Medicine, S-1 W-16, Chuo-ku, Sapporo 060-8543, Japan (e-mail: miura@sapmed.ac.jp).

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ischemia. Wollenberger tongs precooled in liquid nitrogen were used for tissue sampling, and frozen tissues were stored at −80°C until analyses. In *protocol 2*, hearts were assigned to one of seven pretreatments before 35 min global ischemia: *1*) no pretreatment (controls), *2*) PC with two cycles of 5 min ischemia and 5 min reperfusion, *3*) infusion of a Src inhibitor [5 μM 4-amino-5-(methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-d]pyrimidine (PP1); BIOMOL International, Plymouth Meeting, PA], *4*) infusion of a PKCε inhibitor [100 nM PKCε translocation inhibitory peptide (TIP); Calbiochem/EMD, Darmstadt, Germany], *5*) infusion of a p38MAPK inhibitor (1 μM SB-203580; Sigma, St. Louis, MO), *6*) infusion of PKCε-TIP and PC, and *7*) infusion of SB-203580 and PC. Infusion of the protein kinase inhibitors was commenced at 10 min before ischemia in the groups without PC and at 5 min before PC in the group with PC, and infusion was discontinued at the onset of global ischemia. Ventricular tissues were sampled before and after 10 and 35 min of ischemia in the untreated control and PC groups, and the sampling was performed only at 35 min after the onset of ischemia in the inhibitor control groups and inhibitor plus PC groups.

IP and immunoblotting. IP of Cx43 and immunoblotting for kinases were performed as in our previous studies (20, 21). In brief, tissues were homogenized in ice-cold Tris buffer containing 20 mM Tris·HCl, 1 mM EGTA, 5 mM NaN_3, 50 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 mM Na_3VO_4, and a protease inhibitor cocktail (Complete Mini; Roche Diagnostics, Manheim, Germany). The homogenate was first centrifuged at 600 *g* for 10 min, and the supernatant was recentrifuged at 10,000 *g* for 20 min. This 10,000-*g* pellet was used as the intercalated disc-rich fraction, and 1,000 μg of this fraction were solubilized by 500 μl of IP buffer [20 mM Tris·HCl (pH 7.4), 1 mM EGTA , 5 mM NaN_3, 50 mM NaCl, 1 mM PMSF, 50 mM Na_3VO_4, 1% Triton X-100, 0.5% Nonidet P-40, and a protease inhibitor cocktail] and preincubated with 50 μl protein G magnetic beads (New England BioLabs, Ipswich, MA) for 1 h to remove proteins that can bind nonspecifically to the beads. The supernatant was taken and incubated with 5 μg of anti-Cx43 antibodies or anti-phospho-Ser^{368}-Cx43 antibodies for 1 h, and the mixture was then incubated with 50 μl of fresh beads for 1 h. The beads were washed two times using 500 μl of IP buffer, resuspended in 30 μl of SDS sample loading buffer [125 mM Tris·HCl (pH 6.8), 4.3% SDS, 30% glycerol, 10% β-mercaptoethanol, and 0.01% bromphenol blue], and incubated at 70°C for 5 min. Finally, 20 μl of the supernatant was used for immunoblotting for Src, PKCε, p38MAPK, and phospho-Ser^{368}-Cx43. A Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA) was used for protein assay. Samples for immunoblotting were electrophoresed on a 12.5% polyacrylamide gel and then blotted on a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). After blocking with a Tris buffer containing 5% nonfat dry milk and 0.1% Tween 20, the blots were incubated with antibodies against c-Src (Santa Cruz Biotechnology, Santa Cruz, CA), PKCε (BD Biosciences, San Jose, CA), p38MAPK (Cell Signaling Technology, Beverly, MA), p38MAPKα (Cell Signaling Technology), p38MAPKβ (Santa Cruz Biotechnology), Cx43 (BD Biosciences), or phospho-Ser^{368}-Cx43 (Cell Signaling Technology). Peroxidase-linked anti-rabbit IgG F(ab′)2 fragments, anti-mouse IgG F(ab′)2 fragments (Amersham Biosciences, Buckinghamshire, UK), and anti-goat IgG (Santa Cruz Biotechnology) were used as secondary antibodies. PVDF membranes were then used for reblotting with anti-Cx43 antibodies after stripping by using a Re-Blot Western Recycling Kit (CHEMICON International, Temecula, CA). Blotted proteins were visualized by using an ECL Western blotting detection kit (Amersham Biosciences) and quantified by a lumino-image analyzer, LAS-2000mini (Fujifilm, Tokyo, Japan). Densities of Src, PKCε, and p38MAPK signals were normalized by Cx43 in reblots of the same PVDF membranes. Specificities of signals in immunoblottings were confirmed in pilot experiments by negative control experiments (i.e., elimination of primary antibodies) and/or use of commercially available positive controls.

Determination of p38MAPK activity. Because results of immunoblotting experiments (below) showed that an inhibitor of p38MAPK mimicked the effect of PC on Cx43-p38MAPK interaction, p38MAPK activity in the Cx43 immunoprecipitate was determined by the use of exogenous ATF-2 as a substrate. Intercalated disc-rich fractions were prepared from tissue samples taken 35 min after ischemia with or without PC and immunoprecipitated with anti-Cx43 antibody as described above. The Cx43 immunoprecipitates (1,000 μg) were washed two times with 500 μl of kinase buffer (in mM: 25 Tris·HCl, pH 7.5, 5 β-glycerophosphate, 2 dithiothreitol, 0.1 Na_3VO_4, and 10 $MgCl_2$) and then incubated with 200 μM ATP and 1 μl ATF-2 fusion protein (Cell Signaling Technologies) in 50 μl kinase buffer at 30°C for 30 min. The reaction was stopped by addition of 25 μl of SDS sample loading buffer, and the reaction mixture was incubated at 70°C for 5 min. Each supernatant (20 μl) was electrophoresed and immunoblotted for phospho-ATF-2 using anti-phospho-ATF-2 antibody (Cell Signaling Technologies).

Experiment II: Gap Junction Permeability Experiments

Experimental protocol. Rat hearts were isolated and perfused with modified Krebs-Henseleit buffer as in *experiment I*. In *protocol 1*, hearts were subjected to one of the following five pretreatments before ischemia: no pretreatment, PC with two cycles of 5 min ischemia-5 min reperfusion, infusion of 100 nM PKCε-TIP plus PC, infusion of 100 nM scrambled TIP (a negative control of PKCε-TIP; Calbiochem/EMD) plus PC, and infusion of 1 μM SB-203580. Infusion of PKCε-TIP and that of scrambled TIP were commenced 5 min before PC and continued until the onset of sustained ischemia. SB-203580 was infused for 10 min before ischemia. At 5 min after the onset of ischemia, ventricles were subjected to ischemia under a gap junction tracer loading condition (see below) for an additional 20 min. In *protocol 2*, hearts received no pretreatment or infusion of 1 μM SB-203580 before ischemia. The hearts were rendered ischemic as in *protocol 1* except that duration of ischemia under tracer loading was shortened to 10 min.

Determination of gap junction permeability in ischemic myocardium. Gap junction-meditated chemical coupling of cardiomyocytes during ischemia was determined by the use of Lucifer yellow (LY), an anionic gap junction tracer, in vitro as previously reported (19–21). To confirm that the results by use of LY were not specific to anion tracers, we used a cationic tracer, ethidium bromide (EB), in eight post hoc experiments for assessment of the effect of PC. After 5 min ischemia on the Langendorff apparatus, hearts ($n = 30$) were quickly removed from the apparatus, and then a transmural incision was made with a surgical blade in the ventricular wall along the long axis, and the hearts were incubated in anoxic PBS containing 2.5 mg/ml LY and 2.5 mg/ml rhodamine-conjugated dextran (RD) for 20 min (*protocol 1*) or 10 min (*protocol 2*) at 37°C. In additional experiments using EB, the tissue was incubated in the anoxic buffer containing EB (2.5 mg/ml) for 20 min. Next, the hearts were fixed with 1% glutaraldehyde-4% formaldehyde. Histology slides for confocal laser microscopy were made from heart slices, and the area was stained with LY from which the area stained with RD was subtracted and used as an index of gap junction communication. The area data from heart slices were averaged for each heart.

Statistical Analyses

Intergroup differences in protein levels and indexes of gap junction permeability were tested by primarily one-way ANOVA, and two-way ANOVA was used for assessment of the effects of PC and protein kinase inhibitors on interaction of Cx43 and protein kinases at 35 min after ischemia. When ANOVA indicated an overall difference, multiple comparisons were performed by the use of the Student-Newman-Keul's post hoc test. $P < 0.05$ was considered to be statistically significant.

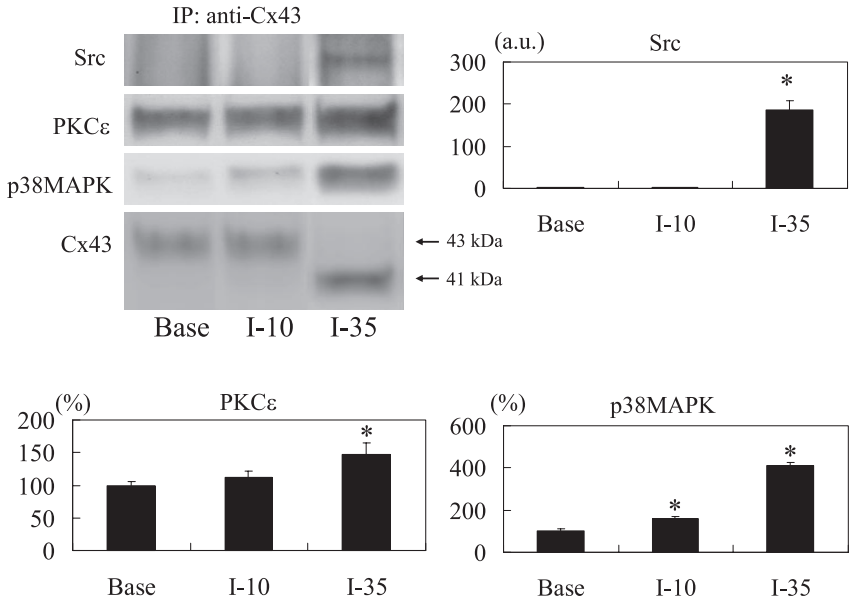

Fig. 1. Effects of ischemia on Src, protein kinase C (PKC) ε, and p38 mitogen-activated protein kinase (MAPK) protein levels in connexin (Cx) 43 immunoprecipitates. Cx43 immunoprecipitates were prepared from the tissues sampled before or after 10 or 35 min ischemia and immunoblotted for Src, PKCε, or p38MAPK. Representative immunoblots and summary of group data are shown. Level of Src protein kinase is expressed in arbitrary units (AU). For PKCε and p38MAPK, protein level is expressed as a percentage of the mean of baseline values. *$P < 0.05$ vs. baseline. IP, immunoprecipitation; Base, baseline; I-10, 10 min after ischemia; I-35, 35 min after ischemia. $N = 6$ in each group.

RESULTS

Experiment I

Effects of ischemia on interactions of Cx43 with protein kinases. Figure 1 shows results in protocol 1: protein levels of Src, p38MAPK, and PKCε in Cx43 immunoprecipitates before and after ischemia. Cx43-Src complex was detected 35 min after ischemia but not under the baseline condition or at 10 min after ischemia. PKCε and p38MAPK were coimmunoprecipitated in the nonischemic myocardium, and their levels increased after the onset of ischemia. The level of Cx43-PKCε complex was elevated by 147 ± 17% at 35 min after ischemia, and the level of Cx43-p38MAPK complex was increased by 158 ± 11 and by 411 ± 13% after 10 and 35 min ischemia, respectively. To identify the isoform of p38MAPK bound to Cx43 in the ischemic myocardium, p38MAPK immunoblotting experiments were repeated using isoform-selective antibodies. As shown in Fig. 2, p38MAPKα, but not p38MAPKβ, was detected in Cx43 immunoprecipitates, and the time course of Cx43-p38MAPKα complex level after ischemia was similar to the results obtained by using the isoform nonselective p38MAPK antibody. In post hoc experiments, we assessed the effect of PC on interaction of Cx43 and p38MAPKβ during ischemia. As in the nonpreconditioned ischemic myocardium (Fig. 2), p38MAPKβ was not detected by immunoblotting in the Cx43 immunoprecipitates prepared from myocardium subjected to PC before 35 min ischemia ($n = 3$ for each treatment, data not shown).

Effects of PC and protein kinase inhibitors on Cx43 binding with protein kinases during ischemia. In *protocol 2*, PC neither increased nor decreased Cx43-Src complex level before ischemia or after 10~35 min ischemia (Fig. 3). Inhibition of Src activity by PP1 tended to reduce Cx43-Src complex level, suggesting a possible role of Src activity in formation of this protein complex.

As shown in Fig. 4, PC per se tended to increase Cx43-PKCε complex level and significantly enhanced ischemia-induced complex formation of Cx43 and PKC-ε. Pretreatment with PKCε-TIP completely inhibited PC-induced enhancement of Cx43-PKCε binding. SB-203580 did not modify Cx43-PKCε interaction during ischemia in the preconditioned or nonpreconditioned myocardium. Immunoblotting for phospho-Ser^{368}-Cx43 showed that PC tended to increase phosphorylation of Cx43 at Ser^{368}, a PKC phosphorylation site after ischemia, compared with that in nonpreconditioned controls. However, the difference did not reach a statistical significance (data not shown). Thus we immunoprecipitated phospho-Ser^{368}-Cx43 in tissue samples and immunoblotted for PKCε. As shown in Fig. 5, PC significantly increased phospho-Ser^{368}-Cx43-PKCε complex after 35 min ischemia by more than twofold. Taken together, these findings indicate that PC augments Cx43-PKCε interaction and phosphorylation of Cx43 by PKC during ischemia.

In contrast to Cx43-PKCε interaction, formation of Cx43-p38MAPKα complex during ischemia was significantly suppressed by PC (Fig. 6). This inhibitory effect of PC on

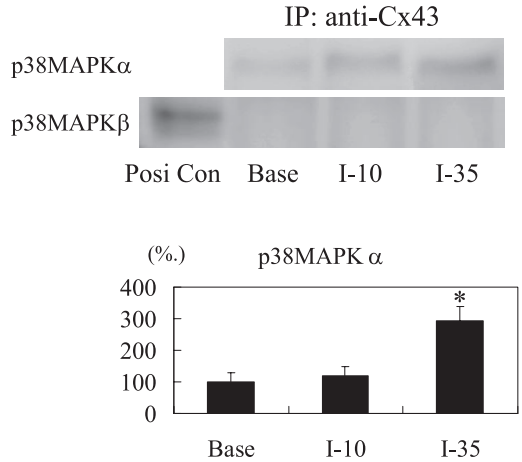

Fig. 2. Effects of ischemia on protein levels of α-isoform and β-isoform of p38MAPK in Cx43 immunoprecipitates. Cx43 immunoprecipitates were prepared from the tissues sampled before or after 10 or 35 min ischemia and immunoblotted for p38MAPKα or p38MAPKβ. Representative immunoblots and summary of group data are shown. Protein level is expressed as a percentage of the mean of baseline values. *$P < 0.05$ vs. baseline. Posi Con, positive control. $N = 6$ in each group.

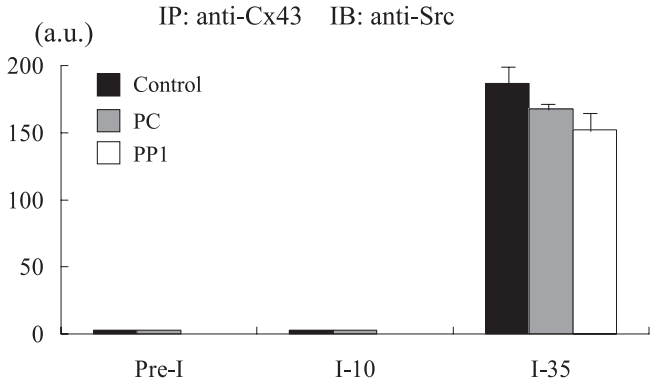

Fig. 3. Effects of preconditioning (PC) on Cx43-Src interaction during ischemia. Levels of Cx43-Src complex in tissues sampled before or after 10 or 35 min ischemia with or without PC are shown. Protein levels of Src in the Cx43 immunoprecipitate are expressed in arbitrary units. IB, immunoblotting; Pre-I, preischemia; I-10, 10 min after ischemia; I-35, 35 min after ischemia. N = 3~6 in each group.

Cx43-p38MAPKα interaction was neither inhibited nor enhanced by PKCε-TIP. However, SB-203580 mimicked the effect of PC on Cx43-p38MAPKα interaction, and the combination of SB-203580 with PC did not further reduce the protein level of Cx43-p38MAPKα complex.

Effects of PC on p38MAPK activity in gap junction Cx43 complex. Because PC reduced the level of p38MAPK protein bound to Cx43 in the ischemic myocardium as did SB-203580 (Fig. 6), we assessed PC-induced changes in p38MAPK activity in the Cx43 protein complex at 35 min after ischemia. p38MAPK activity in Cx43 immunoprecipitates was reduced by PC to 78 ± 4% of the control level (Fig. 7).

Experiment II

In *protocol 1*, PC suppressed gap junction communication assessed by LY during 25 min ischemia to 73 ± 6% of the control level (Fig. 8, *A* and *B*). This inhibitory effect of PC was confirmed by use of a cationic tracer as well; the area stained with EB was significantly reduced to 83.0 ± 3.2% of the control by PC. As shown in Fig. 8*B*, suppression of gap junction permeability by PC was not inhibited by scrambled

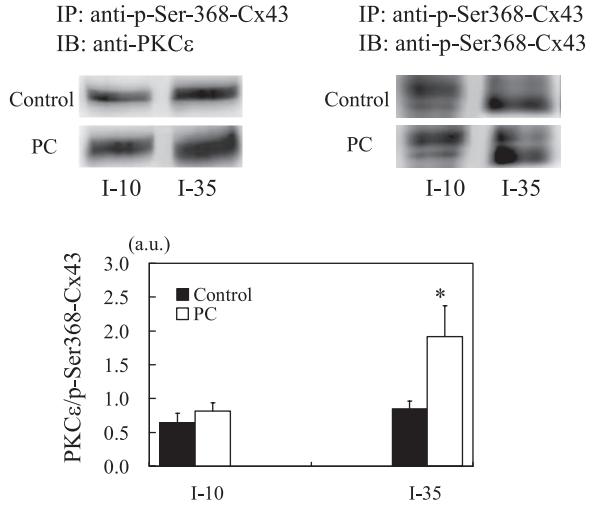

Fig. 5. Effects of PC on phospho-Ser^{368}-Cx43-PKCε interaction. Tissues were sampled after 10 or 35 min ischemia with or without PC, and phospho-Ser^{368}-Cx43 was immunoprecipitated. The immunoprecipitate was immunoblotted for PKCε and then reblotted for phospho-Ser^{368}-Cx43. Representative immunoblots and summary of group data are shown. Level of PKCε was expressed as densitometric ratio of PKCε to p-Ser^{368}-Cx43. *$P < 0.05$ vs. control at the same time point. N = 6 in each group.

TIP but was abolished by pretreatment with PKCε-TIP. SB-203580 increased gap junction communication by 10%, which was not statistically significant. However, in *protocol 2* (Fig. 8*C*), in which gap junction communication in the ischemic myocardium was determined 10 min earlier than in *protocol 1*, the extent of gap junction communication was significantly larger by 50% in the SB-203580-treated group than in untreated controls.

DISCUSSION

Physical Interaction of Cx43 with Protein Kinases During Myocardial Ischemia

Ischemia suppresses gap junction communication and uncouples cardiomyocytes. However, recent studies (19, 27) have shown that chemical coupling via the gap junction is main-

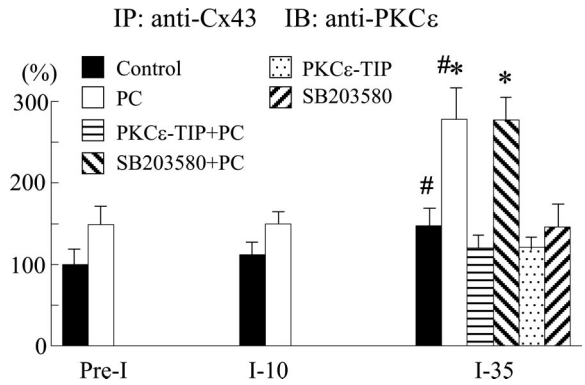

Fig. 4. Effects of PC on Cx43-PKCε interaction. Levels of Cx43-PKCε complex in tissues sampled before or after 10 or 35 min ischemia with or without PC are shown. Protein levels of PKCε in the Cx43 immunoprecipitate are expressed as percentages of the mean of baseline preischemic PKCε levels in the control group. $P < 0.05$ vs. control at the same time point (*) and vs. Pre-I in the same treatment group (#). N = 3~6 in each group.

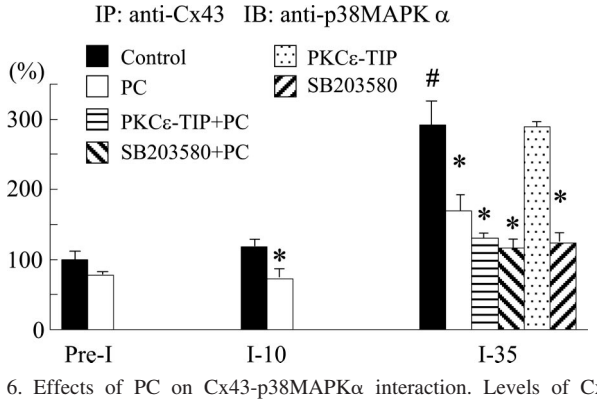

Fig. 6. Effects of PC on Cx43-p38MAPKα interaction. Levels of Cx43-p38MAPKα complex in tissues sampled before or after 10 or 35 min ischemia with or without PC are shown. Protein levels of p38MAPKα in the Cx43 immunoprecipitate are expressed as percentages of the mean of baseline preischemic p38MAPKα levels in the control group. $P < 0.05$ vs. control at the same time point (*) and vs. Pre-I in the same treatment group (#). N = 3~6 in each group.

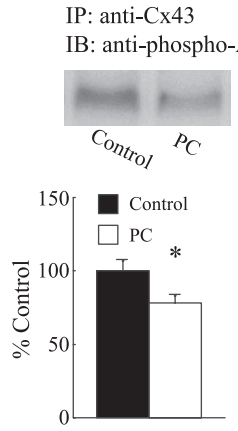

Fig. 7. Effects of PC on p38MAPK activity in the Cx43 immunoprecipitate. Activity of p38MAPK in the Cx43 immunoprecipitates prepared from tissues sampled at 35 min after ischemia with or without PC are shown. Representative immunoblots for phospho-ATF-2 (*top*) and summary of group data (*bottom*) are presented. Levels of ATF-2 phosphorylation are expressed as percentages of the mean in the control group. *$P < 0.05$ vs. control. $N = 6$ in each group.

tained for a considerable time after electrical uncoupling of myocytes, and the reason for the time lag between electrical uncoupling and chemical uncoupling remains unknown. Although phosphorylation of Cx43 by protein kinases is known to be one of the regulatory mechanisms of gap junction communication (8, 9, 12, 18, 23, 32, 35), physical interaction of Cx43 with protein kinases during ischemia in cardiomyocytes has not been systematically examined. In the present study, we found that ischemia significantly increased binding of PKCε, p38MAPK, and Src to Cx43 with different time courses, whereas Cx43 was dephosphorylated (Fig. 1). The results for Src are consistent with a recent finding by Li et al. (16) that Cx43 in astrocytes selectively bound to dephosphorylated Cx43 during simulated ischemia. Nevertheless, the present results suggest that PKCε, p38MAPK, and Src have distinct roles in regulation of the gap junction during ischemia in terms of chemical and/or electrical intercellular communication.

PKCε and PC-Induced Suppression of Gap Junction Permeability

In our previous studies, PC attenuated Cx43 dephosphorylation during ischemia in a PKC inhibitor-sensitive manner (19), and activation of the δ-opioid receptor induced both formation of the Cx43-PKCε complex and phosphorylation of Cx43 at Ser^{368}, a PKC target site (20). Consistent with these findings, PC significantly enhanced physical interaction of PKCε with Cx43 during ischemia in the present experiments (Fig. 4). Level of phospho-Ser^{368}-Cx43-PKCε complexes was also increased by PC (Fig. 5). Ser^{368} in Cx43 is known to be a PKC targeting site in Cx43, and our previous study has shown that phosphorylation of Cx43 at Ser^{368} is sensitive to a PKCε selective inhibitor, PKCε-TIP (20). Furthermore, PC-induced suppression of gap junction permeability was abolished by an inhibitor of PKCε translocation, PKCε-TIP, as shown in Fig. 8. No effect of PKCε-TIP alone on gap junction permeability in the nonpreconditioned ischemic rat myocardium has been confirmed in our previous study (20). Taken together, these results support the notion that translocation of PKCε to the gap junction and direct phosphorylation of Cx43 by this kinase is a primary mechanism of PC-induced suppression of the gap junction-mediated chemical coupling in the ischemic myocardium.

Because significant effects of PC on interaction of Cx43 with protein kinases were observed at 10 and/or 35 min after ischemia (Figs. 4~6), we selected a time point for determination of gap junction permeability between these two time points (i.e., 25 min after ischemia) (Fig. 8). To exclude the possibility that the effect of PC on gap junction permeability is markedly different between 25 and 35 min after ischemia, we performed post hoc experiments in which gap junction permeability was determined at 35 min after the onset of ischemia in hearts with or without PC ($n = 3$ for each group). In the control myocardium, the area stained with LY at 35 min ischemia was 13% larger compared with that at 25 min after ischemia, and PC reduced the gap junction communication by 36%. We also determined gap junction permeability at 10 min after ischemia. The myocardium stained with LY after 10 min ischemia was ~25% of that after 35 min ischemia in both preconditioned and nonpreconditioned myocardium ($n = 3$ for each group). These results of additional experiments are also consistent with the

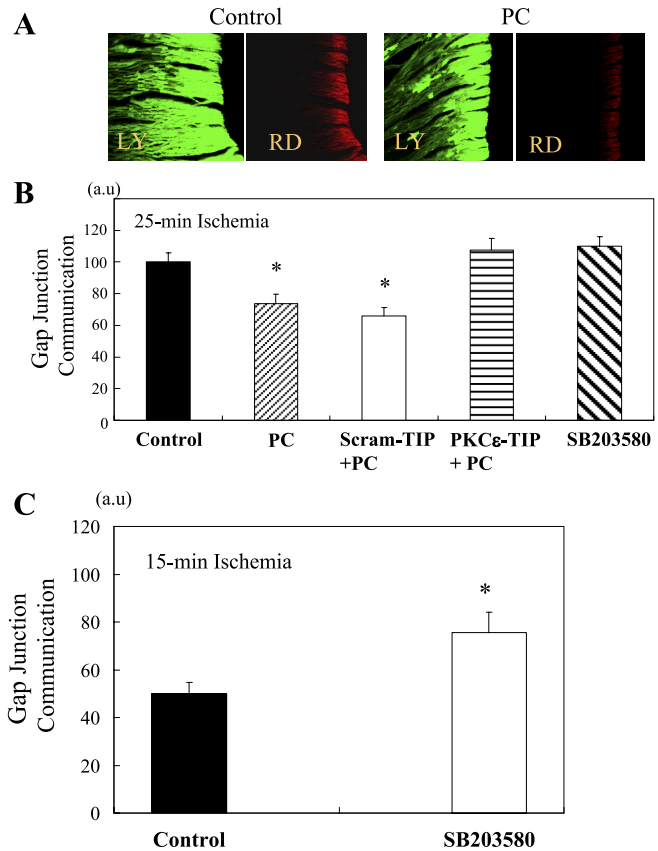

Fig. 8. Effects of PC and SB-203580 on gap junction permeability in ischemic myocardium. Size of area where Lucifer yellow (LY) was transported via gap junctions during ischemia ex vivo was used as an index of gap junction permeability. Areas stained with rhodamine-conjugated dextran (RD) indicate those with disruption of sarcolemma, which was made for LY loading. RD-stained areas were subtracted from LY-stained area for determination of gap junction permeability. *A*: representative images of the control and preconditioned hearts. *B*: group mean data in *protocol 1* (25 min ischemia protocol). Scram-TIP, scrambled TIP. *$P < 0.05$ vs. control. *C*: Group mean data in *protocol 2* (15 min ischemia protocol). $N = 3$~6 in each group.

notion that suppression of gap junction communication by PC is in parallel with the increase in Cx43-PKCε interaction after ischemia.

The mechanism upstream of PKC in gap junction modulation by PC was not explored in the present study. However, numerous earlier studies (2, 14, 34) have established that PC activates several classes of G protein-coupled receptors (such as bradykinin, adenosine, and δ-opioid receptors), which provoke activation of PKC. Although the relative importance of each class of receptors in PC differs depending on animal species, the δ-opioid receptor is a major receptor responsible for triggering the PC mechanism in rat hearts (10). Our recent study (20) showed that activation of the δ-opioid receptor before ischemia significantly suppressed gap junction permeability in the ischemic myocardium as did PC in the present study. Thus the δ-opioid receptor appears to be mainly responsible for PKCε activation in PC-induced gap junction regulation, although other receptors such as adenosine receptors and bradykinin receptors (17, 33) may also be contributory.

Effects of PC on p38MAPKα-Cx43 Interaction and its Functional Outcome

Earlier studies (11, 22, 29, 31) suggest that activation of p38MAPK during PC for triggering the PC mechanism and suppression of p38MAPK activity during sustained ischemia are important for PC to be protective. However, the effect of PC on interaction of p38MAPK with gap junction Cx43 has been examined in only one study (30). Using quantitative immunohistochemistry, Schulz et al. (30) found that PC increased, by ~20%, colocalization of both p38MAPKα and p38MAPKβ with gap junction Cx43 after 85 min ischemia in pig hearts in situ. Because p38MAPK has been shown to reduce gap junction permeability (23, 35), the findings by Schulz et al. (30) suggest possible contribution of this kinase to gap junction suppression by PC. However, in the present study, binding of p38MAPKα to Cx43 after 10 and 35 min ischemia was actually reduced by PC in rat hearts (Fig. 6), and there was no interaction between the β-isoform of p38MAPK and Cx43 after ischemia regardless of PC. Activity of p38MAPK in the Cx43 immunoprecipitate from the myocardium subjected to 35 min ischemia was significantly reduced by PC, and the effect of PC on p38MAPKα-Cx43 interaction was mimicked by SB-203580, a p38MAPK inhibitor. These results suggest that, in rat hearts, at least, PC suppresses interaction of p38MAPKα with Cx43 by reduction of p38MAPK activity during sustained ischemia. We do not have a clear explanation for the apparent discrepancy between our results and those of the study by Schulz et al. (30), although methodological differences in the two studies, including animal species and methods for analysis of protein-protein interaction, may be responsible.

Because p38MAPK has been shown to inhibit gap junction communication (23, 24), the possible functional outcome of reduced binding of p38MAPK to Cx43 complex is an increase in gap junction permeability. In fact, gap junction communication assessed 15 min after ischemia was significantly enhanced by SB-203580 (Fig. 8*C*). Such an effect of SB-203580 was statistically insignificant at 25 min after ischemia, suggesting that other regulatory mechanisms cancel the effect of p38MAPK inactivation. This is not surprising, since a number of protein kinases and phosphatases are involved in phosphorylation-mediated regulation of the gap junction function (8, 12). Nevertheless, the present findings suggest that suppressed p38MAPKα interaction with Cx43 by PC partly counterbalances PKCε-mediated inhibition of gap junction permeability during the early phase of sustained ischemia.

Relationship Between Modulation of Cx43 by PKCε and That by p38MAPKα

Because binding of a protein kinase to a target protein potentially modifies its conformation and affinity to other kinases, we examined whether PKCε translocation to Cx43 is necessary for suppression of p38MAPKα binding to Cx43 or vice versa in the preconditioned myocardium. As shown in Figs. 4 and 6, enhanced Cx43-PKCε interaction and suppressed Cx43-p38MAPKα interaction by PC were insensitive to SB-203580 and PKCε-TIP, respectively. Thus modulations of Cx43 protein by PKCε and p38MAPKα after PC are likely to be independent processes.

Src and PC-Induced Changes in Gap Junction Permeability

Src tyrosine kinase is activated both during PC, presumably by transactivation of the epidermal growth factor receptor, and subsequent sustained ischemia, and inhibition of Src activity during the trigger phase of PC abrogates protection of PC (2, 5, 11, 31, 34). However, PC did not significantly change Cx43-Src interaction (Fig. 3), and Cx43-Src binding did not precede that of Cx43-PKCε or Cx43-p38MAPKα in the preconditioned myocardium (Figs. 4 and 6). These results suggest that physical interaction of Src is not required for Cx43 to form a complex with PKCε or p38MAPKα, and thus it is unlikely that Cx43-Src interaction participates in PC-induced modulation of the gap junction permeability during ischemia.

Contribution of Suppressed Gap Junction Permeability to PC-Induced Protection

Several lines of evidence indicate that gap junctions provide routes for myocardial injury to spread within the region subjected to ischemia-reperfusion. Intracellular Na^+ overload, which occurs during ischemia and primes the cardiomyocyte for Ca^{2+} overload at the time of reperfusion, has been shown to propagate via gap junctions to adjacent cardiomyocytes (28). Administration of gap junction inhibitors before ischemia or at the time of reperfusion significantly limits infarct size (7, 19, 20, 26). Furthermore, involvement of gap junction modulation in PC has been suggested by the findings that both PC and PC mimetics suppress gap junction permeability during ischemia. Furthermore, results of the present study and our previous studies (19, 20) also indicate that phosphorylation of Cx43 by PKCε is a primary mechanism of PC-induced inhibition of the gap junction communication during ischemia.

However, inhibition of gap junction communication is not the only mechanism of PC-induced protection. PC can protect isolated cardiomyocytes (1), and our previous study has shown that the infarct size-limiting effect of pharmacological PC is only partly eliminated by inhibiting PKCε-mediated suppression of gap junction permeability (20). It is also notable that circumstantial evidence suggests a role of gap junction communication in the trigger phase of PC, as discussed previously (19). The present results argue against involvement of Src and p38MAPKα in PC-induced gap junction inhibition during

ischemia. However, contribution of these kinases to PC by other mechanisms is supported by earlier findings that inhibitors of Src and p38MAPK abolished infarct size-limiting effects of PC (11, 22, 31).

Study Limitations

It is technically difficult to prepare intercalated disc-rich fractions without contamination of the mitochondria. In our preparations, level of prohibitin, a marker of mitochondria, in intercalated disc-rich fractions was ~40% of mitochondria fractions freshly prepared by using collagenase and differential centrifugation. However, Cx43 level in the mitochondrial fraction was ~20% of that in the intercalated disc-rich fraction (data not shown), being consistent with an earlier report (4). Thus the mitochondrial Cx43 would have been <10% of total Cx43 in the present intercalated disc-rich fraction. Also data on Cx43-p38MAPK interaction argue against significant contribution of mitochondrial Cx43 to the present findings. If Cx43 immunoprecipitated from the intercalated disc fraction is actually mainly from mitochondria, PKCε-TIP should reduce the Cx43-p38MAPK level in the preconditioned myocardium, since activated PKCε in the mitochondria has been shown to increase recruitment of p38MAPK to the mitochondria in cardiomyocytes (3). Such an effect of PKCε-TIP was not detected in the present experiments (Fig. 6). Furthermore, results of gap junction permeability experiments (Fig. 8) argue for the notion that changes in protein kinase levels in the Cx43 immunoprecipitate represent those in the gap junctional Cx43.

Because there is no tool to selectively inhibit binding of p38MAPKα to Cx43, the functional outcome of suppression of Cx43-p38MAPKα interaction by PC has not been fully characterized in the present study. Because SB-203580 mimicked the effect of PC on Cx43-p38MAPKα interaction, we used this inhibitor to examine the relationship between gap junction permeability and suppressed p38MAPKα binding to Cx43 in the ischemic myocardium. However, we cannot exclude the possibility that mechanisms other than suppression of p38MAPKα-mediated inhibition of the gap junction were also involved in the increase in gap junction permeability during ischemia by SB-203580.

Conclusion

PC has distinct effects on interaction of Cx43 with Src, p38MAPK, and PKCε during ischemia. Translocation of PKCε to gap junction Cx43 and their complex formation is a primary mechanism of PC-induced chemical uncoupling of cardiomyocytes during ischemia. Suppression of Cx43-p38MAPKα complex formation by PC may be a counterbalancing mechanism during the early phase of ischemia against PKCε-mediated gap junction suppression.

GRANTS

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