

Delta Opioid Receptor Stimulation Mimics Ischemic Preconditioning in Human Heart Muscle

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OBJECTIVES	The objective of this study was to examine whether the delta (δ) opioid receptor isoform is expressed in the human heart and whether this receptor improves contractile function after hypoxic/reoxygenation injury.
BACKGROUND	Delta opioid receptor agonists mimic preconditioning (PC) in rat myocardium, corresponding to known cardiac δ opioid receptor expression in this species.
METHODS	The messenger RNA transcript encoding the δ opioid receptor was identified in human atria and ventricles. To evaluate the cardioprotective role of the opioid receptor, human atrial trabeculae from patients undergoing coronary bypass grafting were isolated and superfused with Tyrode's solution. A control group underwent 90 min of simulated ischemia and 120 min of reoxygenation. A second group was preconditioned with 3 min simulated ischemia and 7 min reoxygenation. Additional groups included: superfusion with the δ receptor agonist (DADLE) (10 nM), with the δ receptor antagonist naltrindole (10 nM) and with the mitochondrial K_{ATP} channel blocker 5-hydroxydecanoate (5HD) (100 μ M) either with or without PC, respectively. A final group was superfused with 5HD before DADLE. The end point used was percentage of developed force after 120 min of reoxygenation.
RESULTS	Results, expressed as means \pm SEM, were: control = 32.6 \pm 3.8%; PC = 50.5% \pm 1.8%; DADLE = 46.0 \pm 3.9%*; PC + naltrindole = 25.5 \pm 3.9%; naltrindole alone = 25.5 \pm 4.3%; 5HD + PC = 28.9 \pm 7.4%; 5HD alone = 24.1 \pm 3.0%; 5HD + DADLE = 26.9 \pm 4.4% (*p < 0.001 vs. controls).
CONCLUSIONS	Human myocardium expresses the δ opioid receptor transcript. Stimulation of this receptor appears to protect human muscle from simulated ischemia, similar to PC, and via opening of the mitochondrial K_{ATP} channel. (J Am Coll Cardiol 2000;36:2296-302) © 2000 by the American College of Cardiology

Ischemic preconditioning (PC) is the phenomenon, widely demonstrated in many species, whereby the myocardium is protected from a major ischemic insult by a prior, brief period of ischemia or hypoxia followed by reperfusion or reoxygenation. First demonstrated in 1986 by Murry et al. (1), the mechanisms underlying PC are still not fully clarified but are believed to involve release of adenosine (2,3), bradykinin (4), noradrenaline (5) and endothelin (6), which then trigger intracellular enzyme cascades and may ultimately lead to the opening of the mitochondrial K_{ATP} channel (7-9). More recently the role of opioids in cardioprotection by PC has been examined. In the rat model of myocardial infarction, pharmacological PC by morphine reduces infarct size similarly to ischemic PC (10) through a mechanism that is inhibited by the sulphonylurea, glibenclamide. This effect suggests an involvement of the ATP sensitive potassium channel (K_{ATP}) (10,11). Importantly, glibenclamide can also abolish the effects of PC during angioplasty in humans (12), suggesting a role for this channel in classic PC. More recently, emphasis has shifted

from the sarcolemmal to the mitochondrial K_{ATP} channel that is inhibited by 5-hydroxydecanoate (5HD) (8,9).

The three main opioid receptor subtypes are μ , κ and δ . Differential tissue specific opioid receptor subtype expression has been described in rats (13). Furthermore, in rat myocardium Gross and colleagues (14) have shown that stimulation of the δ_1 opioid receptor subtype mediates cardioprotection similar to PC. However, the distribution of the subtypes in the human myocardium is not known. In this study our aims were, first to delineate by using semi-quantitative reverse transcriptase-polymerase chain reaction (PCR), the relative expression levels of genes encoding the opioid receptor isoforms in human atria and ventricular tissue. Our second aim was to establish the role of δ opioid receptors in cardiac protection in an isolated human muscle preparation, using a δ receptor agonist (DADLE) (D-Ala2-Leu-enkephalin), and naltrindole, a δ receptor antagonist. Our third aim was to ascertain the potential role of the mitochondrial K_{ATP} channel in any protection achieved by δ opioid stimulation. Furthermore, we have recently demonstrated that cardiac kappa receptors appear to mediate an anti-PC signaling in the rat heart (15). Thus, it is important to know whether these receptors exist in the human heart. If so, kappa-mediated inhibitory signals could lessen or abrogate any potential beneficial effects of opioid receptor agonists that exhibit a wide range of receptor subtype activity, such as morphine.

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Abbreviations and Acronyms

DADLE	= delta opioid agonist
naltrindole + PC	= naltrindole plus preconditioning
PC	= preconditioning
PCR	= polymerase chain reaction
5HD	= 5-hydroxydecanoate

METHODS

RNA isolation and RT-PCR analysis of human atria and ventricular tissue. Right atrial appendage biopsies were taken from patients undergoing elective coronary bypass grafting or aortic valve replacement. Ventricular tissue was used from patients who were undergoing mitral valve replacement. Tissue was excluded from patients with heart failure, arrhythmias or on antiarrhythmic or oral hypoglycemic therapy. RNA extraction and quantification were performed as described previously (16). Using the GeneAmp EZrTth RNA PCR Kit (Perkin Elmer; New Jersey) we determined the presence of μ , κ and δ opioid receptors. Specific PCR primers for the human μ , κ and δ opioid receptors were designed using the published receptor sequences (Genebank Accession No's: NM000914, U11053 and U07882, respectively). These reactions produced PCR products of expected sizes 1,347 base pair, 417 bp and 370 bp, respectively. Restriction digestions were performed using HIND III (Promega, Madison, Wisconsin) to confirm the μ receptor PCR product. The δ and κ PCR products conformed to the expected size on gel electrophoresis. Polymerase chain reaction products were compared using densitometric analysis using 0.5, 1.0 and 2.0 mg of total RNA with each set of primers using different cycle numbers (30, 35 and 40 cycles). Control primers encoding glyceraldehyde-phosphate dehydrogenase were used concomitantly under the same conditions. The κ transcript was not visualized after the 40 cycles of PCR. To evaluate whether this transcript was present in very low copy number in the human atria and ventricles, nested PCR with addition primers was performed.

Human atrial trabeculae model. Atrial trabeculae were taken from patients with chronic stable angina undergoing elective coronary bypass grafting or aortic valve replacement. Exclusion criteria were the same as described for RNA isolation. Prior ethical approval had been granted by the Ethics and Clinical Investigation Panel of the Middlesex Hospital. The method of harvesting atrial trabeculae tissue, placing it in an organ bath and mechanical recording of stimulated contractions were as previously published (17,18). In brief, trabeculae were placed in the organ bath and allowed to equilibrate for 45 to 60 min. All the groups underwent a period of 90 min of simulated ischemia followed by 120 min reoxygenation. The ischemic PC protocol consisted of 3 min of hypoxic, substrate-free superfusion with rapid pacing (3 Hz) followed by 7 min of reoxygenation. A third group were superfused with

DADLE (10 nMol), for 5 min followed by a 7 min washout period before the 90 min period of simulated ischemia and reoxygenation (n = 9). The δ receptor antagonist, naltrindole (10 nMol), was superfused for 10 min before and then during simulated ischemic PC (n = 7). The same dose of naltrindole was also superfused alone for 20 min before simulated ischemia and reoxygenation (n = 6). The mitochondrial K_{ATP} channel blocker, 5-hydroxydecanoate (5HD) (100 μ M), was superfused before PC with simulated ischemia (n = 6) before superfusion with DADLE (n = 6) and alone (n = 5). Figure 1 outlines the experimental protocols followed. Developed force was measured throughout the experiment. The end point was taken as developed force as a percentage of baseline developed force after 2 h of reperfusion.

Statistical analysis. All results are expressed as group means \pm standard error of the mean. Differences between groups were evaluated by analysis of variance. Fisher protected least significant difference post hoc test was used for multiple comparisons between the groups. A p value < 0.01 was considered significant.

RESULTS

RT-PCR amplification of μ , κ and δ opioid receptor messenger RNA. Gel electrophoresis of PCR products from human right atrial RNA were analyzed on a 2% agarose gel stained with ethidium bromide. The results are shown in Figure 2. The band consistent with that expected for the δ and μ opioid receptor were reproducibly obtained. We concluded that the δ and μ opioid receptors are present in human atrial tissue. We were unable to detect any κ opioid receptor using the primary round of PCR. When using nested primers, however, a faint band of appropriate size was found. These data suggest that the κ receptors are present in the human atria but at a much lower copy number than for δ and μ receptors. However, due to the low abundance of this κ receptor isoform, we cannot exclude that the PCR product amplified comes from neuronal tissue within the myocardium as opposed to true myocardial expression of this receptor. We repeated our investigation for μ , κ and δ opioid receptors in human right ventricular tissue obtained during mitral valve replacement. Our results show the presence in human ventricular tissue of μ and δ receptors at a similar copy number to human atrial tissue, with evidence of a lower copy number of the κ receptors when using the second round of nested PCR (Fig. 2).

Human atrial trabeculae model. Fifty-two samples were obtained from 27 patients with stable ischemic heart disease (20 men and 7 women; age range 42 to 80 years, mean age 62.7 years). No trabeculae were excluded in this study. If two suitable trabeculae could be dissected from one atrial appendage, then each trabeculae was allocated to one of the eight groups (two sets of apparatus were used simulta-

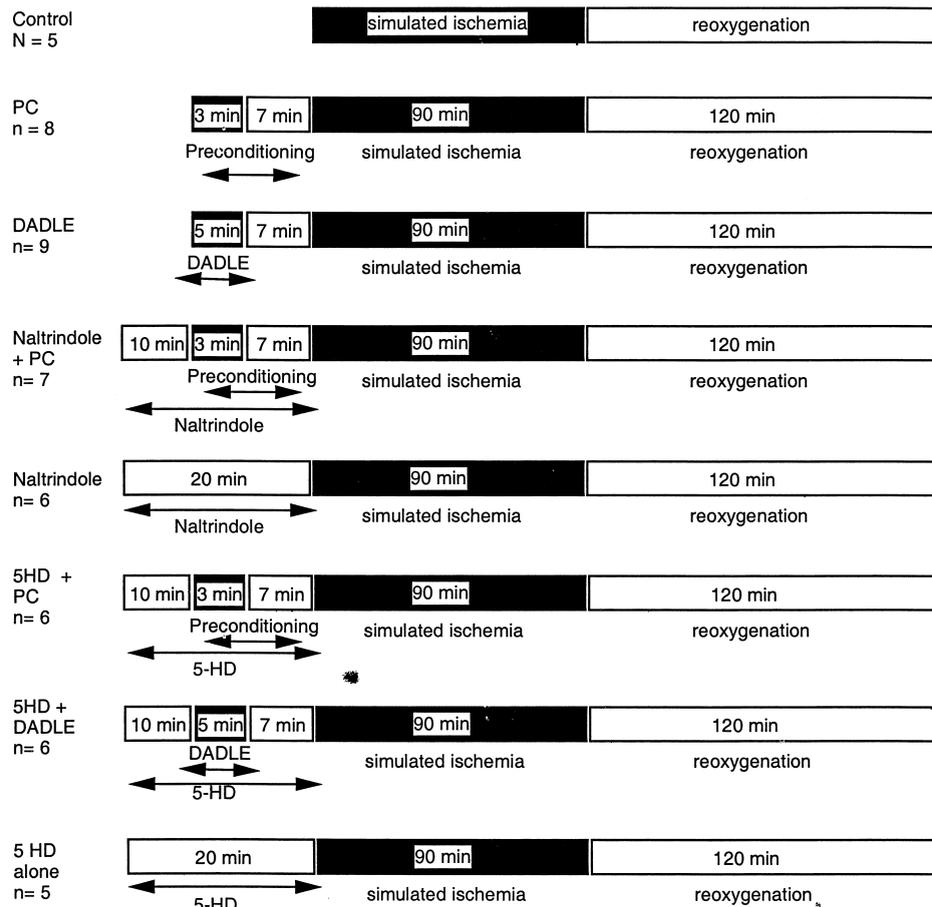


Figure 1. Experimental protocols for eight experimental groups. All protocols were preceded by a stabilization period of 45 to 60 min. This was followed by 90 min of simulated ischemia (superfusion with hypoxic, substrate free Modified Tyrodes solution and pacing at 3 Hz) and 120 min of superfusion with reoxygenated Tyrodes solution. Control = C; preconditioning = PC; delta opioid agonist = DADLE; naltrindole + preconditioning = naltrindole + PC; 5-hydroxydecanoate = 5HD. Solid box = simulated ischemia; open box = normoxic superfusion.

neously) as per previous studies (17,18). After an initial period of stabilization, samples, apart from those acting as controls, were pretreated before undergoing a period of simulated ischemia, which involved superfusion with a hypoxic, substrate free modified Tyrode's solution and pacing at 3 Hz. This was followed by reperfusion with an oxygenated solution for 120 min (Fig. 1). Baseline resting force was manipulated to the same value in all samples, and there was no significant difference in developed force at the end of the stabilization period between groups (Table 1). Experimental data are presented graphically as a percentage of baseline developed force with developed force at the end of the stabilization period taken as 100% developed force.

Figure 3 demonstrates the role of the δ opioid receptor. Preconditioning alone and with concomitant naltrindole caused an initial fall in the developed force of the trabeculae to $16.4 \pm 3.0\%$ and $30.9 \pm 6.4\%$, respectively. In both groups values returned to baseline before the onset of simulated ischemia. Superfusion with DADLE or naltrindole gave values for developed force of $109 \pm 4.9\%$ and $93.1 \pm 5.6\%$ (both NS vs. controls) of values at the onset of simulated ischemia. During the hypoxic period, there were

no significant differences between the functions of all groups studied. After the 120 min superfusion with reoxygenated modified Tyrode's solution, there was greater recovery of function in the groups preconditioned by simulated ischemia and DADLE (control, $32.6 \pm 3.8\%$; PC, $50.5 \pm 1.8\%$; DADLE, $46 \pm 3.9\%$; $p < 0.001$ vs. controls; no significant difference between PC and DADLE groups). The recovery values for the control group, the group superfused with naltrindole alone and the group combining naltrindole with PC did not differ from each other (naltrindole, $25.3 \pm 4.3\%$; naltrindole + PC, $25.5 \pm 3.9\%$).

Figure 4 compares the involvement of the K_{ATP} channel in both simulated ischemic and opioid PC. The trabeculae that were preconditioned with simulated ischemia and were preconditioned with 5HD + PC both decreased function before the onset of 90 min simulated ischemia (PC, $16.4 \pm 3.0\%$; 5HD + PC, $24.9 \pm 5.6\%$) compared with the other groups. After 120 min reoxygenation modified Tyrode's solution, the trabeculae preconditioned with simulated ischemia and the δ opioid agonist, DADLE, recovered to $50.5 \pm 1.8\%$ and $46 \pm 3.9\%$, respectively ($p < 0.001$). When trabeculae preconditioned with simulated ischemia

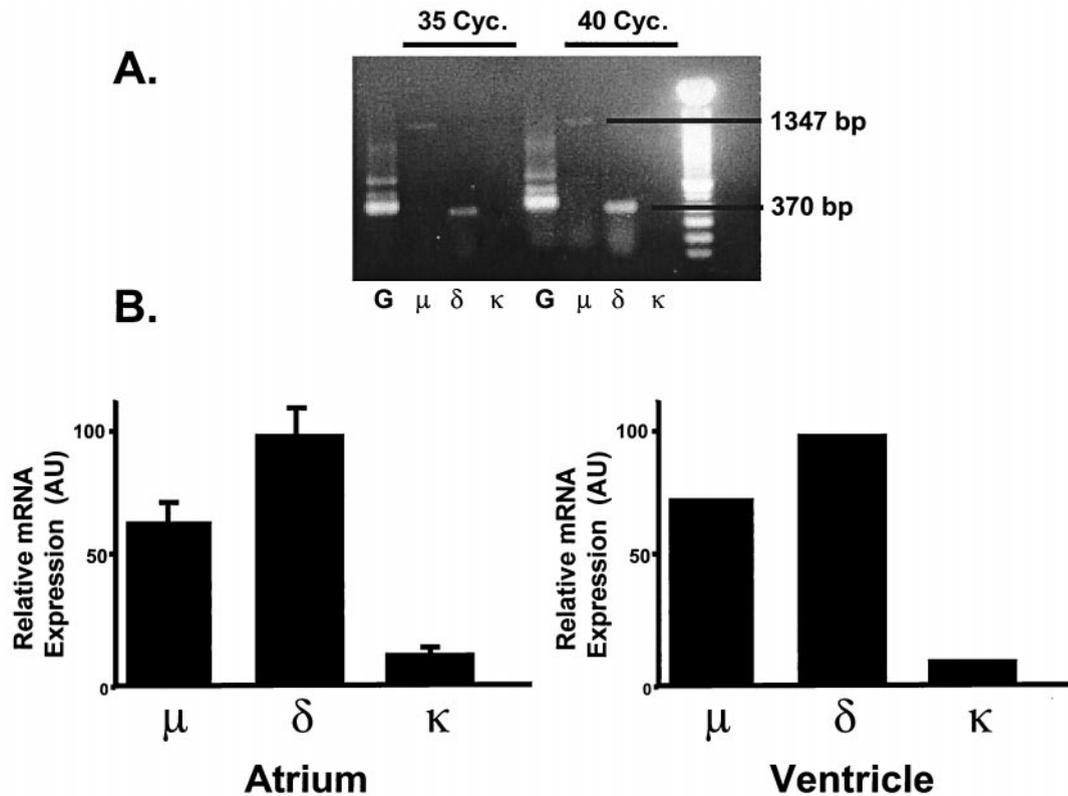


Figure 2. (A) Representative RT-PCR reactions at 35 cycles (Cyc) and 40 cycles, respectively. The control primers (GAPDH ([G]) have multiple bands due to the different optimal annealing temperatures of the GAPDH primers compared with the opioid-receptor subtypes. The μ and δ receptor PCR products are shown at the different cycle lengths. The κ receptor nested PCR data are not shown. (B) This histogram demonstrates the relative intensities (expressed in arbitrary units [AU]) of the PCR products of the opioid receptor subtypes in both human atrial and ventricular tissue. The AU for the μ and δ receptor subtypes reflect the mean PCR product generated after 40 cycles of PCR, and the AU for the κ receptor reflects the PCR products after the second round of nested PCR. bp = base pair; MRNA = messenger RNA; PCR = polymerase chain reaction.

and DADLE were also superfused with 5HD, the protection from preconditioning was lost (5HD + PC, $28.9 \pm 7.4\%$; 5HD + DADLE, $26.9 \pm 4.8\%$). Trabeculae superfused with 5HD initially without additional preconditioning recovered to $24.1 \pm 3.9\%$ of their baseline value. For these three groups the functional recovery attained did not differ from controls ($32.6 \pm 3.8\%$).

DISCUSSION

That activation of opioid receptors may confer a degree of cardioprotection in humans is a potentially important concept. This study is the first to show that stimulation of the

opioid δ receptor in superfused human atria can achieve protection similar to that obtained by PC induced by simulated ischemia, as evidenced by improved reoxygenation mechanical function. We found that short periods of exposure to the δ opioid receptor agonist, DADLE, improved contractile function to a similar extent as when preconditioned with hypoxia. The protection was removed by the blocker of the mitochondrial ATP-sensitive potassium channel (K_{ATP}), 5-hydroxydecanoate, indicating a similarity to the effects of ischemic PC in other models (8). Our semiquantitative analysis using RT-PCR confirmed the presence of the messenger RNA transcript encoding the δ opioid receptors in the human atria and ventricles. Therefore, it is reasonable to propose that δ -opioid receptor stimulation by DADLE can achieve a PC-mimetic effect in human tissue, as previously shown by Gross and colleagues (10,11) for similar receptor stimulation in the intact rat heart and by ourselves and others (15,19) in the isolated rat heart and the isolated rabbit heart (20).

Importance of opioid receptor subtypes. Since three opioid receptor genes have been described, we designed specific primer pairs based on published sequences of genes. The RT-PCR products from these primer pairs were the predicted size when analyzed by DNA gel electrophoresis. We readily found transcripts encoding the δ and μ receptors

Table 1. Baseline Data

Group	Developed Force (g)
Control	0.98 ± 0.19
PC	0.87 ± 0.06
DADLE	1.02 ± 0.12
Naltrindole + PC	0.89 ± 0.10
Naltrindole	0.90 ± 0.15
5HD	0.99 ± 0.09
5HD + PC	0.76 ± 0.07
5HD + DADLE	0.85 ± 0.10

Values are mean \pm SEM.

DADLE = delta opioid agonist; PC = preconditioning; 5HD = 5-hydroxydecanoate.

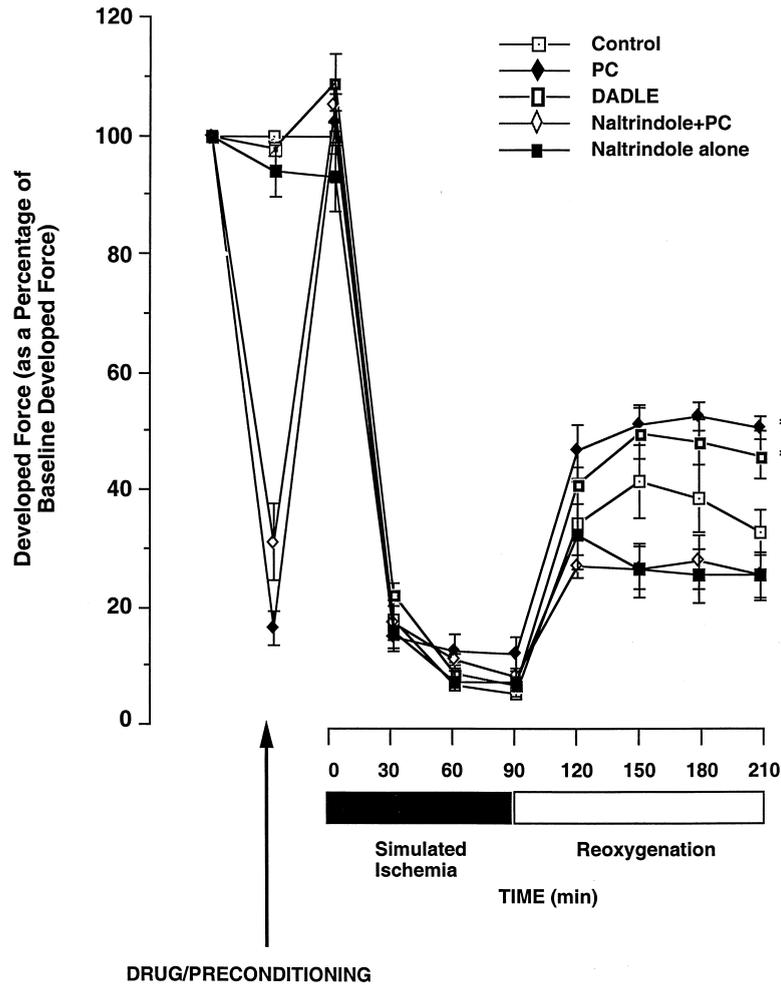


Figure 3. Developed force presented as a percentage of baseline developed force against time. This graph shows that the effect of PC with hypoxia may be simulated by delta-opioid receptor agonism (DADLE) and that ischemic PC is blocked by the delta opioid receptor antagonist, naltrindole. $p < 0.001$ vs. control. C = control; DADLE = δ opioid agonist; PC = preconditioning; naltrindole+PC = naltrindole plus preconditioning.

with a putatively lower copy number of the κ receptor transcript. This opioid receptor distribution pattern differs from the rat heart where similar work has shown the presence of δ receptors, lesser evidence of κ receptors but no μ receptors (13). Morphine has been shown to mimic the effects of PC (10), and this cardioprotection is mediated by the δ opioid receptor (21). Recently Gross' group has suggested that it is the δ subtype that mediates PC (22). As such we used DADLE, the specific δ opioid receptor agonist, rather than morphine, and naltrindole, the δ opioid receptor antagonist, rather than naloxone, to target the δ opioid receptor. Nonetheless, as with most pharmacological agents, it must be appreciated that specificity is always a problem, and it needs to be appreciated that no agent is completely specific under such experimental conditions.

For functional studies, human atrial trabeculae were used because atrial tissue was easily accessible. Sampling such tissue is routinely part of coronary artery bypass grafting, and the right atrium has the advantage of being relatively disease-free. Use of an in vitro model avoids confounding factors such as presence of collateral flow, concomitant

physiological responses and additional and possibly unpredictable effects of the drugs used on other organ systems. The human atrial model of PC is reproducible between investigators and appears to follow all the criteria used to examine PC in animal models, that is, it responds to adenosine stimulation, appears to be mediated by a PKC-type mechanism and involves the K_{ATP} channel (18). The end point of injury used is the recovery of developed force, and not limitation of infarct size, and, therefore, we are unable to say whether or not there is a decrease in myocyte necrosis or whether the protection observed is as a consequence of stunning. In addition it must be noted that we use simulated ischemia, which differs from true ischemia in a number of ways. This is a superfused preparation, which enables removal of catabolites during the simulated ischemia, in addition, because we use a buffer preparation, not blood, there is no exposure to blood borne carriers such as complement, platelets or leukocytes.

As we are fully aware morphine is a drug that is used in the management of pain that accompanies acute myocardial infarction as well as in the perioperative period after

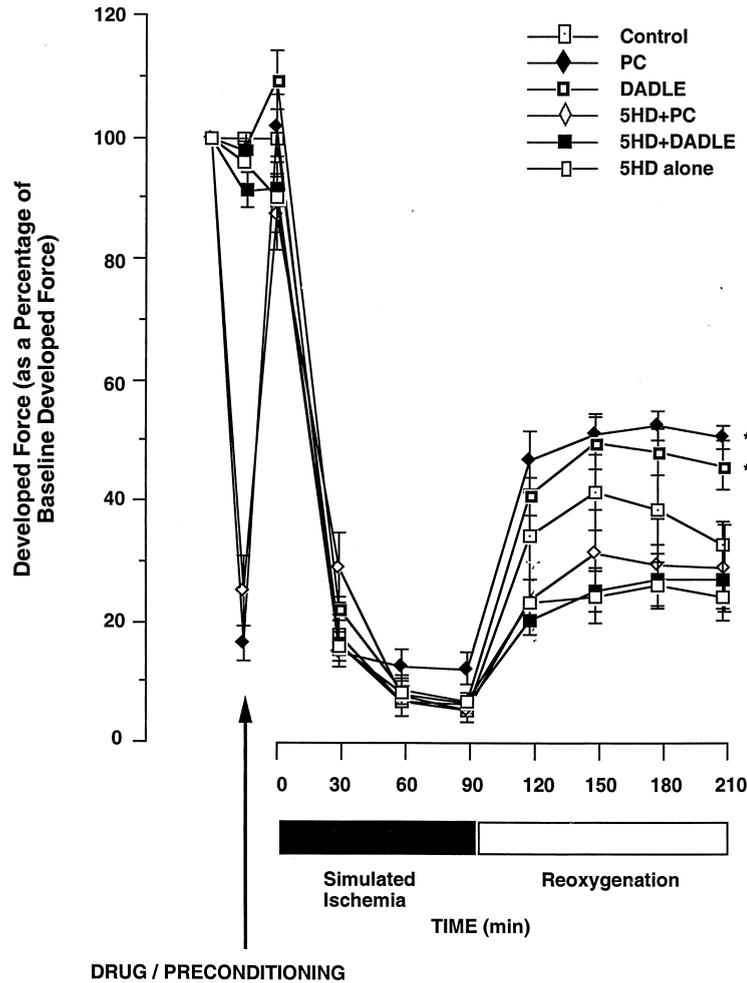


Figure 4. Developed force presented as a percentage of baseline developed force against time. This graph shows that the effect of PC with both simulated ischemia and DADLE. Both the effects of PC and DADLE are blocked by the mitochondrial K_{ATP} channel blocker 5HD. $p < 0.001$ vs. control. C = control; PC = preconditioning; DADLE = delta opioid agonist; 5HD = 5-hydroxydecanoate; 5HD+PC = 5 hydroxydecanoate plus preconditioning; 5HD+DADLE = 5 hydroxydecanoate plus delta opioid agonist.

coronary artery bypass surgery. It is an agent that mainly stimulates the μ (which is associated with the pain receptor) in addition to having effects on the δ and κ receptors (23). Our results, therefore, raise the possibility that such administration of morphine might, besides giving pain relief, also protect the myocardium against further episodes of recurrent ischemia. But are doses of morphine given in clinical practice likely to evoke a PC-like effect? In the isolated rabbit heart, morphine concentrations of 0.3 μ M can give such protection, and in the rabbit heart in situ, 3mg/kg is required (23). These doses are estimated by Miki et al. (24) to be much higher than those clinically used. In the intact rat, however, a total dose of 0.3 mg/kg could achieve a PC effect (14). In humans, the standard doses are 2 to 10 mg/70 kg, that is, up to 0.14 mg/kg. The maximum doses that have been used are 2 to 3 mg/kg, which are regarded as remarkably large but well tolerated (25). According to a preliminary report (26), morphine in a dose of only 15 μ g/kg gave pharmacologic PC during repeat coronary angioplasty. Of interest, 10 nM DADLE achieved

protection in both isolated rat heart experiments (15) and in the present series on superfused human atria. Most recently it has been shown that the opioid receptor antagonist naloxone abolished the adaptation to ischemia observed in humans after two sequential coronary balloon inflations (27). Therefore, there is a real possibility that morphine, as clinically used, may have a pharmacological PC effect, especially at high doses.

The relevance of other receptor subtypes. The role of the κ receptor in opioid PC is still unclear although cardioprotective effects of κ receptor stimulation have been demonstrated in rat ventricular myocytes subjected to metabolic inhibition (28). In our own rat heart experiments (15) using infarct size as the end point, we found that a high dose of the delta-agonist DADLE did not protect the myocardium. This lack of a cardioprotective effect was abolished by simultaneous administration of the κ -receptor blocker, bremazocine. Since we know that κ receptors are present in rat heart (29), the suggestion is that κ receptor stimulation can oppose the protection achieved by δ stimulation in the

rat heart (15). The fear that nonspecific κ receptor stimulation by morphine or DADLE could lessen the degree of protection is real in the rat, but, importantly, our data show that the κ receptor is only present in very small amounts in the human heart. If this is correct than any potential fear that agents that exhibit a wide range of receptor subtype activation, such as morphine, may not necessarily prove detrimental or oppose the potential PC effect of such opioid receptor activation.

Clinical relevance. Our data, taken together with those in rats and rabbits, show that opioid receptor stimulation has clinical potential and warrants further exploration in humans. There are a number of outstanding questions. First, do the doses of morphine as clinically used achieve PC-like protection? Moreover, could a pharmacologic dose of morphine be combined with a lesser degree of ischemia to give added PC as postulated in the case of protection mediated by angiotensin-converting enzyme inhibition (30)? A possible future clinical application of our data lies in the development of δ -opioid agonists that could have fewer central side-effects, yet with more direct protective effects on the human myocardium. Specifically, it is the δ_1 receptor subgroup that gives protection in the rat heart (14). Agonists of this subgroup may have clinical application.

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