G Protein–Coupled Receptor Internalization Signaling Is Required for Cardioprotection in Ischemic Preconditioning

Haiyan Tong, Howard A. Rockman, Walter J. Koch, Charles Steenbergen, Elizabeth Murphy

Abstract—The present study is designed to explore the role of G protein–coupled receptors (GPCRs) in the protection afforded by ischemic preconditioning (PC). We used TG mice with cardiac-specific overexpression of a Gβγ-sequestering peptide, βARKct (TG βARKct mice), to test whether the protection of PC is Gβγ-dependent. To test the role of G, protein, we used wild-type mice pretreated with the G inhibitor pertussis toxin. Recovery of left ventricular developed pressure and infarct size were measured as indices of protection. PC induced protection in wild-type mice, but this protection was blocked by pertussis toxin treatment and was also blocked in TG βARKct mice. To determine the mechanism of Gβγ-induced protection in PC, we investigated one of the downstream targets of Gβγ, the PI3K pathway. PC-induced phosphorylation of p70S6K was not blocked in TG βARKct mice; therefore, we investigated other targets of Gβγ. Recent studies suggest a role for Gβγ in GPCR internalization. We found that βARKct, a specific PI3K inhibitor wortmannin, and bafilomycin A₁, which all block receptor recycling, all blocked the protective effect of PC. To additionally test whether PI3K is involved in PC-activated receptor internalization and endosomal signaling, we used TG mice with cardiac-specific overexpression of a catalytically inactive mutant PI3Kγ, which disrupts the recruitment of functional PI3K to agonist-activated GPCRs in vivo. We found that the catalytically inactive mutant of PI3Kγ blocks the protection of PC. In summary, these data suggest the novel finding that the cardioprotective effect of PC requires receptor internalization. (Circ Res. 2004;94:1133-1141)

Key Words: Gβγ • phosphatidylinositol 3-kinase • transgenic mice • isolated heart • ischemic preconditioning

Brief intermittent periods of ischemia and reperfusion, termed ischemic preconditioning (PC), can increase myocardial resistance to ischemic injury.¹ PC has been shown to reduce arrhythmias, infarct size, and postischemic contractile dysfunction. PC activates a complex cascade of signaling events that ultimately results in cardioprotection. These include activation of G protein–coupled receptors (GPCRs), tyrosine kinases, phosphatidylinositol 3-kinase (PI3K), and protein kinase C (PKC), generation of reactive oxygen species, and opening of the mitochondria K⁺ channel. However, the mechanism by which PC initiates these intracellular cascades is not fully understood.

Binding of agonists to GPCRs activates G proteins and promotes the dissociation of G proteins into both Gα and Gβγ subunits. Both Gα and Gβγ subunits activate target effectors.² Gβγ-dependent targets include activation of certain isoforms of adenylate cyclase,³ PI3K,⁴ and β-adrenergic receptor kinase (βARK1 or GRK2).⁵ The Gβγ binding domain of βARK1 (βARKct) has been used to sequester Gβγ in cells and in vivo to study Gβγ-dependent processes.⁵,⁶ Recent studies suggest that homologous desensitization of GPCRs that is triggered by GRK phosphorylation and β-arrestin binding targets receptors to endosomes through an internalization process.⁷ Internalization of GPCRs involves sequential binding of β-arrestin, the clathrin adaptor AP-2, and clathrin. PI3K and its phosphoinositide products play a critical role in recruitment of β-arrestin GPCR complexes to endosomes.⁸ A recent study has demonstrated that PI3K regulates GPCR trafficking by promoting the recruitment of AP-2 to the receptor/β-arrestin complex.⁹ β-arrestin has recently been shown to be a scaffolding protein that brings other signaling molecules in contact with GPCRs in the endosome during receptor recycling.¹⁰ There is increasing evidence that in addition to leading to receptor desensitization, the βARK1 (GRK2)/β-arrestin interaction can lead to activation of signals such as the mitogen-activated protein kinase (MAPK) pathway (extracellular signal–regulated kinase [ERK] and c-Jun N-terminal kinase [JNK]).¹¹

Because there are data suggesting a role for GPCR in PC, particularly GPCRs that act via G, we wanted to examine the role of Gβγ, PI3K, and G, in cardiac protection. To accomplish this, we used mice with cardiac-specific βARKct overexpression, mice with cardiac-specific overexpression of a catalytically inactive mutant of PI3Kγ (PI3Kγ₅₃₁₀₃₁), and...
pertussis toxin to inhibit G_i signaling. We present data suggesting that G\textsubscript{\textgamma} mediates PC via an endosomal pathway that results in cardioprotection.

### Materials and Methods

#### Experimental Animals

All animals received humane care in accordance with National Institutes of Health guidelines (publication No. 8523, revised 1996). Adult wild-type (WT; Taconic, Germantown, NY) and transgenic (TG; Duke University, Durham, NC) mice of either sex were used for this study, and we found no sex-dependent differences. Sixteen adult heterozygous TG mice with cardiac-specific overexpression of a G\textsubscript{\textgamma} sequestration peptide βARKct\textsuperscript{6} (TG βARKct) with a body weight (BW) of 37.5±2.6 g and 18 WT littermates with a BW of 32.5±2.0 g were used. Sixty-four adult male B6/129 mice of BW 34.5±1.0 were used for the pertussis toxin (PTX) and bafilomycin A\textsubscript{1} (BF) experiments. We also used TG mice with cardiac-specific overexpression of PI3KY\textsubscript{res}\textsuperscript{12}. These mice were backcrossed into C57Bl/6 background and had 180-fold overexpression of the PI3KY\textsubscript{res} protein level relative to WT littermates. Ten adult heterozygous TG PI3KY\textsubscript{res} mice of BW 25.7±2.0 g and 10 WT littermates of BW 26.8±2.2 g were used.

#### Isolated Mouse Heart Preparation

Mice were anesthetized with intraperitoneal pentobarbital sodium, and the hearts were isolated and perfused in the Langendorff mode, as described previously.\textsuperscript{13} Hearts were perfused for 30 minutes (control) or preconditioned (4 cycles of 5 minutes of ischemia and 5 minutes of reperfusion) before being subjected to 20-minute no-flow ischemia and 2 hours of reperfusion. For the PTX experiments, WT mice were given an intraperitoneal injection of PTX at a dose of 100 μg per kg BW 24 hours before isolation and perfusion of the hearts. To confirm that this protocol blocks G\textsubscript{i} activity, we demonstrated that addition of 1 mmol/L carbachol did not result in a bradycardic effect in PTX-treated hearts. Four groups of hearts (control, PC, PC/PTX, and PTX) were used for the PTX experiments. Four groups of hearts (control, PC, PC/BF, and BF) were used for the BF experiments. In the PC/BF group, hearts were treated with 50 nmol/L BF 5 minutes before and throughout PC. In the BF group, hearts were treated with 50 nmol/L BF for 25 minutes. Another inhibitor of receptor endocytosis, monodansylcadaverine (MDC) (100 μmol/L), was used with the same protocol as BF. Recovery of left ventricular developed pressure (LVDP), expressed as a percentage of the initial preischemic LVDP before PC or ischemia, was measured at 1 hour of reperfusion.

#### Necrosis Measurement

At the end of 2 hours of reperfusion, hearts were perfused and incubated with a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC). Area of necrosis (percent of whole heart) was measured and quantitated as described previously.\textsuperscript{13}

#### Western Blot Analysis

Hearts were snap frozen at the end of the treatment period, at the point in the protocol where the 20-minute period of ischemia would have begun, and processed as described previously.\textsuperscript{14} Immunoblotting and detection of phospho-ERK and phospho-p70S6K were performed as described previously.\textsuperscript{14} Immunoblots were incubated with the phospho-ERK or phospho-p70S6K antibodies (1:1000 dilution) (Cell Signaling, Beverly, Mass). The immunoreactive bands were analyzed by densitometry.

#### Statistical Analysis

Data are expressed as mean±SE. One-way ANOVA was used to compare differences in recovery of LVDP, infarct size, and optical density data on multiple groups, followed by Tukey’s post hoc test for multiple comparisons. The statistical significance level was determined at P<0.05.

### Results

#### Overexpression of βARKct in Mice Shows Impaired PC Effect

We used TG mice with cardiac-specific overexpression of a peptide that contains the G\textsubscript{\textgamma} binding domain (βARKct) and acts as an inhibitor of G\textsubscript{\textgamma} function, including the inhibition of βARK1.\textsuperscript{6} We used this TG model as a tool to study the GPCR signaling pathway through G\textsubscript{\textgamma} in the intact heart. There were no significant differences in LVDP, heart rate, and coronary flow rate among the groups at the end of the control period (see online Table 1, available in the online data supplement at http://circres.ahajournals.org).

To investigate the effects of overexpression of βARKct on PC, we examined whether the PC-induced improvement in postischemic function occurs in TG βARKct hearts. As shown in Figure 1A, PC resulted in improved recovery of postischemic LVDP in WT mice (41.0±6.9% for PC versus 18.6±4.6% for non-PC control, P<0.05). However, PC did
not improve the recovery of postischemic LVDP in TG βARKct hearts (18.7±4.9% for PC versus 22.2±13.2% for non-PC control). We also examined the effect of overexpression of βARKct on infarction. As shown in Figure 1B, in WT hearts, PC results in significantly less necrosis than non-PC hearts (63.0±5.6% in non-PC hearts versus 33.8±2.0% in PC hearts, P<0.05). However, PC did not reduce infarct size in TG βARKct mice (59.0±6.1% in non-PC hearts versus 52.2±4.7% in PC hearts). Taken together, these data suggest that overexpression of a Gβγ inhibitor impaired the protection afforded by PC.

Intracellular pH (pHi) measured by 31P NMR spectra decreased during ischemia in all hearts (Figure 2). PC significantly reduced acidification during the 20-minute period of ischemia in both WT and TG hearts. pHi fell to 6.3±0.11 in WT control hearts compared with 6.6±0.04 in WT PC hearts (P<0.01). In TG hearts, pHi fell to 6.2±0.09 in control hearts compared with 6.6±0.02 in PC hearts (P<0.01) (Figure 2A). High-energy phosphates ATP and creatine phosphate measured by 31P NMR spectra were not significantly different among the groups during the sustained 20-minute ischemia and the 1-hour reperfusion periods (data not shown).

Figure 2. Time course of change in pHi during the preischemic period, 20 minutes of ischemia (0 to 20 minutes), and 40 minutes of reflow. A, TG βARKct hearts. B, BF hearts. *P<0.05, WT/control vs WT/PC; #P<0.05, TG/control vs TG/PC.

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Figure 3. A, Phospho-p70S6K levels at the start of sustained ischemia in WT mouse hearts with or without wortmannin treatment. Averaged optic densitometry data shown are mean±SE of 4 hearts in each group and are normalized to the level of p70S6K phosphorylation in WT/control hearts. B, Phospho-ERK1/2 in WT mouse hearts with or without wortmannin treatment at the start of sustained ischemia. Averaged densitometry data shown are mean±SE of 3 hearts in each group and are normalized to the level of ERK1/2 phosphorylation in WT/control hearts. *P<0.05 compared with WT/control; #P<0.05 compared with WT/PC hearts.

Signaling Pathways Downstream of Gβγ: Endosomal Signaling
These data suggest that Gβγ is important in the protection afforded by PC. Because Gβγ can activate PI3K, and PI3K has been shown previously to be involved in PC, we hypothesized that Gβγ signaling during PC would activate PI3K signaling pathways such as p70S6K phosphorylation. Figure 3A shows that wortmannin, a PI3K inhibitor, blocked PC-induced phosphorylation of p70S6K in WT hearts. However, to our surprise, we found that the PC-induced phosphorylation of p70S6K was not blocked in TG βARKct hearts (Figure 4A), suggesting that the cardioprotective effect of
GPCR-Gβγ activation during PC is not through this signaling pathway.

We considered other Gβγ-mediated pathways. Gβγ recruits βARK1 (GRK2), which phosphorylates GPCRs, leading to recruitment of β-arrestin.15 β-arrestin binding to GPCRs dissociates the receptors from G proteins, thereby terminating the signaling.15 β-arrestin has also recently been shown to be a scaffolding protein that recruits additional signaling molecules to GPCRs in the endosomes, leading to unique signaling.10 For example, this pathway has been shown to lead to activation of the ERKs and JNKs.11 Several studies have shown that PC leads to activation of the ERKs16,17; we therefore examined ERK activation as a marker of endosomal signaling. Figure 4B shows that PC-induced phosphorylation of ERK1/2 is blocked in TG βARKct mice, suggesting that Gβγ initiates MAPK signals involved in PC. We also found that PC-induced phosphorylation of ERK1/2 is blocked by wortmannin (Figure 3B), suggesting that ERK/MAPK activation is PI3K-dependent.

Gβγ can activate ERK signaling by several mechanisms, one of which involves transactivation of the EGF receptor (EGFR).18 Activation of MAPK by the EGFR has been shown to depend on internalization of the EGFR.18 GPCRs have also been shown to activate MAPK via internalization of the GPCR by a mechanism that involves the scaffolding protein β-arrestin.18 In both of these mechanisms, activation of MAPK requires internalization of the receptor and is inhibited by dynamin mutants, potassium depletion, hypertonic sucrose, MDC, and BF.18–20 Because potassium depletion and hypertonic sucrose were likely to have adverse and complex effects in a perfused heart model, we tested whether BF, a vacuolar-type H+-ATPase inhibitor that has been shown to block endosomal recycling,19,21 would block the protective effect of PC. Treatment with BF had no effect on preischemic LVDP, heart rate, or coronary flow rate (see online Table 1). As shown in Figure 5A, PC resulted in a significant improvement in recovery of postischemic LVDP compared with control (46.0±2.0% versus 21.3±4.9%, P<0.01). Treatment with BF blocked the PC-induced improvement in postischemic LVDP (17.5±2.2%; P<0.001).
compared with PC). Postischemic function in the group treated with BF alone (10.6±3.9%) was slightly but not significantly lower than that of the control non-PC group. Figure 5B shows the effect of BF on infarction. PC hearts have significantly smaller infarcts compared with control non-PC hearts (49.7±5.7% infarct in control versus 33.0±2.0% in PC hearts, P<0.05). However, treatment with BF blocked the PC-induced reduction in infarct size (50.7±5.9%, P<0.05 compared with PC). Infarct size in the group treated with BF alone (57.4±2.5%) was not significantly different from that of the control group (P>0.05). Figure 6A shows that the PC-induced phosphorylation of ERK1/2 was also blocked by BF, demonstrating the importance of endosomal trafficking in ERK1/2 activation during PC.

PC significantly reduced acidification during the 20-minute period of ischemia (6.36±0.04 for control versus 6.57±0.04 for PC, P<0.01), and BF did not block the PC-induced reduction in acidification (6.58±0.05, P>0.05 compared with PC). pH fell to 6.31±0.04 in the BF-alone group, which was not significantly different compared with the control non-PC group (Figure 2B). A previous study by Gottlieb et al22 reported that 50 nmol/L BF blocked the protective effect of PC through the attenuation of pH during metabolic inhibition, which they attributed to BF inhibition of vacuolar proton pumps. However, the effect of BF on pH was achieved by using 500 nmol/L of BF, a dose 10 times higher than that used to block PC. In contrast with their finding, in the present study, we found that 50 nmol/L BF blocked the protective effect of PC without affecting pH.

Another mechanistically distinct inhibitor of receptor endocytosis, MDC, which inhibits clathrin-mediated endocytosis by stabilizing clathrin cages, has been shown to inhibit agonist-stimulated internalization of GPCR and GPCR-mediated ERK1/2 phosphorylation.20 We used MDC as an alternative approach to explore the role of receptor endocytosis in PC. PC resulted in a significant improvement in recovery of postischemic LVDP compared with control (52.6±6.6% for PC versus 16.5±1.5% for non-PC control, P<0.01), which was blocked by MDC (23.0±5.5%; P<0.01 compared with PC). Recovery of postischemic LVDP in the group treated with MDC alone (14.3±4.1%) was not significantly different from that of the control group. These data imply that the protective effect of PC involves receptor desensitization or internalization.

**Role of G**<sub>i</sub> **in PC**

Because cardioprotection induced by PC seems to be mediated primarily by G<sub>i</sub>-dependent signaling, we next wanted to determine the role of G<sub>i</sub> in PC. We used the G<sub>i</sub> inhibitor PTX to block the dissociation of α and βγ subunits. Pretreatment with PTX had no effect on hemodynamics in the hearts (see online Table 1).

To investigate the effects of PTX treatment on recovery of cardiac function, we examined whether PTX pretreatment blocks the protection afforded by PC. As shown in Figure 7A, PC hearts showed improved recovery of postischemic LVDP compared with control non-PC hearts (66.2±8.3% for PC versus 34.4±7.5% for non-PC control, P<0.05). Pretreatment with PTX blocked the PC-induced improvement in postischemic LVDP (34.2±10.2%, P<0.05 compared with PC). Postischemic function in the group treated with PTX alone (45.7±7.4%) was not significantly different from that of the control group (P>0.05). We examined the effect of PTX pretreatment on infarction. As shown in Figure 7B, PC hearts have significantly smaller infarcts compared with control non-PC hearts (53.8±5.7% infarct in control versus 25.7±2.6% in PC hearts, P<0.01). Furthermore, PTX blocked the PC-induced reduction in infarct size (57.0±7.2%, P<0.01 compared with PC). Infarct size in the group treated
with PTX alone (53.6±9.7%) was not significantly different from that of the control group (P>0.05). Taken together, these data suggest that PTX blocked the protective effect of PC. We also found that the PC-induced phosphorylation of ERK1/2 was blocked by PTX (Figure 6B).

Overexpression of Mutant PI3Kγ<sub>inact</sub> in Mice Shows Impaired PC Effect

To additionally test whether receptor desensitization/internalization was important in PC, we used TG mice with cardiac-specific overexpression of a catalytically inactive mutant of PI3Kγ. Previous studies have shown that PI3Kγ<sub>inact</sub> disrupts the interaction between functional PI3K and βARK1 and blocks agonist-stimulated GPCR internalization<sup>12</sup> without affecting Gβγ-mediated recruitment of βARK1 or β-arrestin translocation to ligand-activated GPCRs.<sup>9,12</sup> We used this TG mouse model as a tool to study the involvement of PI3K distal to Gβγ in receptor internalization during PC in the intact heart. There were no significant differences in LVDP, heart rate, and coronary flow rate among the groups at the end of the control period (see online Table 1).

To investigate the effects of overexpression of PI3Kγ<sub>inact</sub> on PC, we examined whether the PC-induced improvement in postischemic function occurs in TG PI3Kγ<sub>inact</sub> mice. As shown in Figure 8A, PC resulted in improved recovery of postischemic LVDP in WT mice (40.2±5.6% for PC versus 21.8±2.9% for non-PC control, P<0.05). However, PC did not improve the recovery of postischemic LVDP in TG PI3Kγ<sub>inact</sub> mice (25.2±2.5% for TG/PC versus 26.6±5.8% for non-PC TG/control). We also examined the effect of overexpression of PI3Kγ<sub>inact</sub> on infarction. As shown in Figure 8B, in WT mice, PC hearts have significantly less necrosis than non-PC hearts (69.6±7.0% in non-PC hearts versus 39.0±4.8% in PC hearts, P<0.01). However, PC did not reduce infarct size in TG PI3Kγ<sub>inact</sub> mice (63.8±1.7% in non-PC hearts versus 58.4±7.9% in PC hearts). WT PC hearts have significantly less necrosis than PC hearts from TG PI3Kγ<sub>inact</sub> mice (P<0.05), whereas non-PC WT and TG hearts were not significantly different. Taken together, these data suggest that cardiac-specific overexpression of an inactive mutant of receptor-localized PI3Kγ impaired the protection afforded by PC.

Discussion

There are considerable data suggesting that the cardioprotective effect of PC is mediated by GPCRs.<sup>23</sup> GPCRs, including adenosine receptors, opioid receptors, bradykinin receptors, acetylcholine receptors, and angiotensin receptors, have been reported to mediate cardioprotection. In this study, we sought
to determine the role of $G_s$, $G_{i}$, $G_{i,2}$, and receptor internalization in PC. We report the novel finding that $G_{i,2}$-dependent receptor desensitization or internalization is required for PC. Data additionally show that $G_{i,2}$ inhibition does not block the PC-induced increase in p70S6K phosphorylation, suggesting that activation of p70S6K is not sufficient for PC and that p70S6K phosphorylation does not involve a $G_{i,2}$-stimulated pathway. We also are the first to show that $G_{i,2}$, $G_i$, and an endosomal pathway mediate the PC-induced activation of ERK. We find that BF, an inhibitor of endosomal recycling, blocks PC and the PC-induced activation of ERK. In additional support of the role of receptor internalization in PC, we show that hearts with impaired receptor internalization, attributable to overexpression of PI3K, do not exhibit cardioprotection in response to PC. These data provide evidence that PC signals protection through a $G_{i,2}$ signaling pathway that leads to receptor internalization.

**PC is $G_{i,2}$-Dependent**

The data in this study demonstrated that TG $\beta$ARKct hearts, which exhibit impaired $G_{i,2}$ signaling, are not protected by PC using both functional recovery and necrosis as indices of protection. In the absence of PC, TG $\beta$ARKct hearts have ischemia/reperfusion injury similar to WT. These data suggest that the protective effect of PC is dependent on a $G_{i,2}$-mediated process. Because we have shown previously that the protective effect of PC is dependent on PI3K, we wanted to examine whether the $G_{i,2}$-mediated protection was attributable to activation of PI3K. To accomplish this, we measured whether TG $\beta$ARKct hearts exhibited PC-induced activation of p70S6K and PKB, kinases directly downstream of PI3K. Surprisingly, we found that $\beta$ARKct did not block the PC-induced activation of p70S6K (Figure 4A) or PKB (data not shown). These data suggest that activation of p70S6K and PKB are not sufficient for the protection afforded by PC. The most straightforward interpretation of the data is that activation of kinases downstream of PI3K is not protective in the absence of a $G_{i,2}$-mediated process. However, there must be a $G_{i,2}$-independent mechanism for PI3K activation, because p70S6K and PKB are phosphorylated during PC in TG $\beta$ARKct hearts. $G_s$ is also released during G protein activation. The precise role of $G_s$ in PC is an important area that has not been rigorously tested. Our studies focus on the role of $G_{i,2}$ in PC; the role of $G_s$ in PC needs additional study.

**How Does $G_{i,2}$ Mediate PC?**

Our data suggest that $G_{i,2}$ is important in PC by a mechanism that is independent of PI3K signaling via p70S6K and PKB. We therefore considered other $G_{i,2}$-mediated mechanisms. $G_{i,2}$ recruits $\beta$ARK1 (GRK2), which phosphorylates GPCRs and recruits $\beta$-arrestin, disrupting receptor coupling to G proteins and targeting the receptor to the endosomes resulting in receptor desensitization and downregulation. Thus, there are two general mechanisms by which inhibition of $\beta$ARK1 or inhibition of receptor internalization could block PC. First, because $\beta$ARKct inhibits $\beta$ARK1 (GRK2) activity and therefore inhibits GPCR desensitization and internalization in the heart, it is possible that part of the mechanism whereby $\beta$ARKct attenuates the protective effect of PC is by enhancing signaling through GPCRs that oppose the actions of receptors implicated in PC. PC has generally been linked to receptors, such as adenosine receptors that signal through $G_i$. There are data in the literature suggesting that $G_{i}$-linked receptors can oppose the action of $G_i$. The data are consistent with the hypothesis that $G_{i,2}$-mediated desensitization of receptors that oppose PC (for example $G_s$) is necessary for the protective effect of PC. $\beta$-adrenergic receptors are classic $G_s$-linked receptors, but other receptors such as adenosine A2 receptor are G$i$-linked, and their desensitization is regulated by $\beta$ARK1 (GRK2). Second, $\beta$ARKct could lead to inhibition of receptor-mediated endosomal signaling via receptors that mediate PC. There are data showing that $\beta$ARK1 (GRK2) regulates adenosine A1 and A2, trafficking to the endosomes. In addition to receptor desensitization, $G_{i,2}$ and $\beta$-arrestin via a mechanism that requires endosomal targeting have been shown to initiate signaling pathways that lead to activation of endosome-dependent kinases such as ERK and JNKs. Consistent with PC-induced activation of endosomal signaling, our data show that sequestration of $G_{i,2}$ or inhibition of endosomal trafficking with BF or MDC blocks activation of endosomal signaling, as indicated by inhibition of PC-induced ERK activation. These data suggest that PC-induced ERK activation is via an endosome-dependent mechanism. The data herein provide novel insights into the mechanism by which PC activates the ERK pathway. We find that inhibition of $G_{i,2}$, inhibition of the endocytic pathway with BF, inhibition of PI3K with wortmannin, and inhibition of $G_s$ with PTX all block the protective effect of PC and phosphorylation of ERK, consistent with GPCR-mediated activation of ERK during PC, via either EGFR transactivation or the $\beta$-arrestin signaling. Src is also required for activation of MAPK by both these pathways, and inhibition of Src with PP1 has been shown to block PC. Interestingly, Krieg et al have reported that transactivation of the EGFR is important in acetylcholine-mediated cardioprotection. Thus, our data show that sequestration of $G_{i,2}$ blocks the protective effect of PC and blocks ERK activation, but the data do not prove that the $G_{i,2}$-dependent signaling mediates PC via activation of ERK. ERK is a marker of endosomal signaling, and other pathways of endosomal signaling may also be important. Furthermore, as discussed previously, sequestration of $G_{i,2}$ and inhibition of endosomal trafficking would also block receptor desensitization, and this could also be involved in cardioprotection.

The role of ERK in PC is controversial. Mocanu et al reported that the MEK-1 inhibitor PD 98059 blocked the phosphorylation of ERK1/2 but not the reduction of infarct size. However, Fryer et al showed that PD 98059 blocked both protection and ERK1/2 activation induced by PC. Inhibition of ERK has been shown to block PC, and ERK is reported to be a component of a PC-induced signaling complex associated with the mitochondria.

**PI3K Is Required for Receptor Endocytosis in PC**

To examine additionally the role of PI3K in PC signaling via receptor desensitization and internalization, we studied mice with cardiac-specific overexpression of PI3K$\gamma_{inact}$. It has
been shown previously that overexpression of PI3Kγsurf prevents β-AR desensitization and internalization. Therefore, if receptor internalization is important in essential signaling pathways of PC, the protection afforded by PC should be blocked in these mice. Consistent with this hypothesis, our data show that TG PI3Kγsurf mice are not protected by PC using both functional recovery and necrosis as end points. Studies have shown that agonist-dependent recruitment of PI3K to the membrane is an important step in the process of receptor sequestration and links PI3K to GPCR activation and sequestration. PI3Kγ has also been shown to regulate β2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/β-arrestin complex. PI3Kγsurf has been shown to block agonist-stimulated GPCR internalization to endosomes, because the active endogenous PI3Kγ is displaced by mutant, catalytically inactive PI3Kγ. Taken together, these data suggest that the protective effect of PC is dependent on receptor internalization that requires functional receptor-localized PI3K.

### Relationship of Receptor Internalization to Classic PC Signaling Pathways

PKCe, Src tyrosine kinase, and MAPK are known to play crucial roles in cardioprotection of PC. The precise relationship between PKC, Src, and receptor internalization will require additional studies. However, there are reports that PKC can affect the desensitization of GPCRs by phosphorylating βARK1 (GRK2) and altering its activity. PKC has also been reported to regulate membrane trafficking of several transporters such as the Na,K-ATPase. These data would suggest that PKC might be involved in receptor internalization acting upstream of endosomal signaling. This would be consistent with studies by Ping et al. reporting that PC-induced activation of MAPK is mediated by PKC, because PKC inhibitors blocked PC-induced activation of MAPK. Src is also reported to be involved in PC, and Src and other signaling molecules are recruited to the endosome by β-arrestin and play a role in subsequent ERK activation. Ping et al. also demonstrated that Src and Lck induced PKC-dependent cardioprotection, and they additionally showed that PKCe forms modules with Src or Lck, which are essential for PC-induced cardioprotection (see online Figure 1, available in the online data supplement at http://circres.ahajournals.org). Taken together, these data are consistent with a role for PKC and Src in regulating receptor trafficking.

### Conclusions

Our data indicate that (1) Gβγ signaling is required for the protective effect of PC and PC-induced activation of ERK; (2) inhibition of Gβγ signaling does not block PC-induced phosphorylation of p70S6K, suggesting that other PC signals activate PKB/Akt and p70S6K; and (3) receptor-localized PI3K is required for PC.

### Acknowledgments

This work was supported in part by NIH grants HL-39752 (to C.S.), HL-56687 (to H.A.R.), and HL-61690 (to W.J.K.). H.T. and E.M. were supported by the National Institute of Environmental Health Sciences intramural program. We thank Diane Magnuson for helping with Western blot analysis.

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Circ Res. 2004;94:1133-1141; originally published online March 18, 2004;
doi: 10.1161/01.RES.0000126048.32383.6B
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Table 1. Hemodynamics before sustained ischemia

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>LVDP (mmHg)</th>
<th>HR (bpm)</th>
<th>Flow rate (ml/min)</th>
<th>dP/dT-max</th>
<th>dP/dT-min</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG βARKct (WT/C)</td>
<td>5</td>
<td>110±10</td>
<td>323±20</td>
<td>3.3±0.4</td>
<td>4323±644</td>
<td>-4245±1110</td>
</tr>
<tr>
<td>TG βARKct (WT/PC)</td>
<td>5</td>
<td>125±9</td>
<td>342±20</td>
<td>3.8±0.2</td>
<td>4510±573</td>
<td>-4179±323</td>
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<tr>
<td>TG βARKct (TG/C)</td>
<td>4</td>
<td>130±16</td>
<td>380±11</td>
<td>3.7±0.3</td>
<td>5030±900</td>
<td>-4939±809</td>
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<tr>
<td>TG βARKct (TG/PC)</td>
<td>4</td>
<td>116±12</td>
<td>355±25</td>
<td>4.0±0.1</td>
<td>4173±490</td>
<td>-4242±640</td>
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<tr>
<td>BF (WT/C)</td>
<td>4</td>
<td>109±6</td>
<td>333±15</td>
<td>3.4±0.1</td>
<td>3385±847</td>
<td>-3637±887</td>
</tr>
<tr>
<td>BF (WT/PC)</td>
<td>4</td>
<td>115±11</td>
<td>404±12</td>
<td>3.3±0.3</td>
<td>4310±750</td>
<td>-3231±1697</td>
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<tr>
<td>BF (PC/BF)</td>
<td>4</td>
<td>124±10</td>
<td>387±30</td>
<td>3.2±0.2</td>
<td>4239±617</td>
<td>-3360±476</td>
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<tr>
<td>BF (BF)</td>
<td>5</td>
<td>118±8</td>
<td>403±32</td>
<td>3.6±0.2</td>
<td>4019±528</td>
<td>-3672±486</td>
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<tr>
<td>PTX (WT/C)</td>
<td>8</td>
<td>121±6</td>
<td>343±14</td>
<td>3.9±0.1</td>
<td>4482±424</td>
<td>-4133±597</td>
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<td>PTX (WT/PC)</td>
<td>6</td>
<td>116±4</td>
<td>392±15</td>
<td>3.4±0.2</td>
<td>3820±96</td>
<td>-4003±497</td>
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<tr>
<td>PTX (PC/PTX)</td>
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<td>113±10</td>
<td>388±15</td>
<td>3.4±0.2</td>
<td>3767±401</td>
<td>-3832±376</td>
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<tr>
<td>PTX (PTX)</td>
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<td>112±11</td>
<td>347±7</td>
<td>3.5±0.5</td>
<td>3853±277</td>
<td>-3561±604</td>
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<tr>
<td>TG PI3Kγ_{inact} (WT/C)</td>
<td>5</td>
<td>107±6</td>
<td>358±21</td>
<td>2.9±0.1</td>
<td>3589±148</td>
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<tr>
<td>TG PI3Kγ_{inact} (WT/PC)</td>
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<td>2.9±0.1</td>
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<tr>
<td>TG PI3Kγ_{inact} (TG/C)</td>
<td>5</td>
<td>108±12</td>
<td>356±19</td>
<td>2.9±0.1</td>
<td>3396±499</td>
<td>-2497±382</td>
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<tr>
<td>TG PI3Kγ_{inact} (TG/PC)</td>
<td>5</td>
<td>122±15</td>
<td>409±21</td>
<td>2.9±0.1</td>
<td>4200±437</td>
<td>-3147±564</td>
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</tbody>
</table>

Values are mean ± SE. LVDP = left ventricular developed pressure; HR = heart rate; Flow rate = coronary flow rate; dP/dT-max = maximum 1st derivative of the change in left ventricular pressure/time; dP/dT-min = minimum 1st derivative of the change in left ventricular pressure/time.
Proposed schematic diagram of the mechanism of GPCR-dependent protection in PC.

Following PC stimulation agonists bind to GPCR and G\(\beta\gamma\) release from receptors. G\(\beta\gamma\) recruits \(\beta\)ARK1 which recruits PI3K to the activated receptor complex, leading to receptor desensitization and internalization. Endosomal receptor activates ERK cascade through either EGFR transactivation or \(\beta\)-arrestin-dependent pathways. GPCR also activates ERK through G protein-dependent pathway that involves gene transcription.