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# Opposing effects on infarction of delta and kappa opioid receptor activation in the isolated rat heart: implications for ischemic preconditioning

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**Abstract** δ-Opioid receptors are known to participate in the protection found following ischemic preconditioning (IPC), but the role of κ-receptors in IPC is currently controversial. Langendorff-perfused rat hearts received 35 min regional ischemia and 2 h reperfusion. PC (2 cycles 5 min global ischemia) substantially reduced infarct size. Pharmacological PC with the δ-agonist DADLE (10 nmol/L) had similar protective effects. However, higher dose DADLE (1 μmol/L) had a less beneficial effect, and in conjunction with the δ-antagonist naltrindole unexpectedly increased infarct size (61.5 ± 2.0 %, $p<0.05$ v 45.9 ± 2.4 % in controls) suggesting a non-δ effect. The universal κ-opioid agonist bremazocine (30 nmol/L) increased infarct size (61.3 ± 1.6 %, $p<0.05$ v controls), an effect abrogated by the selective $\kappa_1$-antagonist nor-binaltorphimine (BNI).

Since opiates are known to have anti-adrenergic effects, which hypothetically may help to mediate IPC, cyclic AMP levels were measured in DADLE and in bremazocine-treated hearts. Decreased levels of cyclic AMP at the start of the regional ischemic period were found in low dose DADLE hearts (0.485 ± 0/020, n = 8, vs controls, 0.654 ± 0.025 nmol/g wet weight, $p<0.001$), but not in high dose DADLE nor in bremazocine treated hearts.

Thus, in the isolated rat heart $\kappa_1$-opioid receptor activation exacerbates infarct size through an as yet unknown mechanism, suggesting that there could be an "anti-preconditioned state". In contrast, δ-activity mediates protection which may be associated with a reduction of tissue cyclic AMP levels.

**Key words** Delta opioid receptor – kappa opioid receptor – preconditioning – regional ischemia – infarct size

## Introduction

Ischemic preconditioning (IPC) of the myocardium is a phenomenon whereby brief periods of sublethal ischemia will delay the onset of necrosis during a subsequent lethal ischemic insult (15, 40). Strong experimental evidence already implicates a role for opioid receptors in IPC against infarction in the intact rat heart. Morphine mimics the effects of IPC (30), and this cardioprotection is mediated by the delta (δ)-receptor (27). Recently, Schultz et al. (28) have suggested that it is the $\delta_1$-subtype that mediates IPC using the $\delta_1$-selective antagonist BNTX (28) and the novel $\delta_1$-selective agonist TAN-67 (31).

In addition to the δ-opioid receptor, functional and binding data demonstrate that δ- and κ-, but not μ-opioid receptors are present in the rat myocardium (1, 34, 43). Previous experiments with selective μ-antagonists have excluded a role for these opioid receptors in the triggering of IPC (28). Regarding κ-receptors, data are controversial. Stimulation of κ-receptors

prior to coronary artery occlusion increases the severity of ischemic arrhythmias in the rat heart (10, 37), while κ-receptor activation did not appear to play a role in the reduction of infarct size by PC (28). Xia et al. (39) could not find a role for κ-receptors in the effect of PC in decreasing post-ischemic arrhythmias. Conversely, in cultured heart cells, PC by metabolic inhibition was mediated by κ-receptors and inhibited by the $\kappa_1$-receptor blocker, NBI (38). We were, therefore, alerted to the possibility that κ-receptor stimulation or inhibition could modulate the expected infarct-size, thereby reducing the effects of δ-opioid stimulation.

First, we used the isolated rat heart model of PC to obviate the role of any extracardiac opioid receptors, and so that we could be sure of the concentrations of the receptor agonists and antagonists reaching the heart. We chose infarct size as the classic endpoint of PC used in the initial description of Murry et al. (15) and also to allow comparison of our results with those of Schultz and co-workers in their extensive studies. Secondly, we examined the possibility that activation of κ-receptors may have effects on infarction modulating those of the δ-receptor agonist. Therefore, we studied the effects of the universal κ-receptor agonist, bremazocine, and the specific $\kappa_1$-receptor antagonist, nor-binaltorphimine (BNI) (44). Thirdly, because δ-receptors can have physiological antiadrenergic effects by linking to $G_i$ (19), and because a decreased cyclic AMP and increased cyclic GMP level may mediate protection by IPC (11, 18, 24), we argued that modification of myocardial nucleotide levels could be one mode of action of δ-receptor agonists. Therefore, in selected groups of perfused hearts, myocardial levels of cyclic AMP and cyclic GMP were measured.

## Methods

### Isolated rat heart model

The model used was modified from that described by Bugge and Ytrehus (2), who demonstrated that pre-treatment with three cycles of 5 min global ischemia interspersed with 5 min periods of reflow prior coronary artery occlusion and reperfusion could PC isolated rat hearts against infarction. In preliminary studies, we found infarct reduction with two cycles of PC ischemia with less impairment of myocardial contractility (data not shown). All rats were fed ad libitum and cared for according to published guidelines (US National Institutes of Health, NIH publication No. 85–23, revised 1985). Male Long-Evans rats (260–330 g) were anaesthetised with 60 mg $kg^{-1}$ i.p. sodium pentobarbitone and heparinised (200 IU i.p.). The heart was rapidly excised, immersed in ice cold Krebs-Henseleit buffer solution (NaCl 118 mM, KCl 4.7 mM, $CaCl_2$ 1.8 mM, $KH_2PO_4$ 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25.2 mM and glucose 11.0 mM). The aorta was cannulated and the heart perfused retrogradely in the Langendorff mode at a constant pressure (100 cm $H_2O$) with Krebs-Henseleit buffer (pH 7.4). The perfusate was oxygenated with 95 % $O_2$/5 % $CO_2$ and maintained at 37 °C throughout the experiment. A water-filled latex balloon, connected to a pressure transducer, was inserted into the left ventricle (LV) via the left atrium. LV end-diastolic pressure was set between 4–8 mmHg as baseline. Myocardial temperature was measured by a thermoprobe inserted into a small incision in the pulmonary artery. A 5/0 silk suture was placed around the left coronary artery, close to its origin and the hearts stabilized for 20 min. The ends of the suture were threaded through a small polyethylene button that was clamped against onto the epicardial surface with a hemostat clamp to induce regional ischemia for 35 min. Thereafter the heart was reperfused for 120 min. Heart rate (HR) and left ventricular developed pressure (LVDP = difference between LV systolic and diastolic pressures) were continuously displayed on a Lectromed recorder.

### Experimental protocol

Hearts were assigned randomly to one of fourteen experimental groups. In all experiments, infarction was induced by 35 min of regional ischemia followed by 120 min of reperfusion. After 20 min of equilibration control hearts underwent ischemia and reperfusion only. Ischemic preconditioning (IPC) was elicited by two cycles of 5 min global ischemia interspersed with 5 min reperfusion prior to regional ischemia and reperfusion. In the two DADLE-treated groups, hearts were "preconditioned" with two cycles of 5 min perfusion with buffer containing 10 nmol/L (low DADLE) or 1 μmol/L (high DADLE) of the δ-agonist, interspersed with 5 min drug-free periods. To achieve κ-receptor stimulation, hearts were pretreated with 2 × 5 min perfusions with 30 nmol/L bremazocine (23) separated by 5 min drug-free perfusions. In experiments where an opioid receptor antagonist was used, control hearts were treated with 100 nmol/L naltrindole as the δ-antagonist or 30 nmol/L nor-binaltorphimine as the κ-antagonist (21) for 10 min prior to the induction of regional ischemia. In hearts preconditioned with global ischemia, either 100 nmol/L naltrindole or 30 nmol/L nor-binaltorphimine was introduced into the perfusate 5 min prior to the start of the preconditioning protocol until coronary artery occlusion (Fig. 1). In the low DADLE + naltrindole, high DADLE + naltrindole, bremazocine + nor-binaltorphimine, low DADLE + nor-binaltorphimine and hiDADLE + nor-binaltorphimine groups, perfusion with naltrindole or nor-binaltorphimine commenced 5 min prior to the first cycle of agonist treatment and was stopped just prior to induction of the test ischemia (Fig. 1).

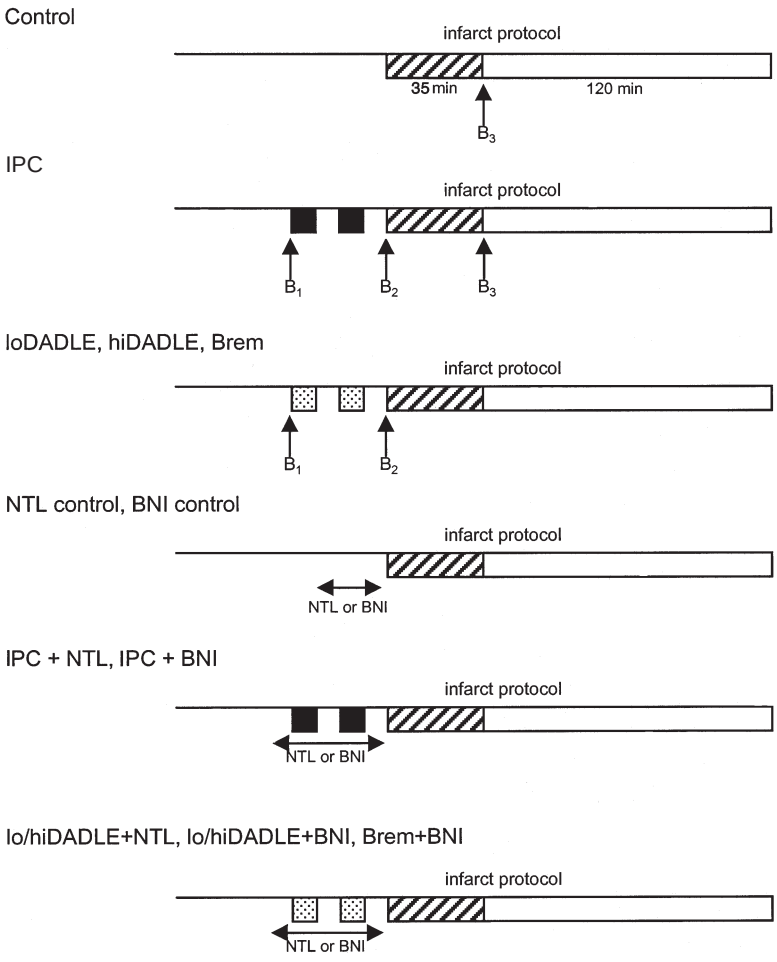


**Fig. 1** Experimental protocol. Control perfused hearts were subject to 20 min of Langendorff mode perfusion, followed by 35 min of regional ischemia, and then 120 min of reperfusion. Ischemic preconditioning: IPC hearts were subject to two cycles of 5 min global ischemia, each followed by 5 min reperfusion, before the onset of regional ischemia for 35 min and then reperfusion. PC procedure: indicates hearts treated for two 5 min cycles with pharmacological agents; B indicates times of biopsies, where $B_1$ = biopsy before addition of pharmaceutical agent, $B_2$ = biopsy after addition of pharmaceutical agent, but before regional ischemia induced by coronary artery ligation; $B_3$ = biopsy at end of period of regional ischemia, just before reperfusion. LoDADLE indicates low concentration of DADLE; HiDADLE indicates high concentration. Brem equals bremazocine. BNI equals nor-binaltorphimine. For concentrations used, see legends to Figs. 2 and 3.

### Measurement of risk zone and infarct size

At the end of the experiment, the silk suture around the coronary artery was securely tied and a 5 mg/ml suspension of zinc-cadmium sulphide fluorescent microspheres (in 0.9 % w/v saline) was slowly infused through the aorta to delineate the myocardial risk zone under ultraviolet light. The heart was then frozen overnight before being cut into 2 mm thick slices (6–7 slices per heart), defrosted, and stained by incubation for 15 min in 1 % w/v triphenyltetrazolium chloride in phosphate buffer (pH 7.4). Slices were then fixed in 10 % v/v formaldehyde solution to enhance the contrast between stained viable tissue and unstained necrotic tissue. The area of the LV at risk and the area of infarcted tissue in the risk zone were determined by an operator blinded to experimental treatment using computerised planimetry (Summa Sketch II; Summa Graphics). The volume of infarcted tissue (I) and the risk zone (R) was then calculated by multiplying each area with the slice thickness and summing the products. The infarct size was expressed as the percentage of the risk zone infarcted (I/R %).

### Cyclic nucleotides

These were measured on freeze-clamped tissue by methods previously described (5, 6). Control biopsy values were obtained after 15 min pre-perfusion, just before the time of anticipated onset of the first period of global ischemia, followed by biopsies in other groups of hearts just before or after the period of regional ischemia (Fig. 1).

### Exclusion criteria

Only hearts with coronary flow within 8–15 ml/min and left ventricular developed pressure (LVDP) > 80 mmHg at the end of the stabilisation period were included in the present study. Verification of coronary artery occlusion was indicated by a reduction in coronary flow of > 30 %. Hearts developing ventricular fibrillation during ischemia-reperfusion that could not be restored to normal sinus rhythm within 2 min were excluded. Any hearts with a risk volume greater than 0.550 $cm^3$ were also excluded from this study.

### Drugs

DADLE, naltrindole, bremazocine and nor-binaltorphimine were obtained from Research Biochemicals International (Natick, MA, USA). 2,3-triphenyl-tetrazolium chloride (TTC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in phosphate buffer (pH 7.4).

### Statistical analysis

All data are presented as means ± SEM. One-way analysis of variance (ANOVA) with Bonferroni corrected t-test (for multiple comparisons) was used to detect differences between groups. P values ≤ 0.05 were considered significant.

## Results

### Exclusions and incidence of ventricular fibrillation

A total of 96 rat hearts were used for this study. Of these, four were excluded during the stabilisation period because of a CF > 15 ml/min (2) or LVDP < 80 mmHg (2). A further three hearts were excluded as a consequence of irreversible ventricular fibrillation during the preconditioning protocol (1 PC, 1 PC + naltrindole and 1 PC + nor-binaltorphimine). Sustained ventricular fibrillation in reperfusion, following regional ischemia, also resulted in the exclusion of three hearts (1 control, 1 PC + naltrindole and 1 DADLE 10 nmol/L). Of the hearts that remained in each group, the incidence of ventricular fibrillation in the reperfusion period was approximately 50 %, with no significant differences between groups. At the end of the experimental protocol, two hearts were excluded due to an excessively large risk volume > 0.550 $mm^3$ (1 control + naltrindole and 1 DADLE 100 nmol/L).

### Hemodynamic data

Heart rate (HR), left ventricular developed pressure (LVDP) and coronary flow (CF) data were monitored throughout the experiment, and hemodynamics measured at various time points throughout the experiment are shown in Table 1. The baseline variables for each of these parameters did not differ among any of the groups. Following the respective treatment protocols, heart rate and coronary flow remained similar among groups (pre-ischemia). However, the ischemic preconditioning protocol with two cycles of 5 min global ischemia + 5 min of reperfusion led to a significant decrease in left ventricular developed pressure in the three preconditioned groups. Drug treatment had no influence on left ventricular developed pressure when compared to the pre-ischemic value in control hearts. Coronary artery occlusion led to a marked reduction in left ventricular developed pressure and coronary flow in all of the experimental groups; the latter was immediately reversed upon reperfusion (data not shown). There were no differences among the groups with respect to any of the hemodynamic

**Table 1** Hemodynamic data for isolated rat hearts

| | Baseline | | | Pre-ischemia | | | Ischemia + 34 min | | | Reperfusion + 120 min | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HR (bpm) | LVDP (mmHg) | CF (ml/min) | HR (bpm) | LVDP (mmHg) | CF (ml/min) | HR (bpm) | LVDP (mmHg) | CF (ml/min) | HR (bpm) | LVDP (mmHg) | CF (ml/min) |
| Control | 294 ± 10 | 112 ± 6 | 13.2 ± 0.3 | 291 ± 12 | 110 ± 6 | 13.6 ± 0.5 | 266 ± 21 | 66 ± 2 | 7.8 ± 0.3 | 254 ± 10 | 79 ± 5 | 9.2 ± 0.5 |
| IPC | 307 ± 16 | 108 ± 3 | 12.7 ± 0.3 | 303 ± 15 | 89 ± 4* | 13.9 ± 0.4 | 303 ± 19 | 56 ± 3 | 7.5 ± 0.3 | 267 ± 18 | 61 ± 2* | 8.0 ± 0.6 |
| loDADLE | 277 ± 12 | 106 ± 4 | 12.9 ± 0.6 | 274 ± 17 | 103 ± 6 | 12.7 ± 0.5 | 269 ± 25 | 55 ± 4 | 7.3 ± 0.3 | 274 ± 20 | 66 ± 2 | 8.5 ± 0.6 |
| hiDADLE | 273 ± 16 | 109 ± 4 | 12.5 ± 0.9 | 260 ± 10 | 106 ± 5 | 12.7 ± 0.3 | 237 ± 10 | 64 ± 2 | 7.1 ± 0.4 | 250 ± 16 | 77 ± 4 | 9.2 ± 0.4 |
| Control + NTL | 280 ± 11 | 111 ± 5 | 12.9 ± 0.7 | 277 ± 16 | 106 ± 3 | 12.2 ± 0.6 | 271 ± 13 | 64 ± 2 | 7.2 ± 0.3 | 271 ± 13 | 70 ± 5 | 8.9 ± 0.6 |
| IPC + NTL | 280 ± 17 | 112 ± 5 | 12.1 ± 0.8 | 260 ± 14 | 100 ± 6 | 12.0 ± 0.6 | 248 ± 16 | 58 ± 5 | 6.4 ± 0.6 | 240 ± 26 | 80 ± 5 | 8.6 ± 0.8 |
| loDADLE + NTL | 300 ± 12 | 114 ± 6 | 13.8 ± 0.8 | 280 ± 16 | 90 ± 3* | 14.9 ± 0.8 | 264 ± 14 | 59 ± 3 | 7.4 ± 0.5 | 280 ± 17 | 63 ± 3* | 9.8 ± 1.0 |
| hiDADLE + NTL | 320 ± 15 | 117 ± 5 | 13.6 ± 0.4 | 272 ± 10 | 95 ± 3 | 13.6 ± 0.6 | 260 ± 13 | 57 ± 2 | 7.8 ± 0.2 | 247 ± 13 | 66 ± 3 | 8.0 ± 0.3 |
| Control + BNI | 307 ± 23 | 107 ± 5 | 12.4 ± 0.4 | 293 ± 18 | 99 ± 4 | 12.0 ± 0.4 | 250 ± 18 | 57 ± 3 | 7.2 ± 0.2 | 257 ± 16 | 77 ± 4 | 8.8 ± 0.2 |
| IPC + BNI | 310 ± 13 | 105 ± 4 | 12.9 ± 0.2 | 300 ± 14 | 106 ± 3 | 12.9 ± 0.3 | 280 ± 22 | 62 ± 3 | 7.3 ± 0.4 | 260 ± 16 | 69 ± 3 | 8.2 ± 0.7 |
| Brem | 307 ± 16 | 105 ± 5 | 13.2 ± 0.4 | 313 ± 13 | 83 ± 2* | 14.5 ± 0.4 | 277 ± 14 | 56 ± 3 | 7.7 ± 0.3 | 300 ± 7 | 62 ± 3* | 8.1 ± 0.3 |
| Brem + BNI | 310 ± 11 | 102 ± 6 | 13.2 ± 0.7 | 297 ± 14 | 98 ± 7 | 12.7 ± 0.7 | 283 ± 19 | 57 ± 5 | 7.4 ± 0.3 | 277 ± 13 | 70 ± 2 | 8.9 ± 0.3 |
| loDADLE + BNI | 287 ± 16 | 104 ± 5 | 12.8 ± 0.4 | 287 ± 11 | 108 ± 9 | 12.6 ± 0.5 | 257 ± 10 | 62 ± 3 | 7.1 ± 0.5 | 253 ± 8 | 71 ± 2 | 9.3 ± 0.7 |
| hiDADLE + BNI | 307 ± 21 | 102 ± 7 | 12.9 ± 0.6 | 290 ± 22 | 98 ± 6 | 12.4 ± 0.7 | 270 ± 10 | 58 ± 2 | 7.6 ± 0.4 | 283 ± 16 | 66 ± 4 | 8.5 ± 0.5 |

Values are mean ± SEM of six observations. PC indicates ischaemic preconditioning; loDADLE, DADLE 10 nmol/L; hiDADLE, DADLE 1 µmol/L; NTL, naltrindole 100 nmol/L; BNI, nor-binaltorphimine 30 nmol/L; BREM, bremazocine 30nmol/L. *p<0.05 vs corresponding value in the control group. Slashed filled blocks: ischemia induced by coronary artery ligation, followed by reperfusion period (open blocks). Solid black blocks in second panel: periods of transient global ischemia. Other filled blocks: as labelled.

**Table 2** Infarct size data for isolated rat hearts

| | Rat weight (g) | Heart weight (g) | Risk volume ($cm^3$) | Infarct volume ($cm^3$) | Infarct/Risk (%) |
|---|---|---|---|---|---|
| Control | 305 ± 12 | 1.05 ± 0.10 | 0.364 ± 0.032 | 0.164 ± 0.012 | 45.9 ± 2.4 |
| IPC | 312 ± 10 | 1.11 ± 0.12 | 0.375 ± 0.024 | 0.070 ± 0.009* | 18.6 ± 1.9* |
| loDADLE | 300 ± 11 | 1.06 ± 0.08 | 0.350 ± 0.034 | 0.070 ± 0.011* | 20.9 ± 2.4* |
| hiDADLE | 299 ± 12 | 1.03 ± 0.11 | 0.386 ± 0.020 | 0.137 ± 0.014* | 35.4 ± 3.0* |
| Control + NTL | 311 ± 13 | 1.10 ± 0.08 | 0.440 ± 0.026 | 0.220 ± 0.016 | 48.6 ± 3.5 |
| IPC + NTL | 304 ± 8 | 1.05 ± 0.11 | 0.418 ± 0.032 | 0.195 ± 0.021 | 47.0 ± 4.3 |
| loDADLE + NTL | 313 ± 10 | 1.14 ± 0.10 | 0.407 ± 0.026 | 0.192 ± 0.018 | 46.8 ± 1.9 |
| hiDADLE + NTL | 308 ± 11 | 1.06 ± 0.07 | 0.405 ± 0.030 | 0.248 ± 0.018* | 61.5 ± 2.0* |
| Control + BNI | 301 ± 9 | 1.03 ± 0.09 | 0.392 ± 0.042 | 0.179 ± 0.019 | 45.7 ± 1.9 |
| IPC + BNI | 295 ± 13 | 0.99 ± 0.12 | 0.416 ± 0.029 | 0.096 ± 0.011* | 23.0 ± 1.8* |
| Brem | 303 ± 9 | 1.05 ± 0.11 | 0.407 ± 0.022 | 0.249 ± 0.014* | 61.3 ± 1.6* |
| Brem + BNI | 297 ± 10 | 1.03 ± 0.09 | 0.353 ± 0.026 | 0.174 ± 0.015 | 48.9 ± 2.3 |
| loDADLE + BNI | 311 ± 8 | 1.12 ± 0.08 | 0.372 ± 0.015 | 0.085 ± 0.006* | 23.0 ± 2.3* |
| hiDADLE + BNI | 296 ± 7 | 1.05 ± 0.13 | 0.366 ± 0.030 | 0.087 ± 0.010* | 24.0 ± 2.8* |

Values are mean ± SEM of six observations. HR indicates heart rate; bpm, beats/min; LVDP, left ventricular developed pressure; CF, coronary flow. Other abbreviations as in legend to Table 1. *p<0.05 vs corresponding value in the control group.

parameters during the ischemic period. At the end of the experiment the hearts in the three PC groups had a significantly lower left ventricular developed pressure than the corresponding controls. There were no differences in either heart rate or coronary flow among groups at the end of the reperfusion period.

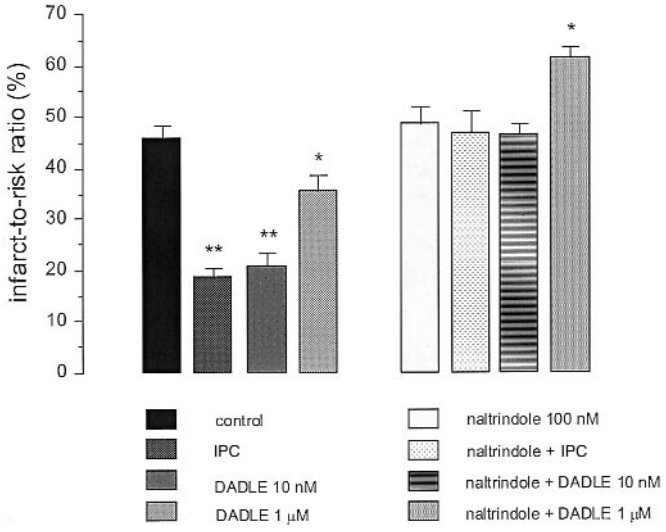


**Fig. 2** Percentage infarction of the risk zone in isolated rat hearts subjected to regional ischemia and reperfusion following ischemic preconditioning (IPC), pretreatment with the δ-receptor agonist DADLE and/or the δ-receptor antagonist naltrindole (NTL). IPC was induced with two cycles of 5 min global ischemia interspersed with 5 min reperfusion. DADLE 10 nmol/L (loDADLE) or 1 μmol/L (hi DADLE) was administered as two 5 min perfusions interspersed with 5 min drug-free periods. NTL (naltrindole, 100 nmol/L) perfusion commenced 5 min prior to IPC or DADLE until coronary artery occlusion. NTL reversed the protective effects of PC and loDADLE and revealed a detrimental effect of hiDADLE. Values are mean ± SEM of six observations. *p<0.05 vs control (one-way ANOVA with Bonferroni correction).

## Myocardial infarct size

Table 2 presents the weights of the animals, heart weight, risk volume and infarct data in each of the experimental groups. There were no significant differences in body weight, heart weight or risk zone size among the various groups. Figure 1 shows mean infarct size ± SEM in control, IPC, low DADLE- and high DADLE-treated hearts, in the absence and in the presence of naltrindole pre-treatment (100 nmol/L). Infarct size as a percentage of the risk zone was 45.9 ± 2.4 % in the control group (Fig. 2). IPC with 2 cycles of 5 min global ischemia interspersed with 5 min reperfusion significantly reduced infarct size to 18.6 ± 1.9 % (p<0.05 vs control hearts). Pharmacological PC with the 10 nmol/L of the δ-receptor agonist DADLE (low DADLE) mimicked the cardioprotective effects of IPC. However, protection against infarction was less marked with a higher concentration of DADLE (1 μmol/L; high DADLE) although infarcts remained significantly smaller than in controls (35.4 ± 3.0 %, p<0.05). Blockade of cardiac δ-receptors with naltrindole did not alter infarct size in control hearts, but abolished the protection afforded by PC and by the lower concentration of DADLE. In the high DADLE hearts, naltrindole reversed protection to reveal a detrimental effect that appeared independent of δ-receptor activation (61.5 ± 2.0 % infarction, p<0.05 vs controls).

Figure 3 depicts the effects of nor-binaltorphimine (30 nmol/L) on infarct size in control, and IPC plus bremazocine-, low DADLE and high DADLE-treated hearts. Pharmacological PC with two 5 min perfusions of the opioid agonist

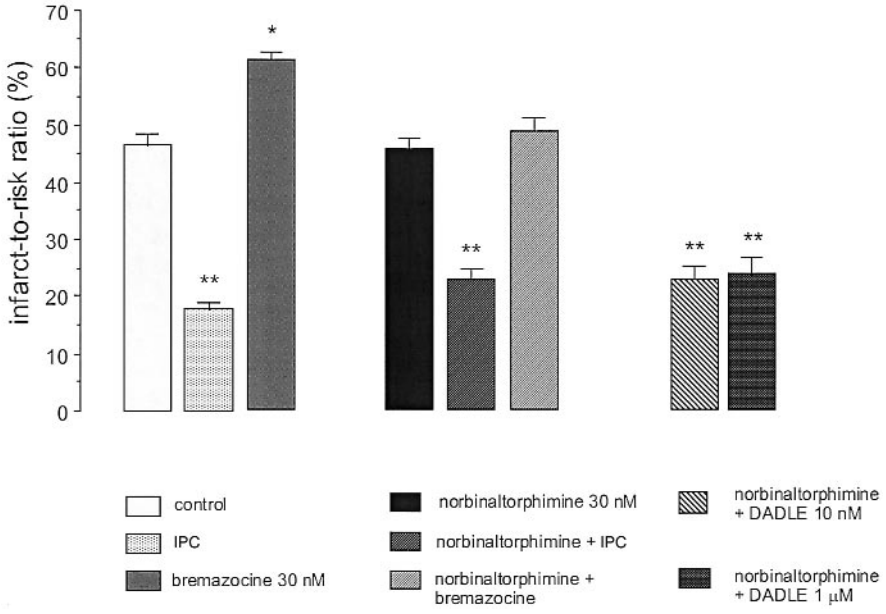


**Fig. 3** Percentage infarction of the risk zone in isolated rat hearts subjected to regional ischemia and reperfusion following ischemic preconditioning (IPC), pre-treatment with the κ-opioid-receptor agonist bremazocine (Brem) and/or the κ-receptor antagonist nor-binaltorphimine (BNI). PC was induced with two cycles of 5 min global ischemia interspersed with 5 min reperfusion. Brem (30 nmol/L) was administered as two 5 min perfusions interspersed with 5 min drug-free periods. BNI (30 nmol/L) perfusion commenced 5 min prior to IPC, Brem or DADLE until coronary artery occlusion. Brem pre-treatment increased infarct size compared to control. BNI did not influence IPC but reversed the effect of Brem, indicating a detrimental role for κ-receptors. Moreover, BNI increased protection observed with hiDADLE suggesting that DADLE is active at both δ- and κ-receptors at micromolar concentrations. Values are mean ± SEM of six observations. *p<0.05 vs control (one-way ANOVA with Bonferroni correction).

bremazocine (30 nmol/L) resulted in an increase in infarct size compared to controls (61.3 ± 1.6 %, p<0.05 vs control). Pre-treatment with the κ-receptor antagonist nor-binaltorphimine did not affect infarct size in control, IPC or in low DADLE hearts. However, nor-binaltorphimine abrogated the effect of breamzocine, returning infarct size to control levels (48.9 ± 2.3 %, p = NS vs control). Moreover, nor-binaltorphimine appeared to reverse the detrimental component of the high DADLE response as infarct size was comparable to that observed in IPC and low DADLE-treated hearts (24.0 ± 2.8, p<0.05 vs control), suggesting that DADLE at micromolar concentrations is active at both κ- and δ-opioid receptors.

### Cyclic nucleotides

After two cycles of global ischemia (Table 3), cyclic AMP levels were unchanged. After two cycles of low DADLE, levels fell from 0.654 ± 0.025 to 0.482 ± 0.020 (p<0.001) nmol/g wet weight. Values with high DADLE, bremazocine and regional ischemia plus IPC (taken at the end of the test ischaemic period) were all unchanged. Cyclic GMP levels were unchanged by IPC, rose with high but not low DADLE, and fell with regional ischemia (with or without IPC). Ratios between the nucleotides, which might have changed in the direction of cyclic GMP (18), were not informative (Table 3).

## Discussion

The major finding of the present study is that DADLE, the selective δ-opioid receptor agonist in a low concentration gave similar protective effects to ischemic preconditioning (IPC) in the isolated rat heart, whereas in a higher concentration the benefit was lessened. The latter effect was mediated by $\kappa_1$-receptor stimulation, as shown by the use of the specific $\kappa_1$-antagonist, nor-binaltorphimine (BNI), and the universal κ-receptor agonist, bremazocine (44). These data lead to our hypothesis that stimulation of these two opioid receptors has opposing effects on myocardial infarct size.

### Role of $\delta_1$-opioid receptors

Previously, extensive work by Schultz and colleagues has shown that: (i) the opioid antagonist naloxone, given just before or just after the trigger ischemic episodes, effectively removed the reduction in infarct size achieved by IPC (25); (ii) morphine mimics the protective effects of IPC by a gliben-

**Table 3** Myocardial cyclic nucleotide contents

| Group | biopsy | n | cyclic AMP nmol $g^{-1}$ wet weight | cyclic GMP nmol $g^{-1}$ wet weight | cyclic AMP/ cyclic GMP ratio |
|---|---|---|---|---|---|
| A. Control | B1 | 10 | 0.654 ± 0.025 | 0.243 ± 0.016 | 2.69 |
| B. IPC | B2 | 7 | 0.543 ± 0.028 | 0.285 ± 0.030 | 1.87 |
| C. DADLE 10 nM | B2 | 8 | 0.485 ± 0.020 | 0.159 ± 0.033 | 3.05 |
| D. DADLE 1 µM | B2 | 5 | 0.566 ± 0.019 | 0.414 ± 0.157 | 1.37 |
| E. Bremazocine 30 nM | B2 | 4 | 0.586 ± 0.049 | 0.178 ± 0.045 | 3.29 |
| F. 35 min ischemia | B3 | 4 | no data | 0.090 ± 0.012 | – |
| G. PC + 35 in ischemia | B3 | 8 | 0.559 ± 0.028 | 0.056 ± 0.002 | 9.98 |

Cyclic nucleotide levels in perfused heart tissue from selected groups at various points in protocols (B for biopsy in Fig. 1).
Column 1 (cyclic AMP): A vs C $p<0.001$ (low concentration DADLE decreases cyclic A level). Other omparisons not significant.
Column 2 (cyclic GMP):
A vs F $p<0.01$ (RI, regional ischemia, drops cyclic GMP level)
B vs F $p<0.001$ (regional ischemia drops cyclic GMP level)
B vs G $p<0.01$ (regional ischemia in presence of PC drops cyclic GMP level)
C vs D $p<0.01$ (high concentration DADLE increases cyclic GMP level)
D vs F $p<0.001$ (regional ischemia in presence of low concentration DADLE decreases cyclic GMP level)
D vs G $p<0.001$ (regional ischemia in presence of low concentration DADLE decreases cyclic GMP level)
PC = preconditioning; RI = regional ischemia. Other abbreviations as in legend to Table 1.

clamide-sensitive mechanism; (iii) ischemic PC and morphine-induced PC both involve δ-receptors (27); and (iv) it is neither µ- nor κ- but $\delta_1$-receptors that are involved in ischemic IPC (28). All of these studies were conducted using the intact rat model, raising the possibility that the opioid receptors involved could be either central or peripheral, and if peripheral, cardiac or non-cardiac in location. Whereas naloxone crosses the blood-brain barrier, its quaternary derivative naloxone methiodide does not. In high doses, the latter compound completely blocked PC indicating that a peripheral rather than central mechanism was involved (29). In demonstrating that the opioid receptors mediating ischemic PC are cardiac as opposed to extracardiac, our study has resolved the remaining important issue concerning the site of action of opioid-mediated cardioprotection in the rat. In contrast to naltrindole, the selective $\kappa_1$-receptor antagonist BNI (21, 44) failed to abolish the anti-necrotic effects of ischemic IPC in the present study. Our finding that IPC was abrogated by naltrindole in the isolated rat heart suggests that the endogenous opioid peptides mediating this protection are derived from the myocardium. Opioid peptides are co-released with catecholamines from autonomic nerve terminals in the heart and vasculature (7). Thus, we propose that IPC triggers the release of opioid peptides from myocytes and/or autonomic nerve terminals which then act directly at cardiac δ-receptors to protect the myocardium against infarction.

Tissue protective effects of δ-opioid receptor activation occur in a number of other situations besides PC. Chien et al. (3) showed that DADLE could extend tissue preservation time in a canine model of multiorgan damage. In addition, Mayfield and D'Alecy (13) demonstrated that activation of $\delta_1$-receptors resulted in an increase in survival time in mice following exposure to severe hypoxia. Thus, it would appear that δ-opioid receptors mediate cytoprotective processes that span the constraints across species and experimental models.

## Cyclic nucleotides

Because of the antiadrenergic effects of opiate stimulation (19), we measured cyclic nucleotides at various critical times during our experimental procedures (Table 3). An increase of cyclic AMP is thought to be detrimental in the setting of myocardial ischemia, whereas a decreased level during the long ischaemic period may be associated with the protection afforded by IPC (11, 24). In general, we could not link our findings on the changes in infarct size to opposite directional changes in cyclic AMP, except for the decreased level of cyclic AMP with low dose DADLE. Nor were measurements of cyclic GMP levels more informative. These levels dropped during prolonged ischemia, and were not increased by IPC. High dose DADLE increased cyclic GMP levels, which should have been protective (18). Bremazocine, which increased infarct size, had no effects on cyclic AMP or cyclic GMP levels, nor on the cyclic AMP to cyclic GMP ratios, even though κ-receptor stimulation decreases forskolin-stimulated cyclic AMP activity (42).

## Other transduction signals

The signal transduction mechanisms involved in PC- or DADLE-mediated protection against infarction were not further investigated in the present study. The cellular mecha-

nisms of IPC-induced protection against infarction in the rat heart have been subject to some controversy. However, current evidence supports a role for $G_{i/o}$ proteins (26), protein kinase C (32), activation of MAP kinases (12) and opening of $K_{ATP}$ channels (22). It is well established that opioid receptors, including cardiac δ-receptors, couple to $G_{i/o}$ proteins upon stimulation (19, 33). It has also been suggested that $\delta_1$-receptor activation results in the opening of $K_{ATP}$ channels (36). Moreover, Schultz et al. (31) found that the infarct-limiting effect of the $\delta_1$-agonist TAN-67 could be abolished by blockade of $K_{ATP}$ channels with glibenclamide, or inhibition of $G_{i/o}$ with pertussis toxin. There is also evidence to suggest that δ-receptors are coupled to the protein kinase C and tyrosine kinase pathways (1, 9), both of which have a proposed involvement in IPC (8, 32, 41). However, whether these pathways contribute to opioid-mediated cardioprotection in the rat heart is not yet established.

## Conflicting data on κ-receptors in preconditioning

DADLE is reported to be a highly potent agonist at δ-opioid receptors (4) although it binds to κ-receptors at micromolar concentrations (43), as supported by our data. Bremazocine is a potent, non-peptide universal agonist at κ-receptors (23), more selective for $\kappa_2$-receptors (44), that increased infarct size. This effect was completely reversed by the selective $\kappa_1$-antagonist, nor-binaltorphimine (NBI).

Data concerning the possible differences between δ- and κ-opioid receptor stimulation in the setting of ischemia-reperfusion appear to be conflicting. On the one hand, activation of $\kappa_1$ receptors in single rat ventricular myocytes with the selective agonist U50,488 results in protein kinase C-dependent mobilization of $Ca^{2+}$ from the sarcoplasmic reticulum (1). During ischemia-reperfusion, such an effect may exacerbate myocardial $Ca^{2+}$ overload and result in extension of necrosis (20) or in reperfusion arrhythmias (6). The κ-agonists, dynorphin and U50-488H, exacerbated ischemic arrhythmias following coronary artery occlusion (10, 37). Moreover, norbinaltorphimine displayed antiarrhythmic activity in the intact rat (14).

Regarding contractile activity, κ-stimulation gives an initial increase in contraction before a decrease, the latter being explained by depletion of the sarcoplasmic reticulum of calcium (35). δ-Stimulation has a similar negative inotropic effect without the biphasic response. Both δ- and κ-receptor stimulation can negatively modulate the β-adrenoceptor activity in the heart (19, 42).

However, none of these data may be directly relevant to effects on ischemic-reperfusion infarct size after IPC, as we measured. In our system, the possible adverse effects of κ-receptor stimulation on infarct size appeared to dominate. Of special interest, κ-receptors, unlike the δ-opioid subtype, are not coupled to the opening of protective $K_{ATP}$ channels thought to play a crucial role in ischemic PC (16, 17). This observation would explain why the κ-antagonist did not abrogate the beneficial effects of δ-stimulation on infarct size in our model nor in that of Schultz et al. (28).

Thus, mechanism of the adverse effects of κ-stimulation in our model are not easy to explain. Concentration-dependent effects have been evoked to explain both pro-and anti-arrhythmic actions, with higher doses acting to increase cell calcium and lower doses, such as below $10^{-6}$ mol/l, to negatively modulate the beta-adrenoreceptor (42). In our studies, however, only 30 nM of the kappa agonist bremazocine increased infarct size, an effect removed by NBI 30 nM.

## Reservations

A major reservation is that we used only one, low concentration of each of the drugs acting on the κ-receptors, bremazocine and NBI. However, we did show that the low concentrations of (30 nM) of bremazocine and NBI had completely opposing effects on infarct size. Thus, we reasoned that a low concentration giving a positive answer would support our hypothesis that adverse effects of high concentrations of DADLE were mediated by the κ-receptors. In general, it is high and not low concentrations of agonists or antagonists that are thought to have non-specific effects. Regarding the cyclic nucleotides, a reservation is that we did not measure these at all time points, but our data were not sufficiently promising to pursue the missing data. A third major reservation is that the signal systems responding to κ-stimulation and mediating its adverse effects were not clarified, and remain the subject for future work.

## Hypothesis: is there an anti-preconditioned state?

Our data raise the possibility that there is an anti-preconditioned state, mediated by κ-receptor stimulation probably involving a “memory” signal system. Such a state, if it exists, may be important in modulating the effects of preconditioning. For example, and again hypothetically, the opioid activation response to acute stress may include induction of a κ-mediated memory effect to lessen or balance the δ-mediated protective effect.

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## References


1. Bian J-S, Zhang W-M, Wong TM (1998) Effects of κ-opioid receptor stimulation of the heart and the involvement of protein kinase C. Br J Pharmacol 124: 600–606
2. Bugge E, Ytrehus K (1995) Inhibition of sodium-hydrogen exchange reduces infarct size in the isolated rat heart – a protective additive to ischaemic preconditioning. Cardiovasc Res 29: 269–274
3. Chien S, Oltegen PR, Diana JN et al. (1994) Extension of tissue survival time in multi-organ block preparation with a delta opioid DADLE ([D-Ala$^2$, D-Leu$^5$]-enkephalin). J Thorac Cardiovasc Surg 107: 964–967
4. Corbett AD, Gillan MGC, Kosterlitz HW et al. (1984) Selectivities of opioid peptide analogs and antagonists at the d-receptor. Br J Pharmacol 83: 271–279
5. Du Toit E, McCarthy J, Miyashiro J et al. (1998) Effect of nitrovasodilators and inhibitors of nitric oxide synthase on ischaemic and reperfusion function of rat isolated hearts. Br J Pharmacol 123: 1159–1167
6. Du Toit EF, Opie LH (1994) Antiarrhythmic properties of specific inhibitors of sarcoplasmic reticulum calcium ATPase in the isolated perfused rat heart after coronary artery ligation. J Am Coll Cardiol 23: 1505–1510
7. Holaday JW (1983) Cardiovascular effects of the endogenous opiate system. Annu Rev Pharmacol Toxicol 23: 541–594
8. Imagawa J, Baxter GF, Yellon DM (1997) Genistein, a tyrosine kinase inhibitor, blocks the "second window of protection" 48 h after ischemic preconditioning in the rabbit. J Mol Cell Cardiol 29: 1885–1893
9. Law PY, McGinn TM, Campbell LE et al. (1997) Agonist activation of δ-opioid receptor but not μ-opioid receptor potentiates fetal calf serum of tyrosine kinase receptor-mediated cell proliferation in a cell-line-specific manner. Mol Pharmacol 51: 152–160
10. Lee AY-S, Chen Y-T, Kan M-N et al. (1992) Consequences of opiate agonist and antagonist in myocardial ischaemia suggest a role of endogenous opioid peptides in ischaemic heart disease. Cardiovasc Res 26: 392–395
11. Lochner A, Genade S, Trompe E et al. (1998) Role of cyclic nucleotide phosphodiesterases in ischemic preconditioning. Mol Cell Biochem 186: 169–175
12. Maulik N, Watanabe M, Zu Y et al. (1996) Ischemic preconditioning triggers the activation of MAP kinases and MAPKAP kinase 2 in rat hearts. FEBBS lett 396: 233–237
13. Mayfield KP, D'Alecy LG (1994) Delta-1 opioid agonist acutely increases hypoxic tolerance. J Pharmacol Exp Ther 268: 683–688
14. McIntosh M, Kane K, Parratt J (1992) Effects of selective opioid receptor agonists and antagonists during myocardial ischaemia. Eur J Pharmacol 210: 37–44
15. Murry CE, Jennings RB, Reimer KA (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischaemic myocardium. Circulation 74: 1124–1136
16. Ocana M, Baeyens JM (1993) Differential effects of $K^+$ channel blockers of antinociception induced by $a_2$-adrenoceptor, GABAB and κ-opioid receptor agonists. Br J Pharmacol 110: 1049–1054
17. Ocana M, Barrios M, Baeyens JM (1996) Cromakalim differentially enhances antinociception induced by agonists of alpha$_2$ adrenoceptors, γ-aminobutyric acid$_B$, alpha, mu and kappa opioid receptors. J Pharmacol Exp Ther 276: 1136–1142
18. Parratt JR (1995) Possibilities for the pharmacological exploitation of ischaemic preconditioning. J Mol Cell Cardiol 27: 991–1000
19. Pepe S, Xiao R, Hohl C et al. (1997) "Cross-talk" between opioid peptide and adrenergic receptor signalling in isolated rat heart. Circulation 95: 2122–2129
20. Piper HM, Garcia-Dorado O, Ovize M (1998) A fresh look at reperfusion injury. Cardiovasc Res 38: 291–300
21. Portoghese PS, Lipowski AW, Takemori AE (1987) Binaltorphimine and nor-binaltorphimine potent and selective kappa opioid receptor antagonists. Life Sci 40: 1287–1292
22. Qian YZ, Levassuer JE, Yoshida K et al. (1996) $K_{ATP}$ channel in rat heart: blockade of ischemic and acetylcholine-mediated preconditioning by glibenclamide. Am J Physiol 271: H23–H28
23. Romer D, Buscher H, Hill RC et al. (1980) Bremazocine: a potent, long-acting opiate kappa-agonist. Life Sci 27: 971–978
24. Sandhu R, Diaz RJ, Mao GD et al. (1997) Ischemic preconditioning. Differences in protection and susceptibility to blockade with single-cycle versus multicycle transient ischemia. Circulation 96: 984–995
25. Schultz JE, Rose E, Yao Z et al. (1995) Evidence for involvement of opioid receptors in ischemic preconditioning in rat hearts. Heart Circ Physiol 37: H2157–H2161
26. Schultz JJ, Hsu AK, Barbieri JT et al. (1998) Pertussis toxin abolishes the cardioprotective effect of ischemic preconditioning in the intact rat heart. Am J Physiol 275: H495–H500
27. Schultz JJ, Hsu AK, Gross GJ (1997) Ischemic preconditioning and morphine-induced cardioprotection involve the delta (δ)-opioid receptor in the intact rat heart. J Mol Cell Cardiol 29: 2187–2195
28. Schultz JJ, Hsu AK, Gross GJ (1998) Ischemic preconditioning in the intact rat heart is mediated by $\delta_1$-but not μ- or κ-opioid receptors. Circulation 97: 1282–1289
29. Schultz JJ, Hsu AK, Gross GJ (1997) Ischemic preconditioning is mediated by a peripheral opioid receptor mechanism in the intact rat heart. J Mol Cell Cardiol 29: 1355–1362
30. Schultz JJ, Hsu AK, Gross GJ (1996) Morphine mimics the cardioprotective effects of ischemic preconditioning via a glibenclamide-sensitive mechanism in the rat heart. Circ Res 78: 100–1104
31. Schultz JJ, Hsu AK, Nagase H et al. (1998) TAN-67, a $\delta_1$-opioid receptor agonist, reduces infarct size via activation of $G_{i/o}$ proteins and $K_{ATP}$ channels. Am J Physiol 274: H909–H914
32. Speechly-Dick ME, Mocanu MM, Yellon DM (1994) Protein kinase C. Its role in ischemic preconditioning in the rat. Circ Res 75: 586–590
33. Standifer KM, Pasternak GW (1997) G proteins and opioid receptor-mediated signalling. Cell Signal 9: 237–248
34. Ventura C, Bastagli L, Bernadi P et al. (1989) Opioid receptors in rat cardiac sarcolemma: effect of phenylephrine and isoprotenerol. Biochim Biophys Acta 987: 69–74
35. Ventura C, Capogrossi MC, Spurgeon HA et al. (1991) Kappa-opioid peptide receptor stimulation increases cytosolic pH and myofilament responsiveness to $Ca^{2+}$ in cardiac myocytes. Am J Physiol 261: H1671–H1674
36. Wild KD, Vanderah T, Mosberg HI et al. (1991) Opioid δ receptor subtypes are associated with different potassium channels.


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Eur J Pharmacol 193: 135–136
37. Wong TM, Lee AY, Tai KK (1990) Effects of drugs interacting with opioid receptors during normal perfusion or ischemia and reperfusion in the isolated rat heart – an attempt to identify cardiac opioid receptor subtype(s) involved in arrhythmogenesis. J Mol Cell Cardiol 22: 1167–1175
38. Wu S, Li Y, Wong TM (1999) Cardioprotection of preconditioning by metabolic inhibition in the rat ventricular myocyte. Circ Res 84: 1388–1395
39. Xia Q, Zhang W, Shen Y et al. (1996) Decreased affinity of kappa receptor binding during reperfusion following ischaemic preconditioning in the rat heart. Life Sci 58: 1307–1313
40. Yellon DM, Baxter GF, Garcia-Dorado D et al. (1998) Ischaemic preconditioning: present position and future directions. Cardiovasc Res 37: 21–33
41. Ytrehus K, Liu Y, Downey JM (1994) Preconditioning protects ischemic rabbit heart by protein kinase C activation. Am J Physiol 266: H1145–H1152
42. Yu X-C, Wang H-X, Pei J-M et al. (1999) Anti-arrhythmic effect of κ-opioid receptor stimulation in the perfused rat heart: involvement of a cAMP-dependent pathway. J Mol Cell Cardiol 31: 1809–1819
43. Zhang WM, Jin WQ, Wong TM (1996) Multiplicity of kappa opioid receptor binding in the rat cardiac sarcolemma. J Mol Cell Cardiol 28: 1147–1154
44. Zhang W-M, Song W, Yu X-C et al. (1999) Effects of U50488 and bremazocine on [$Ca^{2+}$] and cAMP in naive and tolerant rat ventricular myocytes: evidence of kappa opioid receptor multiplicity in the heart. J Mol Cell Cardiol 31: 355–362