H$_2$S preconditioning-induced PKC activation regulates intracellular calcium handling in rat cardiomyocytes

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Pan T-T, Neo KL, Hu L-F, Yong QC, Bian J-S. H$_2$S preconditioning-induced PKC activation regulates intracellular calcium handling in rat cardiomyocytes. Am J Physiol Cell Physiol 294: C169–C177, 2008. First published November 7, 2007; doi:10.1152/ajpcell.00282.2007.—The present study was aimed to investigate the regulatory effect of protein kinase C (PKC) on intracellular Ca$^{2+}$ handling in hydrogen sulfide (H$_2$S)- preconditioned cardiomyocytes and its consequent effects on ischemia challenge. Immunoblot analysis was used to assess PKC isoform translocation in the rat cardiomyocytes 20 h after NaHS (an H$_2$S donor, 10$^{-3}$ M) preconditioning (SP, 30 min). Intracellular Ca$^{2+}$ was measured with a spectrofluorometric method using fura-2 ratio as an indicator. Cell length was compared before and after ischemia-reperfusion insults to indicate the extent of hypercontracture. SP mediated translocation of PKC$\alpha$, PKC$\varepsilon$, and PKC$\delta$ to membrane fraction but only translocation of PKC$\varepsilon$ and PKC$\delta$ was abolished by an ATP-sensitive potassium channel blocker glibenclamide. It was also found that SP significantly accelerated the decay of both electrically and caffeine-induced intracellular [Ca$^{2+}$]$_i$ transients, which were reversed by a selective PKC inhibitor chelerythrine. These data suggest that SP facilitated Ca$^{2+}$ removal via both accelerating uptake of Ca$^{2+}$ into sarcoplasmic reticulum and enhancing Ca$^{2+}$ extrusion through Na$^+$/Ca$^{2+}$ exchanger in a PKC-dependent manner. Furthermore, blockade of PKC also attenuated the protective effects of SP against Ca$^{2+}$ overload during ischemia and against myocyte hypercontracture at the onset of reperfusion. We demonstrate for the first time that SP activates PKC$\alpha$, PKC$\varepsilon$, and PKC$\delta$ in cardiomyocytes via different signaling mechanisms. Such PKC activation, in turn, protects the heart against ischemia-reperfusion insults at least partly by ameliorating intracellular Ca$^{2+}$ handling.

protein kinase C isoforms; ischemia and reperfusion; cardioprotection; ATP-sensitive potassium channel

H$_2$S has long been known only as a pollutant until recently it was found as a biological mediator natively generated in mammalian tissues. Researchers have since boosted focus on its physiological relevance, which led to the recognition that H$_2$S is under the growing family of the gasotransmitters (29). Its physiological relevance, which led to the recognition that H$_2$S is under the growing family of the gasotransmitters (29). H$_2$S has long been known only as a pollutant until recently it was found as a biological mediator natively generated in mammalian tissues. Researchers have since boosted focus on its physiological relevance, which led to the recognition that H$_2$S is under the growing family of the gasotransmitters (29).

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Fig. 1. Protein kinase C (PKC) inhibition reversed the late cardioprotection induced by H2S preconditioning (SP) on cell viability and cellular injury. Experimental design: the ventricular myocytes were preconditioned with NaHS for 30 min and then incubated for 20 h followed by exposure to severe ischemia for 10 min. At 10 min into reperfusion with normal medium, the nonblue cells per total myocytes were counted. VP, control; SP, cells were treated with 100 µM NaHS (30 min) for preconditioning; Che + SP, (3 µM) were added in the medium 15 min before and during SP; Che alone, cells were incubated with 3 µM chelerythrine for 45 min. B: cell viability determined by trypan blue assay. Values were presented as nonblue cells per total myocytes counted; n = 5–13 cultures of ∼200–500 cells each. *p < 0.05 vs. VP; +p < 0.05 vs. SP. C: LDH release. Values were presented as supernatant LDH activity/total LDH activities (supernatant + cell lysate) normalized to 100% of VP group (control); n = 7 experiments. Values are means ± SE; **p < 0.01 vs. VP, +p < 0.05 vs. SP.

Fig. 2. Ischemic preconditioning (IP) and SP activated PKC isoform translocation to membrane fraction. A and D: PKCα; B and E: PKCε; C and F: PKCδ. Samples were harvested after 20 h of culture. Equal amounts of protein from each sample were loaded on 8% SDS-PAGE and subjected to electrophoresis and immunoblotting. A–C: representatives of five separate experiments for each isoform, D–F: correspondent membrane-to-cytosol ratios as indexes of PKC isoform translocation. They are calculated by relative densitometry and normalized to 100% of VP group. The data are presented as means ± SE; n = 5 experiments. *p < 0.05 **p < 0.01 vs. VP.
Taken together, there is no information so far available about the effect of H2S on PKC and how the cardioprotective signals are transduced toward and forward. Thus in the present study, we intended to provide a close inspection on PKC and its upstream and downstream connections in the signal transduction pathway of H2S preconditioning.

MATERIALS AND METHODS

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of National University of Singapore.

Drugs and chemicals. Fura 2-AM, type II collagenase, protease, lactate, 2-deoxyglucose (2-DOG), sodium dithionite (Na2S2O4), sodium hydrosulfide (NaHS), caffeine, trypan blue dye, laminin, medium 199, lactate dehydrogenase (LDH) assay kit, and chelerythrine chloride were purchased from Sigma Chemical. Glibenclamide was obtained from Tocris Cookson. All chemicals were dissolved in deionized water except fura 2-AM and glibenclamide, which were dissolved in DMSO at a final concentration 0.1% (wt/vol).

Cardiac myocytes preparation. Cardiac myocytes were isolated from the hearts of adult male Sprague-Dawley rats using a collagenase perfusion method as described before (17). The heart was quickly excised from Pentobarbitone-anesthetized rats (250–300 g body weight), mounted via the aorta on a Langendorff apparatus, and retrogradely perfused with Ca2+-free Tyrode buffer at 37°C for 5 min. The heart was then perfused for another 25–30 min with circulating Ca2+-free Tyrode solution containing 0.84 mg/ml collagenase (type II) and 0.28 mg/ml protease. Thereafter, the ventricular tissue was cut into fragments and shaken gently to dissociate cardiac myocytes in Ca2+-Tyrode solution. The cell suspension was filtered, centrifuged, and washed three times. More than 80% of the cells were rod shaped and impermeable to trypan blue. The Ca2+ concentration of the Tyrode solution was then increased gradually to 1.25 mM in 45 min. Cells were allowed to stabilize for 30 min at room temperature.

Induction of simulated ischemia. Severe ischemia was induced by ischemia buffer containing (mM) 20 2-DOG (an inhibitor of glycolysis), 5 sodium lactate, 20 Na2S2O4 (an oxygen scavenger), 137 NaCl, 15.8 KCl, 0.49 MgCl2, 0.9 CaCl2, and 4 HEPES. The pH was adjusted to 6.6 to mimic acidosis. For simulation of ischemia preconditioning (IP), the concentrations of 2-DOG and Na2S2O4 were halved.

Experimental protocol. Myocytes were subjected to H2S preconditioning (SP) by incubation with 100 μM NaHS for 30 min. The concentration was adopted based on our previous study in which a maximum protection was observed at 100 μM (17). For ischemia preconditioning (IP), myocytes were subjected to simulated ischemia preconditioning as described above. The control group (VP) did not receive any pretreatment. Cells in all groups were washed several times before being cultured with Dulbecco’s modified Eagle’s medium (DMEM) in

Fig. 3. Blockade of ATP-sensitive (KATP) channel diminished SP-induced PKCε but not PKCα and PKCδ translocation. Glibenclamide (gliben, 10 μM), a selective KATP channel blocker, was applied 10 min before and during SP treatment. A–C: representatives of five separate experiments for each isoform. D–F: correspondent membrane-to-cytosol ratios calculated by relative densitometry and normalized to 100% of VP group as indexes of PKC isoform translocation. Data are presented as means ± SE; n = 5–7 experiments. *P < 0.05 **P < 0.01 vs. VP, +P < 0.01 vs. SP.
a CO$_2$ incubator for 20 h. Samples were then harvested for Western blot analysis or intracellular Ca$^{2+}$ transient recording. Or the cells were subjected to severe ischemia with ischemia buffer for 10 min after the 20-h culture, followed by 10 min reperfusion with normal medium, after which cell viability and cellular injury were assessed. Resting Ca$^{2+}$ elevation was traced real-time during ischemia for examination on cytosolic Ca$^{2+}$ accumulation. Cell length was compared before ischemia and after the onset of reperfusion for evaluation on hypercontracture. To study the involvement of PKC, the PKC inhibitor chelerythrine chloride (3 \mu M) was added into the cell medium 15 min before and during SP preconditioning (Fig. 1A). To study the sequence of signaling events between PKC activation and K$_{ATP}$ opening, cells were treated with glibenclamide (10 \mu M, a blocker of K$_{ATP}$ channel) 15 min before and during SP.

**Trypan blue assay.** Trypan blue exclusion was assessed as an index of cell viability (32). Cells were incubated in 0.4% (wt/vol) trypan blue dye for 3 min. Viable cells excluded the dye and thus remained white while the dead cells were stained blue. Cells were counted in a double-blinded manner under a light microscope at 10 magnification. Cell viability was expressed by the percentage of nonblue cells in each visual field.

**LDH assay.** LDH release was measured after 10 min reperfusion as a cellular injury index (16). Both culture medium and cell lysates (prepared with lysis buffer containing 1% Triton-X100) were collected for determination of LDH activity. LDH assay was performed using a commercially available kit (Sigma). The assay was based on the reduction of NAD catalyzed by LDH. The reduced NAD (NADH) was utilized in the stoichiometric conversion of a tetrazolium dye. The absorbance at a wavelength of 490 nm was measured spectrophotometrically with a microplate reader (Tecon Systems). The background absorbance at 690 nm was subtracted from the absorbance at 490 nm. The results were presented as LDH released into the medium in terms of percentage of the total LDH activity (medium + cell lysate), normalized to 100% for VP group.

**Measurement of [Ca$^{2+}$]$_i$.** Ventricular myocytes were incubated with fura 2-AM (4 \mu M) for 35 min. The loaded cells were transferred to a superfusion chamber on the stage of an inverted microscope (Nikon TS100), which was coupled with a dual-wavelength excitation spectrofluorometer (Photon Technology International). The myocytes were perfused with Krebs bicarbonate buffer containing (in mM) 118 NaCl, 5 KCl, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 1.25 CaCl$_2$, 25 NaHCO$_3$, and 11 glucose; pH 7.4. To generate electrically induced [Ca$^{2+}$]$_i$ transients (E[Ca$^{2+}$]$_i$), myocytes were stimulated at 0.2 Hz with a stimulator (Grass S88), whereas the caffeine-induced [Ca$^{2+}$]$_i$ transients (C[Ca$^{2+}$]$_i$) were generated by adding 10 mM caffeine directly to the incubation buffer. Resting Ca$^{2+}$ level was recorded without any above stimulation during ischemia challenge. Fluorescence signals obtained at 340 nm (F$_{340}$) and at 380 nm (F$_{380}$) excitation wavelengths were recorded and stored in a computer for data processing. The F$_{340}$-to-F$_{380}$ ratio was used to represent [Ca$^{2+}$] changes in the myocytes.

**Measurement of cell length.** Cardiomyocytes were placed on the stage of an inverted microscope (Nikon TE2000-S). The cell image was taken with a digital camera (Nikon DS-5M-L1) connected to the microscope with a 20 objective and analyzed with NIS-documentation software (Nikon).

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**Fig. 4.** SP accelerated sarcoplasmic reticulum (SR)-Ca$^{2+}$ uptake rate in single ventricular myocytes through a K$_{ATP}$-PKC pathway. *A*: typical transients of electrically induced intracellular Ca$^{2+}$ concentration (E[Ca$^{2+}$]$_i$) in VP, IP, SP, and SP + chelerythrine (SP+Che), and SP + glibenclamide (SP+Gliben). Half-decay time ($t_{50}$, B) and 90% decay time ($t_{90}$, C) of E[Ca$^{2+}$]$_i$ indicate the rate of Ca$^{2+}$ uptake to SR via SERCA. Data are presented as means ± SE; n = 14 (VP), 9 (IP), 11 (SP), 7 (Che), and 8 (Gliben) samples. *P < 0.05 vs. VP, **P < 0.01 vs. VP, +P < 0.05 vs. SP, ++P < 0.01 vs. SP.
**Cell fractionation and Western blot analysis.** A cell fractionation technique was adopted from the literature (11, 30). After 20 h of incubation, cardiomyocytes were lysed with 150 µl ice-cold lysis buffer containing 125 mM NaCl, 25 mM Tris (pH 7.5), 5 mM EDTA, 1% NP-40, and protease inhibitors and shaken on ice for 1 h. The cell lysate was centrifuged at 1,000 g at 4°C for 10 min for rough partition between cytosolic and membrane fractions. The supernatant was centrifuged at 16,000 g at 4°C for 15 min to get rid of contaminating pellet materials and collected as cytosolic fraction. The initial pellets were resuspended in 100 µl cell lysis buffer containing 1% Triton X-100 and shaken on ice for another 60 min and were then centrifuged at 16,000 g at 4°C for 15 min. The second supernatant was collected as membrane fraction. Epitopes were exposed by boiling the protein samples at 90°C water for 5 min. Each fraction was analyzed for protein content by the Bradford assay. Equal amounts of protein were loaded and electrophoresed with 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was probed with antibody against PKC ε (Santa Cruz Biotechnology), PKC ε, and PKC δ (Cell Signaling Technology). Immunoreactivity was detected using an ECL advance Western blot detection kit (Amersham Biosciences).

**Statistical analysis.** Values presented are means ± SE. Statistic comparisons were performed by one-way ANOVA and Bonferroni for post hoc analysis. The significance level was set at \( P < 0.05 \).

**RESULTS**

**Effect of SP on cell viability and cellular injury in the presence and absence of a PKC inhibitor.** We have previously reported that NaHS (1–1,000 µM) decreases ischemia-induced myocyte death in a dose-dependent manner, and the maximum effect is reached at 100 µM (17). Thus, in the present study, we used 100 µM as the concentration for H₂S preconditioning. As shown in Fig. 1B, SP significantly increased cell viability compared with that in VP group. To determine the role of PKC in the SP-induced cardioprotection, chelerythrine (3 µM), a selective PKC inhibitor, was applied 15 min before and during SP preconditioning (Fig. 1A). We found that chelerythrine, which alone had no effect, significantly attenuated the cardioprotective effect of SP on cell viability (Fig. 1B).

LDH release was measured as an index of cellular injury (16), which was presented as the ratio of the medium LDH activity over the total LDH activity (medium ± intracellular) and normalized to 100% of control. As shown in Fig. 1C, SP significantly reduced the LDH release induced by severe ischemia, and this effect was reversed by pretreatment with chelerythrine (3 µM), which itself had no effect on LDH release.

**Effect of SP on translocation of PKC isoforms.** To determine the activated PKC isoforms in the delayed phase of cardioprotection induced by SP and IP, subcellular distributions of three main PKC isoforms present in the heart, α, ε, and δ, were examined 20 h after SP and IP with Western blotting experiments. As shown in Fig. 2, A–C, SP induced all three isoforms of PKC translocation from cytosol to membrane. The membrane-to-cytosol ratios of PKC-α, ε, and δ increased approximately twofolds in SP (Fig. 2, D–F). Interestingly, IP only induced translocation of PKCε and PKCδ, but had no effect on PKCα translocation. These data suggest that the SP and IP may
employ different subsets of PKC isoforms to mediate their cardioprotection.

Effect of SP on translocation of PKC isoforms in the absence and presence of a K\textsubscript{ATP} channel blocker. Glibenclamide (10 μM), a K\textsubscript{ATP} channel blocker, was used to examine whether SP-induced translocation of PKC isoforms is secondary to the opening of K\textsubscript{ATP} channels. Similar to the protocol shown in Fig. 1A, cardiac myocytes were treated with glibenclamide 15 min before and during SP treatment. As shown in Fig. 3, glibenclamide blocked SP-induced translocation of PKCε but did not affect translocation of PKCα and PKCδ, suggesting that only PKCε among the three isoforms examined is downstream to K\textsubscript{ATP} channel in the signaling pathway of SP.

Effect of SP on the rate of SR-Ca\textsuperscript{2+} uptake in single cardiomyocytes. Electrical stimulation mimics the arrival of an action potential generated from the sinoatrial node of the heart, triggering the same cascade of events; i.e., membrane depolarization, influx of Ca\textsuperscript{2+}, and Ca\textsuperscript{2+} release from the SR (3, 5). The decline rate of [Ca\textsuperscript{2+}]\textsubscript{i}, is mainly determined by Ca\textsuperscript{2+} uptake to SR via SERCA, which is responsible for the removal of ~90% Ca\textsuperscript{2+} from the cytosol (2). Thus half-decay time (t\textsubscript{50}) and 90% decay time (t\textsubscript{90}) of [Ca\textsuperscript{2+}]\textsubscript{i} were measured as indicators of SR uptake rate. As shown in Fig. 4, both t\textsubscript{50} and t\textsubscript{90} were significantly shortened by SP and IP when compared with those observed in VP. Cotreatment with 3 μM chelerythrine or 10 μM glibenclamide during SP reversed this effect, suggesting that SP accelerated the rate of SR-Ca\textsuperscript{2+} uptake through a K\textsubscript{ATP}-PKC pathway.

Effect of SP on the rate of Ca\textsuperscript{2+} extrusion via NCX in single cardiomyocytes. Because caffeine keeps the RyR open, SR is unable to sequester Ca\textsuperscript{2+} during its application. The decline of [Ca\textsuperscript{2+}]\textsubscript{i}, therefore depends on Ca\textsuperscript{2+} extrusion through NCX(22). The rate of extrusion can be reflected by decay of $C$[Ca\textsuperscript{2+}]\textsubscript{i} (t\textsubscript{50} and t\textsubscript{90}). As shown in Fig. 5, $B$ and $C$, SP and IP significantly shortened both t\textsubscript{50} and t\textsubscript{90} of the decay of C[Ca\textsuperscript{2+}]\textsubscript{i}. These effects were reversed by either inhibition of PKC with chelerythrine (3 μM) or blockade of K\textsubscript{ATP} with glibenclamide (10 μM). We also examined the SR-Ca\textsuperscript{2+} load by measuring the amplitude of C[Ca\textsuperscript{2+}]\textsubscript{i} since caffeine depletes the intracellular Ca\textsuperscript{2+} store at a burst. The amplitude did not differ between groups, which excluded the possibility of a less SR-Ca\textsuperscript{2+} load as a contributory factor to the faster clearing of cytosolic Ca\textsuperscript{2+}.

Effect of SP on cytosolic Ca\textsuperscript{2+} accumulation during ischemia and hypercontracture after reperfusion. Resting Ca\textsuperscript{2+} elevation was traced during ischemia to investigate whether the Ca\textsuperscript{2+} handling preameliorated by SP is effectual to attenuate Ca\textsuperscript{2+} accumulation in the cytoplasm during ischemia challenge. As shown in Fig. 6. $A$ and $B$, when compared with the tracing in VP, the increase in resting Ca\textsuperscript{2+} level was lowered to a marked extent in SP. Pretreatment with chelerythrine during SP reversed this effect.

Hypercontracture refers to the sustained maximal contractile activation of myofibrils resulted from the combination of excessive cytosolic Ca\textsuperscript{2+} accumulation during ischemia and energy resupply after reperfusion(23). We determined cell-length shortening in an attempt to investigate whether the PKC-dependent protective effect of SP against the above ischemia-induced Ca\textsuperscript{2+} accumulation may also alleviate hypercontracture. As shown in Fig. 6C, in VP group myocyte length was shortened to about half of their initial length after reperfusion. SP significantly attenuated this detrimental shortening, and this effect was abolished by inhibition of PKC with chelerythrine.

DISCUSSION

As one of the top killers of humans, ischemic heart disease claims hundreds of thousands of lives every year throughout the world. To fulfill an increasing need for an effective and practical intervention strategy, it is important to understand the mechanisms underlying a potent cardioprotection. We recently
reported that H₂S preconditioning produced delayed cardioprotection against lethal ischemia (17). However, the signaling mechanism remains obscure except a potential involvement of sarcolemmal Kₐ₅p channel and NO (17). In the current study we found that SP-induced PKC isoform activation accelerates the rectification of elevated [Ca²⁺], and thereby increases the susceptibility of cardiomyocytes to ischemia-induced Ca²⁺ overload and consequent series of damages induced by lethal ischemia-reperfusion insults (Fig. 7). These findings disclose a novel mechanism for SP-induced cardioprotection and also provide considerable implication for other PKC-related anti-ischemia interventions.

PKC isoform translocation. Although PKC activation tends to be coupled with the genesis of late phase of cardioprotection induced by various types of preconditioning, the specific PKC isoforms involved in SP is still unknown. PKCα, PKCe, and PKCβ are the three main isoforms expressed in adult cardiomyocytes and also the most important ones involved in cardioprotection of ischemic preconditioning (9, 20). For this reason, we examined the effect of SP on these three isoforms before ischemia insults, which itself can stimulate PKC translocation. We found that H₂S preconditioning motivated translocation of the three isoforms, PKCα, PKCe, and PKCβ, to membrane fraction at 20 h after preconditioning. Such translocation before ischemia attack may act as an essential step to switch the cells into a state tolerant to ischemia insults and failure of such translocation results in the loss of cardioprotection as what we observed in the presence of PKC inhibitor (Fig. 1).

Individual PKC isozymes are believed to mediate characteristic cell functions, as upon stimuli they are directed to distinct subcellular membrane regions by isozyme-specific receptors for activated C kinase (RACK) (12). By binding to their specific RACKs, the activated isozymes are anchored close to their particular substrates. In the present study, we employed IP as a reference model due to its recognized stimulatory effect on PKC and intracellular Ca²⁺. Of great importance, we addressed the mechanism in this study how the SP-activated PKC mediates the cardioprotection. By monitoring the resting Ca²⁺ level in single cardiomyocytes, we observed that SP lowered elevation in [Ca²⁺], during ischemia in a PKC-dependent manner. Such a timely rectification on elevated [Ca²⁺], during ischemia challenge could be therapeutically important, as uncontrolled elevation in [Ca²⁺], could induce irreversible injuries like mitochondria dysfunction (36), membrane degradation, and contractile derangement (37). If the ischemia is followed by reperfusion, the myocytes will exhibit hypercontracture at the onset of reperfusion due to massive stimulation on the contractile machinery by accumulated Ca²⁺ (16). In perfused myocardium, this hypercontracture is manifest by contraction band necrosis (17). Even if the necrotic myocardium can be replaced by scar tissues in a subsequent remodeling process, the aokinetic fibrotic tissue will impair

PKC and Kₐ₅p. In our previous study, Kₐ₅p channel has been shown to be involved in the late phase of cardioprotection induced by SP (17). Since H₂S has been proposed to have a direct effect on Kₐ₅p channels (27), it raises the question whether SP-induced activation is secondary to the opening of Kₐ₅p channels. Unexpectedly, we observed that blockade of Kₐ₅p channel only diminished the SP-induced translocation of PKCe but failed to affect the translocation of PKCo and PKCδ to a noticeable extent. Thus Kₐ₅p channel opening may only be necessary for PKCe activation in the SP signaling pathway.

It is until recently that individual PKC isoforms were found located differently in relation to Kₐ₅p channel in the cardioprotection signaling pathway. Hassouna et al. (8) demonstrated that PKCe is located upstream, whereas PKCα is downstream to mitochondrial Kₐ₅p channel in IP signaling pathway. This implies a considerable diversity regarding PKC activation in the cardioprotective signaling mechanisms despite the similarity of key players. PKC can also be activated by other signaling molecules like NO or Ca²⁺ (14, 18). More studies are warranted to test whether SP-induced activation of PKCo and PKCδ are through provoking release of these signaling molecules.

PKC and intracellular Ca²⁺ handling. Of great importance, we addressed the mechanism in this study how the SP-activated PKC mediates the cardioprotection. By monitoring the resting Ca²⁺ level in single cardiomyocytes, we observed that SP lowered elevation in [Ca²⁺]i during ischemia in a PKC-dependent manner. Such a timely rectification on elevated [Ca²⁺]i during ischemia challenge could be therapeutically important, as uncontrolled elevation in [Ca²⁺]i, could induce irreversible injuries like mitochondria dysfunction (36), membrane degradation, and contractile derangement (37). If the ischemia is followed by reperfusion, the myocytes will exhibit hypercontracture at the onset of reperfusion due to massive stimulation on the contractile machinery by accumulated Ca²⁺ (16). In perfused myocardium, this hypercontracture is manifested by contraction band necrosis (17). Even if the necrotic myocardium can be replaced by scar tissues in a subsequent remodeling process, the aokinetic fibrotic tissue will impair

Fig. 7. Proposed signaling pathway for SP-induced cardioprotection (yellow route). H₂S activates different PKC isoforms directly (dashed line) or indirectly through the opening of Kₐ₅p or other unknown mechanisms (dashed line). The activated PKC isoforms stimulate the Ca²⁺ handling proteins (i.e., NCX and SERCA) and thereby facilitate the clearing of cytosolic Ca²⁺. During ischemia, the faster clearing of cytosolic Ca²⁺ induced by SP attenuates the Ca²⁺ accumulation and reduces hypercontracture.

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pumping function of the heart and when substantial enough will lead to heart failure (38).

To further corroborate the effect of H$_2$S on resting Ca$_{2+}$ during ischemia, we examined the myocyte hypercontracture at the onset of reperfusion. Indeed, SP reduced the development of myocyte hypercontracture through a PKC-dependent pathway. These beneficial effects triggered by SP and mediated by PKC could, in turn, at least partly account for the cardioprotection observed in cell viability and cellular injury tests (Fig. 1). It is also predictable that this limitation on the development of Ca$_{2+}$ overloading and hypercontracture in single cells would achieve further significant benefits by preserving contractile functions in the intact heart.

A previous (1) study has demonstrated an effective approach to attenuate myocyte hypercontracture by increasing SERCA activity. Since SR uptake through SERCA presents the dominant route for Ca$_{2+}$ removal in cardiomyocytes, it is plausible that this reduced hypercontracture is due to a faster Ca$_{2+}$ extrusion from cytosol before reperfusion. Enlightened by this finding, we examined the SR-Ca$_{2+}$ uptake rate as well as the minor mechanism for Ca$_{2+}$ removal; i.e., extrusion via NCX. We found that SP accelerated the clearing rate through both of these routes. Again, all these beneficial effects induced by SP were reversed by inhibition of PKC, implying that PKC may phosphorilate these calcium-handling proteins and improve their function.

In conclusion, the present study significantly advanced our understanding on the SP-induced cardioprotection by delineating the essential role of PKC in the context of signaling pathway (Fig. 7). The results demonstrate that SP activates PKCa, PKCe, and PKCb in cardiomyocytes, among which only activation of PKCe is secondary to the K$_{ATP}$ channel opening. Such PKC activation intervenes in the development of Ca$_{2+}$ clearing through SERCA and NCX.

**Limitation and perspective.** The findings in our study beg more research into these issues. First, H$_2$S alone is sufficient to activate three PKC isoforms, but the inhibitor chelerythrine could not distinguish the one or ones that are necessary for the genesis of the late phase of cardioprotection. It is more likely that different isoforms act on different substrates at various subcellular sites and afford the protection from diverse aspects (12). Assigning specific roles to each PKC isoform needs isofrom-specific antagonists with explicit selectivity. Yet some redundant signaling pathways may place additional complexity and difficulty. Second, the signaling pathway mapped in this study is by no means the only signaling chain headed by H$_2$S. Taking PKC as a nodal point, it has a spectrum of triggers that could vary from NO, adenosine, to free radicals, whereas its targets could range from mitogen-activated protein kinases, heat shock proteins, and mitochondria proteins (21). It is more likely to be a signal network rather than single pathways that translate the extracellular stimulus of H$_2$S into final protection. Finally, the cardiomyocytes model is advantageous in monitoring intracellular ionic changes and avoiding confounding effects of other cell types in the heart. However, as always, it needs further corroboration in intact heart model or animal model to extrapolate these findings to clinical trials.