MitoK$_{\text{ATP}}$ channel activation suppresses gap junction permeability in the ischemic myocardium by an ERK-dependent mechanism

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Abstract

Background: Ischemic preconditioning accelerates suppression of gap junction (GJ) permeability during myocardial ischemia, and GJ blockers reduce infarct size. We hypothesized that the mitochondrial ATP-sensitive K$^+$ (mitoK$_{\text{ATP}}$) channel is one of the mechanisms regulating GJ permeability through the mitogen-activated protein kinase ERK, leading to cardioprotection.

Methods and results: In isolated rabbit hearts, tissues were sampled before and after infusion of diazoxide, a selective mitoK$_{\text{ATP}}$ channel opener, and their intercalated disc-rich fractions were obtained for immunoblotting of mitogen-activated protein kinases. GJ permeability in the myocardium was assessed by using Lucifer yellow as a tracer of GJ communication. Infarction was induced by 30-min global ischemia/2 h reperfusion, and infarct size was expressed as a percent of area-at-risk (%IS/AR). Diazoxide (100 μM) induced phosphorylation of ERK1/2 and Ser279/282 of connexin-43, a GJ subunit protein, and phospho-ERK1/2 was co-immunoprecipitated with connexin-43 in the diazoxide-treated myocardium. This ERK1/2 phosphorylation by diazoxide was inhibited by N-2-mercapto-propionyl-glycine, a free radical scavenger. Diazoxide at 10 and 100 μM reduced intercellular transport of Lucifer yellow during ischemia by 44% and 69%, respectively, and this effect of diazoxide on GJ communication was abolished by PD98059, an ERK inhibitor. Pretreatment with 10 μM and 100 μM diazoxide reduced %IS/AR from 57.1 ± 3.7% to 21.5 ± 10.5% and 5.0 ± 1.3%, respectively. PD98059 abolished cardioprotection by 10 μM diazoxide but not that by 100 μM diazoxide.

Conclusions: Opening of the mitoK$_{\text{ATP}}$ channel activates ERK1/2 via free radicals and induces ERK-mediated suppression of GJ permeability. This suppression of GJ permeability may partly contribute to cardioprotection afforded by mitoK$_{\text{ATP}}$ channel activation.

Keywords: Connexins; Gap junction; Ischemia; MAP kinases; Preconditioning

Preconditioning with a brief period of ischemia (i.e., ischemic preconditioning, IPC) enhances myocardial tolerance against infarction. Although the mechanism of IPC has not been fully elucidated, there is substantial evidence suggesting contribution of the mitochondrial ATP-sensitive K$^+$ channel (mitoK$_{\text{ATP}}$ channel) to IPC [1]. Activation of this channel preserves the mitochondrial function [2–4] and suppresses overproduction of cytotoxic reactive oxygen species (ROS) at the time of reperfusion [5,6]. In addition, the mitoK$_{\text{ATP}}$ channel plays a role in the production of ROS, which act as signaling molecules [7,8]. Recently, we found that IPC accelerated closure of gap junctions in the ischemic myocardium and that suppression of gap junction permeability by its pharmacological inhibitors reduced infarct size after ischemia/reperfusion in isolated rabbit hearts [9]. Although the mechanism by which IPC suppresses gap junction permeability in the myocardium has not been elucidated, results of our studies suggest that phosphorylation of connexin-43 (Cx43), a subunit protein of gap...
junctions, by protein kinase C (PKC) is involved in IPC-induced gap junction closure [9,10]. However, it is possible that other protein kinases are also involved in gap junction regulation by IPC, since Cx43 has multiple phosphorylation sites for protein kinases other than PKC [11].

In this study, we hypothesized that ROS generated by the activated mitoK_ATP channel induce ERK-mediated suppression of the gap junction permeability, which contributes to cardioprotection afforded by mitoK_ATP channel activation. The rationale for this possible link between ROS and gap junction is twofold. First, activation of ERK by a mitoK_ATP channel opener, which was ROS-dependent, has been demonstrated in non-cardiac cells [12]. Second, Cx43 has ERK-dependent phosphorylation sites [11,13,14], and there is evidence to suggest that phosphorylation of Cx43 by ERK reduces gap junction permeability [15,16]. To examine our hypothesis, we assessed the effects of a selective mitoK_ATP channel opener, diazoxide, on mitogen-activated protein kinases (MAPKs) and gap junction permeability and examined the possible correlation between gap junction permeability and anti-infarct tolerance in isolated perfused rabbit hearts.

1. Methods

This study conformed to the Guide for the Care and use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

1.1. Experiment 1: effects of a mitoK_ATP channel opener on MAPKs and MEK1/2

1.1.1. Preparation of isolated buffer-perfused rabbit hearts

In this study, the same isolated perfused heart preparation was used in all three series of experiments (i.e., Experiments 1–3). Under pentobarbital anesthesia and mechanical ventilation, hearts of albino rabbits (Japanese White) were excised and perfused with modified Krebs-Henseleit buffer in the Langendorff mode as in our previous studies [3,9,18]. Coronary perfusion pressure and temperature of the buffer were maintained at 75 mm Hg and 38 °C, respectively, and the preparation was stabilized for 20 min before commencement of the protocol.

1.1.2. Treatment protocols

After stabilization, hearts were assigned into protocol 1 and protocol 2. In protocol 1 (Fig. 1A), each heart received 100 μM of diazoxide or vehicle (normal buffer) alone for 10 min and was then subjected to global ischemia. Ventricular tissues (approximately 0.5 g) were sampled by sharp pre-cooled scissors under baseline conditions, immediately before ischemia and at 10 min after the onset of ischemia. Tissues were quickly frozen in liquid nitrogen and stored at −70 °C until use for immunoblotting. In protocol 2 (Fig. 1B), each heart received 10 μM of diazoxide alone, 300 μM of N-2-mercaptopropionyl glycine (MPG), a free radical scavenger, and diazoxide, or 1 mM of MPG and diazoxide. In this protocol, 10 μM was selected as the dose of diazoxide, since 1 mM MPG only slightly inhibited robust activation of ERK1/2 by 100 μM diazoxide in pilot experiments (n = 3). MPG and...
diazoxyde were infused for 20 min and for 10 min, respectively, before ischemia. Myocardial samples were taken under baseline conditions and at 10 min after diazoxide infusion in this protocol (Fig. 1B). Tissues were frozen after sampling as in protocol 1.

1.1.3. Preparation of intercalated disc-rich fractions

Separation of intercalated disc-rich fractions from tissue homogenates and immunoblotting for protein kinases were performed as previously reported [9,18]. In brief, tissues were homogenized in ice-cold Tris buffer containing 20 mM Tris- HCl, 1 mM EGTA, 5 mM Na3HPO4, 50 mM NaCl, 1 mM PMSF, 10 mM β-mercaptoethanol, 100 mM Na3VO4 and a protease inhibitor cocktail (Complete Mini, Roche Diagnostics, Manheim, Germany). The homogenate was first centrifuged at 600 × g for 10 min to separate myofibrils and nuclei, and the supernatant was re-centrifuged at 10,000 × g for 20 min. This 10,000 × g pellet was used as the intercalated disc-rich fraction, and the supernatant was centrifuged at 100,000 × g for 60 min. The 100,000 × g pellet and supernatant were used as a particulate fraction and a cytosolic fraction, respectively. In pilot experiments, we confirmed that Cx43 was almost exclusively present in the intercalated disc-rich fraction, but not in the cytosolic fraction or in particulate fraction. However, this intercalated disc-rich fraction contained a high level of prohibitin, indicating contamination of mitochondria fragments. Protein level in the fraction was determined by a Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA).

1.1.4. Immunoblotting for MEK and MAPKs

Samples of intercalated disc-rich fractions were electrophoresed on a 12.5% polyacrylamide gel and then blotted onto 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 ERK1/2, phospho-ERK1/2, p38MAPK, phospho-p38MAPK, JNK, phospho-JNK, MEK1/2 and phospho-MEK1/2 (Cell Signaling Technologies). PVDF membranes were then stripped by using a Re-Blot Western Recycling Kit (CHEMICON International, Temecula, CA) and used for re-blotting with anti-ERK1/2 antibody (Cell Signaling Technologies). Peroxidase-linked anti-rabbit IgG F(ab')2 fragments (Amersham Biosciences, Buckinghamshire, UK) were used as secondary antibodies. Since ERK1 and ERK2 were activated by diazoxide (see Results), whether diazoxide induces phosphorylation of ERK-dependent sites of Cx43 was assessed by using antibodies against Ser259-phosphorylated Cx43 and Ser282-phosphorylated Cx43 (Santa Cruz Biotechnology, Santa Cruz, CA). These proteins were visualized by using an ECL Western blotting detection kit (Amersham Biosciences) and quantified by using SigmaGel, a gel analysis software (SPSS, Chicago, IL).

1.1.5. Immunoprecipitation (IP) of Cx43

To assess physical interaction between Cx43 and ERK, intercalated disc-rich fractions from diazoxide-treated hearts and untreated control hearts were immunoprecipitated with anti-Cx43 antibody (BD Transduction Laboratories, San Jose, CA). In brief, 1000 µg protein of intercalated disc-rich fractions was solubilized by 500 µl of IP buffer (20 mM Tris–HCl [pH 7.4], 1 mM EGTA, 5 mM NaN3, 50 mM NaCl, 1 mM PMSF, 50 mM Na3VO4, 1% Triton X-100, 0.5% NP-40 and a protease inhibitor cocktail) and pre-incubated with 50 µl protein G magnetic beads (New England Biolabs, Ipswich, MA) for 1 h to remove proteins that can bind non-specifically to the beads. The supernatant was taken and incubated with 5 µg of anti-Cx43 antibodies for 1 h, and the mixture was then incubated with 50 µl of fresh beads for 1 hr. A magnetic field was applied to this IP mixture, and the supernatant was removed. The beads were washed 2 times using 500 µl of IP buffer, re-suspended in 30 µl of SDS sample loading buffer (125 mM Tris–HCl [pH 6.8], 4.3% SDS, 30% glycerol, 10% β-mercaptoethanol, 0.01% bromophenol blue), and incubated at 70 °C for 5 min. Finally, 20 µl of the supernatant was taken after applying a magnetic field to the mixture and was used for immunoblotting by anti-phospho-ERK1/2 antibody (Cell Signaling Technologies). PVDF membranes were then stripped by using a Re-Blot Western Recycling Kit (CHEMICON International, Temecula, CA) and used for re-blotting with anti-ERK1/2 antibody (Cell Signaling Technologies).

1.2. Experiment 2: effects of a mitoKATP channel opener on gap junction permeability during ischemia

1.2.1. Heart preparation and treatment protocol

Rabbit hearts were perfused as in Experiment 1 and were divided into 6 groups. As shown in Fig. 1C, prior to global ischemia, each received a vehicle or diazoxide (10 or 100 µM) with or without co-infusion of 100 µM 5-hydroxydecanoate [5-HD] (a mitoKATP channel inhibitor), 50 µM genistein (a non-selective tyrosine kinase inhibitor), or 10 µM PD98059 (a MEK1/2 inhibitor). Diazoxide was infused for 10 min, and infusion of inhibitors (5-HD, genistein, and PD98059) was commenced 15 min before diazoxide infusion. Genistein was used in addition to PD98059 to inhibit activation of ERK, which requires dual phosphorylation of its tyrosine and threonine residues.

1.2.2. Determination of gap junction permeability

Gap junction permeability in the ischemic myocardium was determined by using Lucifer yellow according to the method of Ruiz-Meana et al. [19] with slight modification as previously reported [9]. In brief, the ventricle of each heart was excised at 5 min after the onset of ischemia and an incision was quickly made in the endocardium. The ventricle was then immediately soaked and incubated in anoxic Krebs-Henseleit buffer containing Lucifer yellow (2.5 mg/ml) and rhodamine-conjugated dextran (2.5 mg/ml), which was continuously bubbled with N2, for 25 min at 38 °C. After this no-flow anoxic incubation, the ventricle was fixed with 1% glutaraldehyde–4% formaldehyde in
0.2 M cacodylate buffer (pH 7.4). Histology slides were prepared from the fixed heart slices, and the areas stained with rhodamine and those stained with Lucifer yellow were determined by analysis of images obtained by confocal laser microscopy. The size of the area stained with Lucifer yellow but not with rhodamine (Fig. 2) was used as an index of gap junction communication. Data from four data sampling areas were averaged for each heart. The validity of this index was confirmed in our previous study by use of heptanol, a gap junction blocker [9], and reduction of the area stained with Lucifer yellow by heptanol was re-confirmed also in this study (Fig. 2G–I).

1.3. Experiment 3: effects of inhibition of ERK on infarct size limitation by activation of the mitoK<sub>ATP</sub> channel

1.3.1. Heart preparation and treatment protocol

Rabbit hearts were isolated, perfused as in Experiment 1, and subjected to 30-min global ischemia and 2-h reperfusion to induce myocardial infarction as shown in Fig. 1C. Prior to ischemia, hearts were randomly divided into 10 groups and received vehicle (normal buffer) or diazoxide (10 or 100 μM) with or without 5-HD, genistein, or PD98059 pretreatment. Doses of 5-HD, genistein and PD98059 were the same as those used in Experiment 2. Diazoxide was infused for 10 min before ischemia, and inhibitors were infused for 25 min from 15 min before diazoxide infusion. After 2-hr reperfusion, ventricles were frozen before infarct size measurement.

1.3.2. Determination of infarct size

Frozen hearts were sectioned into 2-mm-thick slices and incubated in phosphate buffer (100 mM, pH 7.4) containing 1% triphenyltetrazolium chloride for 20 min to visualize infarcts. The uppermost slices containing valves were not used for infarct size analysis. Infarct areas and areas at risk (left ventricular areas) in each heart were determined by NIH Image and multiplied by thickness of the slice to calculate volumes of infarct and risk area. Infarct size was also expressed as a percentage of area at risk (%IS/AR).

1.4. Statistical analysis

One-way analysis of variance (ANOVA) with the Student-Newman-Keul post hoc test was used to test differences in heart weight and infarct size. Differences in time courses of hemodynamic parameters and data of immunoblottings between groups were compared by two-way repeated measures ANOVA. The difference was considered significant if the p value was less than 0.05.

2. Results

2.1. Experiment 1

2.1.1. Effects of diazoxide on MEK and MAPKs

In protocol 1, treatment with 100μM diazoxide increased levels of phosphorylated ERK1 and ERK2 by approximately 1.6-fold (Fig. 3A, Table 1), while there were no significant changes in levels of phospho-p38MAPK and phospho-JNK. Significant phosphorylation of ERK1/2 by diazoxide persisted after 10-min ischemia. There was no significant change in the phospho-ERK level in untreated hearts during the time control period or after 10-min ischemia. Levels of total ERK1/2, p38MAPK and JNK were not significantly changed after diazoxide treatment with or without MPG (data not shown). MPG alone did not modify phospho-ERK1/2 level (n=3, data not shown).

2.1.2. Effects of diazoxide on ERK-Cx43 interaction

The level of Ser<sup>279/282</sup>-phospho-Cx43 was significantly increased by 50% after diazoxide infusion and this change was maintained at 10 min into sustained ischemia (Fig. 4A and B), whereas there was no significant change in the level of Ser<sup>255</sup>-phospho-Cx43 by diazoxide infusion (data not shown). As shown in Fig. 4C, ERK was co-immunoprecipitated with Cx43, but a signal of phospho-ERK was not detected in untreated control samples. However, in the diazoxide-treated myocardium, phospho-
ERK signals were detected in Cx43 immunoprecipitates, and these signals appeared to be diminished at 10 min into ischemia. On the other hand, the level of total ERK co-immunoprecipitated with Cx43 was similar in diazoxide-treated and -untreated samples.

2.2. Experiment 2

2.2.1. Effects of diazoxide on the gap junction permeability

As shown in Fig. 5, diazoxide reduced gap junction permeability in a dose-dependent manner. This effect was abolished by 5-HD, indicating that the effect of diazoxide on gap junction permeability was mediated by opening of the mitoK_{ATP} channel. Furthermore, genistein and PD98059 abolished suppression of gap junction permeability by diazoxide. These findings suggest involvement of tyrosine kinases, including MEK1/2, in diazoxide-induced suppression of gap junction communication.

2.3. Experiment 3

2.3.1. Hemodynamic parameters

Baseline levels of heart rate, coronary flow and left ventricular developed pressure (LVDP) were comparable...
among the study groups. Infusion of diazoxide significantly increased coronary flow, though heart rate and LVDP were unaffected (Table 3). Recovery of LVDP and coronary flow after reperfusion tended to be better in hearts treated with diazoxide alone, whereas the differences in these parameters were not statistically significant compared with those in the untreated controls. Infusion of 5-HD, genistein and PD98059 did not modify heart rate, coronary flow or LVDP.

### 2.3.2. Infarct size data

Infarct size data are presented in Table 4. There was no significant difference between sizes of area at risk in the study groups. In untreated controls, %IS/AR was 57.1 ± 3.7%, and 10 μM and 100 μM diazoxide reduced %IS/AR to 21.5 ± 10.5% and 5.0 ± 1.3%, respectively. Both 5-HD and genistein abolished the infarct size-limiting effect of 100 μM diazoxide, though neither 5-HD alone nor genistein alone modified %IS/AR. PD98059 abolished cardioprotection afforded by 10 μM diazoxide but not that afforded by 100 μM diazoxide. PD98059 alone did not affect %IS/AR.

### 3. Discussion

The results of the present study indicate that opening of the mitoK<sub>ATP</sub> channel induces ERK-mediated suppression of gap junction permeability in the ischemic myocardium. Association of suppression of gap junction permeability with increased tolerance of the myocardium against infarction shown in this and earlier studies [9,20,21] suggests that not only direct protection of cardiomyocytes but also attenuation of injury propagation via the gap

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**Table 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>ERK1 (%)</th>
<th>ERK2 (%)</th>
<th>MEK1/2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diazoxide</td>
<td>5</td>
<td>143.9 ± 8.5*</td>
<td>142.9 ± 8.0*</td>
<td>209.0 ± 15.1*</td>
</tr>
<tr>
<td>300 μM MPG+Diazoxide</td>
<td>4</td>
<td>135.0 ± 9.7*</td>
<td>125.1 ± 18.4</td>
<td>139.3 ± 21.2*</td>
</tr>
<tr>
<td>1 mM MPG+Diazoxide</td>
<td>5</td>
<td>103.8 ± 9.9</td>
<td>97.4 ± 4.8</td>
<td>108.7 ± 8.1</td>
</tr>
</tbody>
</table>

Mean ± SE. Diazoxide = infusion of 10 μM diazoxide for 10 min. MPG + diazoxide = MPG infusion for 10 min followed by co-infusion of MPG and diazoxide for 10 min. *p < 0.05 vs. baseline level.
junction contribute to infarct size limitation by mitoK\(_{\text{ATP}}\) channel activation (Fig. 6).

In this study, diazoxide, a selective mitoK\(_{\text{ATP}}\) channel opener, was found to activate ERK in the myocardium by a ROS-dependent mechanism. This finding is consistent with observations in THP-1 cells, a monocytic cell line, in an earlier study by Samavati et al. [12]. In their study, diazoxide and pinacidil increased the level of phospho-ERK, and the increase in phospho-ERK was associated with ROS production determined by dihydroethidium fluorescence. Furthermore, ERK activation by mitoK\(_{\text{ATP}}\) channel openers was inhibited by \(N\)-acetylcysteine, an ROS scavenger. The results of Experiment 1 in the present study indicate that such a diazoxide-induced ROS-mediated ERK activation occurs also in cardiomyocytes. The results of the present study also showed that a target of ROS generated by activation of the mitoK\(_{\text{ATP}}\) channel is upstream of MEK1/2 in ERK-activating pathways and that this ROS-mediated mechanism is selective to ERK and not shared by JNK or p38MAPK in the myocardium. The mechanism by which ROS from the mitochondria activates MEK1/2-ERK in the myocardium remains unclear, although ROS-induced Ras-independent activation of Raf [22] is a possibility.

The present study showed for the first time that mitoK\(_{\text{ATP}}\) channel opening induces suppression of gap junction permeability in the ischemic myocardium (Fig. 5). The contribution of ERK to this mitoK\(_{\text{ATP}}\) channel-mediated regulation of the gap junction is supported by two lines of evidence obtained in the present experiments. First, elimination of the effect of diazoxide on gap junction permeability by PD98059 and genistein indicates involvement of MEK1/2, a kinase upstream of ERK. Secondly, diazoxide increased the level of 279Ser/282Ser-phosphorylation in Cx43 by 50% (Fig. 4A and B), though significant change was not detected for 255Ser-phosphorylation in Cx43. Furthermore, activated ERK was co-immunoprecipitated with Cx43 (Fig. 4C). 255Ser, 279Ser and 282Ser in Cx43 are ERK phosphorylation sites, and phosphorylation of these residues has been shown to correlate with closure of the gap junction in liver epithelial cells and endothelial cells [15,16]. Taken together with the results of MPG experiments, it is likely that the suppression of gap junction permeability by diazoxide was induced by phos-
Phosphorylation of Cx43 by ERK activated by mitochondria-derived ROS. Like ERK, PKC-ε has been suggested to reduce gap junction permeability by phosphorylation of Cx43 [23,24]. There is pharmacological evidence suggesting that PKC-ε is downstream of the mitoK<sub>ATP</sub> channel in signal pathways of IPC in the human myocardium [25]. However, our earlier study [18] has shown that neither PKC-ε nor PKC-α is translocated from the cytosol compartment to particulate compartments after activation of the mitoK<sub>ATP</sub> channel by 100 μM diazoxide in rabbit hearts. This finding argues against the possibility that PKC was involved in the suppression of gap junction communication by activation of the mitoK<sub>ATP</sub> channel.

Significant protection of the myocardium from ischemia/reperfusion injury by suppression of gap junction permeability has been shown by a series of studies from Garcia-Dorado’s laboratory [19–21]. In our previous study [9], three structurally different gap junction blockers limited infarct size in isolated buffer-perfused rabbit hearts. In addition, the present study showed that both cardioprotection and suppression of inter-cellular transport of Lucifer yellow by 10 μM diazoxide were abolished by PD98059. Taken together, these findings support the notion that ERK-mediated suppression of gap junction permeability contributes to infarct size limitation by diazoxide (Fig. 6). Unfortunately, however, since no selective opener of the gap junction is available, we could not determine how much of the cardioprotection afforded by diazoxide was attributable to suppression of gap junction permeability.

On the other hand, there is evidence indicating direct cell protection induced by mitoK<sub>ATP</sub> channel activation. Openers of the mitoK<sub>ATP</sub> channel have been shown to attenuate mitochondrial damage during ischemia [2–4,26] and to prevent opening of the permeability transition pore upon reperfusion [27,28]. Furthermore, protective effects of mitoK<sub>ATP</sub> channel openers have been demonstrated in isolated cardiomyocytes [29–31], and finally, in the present experiments, the very potent infarct size-limiting effect of 100 μM diazoxide was insensitive to PD98059, whereas the same dose of PD98059 suppressed gap junction permeability. Nevertheless, these results do not exclude the possibility that suppression of the propagation of tissue injury via the gap junction is an additional mechanism of cardioprotection by activation of the mitoK<sub>ATP</sub> channel (Fig. 6). Both PD98059 and genistein similarly inhibited diazoxide-induced suppression of the gap junction communication, indicating the involvement of MEK1/2, a tyrosine/threonine kinase. Nonetheless, we examined in a small number of post hoc experiments the effects of genistein on diazoxide-induced phosphorylation. Hearts (n = 3) were pretreated with 50 μM genistein before infusion of 100 μM diazoxide as in Experiment 2, and tissue samples were taken under baseline conditions, after treatment and at 10 min into global ischemia. There was no significant elevation in levels of phospho-ERK1 and -ERK2 after genistein/diazoxide treatment (121.6 ± 12.0% and 108.2 ± 5.2% of baseline) and at 10 min into ischemia (91.5 ± 5.2% and 119.2 ± 14.3% of baseline). Compared with the marked

Fig. 6. Schematic representation of the proposed role of gap junction modulation in the mechanism of infarct size limitation by IPC and mitoK<sub>ATP</sub> channel activation. GPCR = G-protein coupled receptor, PI3K = phosphatidylinositol-3-kinase, PKG = protein kinase G, mPTP = mitochondrial permeability transition pore. Signaling pathways provoked by IPC other than that reaching the mitoK<sub>ATP</sub> channel are omitted for simplicity in this figure.
increase in phospho-ERK1/2 by diazoxide alone (Table 1), the results of the post hoc experiments indicate that genistein indeed inhibited ERK activation. However, in contrast to PD98059, genistein eliminated cardioprotection afforded by diazoxide at a dose of 100 μM. This finding is consistent with an earlier finding that the same dose of genistein abrogated the infarct size limiting effect of 10 μM diazoxide in buffer-perfused rabbit hearts [32]. Thus, at least one tyrosine kinase other than MEK1/2 is likely to be involved in gap junction-independent mechanism of cell protection by mitoK<sub>ATP</sub> channel opening (Fig. 6). The identity of that tyrosine kinase remains to be investigated.

The results of the present study are consistent with our previous finding that IPC accelerated ischemia-induced suppression of gap junction permeability assessed by Lucifer yellow. However, studies in which cell-to-cell coupling was assessed by using electrical indices such as myocardial impedance and whole tissue resistance have shown that IPC delays uncoupling of ischemic myocytes [33,34]. Although we do not have a clear explanation for this apparent contradiction, different methods for determination of gap junction permeability (i.e., dye-coupling vs. electrical coupling) may be a possible explanation. There are some earlier findings [19,33–35] suggesting that time courses of chemical-coupling and electrical coupling after ischemia in the myocardium are discordant, though these time courses have not been directly compared. For example, in a study by Jain et al. [33], electrical uncoupling was almost maximal after 30 min of global ischemia in isolated rat hearts. In contrast, assessment of the gap junction permeability by Lucifer yellow transport in a similar rat heart preparation indicated that coupling of ischemic myocytes persisted after 30-min ischemia [19]. Furthermore, gap junction permeability assessed by dye coupling and electrical conductance of the gap junction can change in opposite directions. Kwak et al. [35] showed that PKC-activating phorbol ester reduced dye coupling but increased electrical conductance in neonatal cardiomyocytes. The mechanism underlying these different features in the chemical coupling and electrical coupling warrants further investigation.

As a limitation, we cannot exclude the possibility that interaction between Cx43 and MAPKs used in this study partly occurred also in mitochondria. Although we have assumed that contamination of mitochondria in the intercalated disc-rich fraction does not compromise analysis of Cx43-MAPKs interaction, a recent study by Boengler et al. [36] demonstrated the presence of Cx43 in mitochondria. However, Cx43 level in the purified mitochondrial fraction was much lower than that in the intercalated disc fraction [36], and the present study showed that ERK activation by diazoxide was closely associated with changes in gap junction permeability. Thus, it is likely that ERK-mediated Cx43 phosphorylation responsible for gap junction regulation primarily takes place at the intercalated disc.

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