Calcium-sensing receptor: a sensor and mediator of ischemic preconditioning in the heart

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Sun J, Murphy E. Calcium-sensing receptor: a sensor and mediator of ischemic preconditioning in the heart. Am J Physiol Heart Circ Physiol 299: H1309–H1317, 2010. First published September 10, 2010; doi:10.1152/ajpheart.00373.2010.—As a G protein-coupled receptor, the extracellular Ca\(^{2+}\)-sensing receptor (CaSR) responds to changes not only in extracellular Ca\(^{2+}\), but also to many other ligands. CaSR has been found to be expressed in the hearts and cardiovascular system. In this study, we confirmed that CaSR is expressed in mouse cardiomyocytes and showed that it is predominantly localized in caveolae. The goal of this study was to investigate whether CaSR plays a cardioprotective role in ischemic preconditioning (IPC). Hearts from C57BL/6J mice (male, 12–16 wk) were perfused in the Langendorff mode and subjected to the following treatments: 1) control perfusion; 2) perfusion with a specific CaSR antagonist, NPS2143; 3) IPC (four cycles of 5 min of global ischemia and 5 min of reperfusion); or 4) perfusion with NPS2143 before and during IPC. Following these treatments, hearts were subjected to 20 min of no-flow global ischemia and 120 min of reperfusion. Compared with control, IPC significantly improved postischemic left ventricular functional recovery and reduced infarct size. Although NPS2143 perfusion alone did not change the hemodynamic function and did not change the extent of postischemic injury, NPS2143 treatment abolished cardioprotection of IPC. Through immunoblot analysis, it was demonstrated that IPC significantly increased the levels of phosphorylated ERK1/2, AKT, and GSK-3\(\beta\), which were also prevented by NPS2143 treatment. Taken together, the distribution of CaSR in caveolae along with NPS2143-blockade of IPC-induced cardioprotective signaling suggest that the activation of CaSR during IPC is cardioprotective by a process involving caveolae.

cardioprotection; caveolae; caveolin-1

ISCHEMIC PRECONDITIONING (IPC) is a cellular adaptive phenomenon, whereby brief episodes of myocardial ischemia and reperfusion render the heart resistant to subsequent prolonged ischemic injury (29). Through activation of a complex cascade of signaling events, IPC has been shown to reduce infarct size, postischemic contractile dysfunction, and arrhythmias (2, 28, 54). Although mitochondria have been considered as an end effector of IPC (13, 24, 28), the endogenous cellular protection is proposed to be initiated through the sensing of extracellular ischemic signals. The activation of a number of G protein-coupled receptors (GPCRs) in the plasma membrane has been found to elicit an IPC-like phenotype. The GPCRs appear to be activated by adenosine, bradykinin, catecholamine, and opioid, which have been found to be released by myocardium during the short episodes of IPC (2, 7, 55).

The extracellular Ca\(^{2+}\)-sensing receptor (CaSR), initially found and cloned from parathyroid cells, belongs to the GPCR superfamily. Its primary physiological function is to sense very small changes in extracellular Ca\(^{2+}\) and thereby modulate the release of parathyroid hormone (PTH) and maintain a constant blood Ca\(^{2+}\) level. In addition to the parathyroid gland, CaSR is also expressed in diverse tissues, in which it regulates local PTH-related protein (PTHRP) secretion, while exerting paracrine/autocrine regulatory function (43). Recent studies suggest that CaSR is also expressed in the heart and cardiovascular system (1, 40, 47, 52, 57). However, the physiological or pathophysiological role of CaSR in the heart is still unknown. In addition to extracellular Ca\(^{2+}\), CaSR can also be allosterically activated by various stimuli, such as polyvalent cations, pH, ionic strength, \(\alpha\)-aromatic amino acids, and polyamines (38, 39). Therefore, CaSR is capable of sensing not only changes in extracellular Ca\(^{2+}\), but also the local metabolic environment (3). In response to binding of extracellular Ca\(^{2+}\), activation of CaSR has been shown to activate various downstream cellular signaling pathways, including G\(_{q}\)-coupled stimulation of phosphatidylinositol phospholipase C, G\(_{i}\)-coupled inhibition of adenyl cyclase, and activation of multiple protein kinases, such as the extracellular signal-regulated kinase 1/2 (ERK1/2) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT). Notably, these kinase signaling pathways have been also shown to play an important role in IPC (7, 15). Therefore, it is of interest to study whether the activation of CaSR plays a role in IPC.

It has been shown that IPC leads to changes in extracellular pH (8), ionic strength (42), and other ions and metabolites, which can alter the activity of CaSR. In this study, we tested the hypothesis that activation of CaSR might play an important cardioprotective role in IPC. We used a specific antagonist of CaSR, NPS2143, to see whether IPC-induced cardioprotection could be blocked by inhibition of CaSR in a Langendorff perfused mouse heart ischemia-reperfusion (I/R) model.

MATERIALS AND METHODS

Animals and reagents. C57BL/6J wild-type (WT) male mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were adults between 12–16 wk old at the time of experimentation. All animals were treated in accordance with National Institutes of Health guidelines and the “Guiding Principles for Research Involving Animals and Human Beings.” This study was reviewed and approved by the National Heart, Lung, and Blood Institute, Institutional Animal Care and Use Committee. The molecular formula for specific CaSR antagonist, N{[(\(R\))-2-hydroxy-3-(2-cyano-3-chlorophenoxy)propyl]-1,1-dimethyl-2-(2-naphthyl)ethylamine (NPS2143) is shown in Fig. 1A. NPS2143 was initially obtained as a gift from Dr. Jianxin Hu (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) for dose-response experiments and then purchased from Shanghai Haoyuan Chemexpress (Shanghai, China). NPS2143 was dissolved into DMSO as 10 mM of stock solution, and aliquots were diluted into 100-mL perfusion buffer just before use.
Langendorff heart perfusion and I/R protocol.

Mice were anticoagulated with heparin and anesthetized with pentobarbital. Hearts were excised quickly and placed in ice-cold Krebs-Henseleit buffer (KH buffer) containing the following (in mM): 120 NaCl, 11 D-glucose, 25 NaHCO₃, 1.75 CaCl₂, 4.7 KCl, 1.2 MgSO₄, and 1.2 KH₂PO₄. The aorta was cannulated on a Langendorff apparatus, and the heart was perfused in retrograde fashion with KH buffer at a constant pressure of 100 cmH₂O at 37°C. KH buffer was oxygenated with 95% O₂/5% CO₂ and maintained at pH 7.4. After equilibrium perfusion or IPC (4 cycles of 5 min of ischemia and 5 min of reperfusion), perfused mouse hearts were subjected to 20 min of no-flow ischemia, followed by 120 min of reperfusion. As shown in Fig. 1, there were four experimental groups: 1) control-I/R, mouse hearts were equilibrated for 40 min of perfusion before 20 min of no-flow ischemia and 120 min of reperfusion; 2) NPS2143-I/R, after 10 min of perfusion, hearts were perfused with NPS2143 for 30 min before I/R; 3) IPC-I/R, hearts were perfused for 20 min, followed by four cycles of 5 min of ischemia and 5 min of reperfusion before I/R; and 4) NPS2143-IPC-I/R, hearts were perfused for 10 min and then 10 min of perfusion with NPS2143, followed by IPC in the presence of NPS2143. Additional sets of hearts without I/R were collected for sample preparation and immunoblot analysis.

Hemodynamic and myocardial infarct measurements.

To monitor left ventricular contractile function, a latex balloon connected to a pressure transducer was inserted into the left ventricle of Langendorff-perfused hearts. The left ventricular developed pressure (LVDP) was recorded and digitized using a PowerLab system (ADInstruments, Colorado Springs, CO). We used the rate pressure product (RPP = LVDP × heart rate) as a measure of function. The postischemic functional recovery was expressed as percentage of preischemic RPP. For measurement of myocardial infarct size, the hearts were perfused with 1% (wt/vol) of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) after 120 min of reperfusion and incubated in TTC at 37°C for 15 min, followed by fixation in 10% formaldehyde. Infarct size was expressed...
as the percentage of total area of cross-sectional slices through the ventricles.

Cardiomyocyte cell lysate and total homogenate preparation from mouse hearts. Adult mouse cardiomyocytes were isolated by collage- nase perfusion, and cardiomyocyte lysate was prepared as previously described (45). The total heart homogenate was obtained by grinding snap-frozen mouse heart into powder in liquid nitrogen followed by homogenization with a tight-fitting glass Dounce homogenizer (2 × 10 times of stroke) on ice in 1.5 ml buffer containing (in mM) 70 NaCl, 20 HEPES (pH 7.4), 2.5 MgCl₂, 1 EDTA, 0.5% (vol/vol) Triton X-100, an EDTA-free protease inhibitor, and a protein phosphatase cocktail inhibitor tablet (Roche Diagnostics, one mini-tablet per 10 ml). The mixture was kept on ice for 60 min with gentle vortex every 15 min, then homogenized again on ice by two times of 10 strokes using a Dounce glass homogenizer. The homogenous mixtures labeled as total homogenate were snap frozen in liquid nitrogen and stored at −80°C. The protein concentration was determined by BCA protein assay (Pierce-Thermo Scientific, Rockford, IL).

Detergent-free, caveolae-enriched fractionation. The detergent-free purification of the caveolea-enriched fraction was modified from Song et al. (41). Each snap-frozen heart was ground into powder in liquid nitrogen and resuspended in 2.0 ml of 0.5 M Na₂CO₃ (pH 11.5), which contains protease and protein phosphatase inhibitors. The mixture was transferred into a Beckman polyallomer ultracentrifuge tube and homogenized by Polytroon three times for 10-s bursts (at 22,000 rpm) with 30-s intervals on ice. The final homogenization was three replicates of sonication using Sonicator Misonix, each time for 22,000 rpm) with 30-s intervals on ice. The final homogenization was 2.1 ml was adjusted to 45% (wt/vol) sucrose by adding 2.1 ml of 90% (wt/vol) sucrose in MES buffer (in mM): 25 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5, 150 NaCl, 1 EDTA, and protease and protein phosphatase inhibitors. A discontinuous sucrose gradient was loaded with 4.2 ml of 40% and 2.6 ml of 5% sucrose in MES buffer containing 250 mM Na₂CO₃ and ultracentrifuged at 39,000 rpm (~200,000 g) for 18 h at 4°C in Beckman SW40 swing rotor. After ultracentrifugation, the sucrose gradient was collected in 1 ml of fraction from the top to the bottom by Auto Densi-Flow (Labconco, Kansas City, MO). A light-scattering band confined to the 5–40% fraction from the top to the bottom by Auto Densi-Flow was observed and collected as caveolae-enriched fraction.

Coimmunoprecipitation of CaSR and caveolin-3. Aliquots (~200 μg) of total homogenate were incubated with 2 μg of anti-CaSR monoclonal antibody (Sigma, St. Louis, MO) or anti-caveolin-3 monoclonal antibody (BD Biosciences, San Jose, CA) at 4°C for 1 h with gentle agitation. An antigen nonspecific mouse monoclonal IgG₁ (Affinity BioReagents, Golden, CO) was used for negative control for immunoprecipitation. Protein G agarose (25 μl, Invitrogen, Carlsbad, CA) was added and incubated at 4°C overnight with agitation. The agarose pellets were centrifuged at 3,000 g for 2 min at 4°C, the agarose beads were resuspended into immunoprecipitation buffer (in mM): 150 NaCl, 20 imidazole-HCl (pH 7.0), 1 EDTA, 1% (wt/wt) Triton X-100, an EDTA-free protease inhibitor, and a protein phosphatase cocktail inhibitor tablet (Roche Diagnostics, one mini-tablet per 10 ml). After four washes, the agarose beads were resuspended into 50 μl of 1× reducing sample buffer containing 10% (vol/vol) of 2-mercaptoethanol and boiled at 95°C for 3 min, and proteins were extracted from immunoprecipitates after quick centrifugation.

Immunoblot analysis. Equal amounts of protein from total homogenate or equal volumes of the sucrose gradient fractions were boiled in reducing sample buffer and then separated by 4–12% Bis-Tris SDS-PAGE (Invitrogen, Carlsbad, CA). Some samples from cardiomyocyte lysate were also run on nonreducing SDS-PAGE (without 2-mercaptoethanol in sample buffer). After transfer, the nitrocellulose membranes were stained with Ponceau S and then washed with Tris-buffered saline-Tween 20 (in mM): 10 Tris, pH 8.0, 150 NaCl, and 0.1% (vol/vol) Tween 20. Tris-buffered saline-Tween 20 supplemented with 5% (vol/vol) nonfat dry milk was used for the blocking solution and the antibody diluents. The primary polyclonal anti-rabbit antibodies for signaling pathway analysis were obtained from Cell Signaling (Danvers, MA) and used according to the protocol instructions: anti-phospho (p)-AKT (Ser473) and anti-AKT, anti-p-ERK1/2 (Thr202/Tyr204) and anti-ERK1/2, anti-p-glycogen synthase kinase (GSK)-3β (Ser9) and anti-GSK-3β. Anti-mouse and anti-rabbit IgG horseradish peroxidase-conjugated secondary antibodies (Cell Signaling) were used in combination with a chemiluminescent substrate (Amersham Biosciences/GE Healthcare, Piscataway, NJ), according to standard procedures.

Data analysis. Results are expressed as means ± SE. Statistical significance was determined by Student’s t-test or ANOVA, as required. Differences were considered to be significant at P < 0.05.

RESULTS

Presence and distribution of CaSR at caveolae in mouse cardiomyocytes. As shown in Fig. 2A, CaSR is expressed in adult mouse cardiomyocytes. A single anti-CaSR positive band (~130 kDa) was detected under reducing SDS-PAGE, whereas two bands were detected under nonreducing condition, suggesting that CaSR is present in cardiomyocytes as an intermolecular disulfide-linked homodimer (17). An early study
showed that CaSR is localized in caveolae-rich plasma membrane domains of bovine parathyroid cells (22). Caveolae and myocyte-specific caveolin-3-associated signaling complexes have been suggested to play an important role in mediating myocardial IPC (35, 51). Therefore, coimmunoprecipitation of CaSR and caveolin-3 was carried out to study whether CaSR is associated with caveolin-3. As shown in Fig. 2B, the coimmunoprecipitation of CaSR with caveolin-3 suggested that CaSR is associated with the caveolin-3. In addition, CaSR was found to be predominantly distributed in the caveoleae-enriched fraction (fraction 3, see Fig. 6, A and B) isolated from detergent-free sucrose gradient ultracentrifugation, providing further evidence that CaSR is colocalized with caveolin-3 at caveolae in cardiomyocytes. The caveolin scaffolding domain recognizes common sequence motifs within caveolin-binding signaling molecules, i.e., $\Phi X \Phi \dot{X} \dot{X} \Phi \dot{X} \Phi$ or $\Phi \dot{X} \Phi \dot{X} \Phi \dot{X} \Phi \dot{X}$, where $\Phi$ is aromatic amino acid Trp, Phe, or Tyr (6, 32). According to the primary sequence and its topological analysis (http://www.uniprot.org/uniprot/Q9QY96), mouse CaSR has two such caveolin binding motifs, i.e., E/V[LGEI]KE (629–637) and FNEAKFIF (801–809), located at cytosolic regions hinged to the first and the sixth transmembrane domains, as illustrated in Fig. 2C.

Inhibition of CaSR by NPS2143 abolishes IPC-induced cardioprotection. NPS2143 is a highly specific antagonist of CaSR (IC$_{50}$ of 43 nM) developed by Nemeth et al. (30). Even when tested at much higher concentrations (3 $\mu$M), NPS2143 did not affect the activity of a number of other GPCRs (30). We tested concentrations from 50 nM to 1 $\mu$M and found that a concentration of 1 $\mu$M abolished the cardioprotection of IPC (data not shown). Notably, infusion with 1 $\mu$M of NPS2143 in Langendorff-perfused heart had no effect on heart rate, flow rate, or other cardiac hemodynamic functions (Table 1). Since 1 $\mu$M of NPS2143 abolished IPC-induced cardioprotection, we chose this concentration for additional studies. The hemodynamic parameters listed in Table 2 showed that NPS2143 treatment did not change the postischemic cardiac contractile function compared with untreated hearts in the I/R group. IPC significantly increased postischemic LVDP; however, this protection was prevented by NPS2143 treatment (LVDP 57 ± 5 cmH$_2$O in NPS2143 + IPC-I/R vs. 98 ± 6 cmH$_2$O in IPC-I/R). As shown in Fig. 3A, following 20 min of ischemia and 120 min of reperfusion, hearts recovered 40.5 ± 2.4% of their preischemic RPP, and NPS2143 treatment alone did not change the postischemic RPP recovery (42.2 ± 4.1% of preischemic RPP). Consistent with previous studies, IPC significantly increased postischemic contractile functional recovery. Treatment of hearts with 1 $\mu$M of NPS2143 10 min before and during four cycles of IPC abolished the cardioprotective effect of IPC (41.8 ± 2.6 vs. 74.3 ± 3.2% of preischemic RPP in the presence and absence of NPS2143, respectively). Consistent with the results on cardiac functional recovery, myocardial infarct sizes measured by TTC staining showed that IPC significantly reduced myocardial infarct size compared with untreated ischemic hearts (10.7 ± 2.5% in IPC vs. 35.3 ± 2.3% in control hearts). Although NPS2143 treatment alone did not change the infarct size (33.7 ± 4.1%) compared with untreated ischemic hearts, the presence of NPS2143 before and during IPC abolished IPC-induced cardioprotection, i.e., the infarct sizes of this group was 35.4 ± 4.0%, which was comparable to ischemic hearts without IPC (Fig. 3B).

Signaling pathways involved in cardioprotection by CaSR activation. Activation of CaSR has been shown to activate the ERK1/2 pathway in human vascular smooth muscle cell (26) and rat neonatal ventricular cardiomyocytes (47), and activation of the ERK1/2 pathway has been shown to be protective in a number of preconditioning models (14, 36, 44). We, therefore, tested whether activation of ERK1/2 in IPC hearts was abolished by CaSR inhibition via NPS2143 treatment. As shown in Fig. 4A, IPC significantly increased p-ERK1/2, which was abolished by NPS2143 treatment. **$P < 0.01$ vs. I/R ($n = 8$).

CaSR can also lead to activation of phosphatidylinositol phospholipase C-mediated biological effects. In addition, CaSR has also been found to stimulate PI3K in various CaSR-expressing cells (4). PI3K transfers the terminal phosphate of ATP to the 3-OH of the inositol head group and is an important regulator of membrane inositol phospholipids. Moreover, the
lipid product arising from the action of PI3K on phosphatidylinositol-4,5-biphosphate provides a docking site that leads to activation of the downstream serine-threonine kinases, such as AKT. Activation of PI3K/AKT signaling is a well-known survival pathway involving IPC-induced cardioprotection (7, 15, 48). As shown in Fig. 4B, IPC significantly increased p-AKT, while treatment with NPS2143 blocked phosphorylation of AKT in IPC hearts.

Our laboratory previously reported that phosphorylation and inactivation of GSK-3β during IPC is cardioprotective. Phosphorylation of GSK-3β occurs with activation of PI3K/AKT (49). Phosphorylation and inactivation of GSK-3β is reported to result in mitochondrial signaling that leads to inhibition of the mitochondrial permeability transition pore (12, 20, 21, 31). Similar to our laboratory’s previous finding, IPC significantly increased p-GSK-3β, and this effect was prevented by CaSR inhibition upon NPS2143 treatment (Fig. 4C).

Fig. 4. Changes in cardioprotective signaling kinases. The total homogenates of mouse hearts were subjected to reducing 4–12% SDS-PAGE followed with anti-phosphorylated (p) and total extracellular signal regulated kinase 1/2 (ERK1/2; A), AKT (B), and glycogen synthase kinase-3β (GSK-3β; C) IB detection. Left: representative IBs. Right: graphs represent densitometric analysis for the phosphorylated-to-total ratio of signaling molecules. There was a significant increase in the phosphorylated-to-total ratio in IPC hearts. With NPS2143 treatment, phosphorylated signaling molecules were markedly reduced. *P < 0.05 vs. control (n = 5–6).

Change and distribution of caveolin-3, CaSR, and signaling kinases during IPC. Internalization and trafficking of GPCRs has been found to play an important role in IPC (10, 50). It has been shown that endocytosis and trafficking of CaSR can regulate PTHrP secretion (37). In parathyroid cells, it has been shown that m-calpain colocalizes with the CaSR in caveolae and participates in degradation of the CaSR (23). We initially examined levels of CaSR in control and IPC hearts in the presence of NPS2143 in a cytosolic extract, and we found a decrease in CaSR in this cytosolic fraction (data not shown). As described in MATERIALS AND METHODS, we prepared a total heart homogenate without centrifugation so there would be no loss of any cellular components. Equal amounts of the total homogenate isolated from control and IPC hearts in the absence and presence of NPS2143 were separated by reducing SDS-PAGE, followed by anti-CaSR immunoblot. As shown in Fig. 5, IPC and NPS2143 treatments resulted in no change in the amount of CaSR protein in the total homogenate, suggesting IPC and NPS2143 treatments did not lead to CaSR degradation.

To further study whether IPC causes the internalization and trafficking of CaSR into other subcellular compartments, the caveolae-enriched fractionation was analyzed by immunobLOTS. As shown in Fig. 6A, in perfusion control hearts, caveolin-3 was mostly distributed in buoyant caveolae-enriched fractions (fractions 3 and 4), IPC caused a decrease of caveolin-3 in these two fractions, and slightly increased its content in other heavy fractions containing most cytosolic and other subcellular components (Fig. 6, A and C). Interestingly, CaSR had a similar change in pattern as caveolin-3 (Fig. 6, B and D) upon IPC stimulation, suggesting that IPC induces the internalization of CaSR through caveolae-dependent endocytosis, i.e., via “caveosome/signalosome” trafficking (9, 35). Noticeably, the IPC-induced changes of distribution of caveolin-3 and CaSR were blocked by NPS2143 treatment.

It has been recently shown that p-ERK1/2 was exclusively localized at caveolae in adult mouse cardiac myocytes on exposure to stress (53). The p85 subunit of PI3K was also found to coimmunoprecipitate with caveolin-3 in mouse heart lysates (5). These results suggest that these signaling molecules (i.e., p-ERK1/2 and p-AKT) are subcellularly enriched and...
localized at caveolae in cardiomyocytes. In this study, we also analyzed the distribution of p-ERK1/2, p-AKT, and p-GSK-3β in caveolae and other subcellular fractions. In perfusion control hearts, p-ERK1/2 was abundantly distributed in the caveolin-3-enriched fraction. IPC not only dramatically increased the phosphorylation level of ERK1/2 in caveolin-3-enriched fraction, but also induced significant increase of p-ERK1/2 in other heavy fractions containing cytosolic and other subcellular components (Fig. 7A). In contrast to p-ERK1/2, p-AKT (Fig. 7B) and p-GSK-3β (Fig. 7C) were predominantly distributed in heavy fractions in perfusion hearts, and their phosphorylation level was significantly increased in IPC hearts. In addition, IPC also greatly increased caveolae-associated p-AKT, but not p-GSK-3β, which was consistent with other findings, i.e., caveolae-enriched p-ERK1/2 and p-AKT upon stress exposure (5, 53). Interestingly, IPC-induced changes and redistribution of these cellular signals were blocked by NPS2143 treatment, suggesting activation of CaSR is necessary for activating cardioprotective signaling.

DISCUSSION

The primary role of CaSR in parathyroid cells is to control systemic calcium homeostasis; however, the expression of CaSR in other cells or tissues has been demonstrated to play diverse cellular functions beyond the regulation of systemic calcium homeostasis. In this study, we found that a specific antagonist of CaSR, NPS2143, abolished the cardioprotective...
effect of IPC. In addition, the activation of cardioprotective signaling induced by IPC, such as ERK1/2, AKT, and GSK-3β, was blocked by inhibition of CaSR via NPS2143 treatment. These results suggest that the activation of CaSR plays an important role in IPC-mediated cardioprotection.

Presence and distribution of CaSR in cardiomyocytes. CaSR has been found to be expressed in both neonatal (19, 46, 47) and adult (52, 56) rat cardiac myocytes. As shown in Fig. 2A, CaSR was found to be expressed in adult mouse cardiomyocytes. The anti-CaSR immunoblot after reducing or non-reducing SDS-PAGE suggested that CaSR might also form a homodimer in vivo, as found in other cells and tissues (17). Although CaSR is expressed in cardiomyocytes, the detailed subcellular localization of this receptor is not clear. It has been reported that CaSR is localized in caveolea-rich membrane fractions of bovine parathyroid cells (22). Therefore, we carried out a study to see whether CaSR is also localized in the caveolar structure of cardiomyocytes. The results (Fig. 2B) of coimmunoprecipitation of CaSR and caveolin-3 (cardiomyocyte-specific caveolin isoform) demonstrated a protein-protein interaction between CaSR and caveolin-3. Moreover, CaSR was found to be exclusively distributed in caveolin-3-enriched fraction isolated from the detergent-free sucrose gradient ultracentrifugation (Fig. 6, A and B), further suggesting CaSR is localized at caveolae, a unique signaling complex organelle in the plasma membrane of cardiomyocytes (33).

It has been shown that caveolin-binding proteins contain common sequence motifs recognizing caveolin scaffolding domains (6, 32). Through the primary sequence and its topological analysis, we found two such caveolin binding motifs, i.e., EVLGFYIKF (629–637) and FNEAKFITF (801–809), individually located at cytosolic regions hinged to the first and the sixth transmembrane domains. The protein-protein interaction between CaSR and caveolin-3 was illustrated in Fig. 2C. However, given multiple regulatory domains present in both proteins and the presence of a homodimer of CaSR and oligomerization of caveolin, the protein–protein interaction and in vivo regulation are currently difficult to predict and need further characterization.

Preischemic activation of CaSR mediates cardioprotective effects against I/R injury. Ischemia leads to cellular ionic disequilibrium and acidosis, which is corrected by reperfusion. However, the concepts of calcium, pH, energy, oxygen, and redox paradox as molecular mechanisms of myocardial reperfusion injury have been widely debated (55). The changes of ionic strength and pH induced by IPC upon ischemia and reperfusion might promote allosteric activation of CaSR and initiate its downstream signaling cascades (17, 39, 40). Given the ionic channels/transporters distributed in caveolae and the confined space, the subcellular environmental changes in caveolae could be more exaggerated than expected. We hypothesize that the activation of CaSR is involved in IPC-induced cardioprotection. To test this hypothesis, NPS2143, a specific and selective CaSR antagonist (30), was infused into the perfused heart before and during IPC. In this study, we found that the inhibition of CaSR by NPS2143 treatment abolished the IPC-mediated improvement in postischemic contractile function (Fig. 3A), the reduction in myocardial infarct size (Fig. 3B), and increased phosphorylated cardioprotective signaling kinases (Fig. 4). However, a limitation of this study is its reliance on the specificity of this inhibitor.

In tissues other than the parathyroid gland, the primary physiological function of CaSR is to stimulate the release of PTHrP for paracrine/autocrine regulation (43). In recent studies, Monego et al. (27) have found that PTHrP was released in human ventricular cardiomyocytes on ischemic injury (27), and PTHrP has been reported to improve postischemic cardiac contractile function (18, 25). Interestingly, PTHrP expression has been found to be modulated by estrogen, and PTHrP improved postischemic cardiac function to a greater extent in females than in males after I/R injury (11). Therefore, besides activating the cardioprotective signaling kinases, activation of CaSR could also lead to release of PTHrP, which could have additional protective effects.

CaSR, a caveolae-associated signaling pathway involved in IPC-induced cardioprotection? Our laboratory’s previous findings suggested that the internalization and trafficking of GPCRs is required for cardioprotection induced by IPC (50), possibly via a Gβγ- and β-arrestin-mediated endosomal pathway (34). Recent studies suggest that caveolae and their internalized form known as caveosomes or signalosomes play an important role in preconditioning-mediated cardioprotective effects (16, 35, 51). Garlid et al. (9) hypothesize that IPC-activated GPCR migrate to caveolae, where signaling enzymes are scaffolded into signalosomes that bud off the plasma membrane, are internalized, and migrate to mitochondria, thus initiating signaling cascades with the end effect to inhibit the opening of mitochondrial permeability transition pore, thus decreasing cell death. Consistent with this hypothesis, we find the association of CaSR and caveolin-3 in cardiomyocytes (Fig. 2), the loss of IPC-induced cardioprotection by the inhibition of CaSR via NPS2143 treatment (Fig. 3), the decrease of IPC-induced phosphorylated kinase signaling with CaSR inhibition (Figs. 4 and 7), and NPS2143 blockade of
IPC-induced redistribution of proteins in caveolar and other subcellular fractions (Figs. 6 and 7). In general, activation of many different GPCRs that signal via ERK, AKT and GSK-3β lead to cardioprotection. A major question for future study is whether these receptors are all important in cardioprotection, whether some are only protective in certain models, or whether there is some interaction between different GPCRs.

In conclusion, CaSR, the extracellular Ca²⁺ sensing receptor, a member of GPCRs, is expressed in cardiomyocytes and predominantly localized at caveolae. The IPC-induced cardiac protection and activation of downstream protective kinase signaling pathways were abolished by treatment of hearts with a specific CaSR antagonist, NPS2143, suggesting that activation of CaSR during IPC plays an important role in mediating IPC-induced cardioprotection. Therefore, we proposed that CaSR could not only serve as a “sensor”, but also act as a “mediator” of IPC, playing a “priming” role in IPC-induced cardioprotection.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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