Direct Preconditioning of Cardiac Myocytes via Opioid Receptors and K$_{ATP}$ Channels

Bruce T. Liang, Garrett J. Gross

Abstract—Previous studies demonstrated that opioid receptor activation mimics the cardioprotective effect of ischemic preconditioning via K$_{ATP}$ channels in the intact heart. However, it is unknown whether this beneficial effect is exerted at the level of the cardiac myocyte or coronary vasculature or is mediated via the sarcolemmal or the mitochondrial K$_{ATP}$ channel. Thus, the purpose of the present study was to investigate whether opioid receptor stimulation could mimic the cardioprotective effect of preconditioning in a cardiac myocyte model of simulated ischemia. Cardiac ventricular myocytes cultured from chick embryos 14 days in ovo were used as an in vitro model for ischemic preconditioning. A 5-minute exposure of the myocytes to the opioid receptor agonist morphine protected the myocytes during a subsequent 90-minute period of simulated ischemia, which was manifested as a pronounced reduction in the percentage of cardiac cells killed and the amount of creatine kinase released during ischemia. The preconditioning-like effect of morphine was concentration-dependent, reached a maximal effect at 1 μmol/L, and was reversed by naloxone (0.1 to 10 μmol/L). When K$_{ATP}$ channel antagonists, such as glibenclamide, or the mitochondrial selective inhibitor 5-hydroxydecanoic acid were present during preexposure to morphine, they abolished the protective effect of morphine. Thus, cardiac myocytes express functional opioid receptors, and their activation mimics the cardioprotective effect of ischemic preconditioning. These results provide direct evidence that the preconditioning-like effect of morphine in the intact heart can be exerted at the level of cardiac myocytes and is most likely the result of mitochondrial K$_{ATP}$ channel activation. (Circ Res. 1999;84:1396-1400.)

Key Words: myocyte ■ cardioprotection ■ receptor ■ morphine ■ channel

Activation of opioid receptors has been shown to exert a protective effect in various organ systems during hypoxia or ischemia.$^{1,2}$ Recent studies showed that the nonselective opioid receptor antagonist naloxone could abolish the cardioprotective effect of ischemic preconditioning (IPC) in rat$^3$ and rabbit.$^4$ Activation of opioid receptors by morphine can mimic the protective effect of IPC in the rat in a naloxone-reversible manner.$^5$ In addition, this beneficial effect of morphine was abolished by the K$_{ATP}$ channel antagonist glibenclamide, which suggests a role for an opioid receptor–K$_{ATP}$ channel signaling pathway in mediating the cardioprotective effect of morphine.$^5$

Although these studies provided important evidence for a protective function of opioids in the heart, it is unknown whether the beneficial effect of opioids such as morphine is exerted at the level of the central or the peripheral nervous systems or both. In addition, because opioid receptors are present on the vasculature and can mediate hypoxia-induced vasodilatation,$^6$ it is possible that, in addition to the involvement of the nervous system, the vascular opioid receptor may also play a role in mediating the cardioprotective effect of morphine. Recent studies have shown that cardiac myocytes can be directly preconditioned by simulated ischemia,$^7-10$ which suggests that the cardioprotective effect of IPC in the intact heart is exerted, at least partially, at the level of the cardiac myocytes. Thus, it is possible that the preconditioning-like effect of morphine in the intact animal is also mediated by opioid receptors on the cardiac myocyte.

Liang and colleagues$^7-10$ have established a cardiac myocyte model of preconditioning that exhibits characteristics similar to those found in the intact heart model of IPC.$^5$ Thus, the purpose of the present study was to investigate whether cardiac myocytes express functional opioid receptors and whether activation of these receptors can mimic the cardioprotective effect of IPC. Another objective was to elucidate the underlying cellular mechanism by determining whether the mitochondrial K$_{ATP}$ channel is involved in mediating the preconditioning-like effect of opioid receptor stimulation by attempting to block this effect with the mitochondrial selective K$_{ATP}$ channel inhibitor 5-hydroxydecanoic acid (5-HD).$^{11}$

Materials and Methods

Methods

Preparation and Preconditioning of Cultured Ventricular Cells

Cardiac ventricular myocytes were cultured from chick embryos 14 days in ovo, according to a previously described procedure.$^7,8$
Myocytes were cultivated in a humidified 5% CO_2/95% air mixture at 37°C for 3 days, during which time the cells grew to confluence and exhibited rhythmic spontaneous contraction. All experiments were performed on day 3 in culture. For preconditioning studies, the medium was changed to a HEPES-buffered medium that contained (in mmol/L) 139 NaCl, 4.7 KCl, 0.5 MgCl_2, 0.9 CaCl_2, 5 HEPES, and 2% fetal bovine serum, pH 7.4, before the cells were exposed to the various conditions at 37°C. Control cells were maintained in the HEPES-buffered media under room air. Ischemia was simulated by placing the cells in a hypoxic incubator (NuAire) in which O_2 was replaced by N_2. The percentage of O_2 was monitored by both an oxygen gas analyzer (Fyrite, Bacharach) and an oxygen analyzer (model Ox630, Engineered Systems and Designs) and was <1%. Preconditioning was induced by exposing the cells to 5 minutes of simulated ischemia, called IPC, before a second 90-minute ischemia. To study the ability of the opioid receptor agonist morphine to mimic the protective effect of IPC, cells were exposed to different concentrations of morphine for 5 minutes and incubated in fresh drug-free media for 10 minutes before being exposed to 90 minutes of simulated ischemia. Cells not subjected to preconditioning were exposed only to 90 minutes of ischemia (nonconditioned cells). The extracellular pH was similarly maintained at 7.4 by HEPES in both preconditioned and nonconditioned cells. Determination of basal level of cell injury was made after parallel incubation of control cells under a normal percentage of O_2. Cell injury was determined for all cells at the end of the 90-minute ischemia period.

**Quantitative Determination of the Extent of Myocyte Injury**

The extent of hypoxia-induced injury to the ventricular cell was quantitatively determined by the percentage of cells killed and the amount of creatine kinase (CK) released into the media, according to previously described methods.7–10 To quantify the percentage of cells killed, cells were detached after exposure to a trypsin-EDTA Hanks balanced salt solution for 10 minutes for detachment. Viable cells were sedimented by centrifugation (300g for 10 minutes) and resuspended in culture media for counting in a hemocytometer. Only the cells that were alive sedimented, and the cells that were counted represented those that survived.12 None of the sedimented cells subsequently counted included trypan blue. Control experiments performed in previous studies indicated trypsin treatment, reexposure to Ca^{2+}-containing media, or 300g sedimentation did not cause any significant damage to the control normoxia-exposed cells. The trypsin-EDTA media from cells exposed to 90 minutes of hypoxia contained substantial amount of proteins (0.15±0.03 mg, n=9) and CK activity (16±3 U/mg, n=8). Such proteins and CK activity could arise from hypoxia-damaged cells that failed to sediment because of lighter cellular density from the loss of soluble proteins or from hypoxia-exposed cells that were further damaged by the trypsin treatment. In either case, the cell viability assay clearly separated the control healthy cells from the hypoxia-exposed damaged cells. In support of the notion that 90-minute hypoxia caused significant cell injury and loss of membrane integrity, there was also a marked release of LDH (hypoxia-exposed cells, 35.5±2.7 U/mg, n=8, mean±SE versus control cells, 6.1±0.4 U/mg, n=8) and proteins (hypoxia-exposed cells, 0.15±0.03 mg, n=8, versus control cells, 0.03±0.01 mg, n=8) from cells incubated under prolonged hypoxia. Parallel changes in the amount of CK released into the media and in the percentage of cells killed under every experimental condition studied further validated the cell viability assay. In support of the hypothesis that 90 minutes of hypoxia caused significant cell injury and loss of membrane integrity, there was also marked release of LDH and proteins from the cells incubated under prolonged hypoxia. Thus, the cell viability assay separated the hypoxia-damaged from the control normoxia-exposed cells. Parallel changes in the percentage of cells killed and CK released further validated this assay for the percentage of cells killed. The amount of CK was measured as enzyme activity (U/mg), and increases in CK activity above the control level were determined. The percentage of cells killed was calculated as the number of cells obtained from the control group (representing cells not subjected to any hypoxia or drug treatment) minus the number of cells from the treatment group divided by number of cells in the control group multiplied by 100.

**Materials**

Morphine sulfate was obtained from A.H. Robins Co. Naloxone, glibenclamide, and 5-HD were obtained from Research Biochemicals International. Embryonated chick eggs were obtained from Spafas, Inc (Storrs, Conn.).

**Results**

**Opioid Receptor Activation Mimics the Protective Effect of IPC in Cardiac Myocytes**

Simulated ischemia, which was induced by glucose deprivation and hypoxia, caused significant injury to the cardiac ventricular myocytes, as evidenced by the increased release of CK and the appearance of the cells killed (Figure 1). Brief exposure to 5 minutes of simulated ischemia, which does not injure the myocytes, protected cardiac ventricular myocytes against injury induced by the subsequent prolonged ischemia (Figure 1). These data are similar to those obtained previously in both the cardiac myocyte model and the intact heart model of preconditioning.5,7,8 A 5-minute exposure of the myocytes to morphine sulfate (1 μmol/L) followed by a 10-minute drug-free period also protected myocytes against injury during the subsequent 90-minute period of ischemia (Figure 1). There was a significant reduction in the percentage of cells killed and the amount of CK released in myocytes that were preexposed to morphine versus myocytes that had not been exposed to morphine or to IPC (nonconditioned; 1-way ANOVA and posttest comparison, P<0.01). The extent of cardioprotection achieved by the preconditioning-like effect of morphine was similar to that induced by IPC (P>0.1).
To examine the hypothesis that the preconditioning-like effect of morphine sulfate is mediated by an opioid receptor on the cardiac myocyte, the effect of morphine was further characterized. First, previous exposure of the myocytes to various concentrations of morphine resulted in a concentration-dependent cardioprotection, with a reduction in the percentage of cells killed and in the amount of CK released during the prolonged ischemia (Figure 2). Second, the nonselective opioid receptor antagonist naloxone was able to block the preconditioning-like effect of morphine in a concentration-dependent manner. The concomitant presence of naloxone during myocyte exposure to 1 μmol/L morphine antagonized its protective effect during the subsequent prolonged ischemia (Figure 3). Naloxone did not cause additional myocyte injury when it was present during the 90-minute period of simulated ischemia (Table).

To identify the opioid receptor that mediates the preconditioning-like effect of morphine, the ability of the δ-opioid receptor-selective antagonist 7-benzylidenenaltrexone (BNTX) to block the morphine effect was examined. Figure 4 shows that 10 μmol/L of BNTX reversed the morphine-induced preconditioning-like effect in these cardiac myocytes when it was present during the 5-minute exposure to morphine. These data are consistent with the hypothesis that morphine mimics the cardioprotective effect of IPC via activation of the δ-opioid receptor.

Role of KATP Channels in the Preconditioning-Like Effect Mediated by Morphine
To examine the signaling mechanism downstream of the opioid receptor, we examined whether the KATP channel is involved in the mediation of the preconditioning-like effect of morphine. Concomitant presence of either glibenclamide or the mitochondrial selective KATP channel antagonist, 5-HD, during the 5-minute exposure to morphine (1 μmol/L) blocked its cardioprotective effect during the subsequent prolonged ischemia. This was manifested by an increase in

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**Figure 2.** Morphine causes a concentration-dependent preconditioning-like effect in cardiac myocytes. Cardiac ventricular myocytes were cultured as described in Materials and Methods, exposed to different concentrations of morphine for 5 minutes, and incubated in fresh drug-free media for 10 minutes before being exposed to 90 minutes of simulated ischemia. Data were plotted as the percentage of cells killed and as the amount of CK released. Data represent mean±SE of 4 experiments. At each morphine concentration tested, the percentage of cells killed and the amount of CK released were significantly less than those in cells that were not preexposed to morphine (1-way ANOVA followed by posttest comparison, P<0.05).

**Figure 3.** Reversal of the preconditioning-like effect of morphine by naloxone. Cardiac ventricular myocytes were cultured and exposed to the indicated concentrations of naloxone plus 1 μmol/L morphine for 5 minutes. Cells were then incubated in drug-free media for 10 minutes and exposed to a 90-minute period of ischemia. Data were plotted as the percentage of cells killed and as the amount of CK released. Data represent mean±SE of 4 experiments. At 1 or 10 μmol/L of naloxone, the percentage of cells killed and the amount of CK released were significantly higher than obtained in the absence of naloxone (1-way ANOVA and posttest comparison, P<0.05).

**Figure 4.** δ-opioid receptor antagonist BNTX blocked the preconditioning-like effect of morphine. Cardiac ventricular myocytes were cultured and were exposed for 5 minutes to 1 μmol/L morphine, 1 μmol/L morphine plus 10 μmol/L BNTX, or no drug. Cells were then incubated in drug-free media for 10 minutes before being exposed to 90 minutes of simulated ischemia. Data were plotted as the percentage of cells killed and the amount of CK released. Data represent mean±SE of 4 experiments. *Significantly different from cells preexposed to morphine plus BNTX and from cells not preexposed to any drug (1-way ANOVA and posttest comparison, P<0.01).
the percentage of cardiac cells killed (Figure 5) and in the amount of CK released (data not shown) in the presence of the $K_{\text{ATP}}$ channel blockers. The antagonistic effect of the $K_{\text{ATP}}$ channel blockers was evident at concentrations as low as 0.1 μmol/L and was complete at 10 μmol/L.

The potency of 5-HD in blocking the preconditioning-like effect of morphine, in which a significant effect was observed at 0.1 μmol/L, is similar to the potency of glibenclamide. A previous study showed that 5-HD is less potent than glibenclamide. The reason for this difference is unclear but may relate to the possibility that the negatively charged 5-HD may relate to the possibility that the negatively charged 5-HD glibenclamide. The reason for this difference is unclear but it may be the result of stimulation of another opioid receptor at a higher concentration of morphine that antagonizes the protective effect mediated by another receptor at a lower concentration. The present data provide additional evidence to support the concept that the opioid-induced preconditioning effect in the whole animal is at least partially mediated at the level of the cardiac ventricular myocyte.

Because morphine is somewhat selective for the $\mu$-opioid receptors as opposed to $\delta$- and $\kappa$-opioid receptors, it is possible to assume that the effect observed in the present study may be mediated via a $\mu$-opioid receptor. However, recent data obtained by Schultz et al18-20 suggest that this is not the case. These investigators found that the $\delta$-opioid receptor, more specifically, the $\delta_1$-opioid receptor, extend these previous findings and are the first to demonstrate a signaling pathway that involves opioid receptors as opposed to $\delta$- and $\kappa$-opioid receptors, that the $\delta_1$-opioid receptor mediates the preconditioning effect in the whole animal is at least partially mediated at the level of the cardiac ventricular myocyte.

In support of these data, Bell et al21 have recently shown that the $\delta$-opioid receptor mediates the cardioprotective effect of the peptide opioid agonist (D-Ala2, D-Leu5) enkephalin and also mediates IPC in isolated human cardiac muscle. In a group of patients who underwent PTCA, Xenopoulos et al16 found that the $K_{\text{ATP}}$ channel, is responsible for the cardioprotective effect of IPC in the intact rat heart and that the $\delta$-opioid receptor mediates the cardioprotective effect of morphine. The present data, which show that the $\delta_1$-opioid receptor-selective antagonist BNTX can block the morphine-induced preconditioning, are consistent with the hypothesis that activation of a $\delta_1$-opioid receptor also mediates this potentially important effect in the cardiac myocyte.

The present data suggest that functional opioid receptors are present on the chick cardiac ventricular myocyte and that activation of these receptors by the nonselective opioid receptor agonist morphine can produce a preconditioning-like effect. Although the cardiac ventricular myocytes are isolated from chick embryos, extensive characterization of the chick myocyte model for IPC demonstrated that it exhibits characteristics similar to the adult mammalian model of preconditioning. Present data on the opioids and IPC in the chick myocyte model are consistent with data recently published by Schultz et al2 and Miki et al15 in the intact rat and rabbit heart and by Xenopoulos et al16 in humans that underwent percutaneous transluminal coronary angioplasty (PTCA). These data suggest that the cardioprotective effect produced by morphine in vivo is at least partially mediated at the level of the cardiac myocyte. Miki et al15 found that 0.3 μmol/L of morphine, a concentration similar to that which is cardioprotective in the isolated myocyte (0.1 to 1.0 μmol/L), was equally cardioprotective to the effect of IPC in the isolated rabbit heart. In addition, a portion of the cardioprotective effect of morphine was lost at a higher concentration of 10 μmol/L, which suggests that this compound demonstrates a bell-shaped concentration-response curve. The reason for this finding is unclear, but it may be the result of stimulation of another opioid receptor at a higher concentration of morphine that antagonizes the protective effect mediated by another receptor at a lower concentration. The present data provide additional evidence to support the concept that the opioid-induced preconditioning effect in the whole animal is at least partially mediated at the level of the cardiac ventricular myocyte.

Discussion

The present data suggest that functional opioid receptors are present on the chick cardiac ventricular myocyte and that activation of these receptors by the nonselective opioid receptor agonist morphine can produce a preconditioning-like effect. Although the cardiac ventricular myocytes are isolated from chick embryos, extensive characterization of the chick myocyte model for IPC demonstrated that it exhibits characteristics similar to the adult mammalian model of preconditioning. Present data on the opioids and IPC in the chick myocyte are consistent with data recently published by Schultz et al2 and Miki et al15 in the intact rat and rabbit heart and by Xenopoulos et al16 in humans that underwent percutaneous transluminal coronary angioplasty (PTCA). These data suggest that the cardioprotective effect produced by morphine in vivo is at least partially mediated at the level of
receptors and $K_{ATP}$ channels at the level of the ventricular myocyte.

Recent studies have suggested the involvement of a mitochondrial $K_{ATP}$ channel in the mediation of the cardioprotective effect of $K_{ATP}$ channel openers. The $K_{ATP}$ channel blocker diazoxide for activating mitochondrial $K_{ATP}$ channels at an $\approx 1000$-fold lower concentration than that necessary to open the sarcolemmal channel and on the observation that 5-HD blocked the opening of the mitochondrial $K_{ATP}$ channel, although it had no effect on the sarcolemmal channel. The current data, which show that the mitochondrial selective blocker 5-HD abolishes the cardioprotective effect of morphine at low concentrations (1 to 10 $\mu$mol/L), imply that the mitochondrial $K_{ATP}$ channel is the likely distal effector in this response. Although the $K_{ATP}$ channel blockers were present during only the 5-minute exposure to morphine, the protection induced by their preconditioning-like effect was completely abolished. These data raised the possibility that activation of the channel is important in initiating the morphine-mediated preconditioning process. Alternatively, the channel blocker may remain bound to its intracellular mitochondrial site during the subsequent simulated ischemia and thus abolish the cardioprotection. Proof of this concept requires additional study.

It is unknown whether protein kinase C (PKC) plays a role in the mediation of the preconditioning-like effect of morphine in these cardiac myocytes. Miki et al showed that PKC is an important downstream effector in mediating the preconditioning effect of opioids in the intact rabbit heart. It is possible that PKC also acts downstream from the opioid receptor to cause the cardioprotective effect of morphine in the cardiac myocyte. The relationship between PKC and the $K_{ATP}$ channel in the mediation of the morphine effect in the myocyte is unknown. These important questions deserve additional investigation.

In summary, these studies are the first to demonstrate a cardioprotective effect of opioid receptor activation in isolated cardiac myocytes exerted by morphine. Because the nonselective antagonist naloxone blocked the cardioprotective effect of morphine at relatively low concentrations, this suggested that this was an opioid receptor–mediated event. However, the subtype of the opioid receptor involved and the intracellular signaling pathways that lead from the receptor to the mitochondrial $K_{ATP}$ channel in the mediation of the preconditioning-like effect of morphine and other opioids remain unknown but deserve further investigation.

**Acknowledgments**

This study was supported by an Established Investigatorship from the American Heart Association and by grants R01-HL-48225 (B.T.L.) and R01-HL-08311 (G.J.G.) from the National Institutes of Health.

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*Circ Res.* 1999;84:1396-1400
doi: 10.1161/01.RES.84.12.1396

*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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