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Cardioprotective effects of acute and chronic opioid treatment are mediated via different signaling pathways

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Peart, Jason N., and Garrett J. Gross. Cardioprotective effects of acute and chronic opioid treatment are mediated via different signaling pathways. *Am J Physiol Heart Circ Physiol* 291: H1746–H1753, 2006. First published May 26, 2006; doi:10.1152/ajpheart.00233.2006.—A 5-day exposure to morphine exerts a profound cardioprotective phenotype in murine hearts. In the present study, we examined mechanisms by which morphine generates this effect, exploring the roles of G_i and G_s proteins, PKA, PKC, and β-adrenergic receptors (β-AR) in acute and chronic opioid preconditioning. Langendorff-perfused hearts from placebo, acute morphine (AM; 10 μmol/l)-, or chronic morphine (CM)-treated mice (75-mg pellet, 5 days) underwent 25-min ischemia and 45-min reperfusion. After reperfusion, placebo-treated hearts exhibited marked contractile and diastolic dysfunction [rate-pressure product (RPP), 40 ± 4% baseline; end-diastolic pressure (EDP), 33 ± 3 mmHg], whereas AM hearts showed significant improvement in recovery of RPP and EDP (60 ± 3% and 23 ± 4 mmHg, respectively; $P < 0.05$ vs. placebo). Furthermore, CM hearts demonstrated a complete return of diastolic function and significantly greater recovery of contractile function (83 ± 3%, $P < 0.05$ vs. both placebo and AM). Pretreatment with G_i protein inhibitor pertussis toxin abolished AM protection while partially attenuating CM recovery ($P < 0.05$ vs. placebo). Treatment with G_s inhibitor NF-449 did not affect AM preconditioning yet completely abrogated CM preconditioning. Similarly, PKA inhibition significantly attenuated the ischemia-tolerant state afforded by CM, whereas it was ineffective in AM hearts. PKC inhibition with chelerythrine was ineffective in CM hearts while completely abrogating AM preconditioning. Moreover, whereas β_1-AR blockade with CGP-20712A failed to alter recovery in CM hearts, the β_2-AR antagonist ICI-118,551 significantly attenuated postischemic recovery. These data describe novel findings whereby CM preconditioning is mediated by a PKC-independent pathway involving PKA, β_2-AR, and G_s proteins, whereas AM preconditioning is mediated via G_i proteins and PKC.

ischemia-reperfusion; G protein-coupled receptors; rate-pressure product; end-diastolic pressure; morphine

OPIOID RECEPTORS have been shown to regulate cardiovascular function in both normal and diseased myocardium. These receptors have been localized to the central nervous system and peripherally to autonomic presynaptic nerve endings and on cardiac myocytes. Importantly, myocardial cells are sites of opioid peptide synthesis, storage, and release (4), which are elevated during episodes of stress, such as ischemia (11).

The opioid receptor family comprises three primary subtypes, μ, κ, and δ. The opioid receptors are guanine nucleotide binding protein (G protein)-coupled receptors, inhibiting adenylyl cyclase (G_i coupled). Opioid receptor activation has been implicated in ischemic preconditioning (IPC), and, indeed, exogenous activation of opioid receptors has been well documented to afford both acute and delayed cardioprotection against ischemia-reperfusion (16). The mechanisms by which opioid receptor activation affords cardioprotection are typically thought to involve the phosphatidylinositol 3-kinase (PI3K), MAPK, and GSK3-β pathways (15, 16) as well as both the sarcolemmal and mitochondrial ATP-sensitive K^+ ($mitoK_{ATP}$) channels (16). Opioid-mediated delayed preconditioning also involves cyclooxygenase-2 and inducible nitric oxide synthase (34, 45). We recently described a cardioprotective phenotype that occurred after prolonged exposure (5 days) to the opioid agonist morphine (35). This phenotype persists for at least 48 h after complete opioid withdrawal and still exists in the aged myocardium (36).

The opioid receptor family is known to have functional cross talk with other G protein-coupled receptor families, including both adenosine (37) and β-adrenergic receptors (β-AR) (38). The β-AR system exerts various effects on the myocardium via three β-AR subtypes, β_1-AR, β_2-AR, and β_3-AR. β_1-AR is coupled to the stimulatory G protein (G_s), whereas β_2-AR can couple to both G_i and G_s proteins. β_3-AR primarily associates with G_i proteins. The G_s protein coupling is associated with adenylyl cyclase activation and a resultant increase in cAMP production. This increased cAMP subsequently results in the activation of cAMP-dependent PKA, which is responsible for downstream signaling (for review, see Ref. 10). Moreover, β_2-AR activation via isoproterenol administration has been demonstrated to mimic IPC (12, 30).

In light of this, we sought to investigate the mechanisms responsible for chronic opioid-mediated preconditioning while examining the potential roles of both G_i and G_s proteins in the genesis of chronic preconditioning and to determine whether the protection afforded by prolonged morphine exposure is, in part, mediated by β-AR and PKA.

METHODS

The following investigations conformed to the guidelines of and were approved by the Animal Care Committee of the Medical College of Wisconsin (Milwaukee), which is accredited by the American Association of Laboratory Animal Care. Hearts were isolated from fed male wild-type C57/BL6 mice (22.7 ± 0.3 g body wt).

Perfused heart preparation. Mice were anesthetized with 60 mg/kg pentobarbital sodium administered intraperitoneally and perfused as described previously (17). After anesthesia, a thoracotomy was performed, and hearts were rapidly excised into ice-cold perfusion fluid. The aorta was cannulated, and the coronary circulation of all hearts was perfused in a Langendorff mode and maintained at a constant

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pressure of 80 mmHg with modified Krebs-Henseleit buffer containing (in mmol/l) 120 NaCl, 25 $NaHCO_3$, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 15 D-glucose, and 0.5 EDTA. Perfusate was equilibrated with 95% O_2-5% CO_2 at 37°C to give a pH of 7.40 and Po_2 of ~600 mmHg at the tip of the aortic cannula over a 1–5 ml/min range. All perfusate delivered to the heart was passed through an in-line 0.22-μm Sterivex-HV filter cartridge (Millipore, Bedford, MA) to continuously remove microparticulates. The left ventricle was vented with a polyethylene apical drain, and hearts were instrumented for functional measurements as described below. They were then immersed in warmed perfusate inside a water-jacketed bath maintained at 37°C. The temperature of the perfusion fluid was monitored by a needle thermistor at the entry into the aortic cannula, and the temperature of the water bath was assessed with a second thermistor probe. Temperature was recorded using a three-channel Physitemp TH-8 digital thermometer (Physitemp Instruments, Clifton, NJ).

For assessment of isovolumic function, fluid-filled balloons constructed of polyvinyl chloride plastic film were inserted into the left ventricle via the mitral valve. Balloons were connected to a P23 XL pressure transducer (Viggo-Spectramed, Oxnard, CA) by fluid-filled polyethylene tubing, permitting continuous measurement of left ventricular pressure. Balloon volume was increased to an end-diastolic pressure (EDP) of 5 mmHg. Coronary flow was monitored via a cannulating Doppler flow probe (Transonic Systems, Ithaca, NY) placed in the aortic perfusion line and connected to a T206 flowmeter (Transonic Systems). All functional data were recorded at 1 KHz on an 8/s MacLab data acquisition system (ADInstruments, Castle Hill, NSW, Australia) connected to an Apple 7300/180 computer. The ventricular pressure signal was digitally processed to yield peak systolic pressure, diastolic pressure, the positive and negative rate of pressure development, and heart rate.

Hearts were excluded from the study after the initial 25-min stabilization period if they met one of the following functional criteria: *1*) coronary flow >5 ml/min (near maximal dilation or an aortic tear), *2*) unstable (fluctuating) contractile function, *3*) left ventricular systolic pressure below 100 mmHg, or *4*) significant cardiac arrhythmias.

Experimental protocol. After a 20-min stabilization period, hearts were switched to ventricular pacing at a rate of 420 beats/min (Grass S9 stimulator, Quincy, MA). Those hearts receiving acute morphine and antagonist drug treatment were then treated for 10 min before induction of global normothermic ischemia. Baseline measurements were then made and 25 min of ischemia was initiated, followed by 45 min of reperfusion. Pacing was terminated on initiation of ischemia and resumed after 1.5 min of reperfusion. Drug infusion resumed at the onset of reperfusion and continued throughout the reperfusion period. Pertussis toxin (PTX)-treated hearts received 100 μg/kg intraperitoneally 24 h before heart isolation.

Chronic model. To induce chronic preconditioning, mice were briefly anesthetized with halothane, and a small incision was made at the base of the neck. Placebo or morphine (75 mg) pellets (National Institute of Drug Abuse) were inserted in the dorsal subcutaneous space before the site was closed with 9-mm wound clips. Pellets were left in place for 5 days.

Chemicals. PTX, NF-449, H-89, ICI-118,551, and CGP-20712A were purchased from Sigma-RBI (St. Louis, MO). The PKA inhibitor (PKI) 14-22 amide was obtained from Calbiochem (La Jolla, CA). All drugs were infused through a 0.22 μm filter at not more than 1% of coronary flow to achieve the final concentrations indicated. Inhibitor concentrations were based on previous literature reports, concentration-response experiments, or prior work performed by the authors' laboratory.

Statistical analysis. Data are expressed as means ± SE. For all groups, $n \geq 6$. Functional responses to ischemia-reperfusion were analyzed by multiway analysis of variance with repeated measures. When significance was detected, a Newman-Keuls post hoc test was employed for individual comparisons. For all tests, significance was accepted for $P < 0.05$.

RESULTS

Baseline hemodynamics and ischemic tolerance. After pellet implantation for the allotted time, hearts were rapidly excised and perfused. After 20-min equilibration, baseline measurements were taken. No baseline differences were noted between placebo and morphine-treated mice. Global ischemia was induced for 25 min, followed by 45 min reperfusion. On termination of reperfusion, placebo-treated hearts exhibited a poor recovery of left ventricular contractile function [40 ± 4% baseline function, rate-pressure product (RPP)] and an elevated EDP (33 ± 3 mmHg) (Fig. 1). In contrast, mice receiving acute morphine (10 μM) infusion before ischemia and throughout reperfusion exhibited a significant improvement in recovery of both RPP (60 ± 3% baseline, $P < 0.05$ vs. placebo) and EDP (23 ± 4 mmHg, $P < 0.05$ vs. placebo). Additionally, mice implanted with morphine pellet for 5 days demonstrated a further improvement in ischemic tolerance and EDP returned to baseline, whereas left-ventricular contractile function returned to 83 ± 3% of baseline (RPP) after 45-min reperfusion ($P < 0.05$ vs. placebo and vs. acute morphine treatment; Fig. 1).

Archetypal mediators of preconditioning. In a preliminary study, we set out to investigate the role of previously described mediators implicated in the preconditioning phenotype (15, 16, 45). To this end, we employed the nonselective opioid antagonist naloxone (10 μmol/l), the nonselective K_{ATP} channel inhibitor glibenclamide (300 nmol/l), the general nitric oxide synthase inhibitor N^{ω}-nitro-L-arginine methyl ester (100 μmol/l), the inhibitor of the mammalian target of rapamycin, rapamycin (1 nmol/l), and GW-5074 (5 μmol/l), an inhibitor of cRaf1 kinase, an upstream mediator of the MEK/MAPK pathway, including ERK1/2. These antagonists/inhibitors were administered pre- and postischemia, after isolation and perfusion of the heart. As demonstrated in Fig. 1, these compounds failed to antagonize the cardioprotective effects of chronic opioid preconditioning, suggesting that the pathways involved in the mediation of this protection may be dissimilar to that of "acute" preconditioning. On the basis of these observations, we sought to investigate in more depth an alternate receptor-mediated pathway in the genesis of this cardioprotective phenotype.

G proteins. To determine the relative contributions of both the G_i (inhibitory) and G_s (stimulatory) proteins in the phenotype generated with prolonged opioid receptor activation, we utilized selective inhibitors for both proteins. The G_i protein inhibitor PTX was administered intraperitoneally (100 μg/kg) 24 h before excision of the heart, whereas NF-449 (1 μmol/l), a selective G_s protein inhibitor that blocks the binding of GTP to $G_{s\alpha}$, was administered 10 min preischemia and throughout reperfusion, as were the remainder of the inhibitors.

When administered to placebo-treated hearts, PTX failed to afford any shift in recovery, whereas NF-449 led to an improvement in recovery of EDP but not RPP. The NF-449 effects are likely due to the limitation of catecholamine-mediated damage (41). As expected, PTX pretreatment abolished the cardioprotective effects of acute morphine treatment, whereas NF-449 failed to exert any effect (Fig. 2).

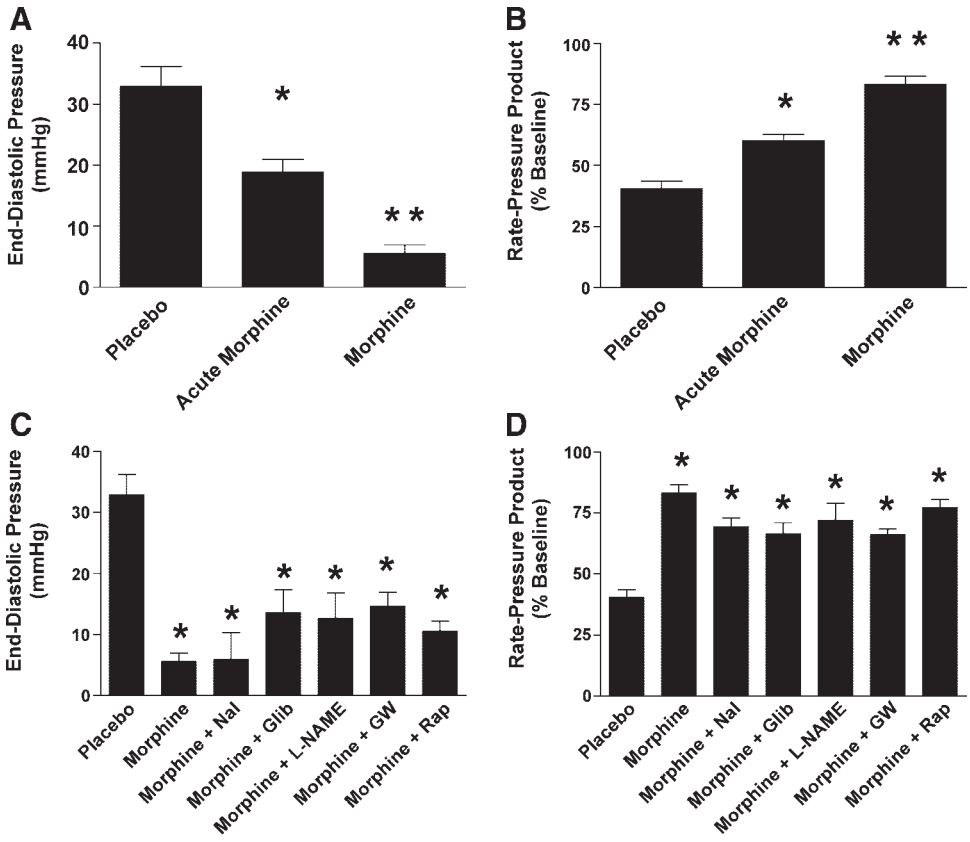

Fig. 1. Recovery of end-diastolic pressure (*A*) and rate-pressure product (*B*) after 25-min ischemia and 45-min reperfusion in hearts treated with acute morphine (10 μM) or hearts from mice exposed to either placebo or morphine for 5 days. Also shown is recovery of end-diastolic pressure (*C*) and rate-pressure product (*D*) in 5-day placebo or morphine hearts in the presence or absence of a variety of "preconditioning" inhibitors: naloxone (Nal; 10 μM), glibenclamide (Glib; 300 nM), N^{ω}-nitro-L-arginine methyl ester (L-NAME; 100 μM), GW-5074 (GW; 5 μM), and rapamycin (Rap; 10 nM). $^{*}P < 0.05$ vs. placebo; $^{**}P < 0.05$ vs. both placebo and acute morphine-treated hearts.

Despite the dependency of opioid receptors on G_i protein coupling, pretreatment with PTX, although partially limiting postischemic functional recovery in chronic morphine-treated hearts ($P < 0.05$ vs. chronic morphine alone), failed to completely abolish the cardioprotective phenotype ($P < 0.05$ vs. placebo; Fig. 2). Conversely, NF-449 treatment completely abrogated the protective response observed with chronic morphine treatment against postischemic dysfunction, as measured by the recovery of RPP and EDP (Fig. 2), implicating a major effect of G_s proteins in the genesis of the chronic morphine preconditioning phenotype.

PKA, PKB, and PKC. Preconditioning is typically thought to be mediated via a PKC-dependent pathway, being downstream of G_i protein coupling. Here we sought to determine the role of PKC in our model of chronic preconditioning with a comparison to that of PKA. Blockade of PKC with the inhibitor chelerythrine (3 μmol/l), with or without the tyrosine kinase inhibitor lavendustin A (1 μmol/l), infused both preischemia and throughout reperfusion completely abrogated the protection afforded by acute morphine, yet it failed to modify the substantial protection afforded by chronic opioid exposure (EDP, 8 ± 1 mmHg; %RPP, 78 ± 5%; $P < 0.05$ vs. placebo, for both parameters; Fig. 3).

To further verify the results obtained with the PKC inhibitor, we further interrogated the involvement of the PI3K/protein kinase B (Akt) pathway in both acute and chronic opioid preconditioning. To this end, wortmannin (100 nmol/l) was infused pre- and postischemia. Wortmannin failed to significantly alter the ischemic tolerance in the placebo heart; however, it abolished the protection produced by acute morphine preconditioning. Moreover, Akt blockade, although attenuating postischemic recovery, failed to completely abolish the chronic

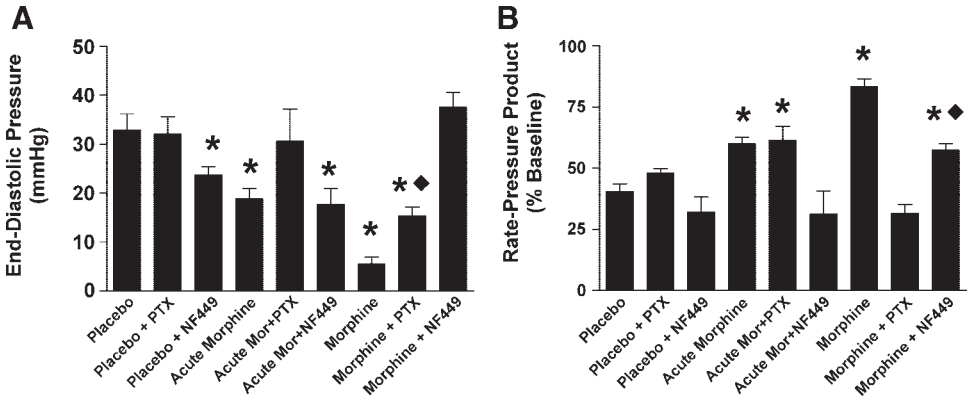

Fig. 2. Relative roles of G_i and G_s proteins in functional protection associated with chronic opioid preconditioning. Recovery of end-diastolic pressure (*A*) and rate-pressure product (*B*; percentage from baseline) after 25-min global ischemia and 45-min normoxic reperfusion in acute morphine (Mor)-, 5-day placebo-treated, or chronic morphine-treated hearts with or without G_i protein inhibitor pertussis toxin (PTX; 100 μg/kg) or G_s protein inhibitor NF-449 (1 μM). All values are reported as means ± SE. $^{*}P < 0.05$ vs. placebo; ◆$P < 0.05$ vs. both placebo and morphine-treated hearts.

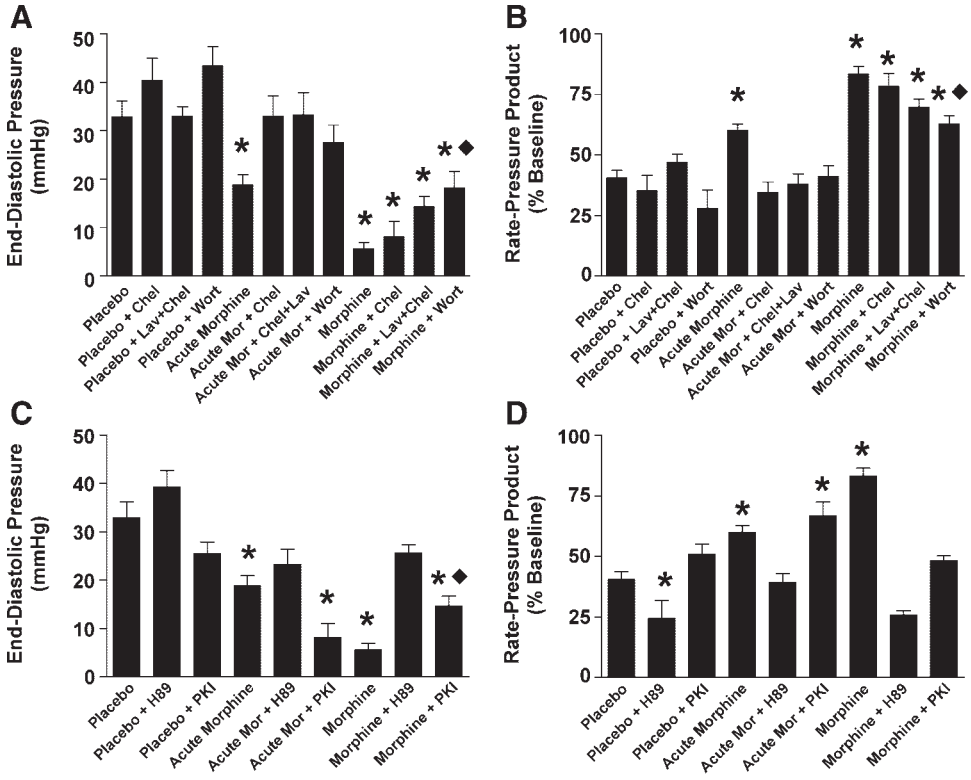

Fig. 3. PKC-independent protective effects of opioid-preconditioning phenotype. Values are shown for recovery of end-diastolic pressure (*A*) and left ventricular rate-pressure product (*B*; percentage from baseline). Data are provided for acute morphine-, placebo-treated, and chronic morphine-treated hearts treated with or without chelerythrine (Chel; 3 μM), chelerythrine (3 μM) + lavendustin A (Chel + Lav; 1 μM), or wortmannin (Wort; 100 nM) for 10 min before ischemia and throughout reperfusion. Also shown is effect of PKA inhibition after chronic preconditioning protocol. Functional recovery of end-diastolic pressure (*C*) and rate-pressure product (*D;* percentage from baseline) after 25-min global ischemia and 45-min normoxic reperfusion in acute morphine-, placebo-treated, or chronic morphine-treated hearts in the presence or absence of PKA inhibitors H-89 (3 μM) and 14-22 amide (PKI; 300 nM). All values are reported as means ± SE. **P* <0.05 vs. placebo; ◆*P* < 0.05 vs. both placebo and morphine-treated hearts.

morphine-mediated phenotype. ($P < 0.05$ vs. chronic morphine alone, $P < 0.05$ vs. placebo; Fig. 3).

Coupling to G_s activates adenylyl cyclase, increasing cAMP production with resultant cAMP-dependent PKA activation. On the basis of the findings with NF-449, we sought to examine a potential dependency between chronic morphine preconditioning and PKA. To this end, we treated hearts with H-89 (3 μmol/l), a cell-permeable PKA inhibitor, or PKI (14-22) amide (300 nmol/l), an alternate PKA inhibitor with enhanced cell permeability due to myristoylation at the NH_2 terminus.

Neither inhibitor significantly altered baseline hemodynamics. In contrast to PKC inhibition, chronic preconditioning failed to limit postischemic diastolic dysfunction (26 ± 2 mmHg, not significant vs. placebo) nor improve contractile recovery (26 ± 2 %RPP, Fig. 3) in the presence of H-89.

Similarly, at the termination of reperfusion, PKI reduced the recovery of %RPP observed in chronically preconditioned hearts (48 ± 2 %RPP) while significantly attenuating the preconditioning-mediated improvement in postischemic diastolic dysfunction (15 ± 2 mmHg, $P < 0.05$ vs. placebo, $P < 0.05$ vs. morphine; Fig. 3), further suggesting an important role for PKA in the preconditioning phenotype generated after prolonged morphine exposure. In contrast, PKI failed to alter the improvement in postischemic recovery of acute morphine-treated hearts. However, H-89 abolished the preconditioning effect of acute morphine treatment, although this effect may be due to nonspecific actions of H-89 as a general kinase inhibitor (29).

β-ARs. The β-ARs, primarily β_1-AR and β_2-AR, are representative G_s-coupled receptors. As such, the next series of experiments explored the potential role for β-AR in both acute and chronic morphine preconditioning. Administration of the β_1-AR antagonist CGP-20712A (300 nmol/l) or 1 μmol/l of the β_2-AR antagonist ICI-118,551 had no effect on hearts preconditioned with acute morphine treatment. Interestingly, both CGP-20712A and ICI-118,551 appear to induce an ischemia-tolerant state in placebo hearts (Fig. 4). Similarly, CGP-20712A failed to alter the postischemic recoveries of hearts from chronic morphine-preconditioned mice (EDP, 7 ± 2 mmHg; %RPP, 74 ± 1% not significant vs. chronic morphine; Fig. 4). On the contrary, the ischemia-tolerant phenotype observed in hearts after prolonged opioid exposure was significantly attenuated in the presence of 1 μM of the β_2-AR antagonist ICI-118,551 (EDP, 16 ± 2 mmHg; %RPP, 53 ± 1%; $P < 0.05$ vs. placebo, $P < 0.05$ vs. chronic morphine, for both parameters; Fig. 4). These data suggest a potential role for β_2-AR, as opposed to β_1-AR, in chronic opioid preconditioning.

Interestingly, concentration-response curves to isoproterenol failed to discern a difference in inotropic or chronotropic responses between either placebo or chronic morphine-treated hearts (data not shown), suggesting a maintenance in the β-AR status quo.

MitoK_{ATP} channel. To further dissociate the respective pathways involved in acute and chronic opioid preconditioning, we examined the function of the mitoK_{ATP} channel, often considered an integral component of both acute and delayed preconditioning. Here, with 300 μmol/l 5-hydroxydecanoate acid (5-HD) infusion bracketing the ischemic episode, the cardioprotective effects of acute opioid treatment were abolished, whereas those of chronic morphine preconditioning were unaltered (Fig. 4), adding further credence to the hypothesis that chronic and acute opioid preconditioning is mediated via distinctly different pathways (Fig. 5).

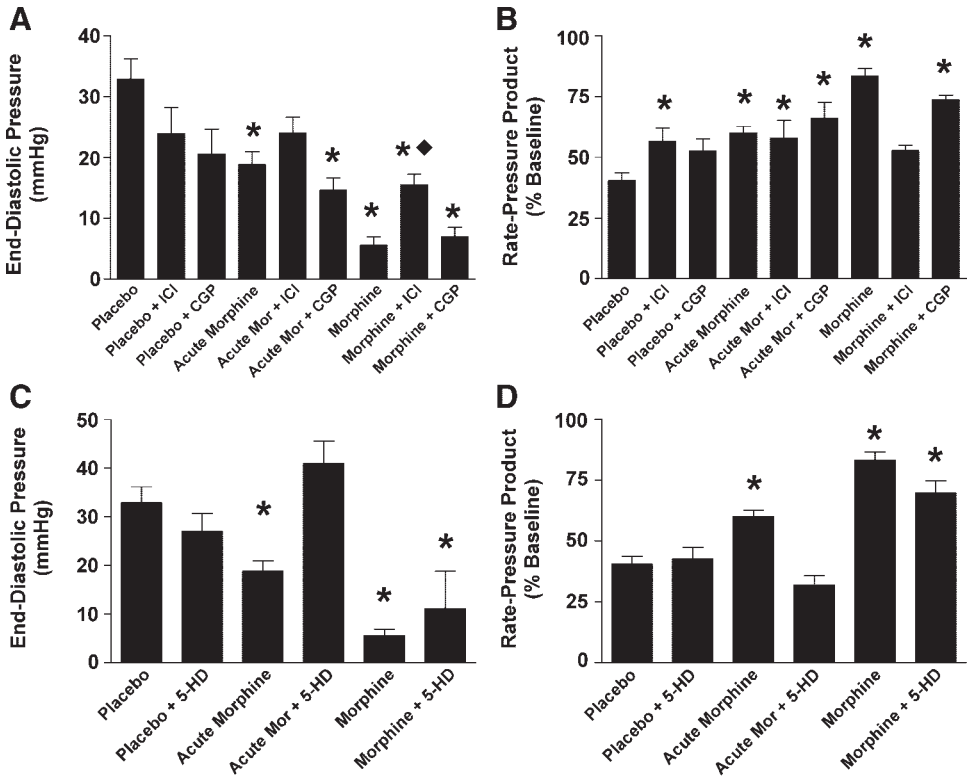

Fig. 4. Relative roles of β-adrenergic receptors (β-AR) β_1-AR and β_2-AR in mediation of ischemic tolerance associated with chronic opioid preconditioning. Recovery of end-diastolic pressure (*A*) and rate-pressure product (*B*; percentage from baseline) in placebo-treated hearts or chronic morphine-treated hearts with or without β_1-AR antagonist CGP-20712A (CGP; 300 nM) or β_2-AR antagonist ICI-118,551 (ICI; 1 μM). Values are also shown for recovery of end-diastolic pressure (*C*) and left ventricular rate-pressure product (*D*; percentage from baseline), in acute morphine-treated hearts or hearts excised for mice exposed to placebo of morphine for 5 days with 5-hydroxydecanote acid (5-HD; 300 μM) present pre- and postischemia. All values are reported as means ± SE. *$P < 0.05$ vs. placebo; ◆$P < 0.05$ vs. both placebo and morphine-treated hearts.

DISCUSSION

We have previously reported a profound and persistent ischemic-tolerant state observed in mice after a chronic (5 day) exposure to morphine. Classical preconditioning is typically thought to be a receptor-mediated process transduced via G_i proteins with PKC activation as a common intermediate effector. Here we demonstrate that our model of chronic opioid preconditioning, being distinctly different from that of acute preconditioning, is mediated via G_s proteins, as evidenced by NF-449 treatment. Furthermore, this G_s-mediated protection is also PKA dependent and may involve activation of β_2-AR.

Involvement of β-AR system. The role of β-AR in preconditioning is controversial. Historically, it was thought that the activation of β-AR by catecholamines released during ischemia and reperfusion is partially responsible for the propagation of ischemic cell damage (41). Regardless, our observation that β_2-AR inhibition limits the cytoprotective effects of chronic opioid exposure is supported by previous studies. Lochner et al. (27) demonstrated that IPC was associated with increases in cAMP during the trigger phase. Ablation of the increases in cAMP abrogated the IPC-mediated myocardial protection. Additionally, numerous studies (12, 30) have reported that preischemic β-AR stimulation with isoproterenol mimics preconditioning. Marais et al. (30) suggested that β-AR preconditioning is mediated via p38 MAPK and heat shock protein 27. Preconditioning may also reduce β-AR internalization (21).

The correlation between the β-AR system and opioid-mediated preconditioning is unclear. However, there is a well-reported "cross talk" interaction between both the β-AR and opioid receptor systems. Endogenous opioid peptides are released concomitantly with catecholamines from sympathetic neuronal terminals in the heart, presumably as an inhibitory control mechanism. These effects are thought to occur as a result of an interaction between the δ-opioid and β_1-AR, because β_2-AR responses were not reversed by a δ-opioid peptide, and this effect appeared to be mediated via a PTX-sensitive G_i protein (38). Interestingly, Jordan et al. (22) reported a robust hetero-oligomerization between the β_2-AR and both the κ- and δ-opioid receptors leading to differential modulation of receptor trafficking and signal transduction. Furthermore, Shan et al. (42) show a decreased G_s protein-dependent cross talk between β-AR and κ-opioid receptors after chronic hypoxia.

Chronic morphine exposure may also modify the β-AR system in the central nervous system. Chronic morphine treatment significantly increases extracellular norepinephrine (13) and leads to an upregulation of β_2-AR (1). Although Genade et al. (14) reported a significant reduction in β-AR responses to isoproterenol in the presence of acute opioid receptor stimulation, the lack of change in our isoproterenol concentration-response curve in chronically treated hearts suggests that the tonic balance of β-ARs remains unchanged in our model.

Moreover, chronic morphine exposure confers a G_i protein-dependent adenylyl cyclase superactivation (3). This increase in adenylyl cyclase is consistent with PKA activation.

Role of PKA. The results of our current study demonstrate that the enhanced ischemia-tolerant state provided by prolonged exposure to morphine was abolished via two structurally distinct PKA inhibitors but was unaffected by PKC blockade, suggesting that this protection is mediated via a PKA-dependent mechanism, independent of PKC. In support of our data, Lou and Pei (28) reported that, whereas acute δ-opioid receptor activation activated PKC in a dose-dependent manner with no effect on PKA, prolonged (24 h) δ-opioid receptor

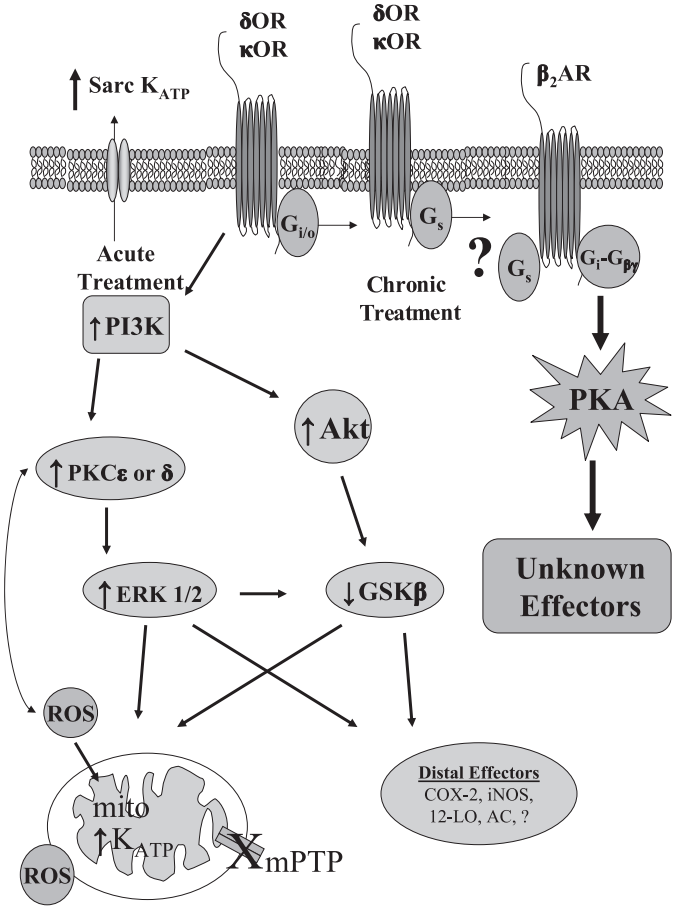

Fig. 5. Basic schematic describing potential pathway/s leading to acute opioid-mediated preconditioning and that proposed for chronic morphine preconditioning. Acute preconditioning follows archetypal scheme typically, and it repeatedly demonstrated for most G protein-coupled receptor-mediated preconditioning and IPC. Acute preconditioning likely involves, but is not restricted to, phosphatidylinositol 3-kinase (PI3K), Akt, PKC, ERK, GSK3-β, and reactive oxygen species (ROS) activation as well as opening of sarcolemmal (Sarc) and/or mitochondrial K_{ATP} ($mitoK_{ATP}$) channel. Recent evidence suggests that protection may be ultimately afforded by inhibition of mitochondrial permeability transition pore formation (mPTP). Mechanisms leading to enhanced ischemic tolerance induced by prolonged morphine exposure are much less clear but may involve a switch from $G_{i/o}$- to G_s-coupled signaling for either opioid (OR) or β_2 adrenergic receptors, PKA activation, and, perhaps, as yet unknown end-effectors. iNOS, inducible nitric oxide synthase; COX-2, cyclooxygendase-2; 12-LO,12-lipoxygeanse; AC, adenylate cyclase.

activation led to a significant increase in PKA activity with subsequent reduction in the basal activity of PKC.

PKA activation, which has been implicated in tolerance to opioid stimulation (44, 49), has also been previously shown to limit ischemia-reperfusion injury (24, 46). Moreover, PKA has also been implicated in IPC (20). Indeed, IPC mediation by PKA is independent of PKC (40), supporting our findings. However, there is controversy regarding the role of PKA in IPC. Makaula et al. (29) reported that PKA blockade, via H-89, elicited an improvement in postischemic function and reduced infarct size in the isolated rat heart and augmented postischemic function induced by IPC, whereas Tong et al. (47) reported that both H-89 and PKI attenuated IPC-derived cardioprotection in the isolated mouse heart. Indeed, Inserte et al. (20) suggested that transient activation of PKA acts as an IPC mimetic and, moreover, that H-89 blocked the effects of IPC. These results are supported by those of Robinet et al. (39), who reported that preconditioning, induced by β1-AR activation, is inhibited by H-89, and by Sanada et al. (40), whereby PKA blockade with H-89 blunted IPC in a canine model of myocardial infarction. Additionally, in this model, PKA activation mimicked IPC. The downstream kinase signaling mediated via PKA may include PI3K and Akt (2), p42/p44 MAPK (6), p38 MAPK (18), and GSK3-β (25), all of which have previously been shown to be involved in opioid-mediated cardioprotection (15, 16).

Relative roles of G_i/G_s proteins. Our current data implicate a role for both inhibitory and stimulatory G proteins in the mediation of chronic preconditioning, with an apparently greater role for the G_s protein, as evidenced by a complete abrogation of myocardial protection after blockade of G_s. This would seem to be counterintuitive because G_s activation is typically considered detrimental to the ischemic myocardium. Indeed, β-AR blockade is a widely used therapeutic modality for heart failure and mice overexpressing G_s-α develop cardiomyopathy with age (23). Additionally, β-AR overexpressing mice also develop cardiomyopathy (9, 26).

Regardless, there is little evidence to support a protective role of G_s protein activation in cardioprotection. However, a recent study by Meino et al. (31) reported that the loss of IPC-mediated protection in postmyocardial infarcted hearts was restored in the presence of adenylyl cyclase, suggesting a role for G_s and β-AR.

After chronic activation of opioid receptors, the opioid receptors may convert from inhibitory (G_i coupled) to excitatory (G_s coupled) (7). There is considerable evidence to support opioid-mediated G_s stimulation (75, 48, 50), regardless of mechanism. Although G_s stimulation may potentially exist as a downstream effect, there are studies that support conformational changes from G_i to G_s. Wu et al. (51) reported that cloned δ-opioid receptors transfected in Chinese hamster ovary cells do indeed undergo a conformational change, or at least undergo some change resulting in increases in cAMP. Moreover, in line with the prolonged protective state that we have reported previously (35), Crain and Shen (8) reveal that chronic morphine-treated, dorsal-root ganglion neurons exhibit an opioid excitatory supersensitivity for up to 3 mo after return to control culture medium.

$MitoK_{ATP}$ channel. The $mitoK_{ATP}$ channel has long been touted as one of the primary mediators of both acute and delayed preconditioning. Indeed, it is considered by many to be the "end-effector" of myocardial preconditioning. In support, there is a vast body of literature describing an integral role for $mitoK_{ATP}$ channels in many previously described models of preconditioning. Moreover, there are numerous reports of a tight link between K_{ATP} channels and the physiological actions of opioids (2, 19, 33, 43, 52). Although our current data fail to discern an association between the mediation phase of chronic opioid preconditioning and the $mitoK_{ATP}$, there exists the possibility of a tangible relationship between $mitoK_{ATP}$ and the "trigger" phase of chronic preconditioning. Nonetheless, the data regarding the $mitoK_{ATP}$ channel presented herein further support the notion that the mediation of chronic opioid preconditioning is markedly different from the acute and delayed modes of preconditioning.

Study limitations. An alternative hypothesis for the apparent inability of the "classic-pathway" inhibitors, administered

acutely, to block the chronic morphine-mediated "preconditioning" may be due to a combination of enhanced PKC and PI3 activation after chronic morphine and, consequently, suboptimal dosing of the inhibitors, rather than a lack of activation of these pathways. However, Lou and Pei (28) report that, in NG 108-15 cells, acute treatment with a δ-opioid agonist [δ-pen2,5enkephalin (DPDPE)] activated PKC with no effect on PKA, whereas prolonged exposure (24 h) to DPDPE reduced the basal activity of PKC while increasing the activity of PKA. Moreover, some of our more recent data, incorporating a coinfusion (from implanted osmotic minipumps) of PKI failed to block the development of chronic morphine-mediated protection, despite the acute effects of PKI, whereas the same coinfusion of wortmannin completely abolished the onset of preconditioning. These unpublished results suggest a differential involvement on both PKA and PI3K/PKC, where PI3K/PKC is involved in the trigger, but not mediation, phase and the opposite is true for PKA. This differential involvement may also support a crucial "upstream" role for these signaling intermediates. Indeed, there is a high likelihood that the mediators that failed during the "mediation" phase of chronic morphine preconditioning protection are integral components of the early onset/trigger phase of the phenotype.

Additionally, our examination of the mitoK_{ATP} channel with 5-HD, which is often cited as being the end-effector in myocardial preconditioning, also considered distal to or the converging point/s of multiple upstream pathways, leads to the presumption that the tolerance observed is unlikely due to a suboptimal dosing of the upstream inhibitors.

We also cannot discount the possibility that, as the mice are still absorbing morphine up until the point of euthanasia, circulating morphine may be having an acute effect. However, there are numerous factors suggesting that this is not the case. First, after excision, the heart is perfused for 30 min before the onset of ischemia, allowing for the complete washout of any circulating morphine. Second, our previous study (35) shows that the protected state is still present at least 48 h after complete opiate withdrawal. Finally, the current study demonstrates that acute and chronic opioid protection is mediated via distinct pathways, namely, PKC dependent and PKA dependent, respectively. As such, we are confident that any potential acute effect of morphine is negligible in this model.

In summary, chronic preconditioning with morphine, which leads to both a persistent and profound cardioprotective phenotype (35) in the murine heart, occurs via a pathway independent of that described for both classical acute and delayed preconditioning. Although the exact mechanisms remain unknown, the protection appears to be mediated by both G_i and G_s proteins, with a greater emphasis on the activation of the G_s protein, a PKA-dependent/PKC-independent pathway, and activation of the β_2-AR. The resolution of these protective pathways elicited by chronic opioid exposure may provide novel therapeutic approaches to myocardial protection, particularly in light of our previous observation that chronic morphine exposure rescues the protective effects of opioid stimulation that is lost in aged hearts (36).

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