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Endocannabinoids protect the rat isolated heart against ischaemia

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1 The purpose of this study was to determine whether endocannabinoids can protect the heart against ischaemia and reperfusion.

2 Rat isolated hearts were exposed to low-flow ischaemia (0.5–0.6 ml min^{-1}) and reperfusion. Functional recovery as well as CK and LDH overflow into the coronary effluent were monitored. Infarct size was determined at the end of the experiments. Phosphorylation levels of p38, ERK1/2, and JNK/SAPK kinases were measured by Western blots.

3 None of the untreated hearts recovered from ischaemia during the reperfusion period. Perfusion with either 300 nM palmitoylethanolamide (PEA) or 300 nM 2-arachidonoylglycerol (2-AG), but not anandamide (up to 1 μM), 15 min before and throughout the ischaemic period, improved myocardial recovery and decreased the levels of coronary CK and LDH. PEA and 2-AG also reduced infarct size.

4 The CB_2-receptor antagonist, SR144528, blocked completely the cardioprotective effect of both PEA and 2-AG, whereas the CB_1-receptor antagonist, SR141716A, blocked partially the effect of 2-AG only. In contrast, both ACEA and JWH015, two selective agonists for CB_1- and CB_2- receptors, respectively, reduced infarct size at a concentration of 50 nM.

5 PEA enhanced the phosphorylation level of p38 MAP kinase during ischaemia. PEA perfusion doubled the baseline phosphorylation level of ERK1/2, and enhanced its increase upon reperfusion. The cardioprotective effect of PEA was completely blocked by the p38 MAP kinase inhibitor, SB203580, and significantly reduced by the ERK1/2 inhibitor, PD98059, and the PKC inhibitor, chelerythrine.

6 In conclusion, endocannabinoids exert a strong cardioprotective effect in a rat model of ischaemia–reperfusion that is mediated mainly through CB_2-receptors, and involves p38, ERK1/2, as well as PKC activation.
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Abbreviations: ACEA, arachidonyl-2′-chloroethylamide; 2-AG, sn-2 arachidonoylglycerol; BCA, bicinchoninic acid; CCP, coronary perfusion pressure; CHO, Chinese hamster ovary; CK, creatine kinase; DMSO, dimethylsulphoxide; DTT, dithiothreitol; EDP, left ventricular end-diastolic pressure; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol *bis*(β-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ERK1/2, extracellurar regulated kinase 1/2; HSP27, heat-shock protein 27; JNK/SAPK, janus kinase/stress-activated protein kinase; JWH015, [2-methyl-1-propyl-1*H*-indol-3-yl]-1-naphthalenylmethanone; K–H, Krebs–Henseleit buffer; LDH, lactate dehydrogenase; MAP kinase, mitogen-activated protein kinase; MAPKAP2/3, MAP kinase-activated protein kinase 2/3; PD98059, 2′-amino-3′-methoxyflavone; PEA, palmitoylethanolamide; PKC, protein kinase C; PMSF, phenylmethylsulphonyl fluoride; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulphinylphenyl)-5-(4-pyridil)1H-imidazole; SDS, sodium dodecyl sulphate; s.e.m., standard error of the mean; TBST, Tris-buffered saline containing 0.1% Tween 20

Introduction

Arachidonoylethanolamide (anandamide) and *sn*-2 arachidonoylglycerol (2-AG) are natural constituents of the plasma membrane that act as CB_1 and/or CB_2 agonists and exhibit pharmacological activity comparable to cannabinoids (Felder & Glass, 1998). Palmitoylethanolamide (PEA), although having low affinity for transfected CB_1 and CB_2 receptors, exerts analgesic effects that are reversed by selective CB_2-receptor antagonists (Lambert & Di Marzo, 1999).

Cannabinoids exert complex cardiovascular effects *in vivo*, some of which being mediated through the sympathetic nervous system (Adams *et al*., 1976; Lake *et al*., 1997). Messenger RNA coding for cannabinoid receptors has been detected in human cardiac tissue (Galiegue *et al*., 1995), and the presence of both CB_1- and CB_2-receptors has recently been confirmed by Western blots in the rat heart (Bouchard *et al*., 2003). Furthermore, PEA and 2-AG have been detected in rat cardiac tissue (Schmid *et al*., 2000). However, little is known about the role played by these endocannabinoids in the heart. It has been reported that the ability of a prior exposure to lipopolysaccharide to limit infarct size in rats is blocked by a CB_2-receptor antagonist (Lagneux & Lamontagne, 2001). A similar contribution of CB_2-receptors in the infarct size-reducing effect of heat stress in rats has been recently

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demonstrated (Joyeux *et al*., 2002). However, until now, it was not known whether cannabinoids exert direct cardioprotective effects. Therefore, the first aim of the present study was to evaluate the cardioprotective effect of endocannabinoids in the rat isolated heart. Secondly, the contribution of protein kinase C (PKC) and mitogen-activated protein kinases (MAP kinases) in this cardioprotective effect was assessed.

Methods

Preparation of hearts

The investigation was performed in accordance with the guidelines from the Canadian Council on Animal Care. The detailed methodology has been described earlier (Bouchard & Lamontagne, 1996; Lagneux & Lamontagne, 2001). Succinctly, male Sprague–Dawley rats (300–350 g) were narcotised by a gradual enrichment of the ambient atmosphere with CO_2 until a complete loss of consciousness and promptly decapitated. Hearts were rapidly excised and mounted on the Langendorff setup and perfused at constant flow by means of a digital peristaltic pump. The flow rate was adjusted to obtain a coronary perfusion pressure of approximately 75 mmHg and was held constant, with the exception of the ischaemic period during which flow was reduced to a value between 0.5 and 0.6 ml min^{-1}. The normal perfusion solution consisted of a modified Krebs–Henseleit (K–H) buffer containing (in mM): NaCl 118, KCl 4, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1, $NaHCO_3$ 24, D-glucose 5, and pyruvate 2, gassed with 95% O_2–5% CO_2 (pH 7.4, 37°C). All drugs (a hundred times the desired final concentration) were administered through a side port of the aortic cannula with syringe pumps at one-hundredth of the coronary flow rate. The turbulent flow created in the reversed-drop-shaped cannula ensured proper mixing of the drugs before entering the aorta. Isovolumetric left ventricular pressure and its first derivative (dP/dt) were measured by a fluid-filled latex balloon inserted into the left ventricle and connected to a pressure transducer. The volume of the balloon was adjusted once during the stabilisation period to obtain a diastolic pressure between 5 and 10 mmHg. The coronary perfusion pressure (CPP) was measured with a pressure transducer connected to another side port of the aortic perfusion cannula. All these data were recorded on a polygraph system (Grass Model 79 polygraph, Astro-Med Inc., Boucherville, QC, Canada).

Experimental protocols

In a first series of experiments, the effect of cannabinoids on functional recovery and biochemical markers of myocardial injury following ischaemia and reperfusion was studied. The hearts in all groups were first subjected to a 20-min stabilisation period, followed by infusion with either SR141716A, a selective CB_1-receptor antagonist (Rinaldi-Carmona *et al*., 1995), SR144528, a selective CB_2-receptor antagonist (Rinaldi-Carmona *et al*., 1998), or K–H buffer. The concentration of SR141716A and SR144528 (1 μM) was selected according to the literature (Randall & Kendall, 1997; Ford *et al*., 2002). After 15 min, infusion with endocannabinoids (PEA, 2-AG, or anandamide, at concentrations of 100, 300, or 1000 nM) or K–H buffer was then started and, 15 min later, hearts were exposed to 120 min of low-flow ischaemia and 20-min reperfusion at the preischaemic flow rate. All drug perfusion lasted throughout the 120-min ischaemic period and was stopped upon reperfusion. In these experiments, coronary effluent samples were collected at the end of the 20-min reperfusion period and stored at −80°C until analysis. Activities of creatine kinase (CK) and lactate dehydrogenase (LDH), two biochemical markers of myocardial infarction, were evaluated with Sigma diagnostic procedures (procedure 520 for CPK and procedure 228-UV for LDH, Sigma-Aldrich, Mississauga, ON, Canada).

In a second experimental series, the effect of cannabinoids on infarct size was studied. Hearts were exposed to a 90-min low-flow ischaemia and 60-min reperfusion to allow the evaluation of infarct size by a staining method. Perfusion with either PEA, 2-AG, the selective CB_1-receptor agonist, ACEA (Hillard *et al*., 1999), the selective CB_2-receptor agonist, JWH015 (Huffman, 2000), or K–H buffer was initiated 15 min before ischaemia, maintained during the entire ischaemic period, and stopped at reperfusion.

In a third experimental series, the contribution of PKC and MAP kinases in the cardioprotective effect of PEA was assessed. After the 20-min stabilisation period, hearts were perfused with either 1 μM chelerythrine, a PKC inhibitor (Herbert *et al*., 1990), 5 μM SB203580, a p38 MAP kinase inhibitor (Cuenda *et al*., 1995), 5 μM PD98059, an ERK1/2 inhibitor (Dudley *et al*., 1995), or K–H buffer. After an additional 15 min stabilisation period, perfusion with PEA was started, following by a 120-min low-flow ischemia and 60-min reperfusion. Additional hearts were treated with either chelerythrine, SB203580, or PD98059 without PEA and exposed to 120-min low-flow ischemia and 60-min reperfusion. Physiological parameters were measured during ischemia and the first 20 min of reperfusion, while infarct size was measured after 60 min of reperfusion. All drug perfusions were stopped upon reperfusion.

In a fourth series of experiments, activation of p38 MAP kinase, ERK1/2, and JNK/SAPK during ischaemia and reperfusion was evaluated by Western blots. After 35 min of stabilisation, hearts were perfused with either PEA or K–H buffer, followed by 120-min low-flow ischaemia and 30-min reperfusion. Hearts (three per group) were collected after either 5, 15, 30, 60, or 120 min of ischaemia, or after 5 or 30 min of reperfusion. Additional hearts were collected during either 15- or 120-min perfusion with PEA or K–H buffer without having been exposed to any ischaemia.

Infarct size determination

Infarct size was determined after 60 min of reperfusion. Atria were removed and the heart was frozen at −80°C for 10 min. It was then cut into 0.6–0.8 mm transverse sections from apex to base (six to seven slices/heart). Once thawed, the slices were incubated at 37°C with 1% triphenyltetrazolium chloride in phosphate buffer (pH 7.4) for 10 min and fixed in 10% formaldehyde solution to distinguish the clearly stained viable tissue from unstained necrotic tissue. Infarct size was determined using a computerised planimetric technique (Scion® image for Windows®) and expressed as a percentage of the total ventricular area which, in a global ischaemia, is equal to the area at risk.

Western blotting

Hearts used to perform Western blots were snap-frozen in liquid nitrogen at the different aforementioned times and kept at −80°C until crushed in a mortar with dry ice in liquid nitrogen. The samples were homogenised on ice with a polytron for 10 s in a lysis buffer containing Tris (pH 7.5) 20 mM, EDTA 1 mM, EGTA 1 mM, β-glycerophosphate 1 mM, NaCl 150 mM, sodium vanadate 1 mM, sodium pyrophosphate 2.5 mM, $MgCl_2$ 4.5 mM, 1,4 dithiothreitol (DTT) 0.5 mM, phenylmethylsulphonyl fluoride (PMSF) 1 mM, Triton X-100 1%, and leupeptin 1 μg ml^{-1}. They were then incubated on ice for 30 min and centrifuged at 12,000 × *g* for 30 min at 4°C. Protein extracts were aliquoted for further experiments and kept at −80°C. Protein quantification was determined by bicinchoninic acid (BCA) assay (Pierce, Rockford, IL, U.S.A.). Protein extracts (20 μg) were separated on a 10% SDS polyacrylamide gel for 90 min and transferred overnight at 4°C to a supported nitrocellulose membrane. After the membrane was blocked for 2 h at room temperature with 5% nonfat dry milk solution in Tris-buffered saline containing 0.1% Tween 20 (TBST), it was probed overnight with either phosphospecific p38 MAP kinase (Thr180/Tyr182) monoclonal antibody 1 : 2000 in 5% nonfat dry milk solution in TBST (New England Biolab #9216, Beverly, MA, U.S.A.), phosphospecific ERK1/2 (p44/42 MAP kinase; Thr202/Tyr204) monoclonal antibody 1 : 2000 in BSA 5% solution in TBST (New England Biolab #9106) or phosphospecific JNK/SAPK (Thr183/Tyr185) monoclonal antibody 1 : 1000 in 5% nonfat dry milk solution in TBST (New England Biolab #9255). Membranes were washed in TBST. They were incubated with anti-mouse IgG horseradish peroxidase-linked antibody (1 : 1000 dilution in 5% nonfat dry milk solution) for 1 h, washed and incubated with Amersham ECL Western blotting detection reagent. Membranes were then stripped in SDS 2%, Tris (pH 6.8) 62.5 mM and 100 mM 2-mercaptoethanol buffer at 60°C for 30 min and reprobed, respectively, with either non-phospho-specific polyclonal p38 MAP kinase antibody 1 : 2000 in 5% nonfat dry milk solution in TBST (New England Biolab #9212), non-phospho-specific polyclonal p44/42 MAP kinase antibody 1 : 2000 in 5% BSA in TBST (New England Biolab #9102), or non-phosphospecific polyclonal JNK/SAPK antibody 1 : 1000 in 5% nonfat dry milk solution in TBST (New England Biolab #9252) for total MAP kinases. An anti-rabbit IgG horseradish peroxidase-linked antibody was used as secondary antibody. Either films or membranes were analysed directly on a Chemilmager 5500 from Alpha Innotech Corporation (San Leandro, CA, U.S.A.). Results were expressed as the proportion of phosphorylated kinase over total kinase, relative (in %) to a group of hearts submitted to 15-min normal K–H perfusion after the stabilisation period (baseline value).

Statistical analysis

Values represent the mean ± s.e.m. Statistical significance of differences between means was evaluated by either one-way (infarct size and biochemical markers) or two-way (functional variables) analyses of variance with either Tukey or Dunnett *post hoc* tests (Systat® for Windows® version 9). Western blot results were compared to the baseline value with one-sample *t*-tests. $P < 0.05$ was considered to be statistically significant.

Drugs

Stock solutions (10 mM) of SR144528 and SR141716A (Sanofi Recherche, Montpellier, France) were prepared in 1 ml 100% dimethylsulphoxide (DMSO), than diluted in water to obtain the desired final concentrations. Anandamide (1 mM, Sigma-Aldrich, Mississauga, ON, Canada) was diluted in 1 ml propylene glycol and 9 ml of K–H buffer. Stock solutions of 2-AG (13.2 mM, Sigma-Aldrich), PEA (16.7 mM, Sigma-Aldrich), ACEA (arachidonyl-2′-chloroethylamide, 5 mg ml^{-1}, Tocris, Ballwin, MO, U.S.A.) and JWH015 ([2-methyl-1-propyl-1*H*-indol-3-yl]-1-naphthalenylmethanone, 5 mg ml^{-1}, Tocris) were prepared in anhydrous ethanol and diluted in K–H buffer to obtain the desired final concentration. PD98059 (2′-amino-3′-methoxyflavone, Calbiochem, La Jolla, CA, U.S.A.) stock solutions were prepared in ethanol. Chelerythrine (Sigma-Aldrich) and SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridil)1*H*-imidazole, Calbiochem) stock solutions were prepared in H_2O. All stock solutions were further diluted in K–H buffer. Ethanol (0.02%) and DMSO (0.02%), at the concentration obtained in the final dilution, had no effect on any of the variables studied.

Results

Effect of endocannabinoids on functional recovery

The baseline values of coronary resistance, left ventricular end-diastolic pressure (EDP), and maximum d*P*/d*t*, measured after treatments but before ischaemia, are shown in Table 1. There was no statistical difference in these values among all groups studied. Maximum d*P*/d*t* decreased rapidly and markedly in all groups of hearts during the ischaemic period (Figures 1 and 2). Left ventricular EDP increased by approximately 20 mmHg after the 120-min low-flow ischaemia (Figures 1 and 2), with no statistical difference among groups. Following reperfusion, maximum d*P*/d*t* remained low and EDP rose rapidly up to 100 mmHg after 2 min of reperfusion in untreated hearts (Figure 1). Treatment with 300 nM of PEA or 2-AG allowed a full recovery of maximum d*P*/d*t* and prevented the increase in EDP during reperfusion (Figures 1 and 2). In the presence of the CB_1-receptor antagonist, SR141716A (1 μM), treatment with PEA still allowed an almost complete recovery of maximum d*P*/d*t* and prevented the increase in EDP upon reperfusion (Figure 1). However, the same CB_1-receptor antagonist halved the beneficial effects of 2-AG on functional recovery (Figure 2). In contrast, treatments with PEA or 2-AG in SR144528-pretreated hearts (1 μM) were unable to prevent the deleterious effect of ischaemia and reperfusion on maximum d*P*/d*t* and EDP (Figures 1 and 2). When given alone (without endocannabinoids), neither SR141716A nor SR144528 had any significant effect on postischaemic ventricular recovery (Figure 3).

In contrast to PEA and 2-AG, anandamide had no effect on maximum d*P*/d*t* recovery and did not prevent the increase in EDP upon reperfusion (Figure 3).

Effect of endocannabinoids on biochemical markers of infarction

The overflow of LDH and CK into the coronary effluent increased markedly in hearts exposed to 120-min low-flow

Table 1 Baseline values measured after treatment, just before ischaemia, for the first series of experiments

Group	n	*Coronary resistance* (mmHg g min ml^{-1})	*EDP* (mmHg)	*max* dP/dt (mmHg s^{-1})
Untreated	6	12.1±0.7	10.8±2.3	1787±57
PEA	5	15.5±1.6	12.0±2.0	1550±77
PEA+SR141716A	5	12.5±1.2	11.9±2.1	1505±53
PEA+SR144528	5	9.2±1.0	8.0±3.0	1540±87
2-AG	6	13.8±1.3	12.9±2.2	1896±239
2-AG+SR141716A	5	13.2±0.6	16.3±1.6	1700±63
2-AG+SR144528	5	10.3±0.8	10.0±0.8	1650±120
Anandamide	4	16.2±1.2	15.0±1.8	1375±144
SR141716A	6	13.0±1.1	11.3±0.9	1775±189
SR144528	6	13.0±1.9	10.5±1.8	1458±61

EDP: left ventricular end-diastolic pressure.

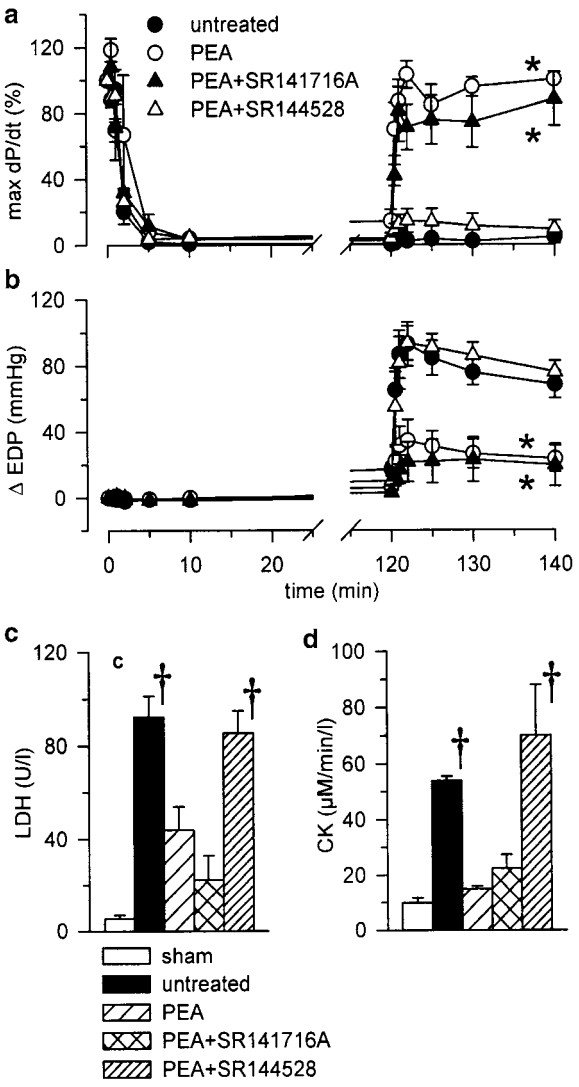

Figure 1 Effects of 300 nM PEA in the absence or presence of the CB_1-receptor antagonist SR141716A or the CB_2-receptor antagonist SR144528 (both at 1 μM) on maximum dP/dt (percentage of baseline value, panel a) and left ventricular end-diastolic pressure (EDP, △ from baseline value, panel b) during the 120-min ischaemia and 20-min reperfusion. Panels (c) and (d) represent, respectively, the coronary effluent activity of LDH and CK at the end of the 20-min reperfusion period. *$P<0.05$ compared with untreated hearts for the entire reperfusion period. †$P<0.05$ compared with sham hearts. The n and baseline values are provided in Table 1.

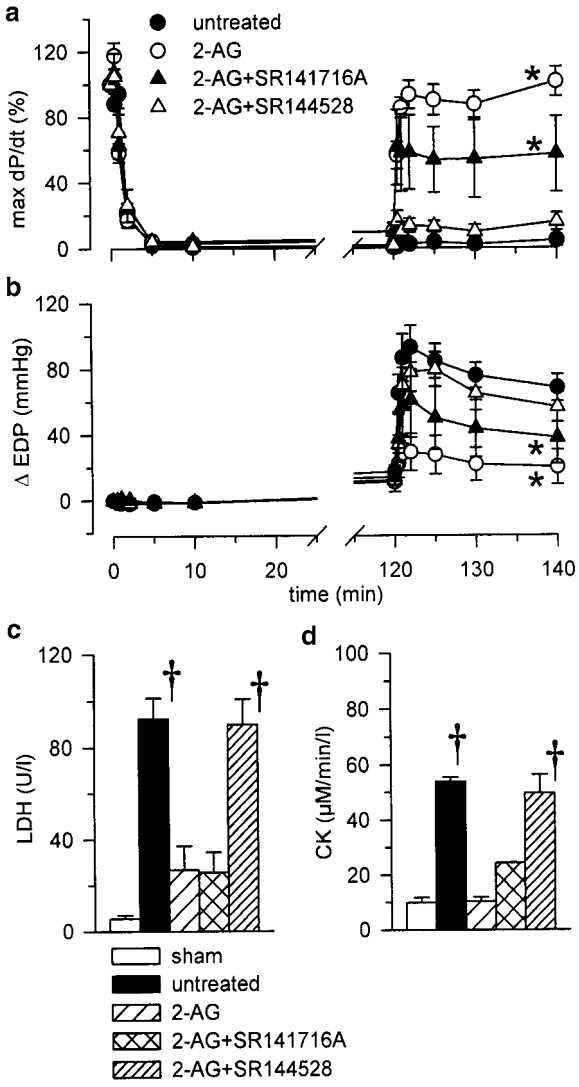

Figure 2 Effects of 300 nM 2-AG in the absence or presence of the CB_1-receptor antagonist SR141716A or the CB_2-receptor antagonist SR144528 (both at 1 μM) on maximum dP/dt (percentage of baseline value, panel a) and EDP (△ from baseline value, panel b) during the 120-min ischaemia and 20-min reperfusion. Panels (c) and (d), represent, respectively the coronary effluent activity of LDH and CK at the end of the 20-min reperfusion period. *$P<0.05$ compared with untreated hearts for the entire reperfusion period. †$P<0.05$ compared with sham hearts. The n and baseline values are provided in Table 1.

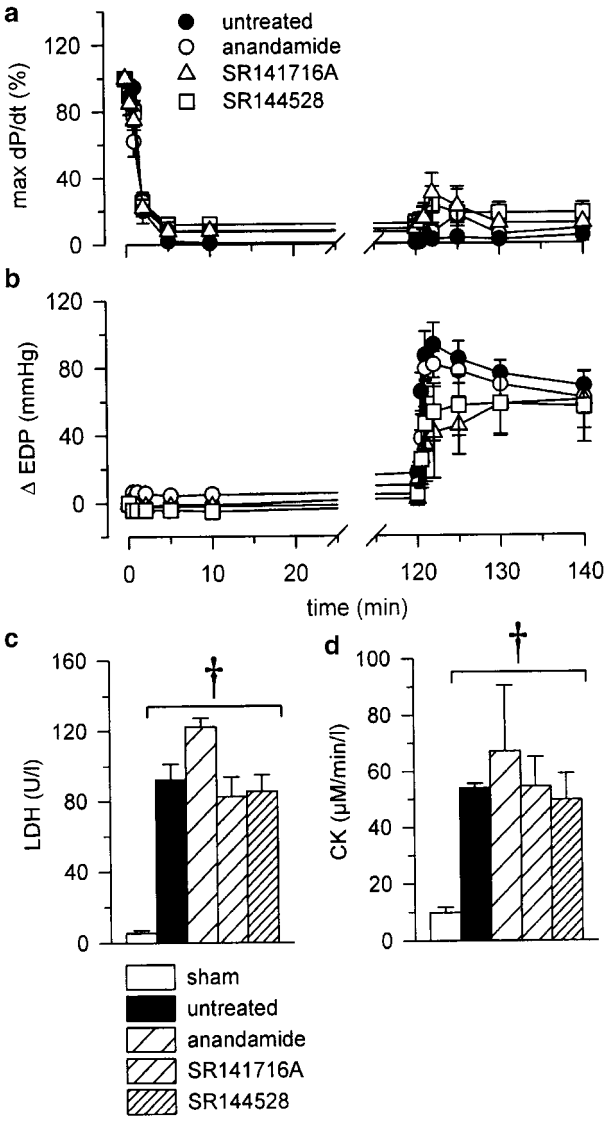

Figure 3 Effects of 1 μM anandamide on maximum dP/dt (percentage of baseline value, panel a) and EDP ($\triangle$ from baseline value, panel b) during the 120-min ischaemia and 20-min reperfusion. The effect of the CB_1-receptor antagonist, SR141716A, and the CB_2-receptor antagonist, SR144528, both perfused at 1 μM without cannabinoid, is also depicted in the figure. Panels (c) and (d) represent, respectively, the coronary effluent activity of LDH and CK at the end of the 20-min reperfusion period. †$P<0.05$ compared with sham hearts. The n and baseline values are provided in Table 1.

ischaemia and 20-min reperfusion, compared with time-matched perfused hearts without ischaemia (Figure 1). Treatment with PEA (Figure 1) or 2-AG (Figure 2) significantly reduced the overflow of both LDH and CK. Similarly to the data on functional recovery, only the CB_2-receptor antagonist, SR144528, blocked the protective effect of PEA (Figure 1) and 2-AG (Figure 2) on LDH and CK leakage from cells, whereas the CB_1-receptor antagonist, SR141716A, had no clear effect. Likewise, in contrast to PEA and 2-AG, anandamide had no effect on LDH and CK overflow upon reperfusion (Figure 3).

Effect of cannabinoids on infarct size

The effect of PEA and 2-AG on infarct size was compared to that of arachidonyl-2′-chloethylamide (ACEA) and JWH015, two selective agonists for CB_1- and CB_2-receptors, respectively. There was no statistical difference in the baseline values of coronary resistance, EDP, and maximum dP/dt, measured after treatment with these cannabinoids (Table 2). In agreement with the results obtained with biochemical markers of infarction, both PEA and 2-AG (300 nM) reduced infarct size, compared with untreated hearts (Figure 4). Two concentrations of ACEA and JWH015 were tested. At the lowest concentration (5 nM), only one-third of ACEA- and JWH015-treated hearts had a small infarct, which widened the scattering of the data and yielded mean values statistically comparable with that of untreated hearts (Figure 4). In contrast, the vast majority of hearts treated with ACEA or JWH015 at the highest concentration (50 nM) had a small infarct, yielding a significantly reduced mean infarct size (Figure 4).

Signalling pathways

The baseline values of coronary resistance, EDP, and maximum dP/dt for this experimental series are shown in Table 3. Similar to the first experimental series, reperfusion of untreated hearts was accompanied by a poor recovery of maximum dP/dt after 20 min of reperfusion and a massive increase in EDP (Figure 5, panels b and d). Likewise, PEA-treated hearts (300 nM) showed a significantly improved recovery of maximum dP/dt at the end of reperfusion and a blunted EDP (Figure 5, panels a and c). Treatment of hearts with the p38 MAP kinase inhibitor, SB203580, alone had no effect on maximum dP/dt recovery, but reduced EDP following reperfusion (Figure 5, panels b and d). However, SB203580 prevented the protection afforded by PEA on both maximum dP/dt and EDP (Figure 5, panels a and c). Similar to SB203580, the PKC inhibitor, chelerythrine, and the ERK1/2 inhibitor, PD98059, had no effect on maximum dP/dt recovery and reduced EDP upon reperfusion (Figure 5, panels b and d). Both chelerythrine and PD98059 halved the PEA-induced protection on maximum dP/dt, the latter being just short of reaching the statistical level of significance (Figure 5, panel a). In contrast, they did not inhibit the effect of PEA on EDP (Figure 5, panel c). None of these signalling pathway inhibitors, administered alone or in combination with PEA, affected the baseline values of coronary resistance, EDP, and maximum dP/dt (Table 3).

Infarct size among the untreated group after 60 min of reperfusion equalled 48 ± 2% of total area (Figure 6). Similar to the second series of experiments, treatment with PEA (300 nM) significantly reduced this value. Infarct size in hearts treated with PEA in the presence of either chelerythrine or PD98059 displayed a wider scattering, with some hearts protected and others not, resulting in mean values not different from either untreated or PEA-treated hearts (Figure 6). In contrast, SB203580 blocked significantly the infarct size reducing effect of PEA (Figure 6). None of the antagonists and inhibitors used in the present study had a significant effect on infarct size when administered alone (Table 4).

Western blot analysis

Western blot was used to assess the phosphorylation level of p38, ERK1/2, and JNK/SAPK (representative blots depicted in Figure 7). The band intensity ratio of the phosphospecific blots over the corresponding non-phosphospecific ones was

Table 2 Baseline values measured after treatment, just before ischaemia, for the second series of experiments

Group	n	*Coronary resistance* (mmHg g min ml^{-1})	*EDP* (mmHg)	*max dP/dt* (mmHg s^{-1})
Untreated	6	11.8±1.0	12.0±3.0	1683±99
ACEA 5 nM	6	13.0±1.8	6.9±2.8	1667±204
ACEA 50 nM	6	11.5±1.7	10.4±3.1	1500±134
JWH015 5 nM	6	9.8±0.9	7.1±1.9	1708±154
JWH015 50 nM	6	10.9±0.4	9.6±2.3	1725±162
PEA	6	12.7±1.3	6.3±1.8	1910±143
2-AG	6	12.1±1.7	10.0±2.7	1988±343

EDP: left ventricular end-diastolic pressure.

Table 3 Baseline values measured after treatment, just before ischaemia, for the third series of experiments

Group	n	*Coronary resistance* (mmHg g min ml^{-1})	*EDP* (mmHg)	*max dP/dt* (mmHg s^{-1})
Untreated	12	11.3±0.9	16.8±1.3	2084±144
PEA	12	11.1±0.4	11.8±3.9	2048±146
PEA + chelerythrine	13	9.1±0.7	12.9±2.2	2054±144
PEA + SB203580	8	9.7±0.7	15.5±5.2	2394±130
PEA + PD98059	10	12.0±1.4	13.8±2.1	2273±194
Chelerythrine	4	12.6±1.5	8.8±3.1	2038±548
SB203580	4	11.5±0.7	10.0±5.8	2225±307
PD98059	4	12.9±2.3	9.4±4.1	2325±442

EDP: left ventricular end-diastolic pressure.

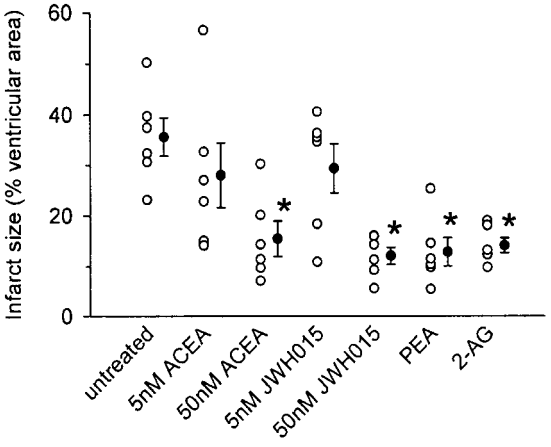

Figure 4 Comparison of the effect of PEA and 2-AG (both at 300 nM) with that of ACEA (5 and 50 nM) and JWH015 (5 and 50 nM), two selective agonists for CB_1- and CB_2-receptors, respectively, on infarct size. The open circles represent individual data, with the corresponding mean±s.e.m. value presented by filled circles. *$P<0.05$, compared with untreated hearts ($n=6$ hearts per group).

expressed relative to the one measured in hearts frozen after 15 min of normal K–H perfusion (defined as baseline values). A 120-min perfusion with normal K–H buffer did not alter the phosphorylation levels of p38, ERK1/2, and JNK/SAPK (Table 5). A simple 120-min perfusion of PEA (300 nM) without ischaemia–reperfusion induced an increase in ERK1/2 phosphorylation compared to baseline, whereas no effect on p38 or JNK/SAPK phosphorylation was observed (Table 5).

Hearts collected after 5 min of ischaemia showed no increase in the phosphorylation level of either p38 or ERK1/2. Phosphorylation levels of p38 increased slightly 15 min after the onset of ischaemia, and remained elevated for the rest of the ischaemic period. Therefore, phosphorylation levels measured from 15 to 120 min of ischaemia were pooled to yield a single representative value. P38 phosphorylation level in untreated ischaemic hearts was short of being statistically significant ($P=0.13$ vs baseline). However, in the PEA-treated group, there was a significant increase in p38 phosphorylation level (Table 5). No significant change was found in ERK1/2 phosphorylation levels during ischaemia, either in the PEA-treated or untreated hearts. Surprisingly, ERK1/2 was significantly more phosphorylated during ischaemia in untreated hearts than in PEA-treated hearts ($P<0.05$). JNK/SAPK showed a significant reduction of phosphorylation levels in the PEA-treated group during ischaemia (Table 5).

Reperfusion was not accompanied by any change in p38 phosphorylation level (Table 5). In contrast, ERK1/2 became strongly phosphorylated during reperfusion in untreated hearts (Table 5). PEA-treated hearts also showed a strong elevation of ERK1/2 phosphorylation level, which was higher than in untreated hearts (Table 5). The JNK/SAPK phosphorylation levels during reperfusion were not statistically different from baseline in either groups (Table 5).

Discussion

In the present study, we have demonstrated that cannabinoids can protect the rat heart from the deleterious effects of ischaemia and reperfusion. Perfusion with 2-AG or PEA, but not anandamide, improved myocardial recovery, decreased the

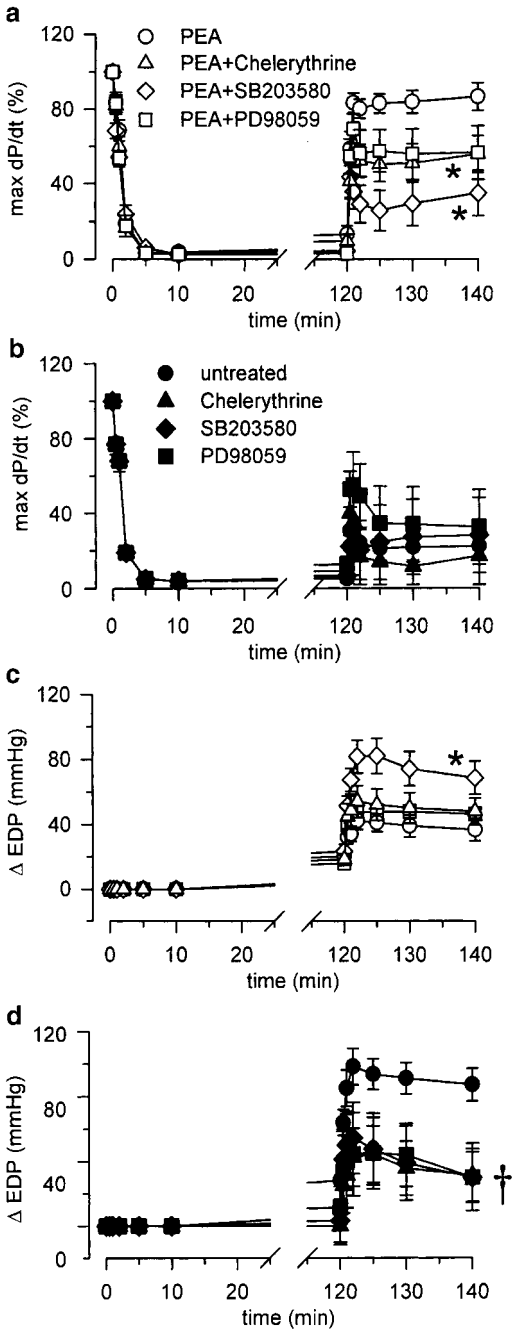

Figure 5 Effects of 300 nM PEA in the absence or presence of either the PKC inhibitor, chelerythrine (1 μM), the p38 MAP kinase inhibitor, SB203580 (5 μM), or the ERK1/2 inhibitor, PD98059 (5 μM) on maximum dP/dt (percentage of baseline value, panel a) and EDP ($\triangle$ from baseline value, panel c) during the 120-min ischaemia and 20-min reperfusion. The effect of chelerythrine, SB203580, and PD98059 alone on maximum dP/dt and EDP is depicted in panels (b) and (d), respectively. *$P<0.05$, compared with PEA-treated hearts. †$P<0.05$ compared with untreated hearts. The n and baseline values are provided in Table 3.

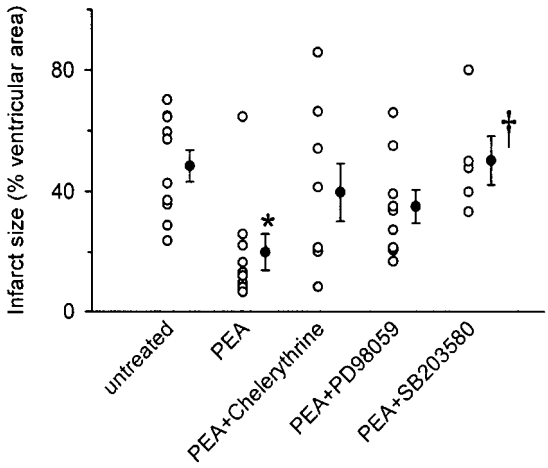

Figure 6 Effects of 300 nM PEA in the absence or presence of either the PKC inhibitor, chelerythrine (1 μM), the p38 MAP kinase inhibitor, SB203580 (5 μM), or the ERK1/2 inhibitor, PD98059 (5 μM) on infarct size. The open circles represent individual data, with the corresponding mean ± s.e.m. value presented by filled circles. *$P<0.05$, compared with untreated hearts. †$P<0.05$ compared with PEA-treated hearts.

Table 4 Effect of the different antagonists and inhibitors on infarct size

	Infarct size (%)	n
Untreated[a]	43 ± 4	16
SR141716A	32 ± 2	6
SR144528	31 ± 3	6
Chelerythrine	37 ± 6	4
SB203580	52 ± 7	4
PD98059	57 ± 7	4

[a]Pooled data from Figures 4 and 6.

levels of CK and LDH, two biochemical markers of ischaemic injury, and reduced infarct size. Although the selective agonist for CB_1-receptors, ACEA, protected the hearts as well as the selective CB_2-receptor agonist, JWH015, the cardioprotective effect of the endogenous cannabinoid, 2-AG, was blocked completely by the CB_2-antagonist and only partially by the CB_1-antagonist. In contrast to 2-AG, PEA acted as selective CB_2-agonist in this model. Involvement of p38, ERK1/2, JNK/SAPK, and PKC in the cardioprotective effect of PEA was also assessed. Using pharmacological tools, an almost total inhibition of the protection on infarct size and functional recovery with the p38 inhibitor, SB203580, was observed. PD98059, the ERK1/2 inhibitor, and chelerythrine, the PKC inhibitor, partially inhibited these effects. PEA was able to activate of ERK1/2 by itself, enhanced the activation of ERK1/2 upon reperfusion, enhanced the activation of p38 during ischaemia, while decreasing that of JNK/SAPK.

A model of low-flow ischaemia was used in the present study. This model has the advantage of allowing a continuous perfusion of cannabinoids in the ischaemic myocardium, ensuring a constant concentration during the entire ischaemic period. This would not have been possible in models of zero-flow ischaemia or regional ischaemia following coronary artery ligation. Some experimental procedures of the present study may potentially influence postischaemic recovery and infarct size. Although the unconscious rats were decapitated before breathing stopped, one cannot rule out that hypoxia following exposure to the CO_2-enriched atmosphere could precondition the hearts. However, alternative methods of

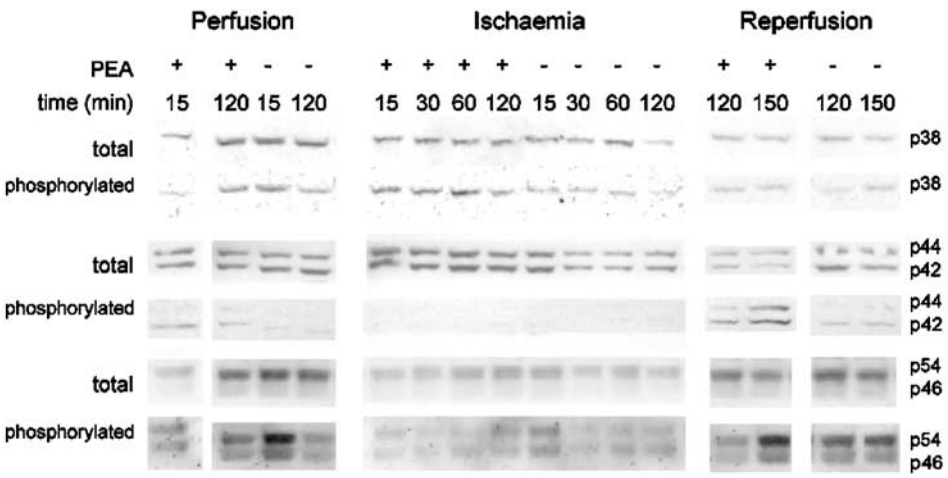

Figure 7 Representative Western blots of phosphorylated and corresponding (after stripping) total p38, p42/44 (ERK1/2), and p46/54 (JNK/SAPK) kinases, either during simple perfusion (without ischaemia), low-flow ischaemia, or reperfusion, in the presence (+) or absence (−) of 300 nM PEA.

Table 5 Phosphorylation level of p38, ERK1/2, and JNK/SAPK kinases

	120-min perfusion	*Ischaemia*	*Reperfusion*
p38 MAP kinase			
Untreated	97±5%	123±14%	123±12%
PEA-treated	110±11%	139±11%*	128±11%
ERK1/2			
Untreated	112±21%	121±13%	387±54%*
PEA-treated	199±20%*	76±14%	953±248%*
JNK/SAPK			
Untreated	107±9%	89±8%	143±23%
PEA-treated	99±12%	65±6%*	142±27%

*$P<0.05$, compared to the baseline value measured after 15 min of normal K–H buffer perfusion and arbitrarily set to 100%.

anaesthesia have limitations of their own, since several anaesthetic agents including narcotics (Schultz *et al*., 1997), barbiturates (Minatoguchi *et al*., 1997), ketamine-xylazine (Walsh *et al*., 1994), and volatile anaesthetics (Cason *et al*., 1997; Toller *et al*., 1999) can either reduce infarct size or interfere with cardioprotective mechanisms. A clear disadvantage of the isolated heart model is the unavoidable ischaemia the heart is exposed to, from the excision to the time the Langendorff perfusion is initiated, which could theoretically induce a preconditioning. However, this period was limited to 30–60 s, which is probably too short to induce a measurable preconditioning (Vegh *et al*., 1992).

Anandamide and 2-AG are both recognised as being endogenous cannabinoids (Felder & Glass, 1998). Therefore, it may appear surprising that anandamide was without effect in the present study. Anandamide can be rapidly taken up by transporters and degraded (Di Marzo, 1999; Piomelli *et al*., 1999). Therefore, we cannot exclude that the perfused anandamide under our experimental conditions is too rapidly eliminated and, therefore, unable to protect the heart. Interestingly, while both PEA and 2-AG are present in the rat heart, anandamide is undetectable in the same tissue (Schmid *et al*., 2000).

To assess the contribution of the two cannabinoid-receptor subtypes in the cardioprotective effect of PEA and 2-AG, selective antagonists were used. SR141716A is a potent and highly selective antagonist for CB_1-receptors, with a K_i value of 2 nM for CB_1-receptors and well above 1 μM for CB_2-receptors (Rinaldi-Carmona *et al*., 1995). Likewise, SR144528 is highly selective for CB_2-receptors, with a K_i values of 0.3 and 437 nM in cell lines expressing either human CB_2- or CB_1-receptors, respectively (Rinaldi-Carmona *et al*., 1998). Therefore, at the concentration used in the present study (1 μM), it is very likely that both antagonists blocked completely their targeted receptors. Although one cannot rule out a partial inhibition of CB_1-receptors with 1 μM SR144528, the contrasting effects observed with the two cannabinoid-receptor antagonists support a high degree of selectivity. Selective agonists for both CB_1- and CB_2-receptors were used as well. ACEA exhibits a K_i of 1.4 nM for CB_1-receptors and over 3000 nM for CB_2-receptors (Hillard *et al*., 1999). The potency and selectivity of JWH015 for CB_2-receptors is slightly less, with K_i values of 13.8 and 383 nM for CB_2- and CB_1-receptors, respectively (Huffman, 2000). Both ACEA and JWH015 reduced infarct size at a concentration of 50 nM, but not 5 nM. This indicates that both CB_1- and CB_2-receptor activation can protect the heart against ischaemia. However, since ACEA is 10 times more potent for its targeted receptor, compared with JWH015, one should expect the former equally effective as the latter at one-tenth the concentration. The fact that both are effective at the same concentration suggests that a higher number of CB_1-receptors need to be activated to produce a comparable protective effect, compared with CB_2-receptors. Alternatively, one cannot rule out the contribution of cannabinoid receptors distinct from CB_1 and CB_2 (Jarai *et al*., 1999; Ford *et al*., 2002) in the cardioprotective effect observed in the present study.

In the present study, PEA was used to study the signalling pathways involved in the cardioprotective effect of cannabinoids, since it was the only endocannabinoid acting through a single CB-receptor subtype. The cardioprotective effects of PEA were blocked by SB203580, which suggest a major role of p38 MAP kinase in these effects. It has been reported that p38 phosphorylation of residue tyrosine 182 alone, as detected by

some antibodies, could not be used as an indicator of p38 activity since phosphorylation of both residues threonine 180 and tyrosine 182 are needed for p38 activation (Nagarkatti & Sha'afi, 1998). Using a phosphospecific monoclonal antibody for p38 phosphorylated on residues threonine 180 and tyrosine 182, we observed that PEA increases p38 phosphorylation only under ischaemic conditions. These results are in agreement with the report that another protective stimulus, ischaemic preconditioning, is accompanied by phosphorylation of p38 after 10 and 20 min of global ischaemia in the rabbit heart (Weinbrenner *et al*., 1997), but not immediately after the preconditioning (Gysembergh *et al*., 2001). In agreement with these results, it has been demonstrated that SB203580 could block the protective effect of ischaemic preconditioning if perfused during ischaemia, whereas it had no effect if perfused only during the preconditioning (Mocanu *et al*., 2000). These results suggest that in the perfused heart, a protective stimulus can trigger a series of events that will lead to p38 phosphorylation only during the subsequent ischaemia. The events downstream of p38 activation leading to cardioprotection are not fully understood, but it is known that p38 activates MAPKAP2/3, which in turn activates HSP27, a key player in cell protection (Landry & Huot, 1995).

Conflicting results on the role of p38 MAP kinase during ischaemia–reperfusion have been reported. First, six isoforms of p38 have been cloned in the human heart: α1, α2, β1, β2, γ, and δ (Sugden & Clerk, 1998). In PC12 cell line, hypoxia caused an increase in phosphorylation of p38 α- and γ-isoforms (Conrad *et al*., 1999). Schneider *et al*. (2001) could not block the protective effect of ischaemic preconditioning with SB202190, a different p38 inhibitor, which is thought to be selective for α- and β-isoforms of p38. In addition, SB203580 has been reported to inhibit, at least partially, the JNK1 kinase in the mouse heart if injected i.p. at a dose of 1 mg kg^{-1} (Tekin *et al*., 2001). Other studies confirm this inhibition *in vitro* (Whitmarsh *et al*., 1997) and *in vivo* (Clerk & Sugden, 1998) at a final concentration of 10 μM (twice the concentration used in the present study). SB203580 was not able to inhibit p38γ activation by hypoxia in cell line (Conrad *et al*., 1999). It is therefore possible that the lack of selectivity of the commercially available antibodies and inhibitors toward the different isoforms contributes to these conflicting results.

In the present study, no increase in JNK/SAPK phosphorylation by either PEA or ischaemia has been observed. Therefore, the inhibition of the cardioprotective effects of PEA by SB203580 cannot be explained by inhibition of JNK/SAPK. Instead, a decrease in JNK/SAPK phosphorylation was observed in PEA-treated heart during ischaemia. Since JNK/SAPK is known to be activated following ischaemic injury (Sato *et al*., 2000), a decrease in injury by PEA could therefore explain this result. Interestingly, TAN-67, a δ_1-opioid receptor agonist and known cardioprotective agent, induced a slight nonsignificant decrease in JNK/SAPK phosphorylation during ischaemia, whereas ischaemic preconditioning showed a significant increase in the same conditions (Fryer *et al*., 2001a). Different mechanisms are to be suspected.

Experiments in CB_2-receptor transfected CHO cells showed that incubation of cells with either CP-55940 or WIN 55212.2 induced an activation of MAP kinase, specifically ERK1/2, through a CB_2-receptor-mediated, G_i/G_0-dependent pathway (Bouaboula *et al*., 1996). These results support our observation of an ERK1/2 phosphorylation in rat hearts perfused with exogenous PEA without any ischaemia.

Activation of ERK1/2 during reperfusion has been reported in numerous studies (Omura *et al*., 1999; Ping *et al*., 1999; Fryer *et al*., 2001b). This activation is enhanced by protective events such as preconditioning and opioid agonists (Fryer *et al*., 2001b). In the present study, PEA enhanced the activation of ERK1/2 during reperfusion. Furthermore, inhibition of ERK1/2 with PD98059 reduced the protective effect of PEA. Interestingly, PD98059 also reduced the protective effect of preconditioning on infarct size (Strohm *et al*., 2000; Fryer *et al*., 2001b). It is well established that PKC is involved in the cardioprotective effect of ischaemic preconditioning (Ping *et al*., 1997; Fryer *et al*., 1999). In the present study, the protective effect of PEA was reduced by the PKC inhibitor, chelerythrine. Therefore, our results show several similarities in the signalling pathway involved in the protective effect of PEA and ischaemic preconditioning.

In conclusion, the data suggest that endocannabinoids afford protection to the rat heart against ischaemia and reperfusion injury. This effect appears to be mediated mainly by CB_2-receptors, and involved PKC, p38, and ERK1/2 activation.

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References

ADAMS, M.D., EARNHARDT, J.T., DEWEY, W.L. & HARRIS, L.S. (1976). Vasoconstrictor actions of delta8- and delta9-tetrahydrocannabinol in the rat. *J. Pharmacol. Exp. Ther.,* **196,** 649–656.

BOUABOULA, M., POINOT-CHAZEL, C., MARCHAND, J., CANAT, X., BOURRIE, B., RINALDI-CARMONA, M., CALANDRA, B., LE FUR, G. & CASELLAS, P. (1996). Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor. Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression. *Eur. J. Biochem.,* **237,** 704–711.

BOUCHARD, J.-F. & LAMONTAGNE, D. (1996). Mechanisms of protection afforded by preconditioning to endothelial function against ischemic injury. *Am. J. Physiol.,* **271,** H1801–H1806.

BOUCHARD, J.-F., LÉPICIER, P. & LAMONTAGNE, D. (2003). Contribution of endocannabinoids in the endothelial protection afforded by ischemic preconditioning in the isolated rat heart. *Life Sci.,* **72,** 1859–1870.

CASON, B.A., GAMPERL, A.K., SLOCUM, R.E. & HICKEY, R.F. (1997). Anesthetic-induced preconditioning: previous administration of isoflurane decreases myocardial infarct size in rabbits. *Anesthesiology,* **87,** 1182–1190.

CLERK, A. & SUGDEN, P.H. (1998). The p38-MAPK inhibitor, SB203580, inhibits cardiac stress-activated protein kinases/c-Jun N-terminal kinases (SAPKs/JNKs). *FEBS Lett.,* **426,** 93–96.

CONRAD, P.W., RUST, R.T., HAN, J., MILLHORN, D.E. & BEITNER-JOHNSON, D. (1999). Selective activation of p38alpha and p38gamma by hypoxia. Role in regulation of cyclin D1 by hypoxia in PC12 cells. *J. Biol. Chem.,* **274,** 23570–23576.

CUENDA, A., ROUSE, J., DOZA, Y.N., MEIER, R., COHEN, P., GALLAGHER, T.F., YOUNG, P.R. & LEE, J.C. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.,* **364,** 229–233.

DI MARZO, V. (1999). Biosynthesis and inactivation of endocannabinoids: relevance to their proposed role as neuromodulators. *Life Sci.,* **65,** 645–655.

DUDLEY, D.T., PANG, L., DECKER, S.J., BRIDGES, A.J. & SALTIEL, A.R. (1995). A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. U.S.A.,* **92,** 7686–7689.

FELDER, C.C. & GLASS, M. (1998). Cannabinoid receptors and their endogenous agonists. *Annu. Rev. Pharmacol. Toxicol.,* **38,** 179–200.

FORD, W.R., HONAN, S.A., WHITE, R. & HILEY, C.R. (2002). Evidence of a novel site mediating anandamide-induced negative inotropic and coronary vasodilatator responses in rat isolated hearts. *Br. J. Pharmacol.,* **135,** 1191–1198.

FRYER, R.M., PATEL, H.H., HSU, A.K. & GROSS, G.J. (2001a). Stress-activated protein kinase phosphorylation during cardioprotection in the ischemic myocardium. *Am. J. Physiol.,* **281,** H1184–H1192.

FRYER, R.M., PRATT, P.F., HSU, A.K. & GROSS, G.J. (2001b). Differential activation of extracellular signal regulated kinase isoforms in preconditioning and opioid-induced cardioprotection. *J. Pharmacol. Exp. Ther.,* **296,** 642–649.

FRYER, R.M., SCHULTZ, J.E., HSU, A.K. & GROSS, G.J. (1999). Importance of PKC and tyrosine kinase in single or multiple cycles of preconditioning in rat hearts. *Am. J. Physiol.,* **276,** H1229–H1235.

GALIEGUE, S., MARY, S., MARCHAND, J., DUSSOSSOY, D., CARRIERE, D., CARAYON, P., BOUABOULA, M., SHIRE, D., LE FUR, G. & CASELLAS, P. (1995). Expression of central and peripheral cannabinoid receptors in human immune tissues and leukocyte subpopulations. *Eur. J. Biochem.,* **232,** 54–61.

GYSEMBERGH, A., SIMKHOVICH, B.Z., KLONER, R.A. & PRZYKLENK, K. (2001). p38 MAPK activity is not increased early during sustained coronary artery occlusion in preconditioned versus control rabbit heart. *J. Mol. Cell Cardiol.,* **33,** 681–690.

HERBERT, J.M., AUGEREAU, J.M., GLEYE, J. & MAFFRAND, J.P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.,* **172,** 993–999.

HILLARD, C.J., MANNA, S., GREENBERG, M.J., DICAMELLI, R., ROSS, R.A., STEVENSON, L.A., MURPHY, V., PERTWEE, R.G. & CAMPBELL, W.B. (1999). Synthesis and characterization of potent and selective agonists of the neuronal cannabinoid receptor (CB1). *J. Pharmacol. Exp. Ther.,* **289,** 1427–1433.

HUFFMAN, J.W. (2000). The search for selective ligands for the CB2 receptor. *Curr. Pharm. Des.,* **6,** 1323–1337.

JARAI, Z., WAGNER, J.A., VARGA, K., LAKE, K.D., COMPTON, D.R., MARTIN, B.R., ZIMMER, A.M., BONNER, T.I., BUCKLEY, N.E., MEZEY, E., RAZDAN, R.K., ZIMMER, A. & KUNOS, G. (1999). Cannabinoid-induced mesenteric vasodilation through an endothelial site distinct from CB1 or CB2 receptors. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 14136–14141.

JOYEUX, M., ARNAUD, C., GODIN-RIBUOT, D., DEMENGE, P., LAMONTAGNE, D. & RIBUOT, C. (2002). Endocannabinoids are implicated in the infarct size-reducing effect conferred by heat stress preconditioning in isolated rat hearts. *Cardiovasc. Res.,* **55,** 619–625.

LAGNEUX, C. & LAMONTAGNE, D. (2001). Involvement of cannabinoids in the cardioprotection induced by lipopolysaccharide. *Br. J. Pharmacol.,* **132,** 793–796.

LAKE, K.D., MARTIN, B.R., KUNOS, G. & VARGA, K. (1997). Cardiovascular effects of anandamide in anesthetized and conscious normotensive and hypertensive rats. *Hypertension,* **29,** 1204–1210.

LAMBERT, D.M. & DI MARZO, V. (1999). The palmitoylethanolamide and oleamide enigmas: are these two fatty acid amides cannabimimetic. *Curr. Med. Chem.,* **6,** 757–773.

LANDRY, J. & HUOT, J. (1995). Modulation of actin dynamics during stress and physiological stimulation by a signaling pathway involving p38 MAP kinase and heat-shock protein 27. *Biochem. Cell Biol.,* **73,** 703–707.

MINATOGUCHI, S., KARIYA, T., UNO, Y., ARAI, M., OHNO, M., HASHIMOTO, K., NISHIDA, Y., WO, D.J. & FUJIWARA, H. (1997). Modulation of cardiac interstitial noradrenaline levels through K(ATP) channels during ischemic preconditioning in rabbits: comparison of the effect of anesthesia between pentobarbital and ketamine + xylazine. *Heart Vessels,* **12,** 294–299.

MOCANU, M.M., BAXTER, G.F., YUE, Y., CRITZ, S.D. & YELLON, D.M. (2000). The p38 MAPK inhibitor, SB203580, abrogates ischaemic preconditioning in rat heart but timing of administration is critical. *Basic Res. Cardiol.,* **95,** 472–478.

NAGARKATTI, D.S. & SHA'AFI, R.I. (1998). Role of p38 MAP kinase in myocardial stress. *J. Mol. Cell Cardiol.,* **30,** 1651–1664.

OMURA, T., YOSHIYAMA, M., , SHIMADA, T., SHIMIZU, N., KIM, S., IWAO, H., TAKEUCHI, K. & YOSHIKAWA, J. (1999). Activation of mitogen-activated protein kinases in *in vivo* ischemia/reperfused myocardium in rats. *J. Mol. Cell Cardiol.,* **31,** 1269–1279.

PING, P., ZHANG, J., CAO, X., LI, R.C., KONG, D., TANG, X.L., QIU, Y., MANCHIKALAPUDI, S., AUCHAMPACH, J.A., BLACK, R.G. & BOLLI, R. (1999). PKC-dependent activation of p44/p42 MAPKs during myocardial ischemia–reperfusion in conscious rabbits. *Am. J. Physiol.,* **276,** H1468–H1481.

PING, P., ZHANG, J., QIU, Y., TANG, X.L., MANCHIKALAPUDI, S., CAO, X. & BOLLI, R. (1997). Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. *Circ. Res.,* **81,** 404–414.

PIOMELLI, D., BELTRAMO, M., GLASNAPP, S., LIN, S.Y., GOUTOPOULOS, A., XIE, X.Q. & MAKRIYANNIS, A. (1999). Structural determinants for recognition and translocation by the anandamide transporter. *Proc. Natl. Acad. Sci. U.S.A.,* **96,** 5802–5807.

RANDALL, M.D. & KENDALL, D.A. (1997). Involvement of a cannabinoid in endothelium-derived hyperpolarizing factor-mediated coronary vasorelaxation. *Eur. J. Pharmacol.,* **335,** 205–209.

RINALDI-CARMONA, M., BARTH, F., HEAULME, M., ALONSO, R., SHIRE, D., CONGY, C., SOUBRIE, P., BRELIERE, J.C. & LE FUR, G. (1995). Biochemical and pharmacological characterisation of SR141716A, the first potent and selective brain cannabinoid receptor antagonist. *Life Sci.,* **56,** 1941–1947.

RINALDI-CARMONA, M., BARTH, F., MILLAN, J., DEROCQ, J.M., CASELLAS, P., CONGY, C., OUSTRIC, D., SARRAN, M., BOUABOULA, M., CALANDRA, B., PORTIER, M., SHIRE, D., BRELIERE, J.C. & LE FUR, G.L. (1998). SR 144528, the first potent and selective antagonist of the CB2 cannabinoid receptor. *J. Pharmacol. Exp. Ther.,* **284,** 644–650.

SATO, M., CORDIS, G.A., MAULIK, N. & DAS, D.K. (2000). SAPKs regulation of ischemic preconditioning. *Am. J. Physiol.,* **279,** H901–H907.

SCHMID, P.C., SCHWARTZ, K.D., SMITH, C.N., KREBSBACH, R.J., BERDYSHEV, E.V. & SCHMID, H.H. (2000). A sensitive endocannabinoid assay. The simultaneous analysis of *N*-acylethanolamines and 2-monoacylglycerols. *Chem. Phys. Lipids,* **104,** 185–191.

SCHNEIDER, S., CHEN, W., HOU, J., STEENBERGEN, C. & MURPHY, E. (2001). Inhibition of p38 MAPK alpha/beta reduces ischemic injury and does not block protective effects of preconditioning. *Am. J. Physiol.,* **280,** H499–H508.

SCHULTZ, J.J., HSU, A.K. & GROSS, G.J. (1997). Ischemic preconditioning and morphineinduced cardioprotection involve the delta (delta)-opioid receptor in the intact rat heart. *J. Mol. Cell. Cardiol.,* **29,** 2187–2195.

STROHM, C., BARANCIK, T., BRUHL, M.L., KILIAN, S.A. & SCHAPER, W. (2000). Inhibition of the ER-kinase cascade by PD98059 and UO126 counteracts ischemic preconditioning in pig myocardium. *J. Cardiovasc. Pharmacol.,* **36,** 218–229.

SUGDEN, P.H. & CLERK, A. (1998). 'Stress-responsive' mitogen-activated protein kinases (c-Jun N-terminal kinases and p38 mitogen-activated protein kinases) in the myocardium. *Circ. Res.,* **83,** 345–352.

TEKIN, D., XI, L., ZHAO, T., TEJERO-TALDO, M.I., ATLURI, S. & KUKREJA, R.C. (2001). Mitogen-activated protein kinases mediate heat shock-induced delayed protection in mouse heart. *Am. J. Physiol.,* **281,** H523–H532.

TOLLER, W.G., KERSTEN, J.R., PAGEL, P.S., HETTRICK, D.A. & WARLTIER, D.C. (1999). Sevoflurane reduces myocardial infarct size and decreases the time threshold for ischemic preconditioning in dogs. *Anesthesiology,* **91,** 1437–1446.

VEGH, A., KOMORO, S., SZEKERES, L. & PARRATT, J.R. (1992). Antiarrhythmic effects of preconditioning in anaesthetised dogs and rats. *Cardiovasc. Res.,* **26,** 487–495.

WALSH, R.S., TSUCHIDA, A., DALY, J.J.F., THORNTON, J.D., COHEN, M.V. & DOWNEY, J.M. (1994). Ketamine–xylazine anaesthesia permits a K_{ATP} channel antagonist to attenuate preconditioning in rabbit myocardium. *Cardiovasc. Res.,* **28,** 1337–1341.

WEINBRENNER, C., LIU, G.S., COHEN, M.V. & DOWNEY, J.M. (1997). Phosphorylation of tyrosine 182 of p38 mitogen-activated protein kinase correlates with the protection of preconditioning in the rabbit heart. *J. Mol. Cell Cardiol.,* **29,** 2383–2391.

WHITMARSH, A.J., YANG, S.H., SU, M.S., SHARROCKS, A.D. & DAVIS, R.J. (1997). Role of p38 and JNK mitogen-activated protein kinases in the activation of ternary complex factors. *Mol. Cell Biol.,* **17,** 2360–2371.

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