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The extracellular regulated kinases (ERK) 1/2 mediate cannabinoid-induced inhibition of gap junctional communication in endothelial cells

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1 Cannabinoids are potent inhibitors of endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxations. We set out to study the mechanism underlying this effect and the possible role of cannabinoid-induced changes in intercellular gap junction communication.

2 In cultured endothelial cells, Δ^9-tetrahydrocannabinol (Δ^9-THC) and the cannabinoid receptor agonist HU210, increased the phosphorylation of extracellular regulated kinases 1/2 (ERK1/2) and inhibited gap junctional communication, as determined by Lucifer Yellow dye transfer and electrical capacity measurements.

3 Δ^9-THC elicited a pronounced increase in the phosphorylation of connexin 43, which was sensitive to PD98059 and U0126, two inhibitors of ERK1/2 activation. Inhibition of ERK1/2 also prevented the Δ^9-THC-induced inhibition of gap junctional communication.

4 Δ^9-THC prevented both the bradykinin-induced hyperpolarization and the nitric oxide and prostacyclin-independent relaxation of pre-contracted rings of porcine coronary artery. These effects were prevented by PD98059 as well as U0126.

5 In the absence of Δ^9-THC, neither PD98059 nor U0126 affected the NO-mediated relaxation of coronary artery rings but both substances induced a leftward shift in the concentration–relaxation curve to bradykinin when diclofenac and N^{ω}nitro-L-arginine were present. Moreover, PD98059 and U0126 prolonged the bradykinin-induced hyperpolarization of porcine coronary arteries, without affecting the magnitude of the response.

6 These results indicate that the cannabinoid-induced activation of ERK1/2, which leads to the phosphorylation of connexin 43 and inhibition of gap junctional communication, may partially account for the Δ^9-THC-induced inhibition of EDHF-mediated relaxation. Moreover, the activation of ERK1/2 by endothelial cell agonists such as bradykinin, appears to exert a negative feedback inhibition on EDHF-mediated responses.

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Abbreviations: CB1, Cannabinoid receptor 1; EDHF, Endothelium-derived hyperpolarizing factor; ERK, Extracellular regulated kinase; HUVEC, Human umbilical vein endothelial cells; MAP kinase, mitogen activated protein kinase; NO, nitric oxide; PCEC, Porcine coronary endothelial cells; PGI_2, prostacyclin; PKC, protein kinase C; Δ^9-THC, Tetrahydrocannabinol

Introduction

Administration of cannabinoids *in vivo* results in hypotension, a response which is partially mediated by central effects and partially by a direct dilatory action on vascular smooth muscle cells (Hillard, 2000). Results from studies using isolated vessels suggest that the latter effect might be mediated by different mechanisms involving endothelium-dependent and -independent pathways (Hillard, 2000).

Although the activation of peripheral cannabinoid (CB) receptors has been reported to elicit the relaxation of isolated blood vessels, cannabinoid-induced relaxation does not appear to be mediated by any of the CB receptors characterized to date. For example, concentrations of cannabinoids exceeding those required to fully activate the CB1 receptor are needed to induce relaxation. Moreover, such responses are largely insensitive to SR141716A, a selective antagonist of the CB1 receptor (Jarai *et al*., 1999). Possible mechanisms underlying this response include metabolism of cannabinoids by cyclo-oxygenase to vasoactive prostaglandins (Fleming *et al*., 1999), as well as inhibitory effects on L-type Ca^{2+}-channels (Gebremedhin *et al*., 1999). It has been suggested that cannabinoids improve gap junctional communication, an effect which might facilitate the diffusion of the endothelium-derived hyperpolarizing factor (EDHF) to smooth muscle cells and facilitate relaxation (Chaytor *et al*., 1999). However, we previously observed that Δ^9-tetrahydrocannabinol (Δ^9-THC), HU210 and the endogenous cannabinoid, anandamide inhibit the EDHF-mediated relaxation and hyperpolarization of porcine and rabbit arteries (Fleming *et al*., 1999).

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In the present study we determined the effect of cannabinoids on EDHF signalling and gap junctional communication in vascular cells and set out to study the underlying signal transduction mechanisms. In particular, we focused on the role of MAP kinases in cannabinoid signal transduction in endothelial cells as the connexin proteins, which form the gap junctional pore, contain potential MAP kinase phosphorylation sides (Zhou *et al*., 1999).

Methods

Materials

Bradykinin was from Bachem Biochemica GmbH (Heidelberg, Germany), PD98059 from Biomol (Hamburg, Germany), caged cAMP and U0126 were from Calbiochem-Novabiochem (Bad Soden, Germany). The connexin 43 (Cx43) monoclonal antibody was from Transduction Laboratories, (Lexington, KY, U.S.A.). The phospho-ERK1/2 (recognizing Thr202/Tyr204) antibodies, as well as the ERK1/2 antibodies were from New England Biolabs Inc (Beverly, MA, U.S.A.). HU210 was from Biotrend (Köln, Germany). SR141716A was a kind gift of Sanofi Pharmaceutical (Berlin, Germany). Δ^9-THC and all other compounds were purchased from Sigma (Deisenhofen, Germany).

Cell culture

Human umbilical vein endothelial cells and porcine coronary artery endothelial cells were isolated and cultured as described (Popp *et al*., 1996).

Determination of gap junctional communication by Lucifer Yellow dye coupling

Sharp microelectrodes were loaded with the fluorescent tracer, Lucifer Yellow (4% in 100 mM lithium chloride) or ethidium bromide (saturated aqueous solution in 100 mM potassium chloride). Glass cover slips containing a confluent endothelial cell monolayer were superfused with a modified Tyrode's solution (mM): NaCl 132, KCl 4, $CaCl_2$ 1.6, $MgCl_2$ 0.98, $NaHCO_3$ 20 NaH_2PO_4 0.36, Glucose 10, Ca^{2+}-EDTA 0.05, containing diclofenac (10 μM) and N^{ω}nitro-L-arginine (L-NA, 300 μM), gassed with 20% O_2, 5% CO_2 and 75% N_2 to give a pO_2 of 140 mmHg and pH 7.4 at 37°C. Cells were impaled with the electrode using a micro-manipulator (5171 Eppendorf, Hamburg, Germany) with the aid of a tickler (IR-283, Neuro Data Instruments, Delaware, PA, U.S.A.), and dye was injected iontophoretically for 30 s (Neurodata). After an additional 60 s, fluorescence was recorded using a fluorescein filter set (excitation 488, emission 512) and a CCD camera (Zeiss Attoflor Ratio Vision). Dye coupling between endothelial cells of a given cell batch was initially determined three times in the absence of a given inhibitor. Thereafter, the inhibitor was applied and dye injections were repeated in the presence of the inhibitor on the same cover slip. This mode of application was chosen as in control experiments, gap junction coupling remained unchanged for at least 90 min.

Determination of gap junctional communication by electrical capacity measurements

Electrical coupling was assessed in clusters of 20–50 endothelial cells superfused with a modified Tyrode's solution containing diclofenac (10 μM) and L-NA (300 μM). Whole cell membrane currents were measured using ruptured patches, with a patch-clamp amplifier (EPC7/EPC9, HEKA Elektronik, Lambrecht, Germany). Patch pipettes were filled with a solution containing (mM): K-aspartate 120, KCl 14, $MgCl_2$ 1, HEPES 10, EGTA 0.1 (pH 7.4) and overlaid with a solution containing (mM): KCl 140, $MgCl_2$ 1, HEPES 10 (pH 7.4). The pipette resistance was between 5–10 MΩ. In the cell-attached mode, the capacitance of the electrode was routinely compensated, and capacitative current transients were elicited by applying a 10 mV (10 ms) voltage step. Changes in the time course of the current transients were used to determine the extent of coupling between cells (Lindau & Neher, 1988; de roos *et al*., 1996). Whole-cell currents were filtered with a low pass filter (1 kHz) and digitized. The area under the transient, which represents the charge accumulated on the membrane condensator, was calculated using specialized software (Pulse, HEKA-Elektronik, Lambrecht, Germany). Since these transients cannot be calculated on the basis of a single exponential function, changes in capacitance are expressed relative to capacitance in unstimulated cells.

Immunoblotting

Cells were lysed in buffer containing Tris/HCl (pH 7.5; 50 mM), NaCl (150 mM), NaF (100 mM), $Na_4P_2O_7$ (15 mM), Na_3VO_4 (2 mM), leupeptin (2 μg ml^{-1}), pepstatin A (2 μg ml^{-1}), Trypsin inhibitor (10 μg ml^{-1}), phenylmethylsulphonyl fluoride (PMSF; 44 μg ml^{-1}) and Triton X-100 (1% v v^{-1}), left on ice for 10 min and centrifuged at 10,000 × *g* for 10 min. Proteins in the Triton X-100-soluble fraction were heated with SDS–PAGE sample buffer and separated by SDS–PAGE, as described (Fleming *et al*., 1998). Proteins were detected using their respective antibodies as described in Results, and were visualized by enhanced chemiluminescence using a commercially available kit (Amersham, Germany).

To re-probe Western blots with alternative primary antibodies the nitrocellulose membranes were incubated at 50°C for 30 min in a buffer containing Tris/HCl (67.5 mM, pH 6.8); β-mercaptoethanol (100 mM) and SDS (2%). After extensive washing in buffer containing Tris (50 mM, pH 7.5) and NaCl (200 mM), the filters were incubated in blocking buffer containing BSA (3%), and subsequently with the primary antibody.

Organ chamber experiments

The main branch of the right porcine coronary artery was dissected free of epicardial fat and cut into rings. Rings were mounted on stainless steel wires connected to force transducers and placed in individual organ chambers containing Krebs buffer of the following composition (in mM): NaCl 119, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, EDTA 0.026, glucose 12, gassed with 95% O_2, 5% CO_2, pH 7.4 at 37°C. Diclofenac (10 μM)

and L-NA (300 μM) was present in some experiments in order to inhibit prostaglandin and NO synthesis, respectively. Passive tension was gradually increased to 8 g. Each ring was challenged twice with K^+-rich Krebs buffer. Precontraction was elicited with the thromboxan analogue U46619, at a concentration adjusted to obtain a similar level of precontraction in each ring (approximately 80% of initial KCl-induced contraction). When a stable contraction plateau was obtained, concentration–relaxation curves were performed to cumulatively increasing concentrations of bradykinin (0.1 nM – 1 μM) in the presence or absence of the CB agonists Δ^9-THC (30 μM) and in the presence or absence of the ERK1/2 kinase inhibitors PD98059 (50 μM) and U0126 (10 μM).

Membrane potential recordings

Segments of porcine coronary arteries were carefully opened longitudinally, pinned to a sylgard base of a heated bath with the intimal side upward and then superfused (5 ml min^{-1}) with a thermostated HEPES Tyrode solution (37°C) of the following composition (in mM): NaCl 140, KCl 4.7, $CaCl_2$ 1.3, $MgCl_2$ 1, HEPES 10, glucose 5 (pH 7.4 with NaOH). The membrane potential was recorded with glass capillary microelectrodes (tip resistance of 80 to 120 MΩ) filled with KCl (3 M) and connected to a high impedance amplifier (intra 767, WPI). Impalements of the smooth muscle cells were performed from the intimal side. Successful impalements were signalled by a sudden negative drop in potential from the baseline (zero potential reference) followed by a stable negative potential for at least 3 min. Bradykinin was applied as bolus injections into the bath to obtain the required concentration. In some experiments the segments were superfused either with HEPES-Tyrode solution or with modified Tyrode bicarbonate solution of the following composition (in mM): NaCl 132, KCl 4, $CaCl_2$ 1.6, $MgCl_2$ 1.2, NaH_2PO_4 0.36, $NaHCO_3$ 23.8, Ca^{2+}-EDTA 0.05, glucose 10 (gassed with 20% O_2/5% CO_2/75% N_2, pH 7.4). All experiments were performed in the presence of L-NA (300 μM), diclofenac (10 μM) and U46619 (0.1 μM).

Connexin 43 metabolic labelling

Endothelial cells were washed in phosphate-free Tyrode's solution and incubated with Tyrode's solution containing $H_2{}^{32}PO_4^-$ (30 μM, 0.125 mCi ml^{-1}) for 8–10 h. Following stimulation, cells were washed twice and lysed in the immunoblotting lysis buffer. Connexin 43 was immunoprecipitated from the supernatant after centrifugation (10 min, 10,000 × *g*): Following pre-clearing of the protein extracts with 50 μl of pansorbin (Calbiochem, Bad Soden, Germany), samples were incubated overnight at 4°C with 4 μg of polyclonal anti-connexin 43. Antibody and antigen/antibody complexes were subsequently recovered by incubation with pansorbin (50 μl, 2 h) followed by extensive washing. Finally, the antibody/pansorbin pellet was boiled in SDS-sample buffer and centrifuged. The supernatant was subjected to SDS–PAGE and transferred to PVDF-membranes (Millipore, Eschborn, Germany). After auto radiography Western blot analysis with a monoclonal connexin 43 antibody was performed.

Statistics

Data are expressed as mean ± s.e.mean and statistical evaluation was performed using Students *t*-test for unpaired data or one-way analysis of variance (ANOVA) followed by a Bonferroni *t*-test, if appropriate. Values of $P<0.05$ were considered statistically significant.

Results

Effects of cannabinoids on gap junctional coupling in endothelial cells

Gap junctional communication, as assessed by electrical capacity measurements in porcine coronary endothelial cells (PCEC), was markedly attenuated by a 15 min incubation with the cannabinoids Δ^9-THC and HU210. The degree of inhibition was similar to that achieved using the gap junction inhibitors 18α-glycyrrhetinic acid (20 μM) and palmitoic acid (20 μM) (Figure 1A,B). Cannabinoids also inhibited the intercellular diffusion of Lucifer Yellow (Figure 1C) and ethidium bromide (data not shown). In the case of Δ^9-THC, the inhibition was reversible, as a 45 min washout period restored gap junctional communication (Lucifer Yellow dye coupling: pre Δ^9-THC: 18.3 ± 2.5 cells, 15 min Δ^9-THC 3 μM: 6.4 ± 0.6 cells, washout 13.1 ± 2.1, $n=12$, $P<0.01$). The inhibition induced by HU210 was irreversible (Lucifer Yellow dye-coupling: pre HU210: 20.0 ± 3.0 cells, 15 min HU210 3 μM: 3.8 ± 0.9 cells, $P<0.01$ washout 1.0 ± 0.0, $n=12$, $P=$ns).

In order to study the involvement of the CB1 receptor, the effect of the CB1 receptor antagonist SR141716A (10 μM) (Howlett *et al*., 1998) on Δ^9-THC-induced inhibition of coupling was investigated. While SR141716A alone had no effect on Lucifer Yellow dye coupling (CTL: 31 ± 3.7 cells *vs* SR141716A: 33 ± 3.0 cells, $n=9$, $P=$ns), it partially prevented the Δ^9-THC-induced inhibition of coupling (Δ^9-THC-induced inhibition without SR141716A 62 ± 6%, with SR141716A 35 ± 9%, $n=12$, $P=0.02$).

Effects of cannabinoids on the phosphorylation of connexin 43

Since the open probability and/or permselectivity of gap junctions can be modulated by the specific phosphorylation of connexin proteins forming the connexon pore, we studied the effect of cannabinoids on the phosphorylation state of connexin 43.

Exposure of endothelial cells to Δ^9-THC-induced a pronounced increase in the incorporation of ^{32}P into connexin 43 as determined by autoradiography of immunoprecipitated connexin 43, as well as a shift in SDS–PAGE. Connexin 43 phosphorylation was prevented by the inhibitor of ERK1/2 activation PD98059, indicating that Δ^9-THC-induced phosphorylation of connexin 43 may be mediated by the activation of ERK1/2 (Figure 2A). Indeed, Δ^9-THC as well as HU210 transiently increased ERK1/2 phosphorylation. Enhanced phosphorylation of ERK1/2 was apparent within 5 min of the addition of either Δ^9-THC or HU210, reached a maximum after approximately 30 min and returned to control levels after 60 min. Cannabinoid-induced activation

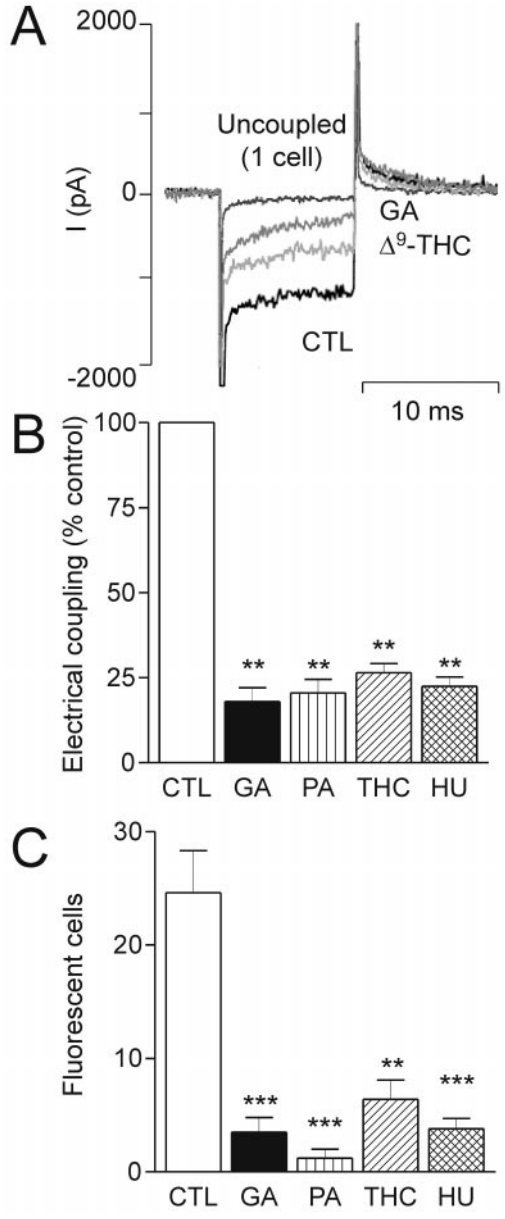

Figure 1 Effect of cannabinoids on the intercellular coupling between endothelial cells. (A) Original tracing showing electrical capacity measurements in cultured porcine coronary endothelial cells. Capacitative current transients (depicted) were elicited by applying a 10 mV (10 ms) voltage step and recording the resulting current. Experiments were performed in the absence (CTL) and presence of 18α-glycyrrhetinic acid (GA, 30 μM) and Δ^9-THC (30 μM). Current measurements on a single isolated cell (one cell) are provided as example for complete uncoupling (B). Statistical analysis showing the effect of Δ^9-THC (30 μM) HU210 (30 μM), 18α-glycyrrhetinic acid (30 μM, aGA) and palmitoic acid (PA, 10 μM) on electrical capacity measurements in cultured porcine coronary endothelial cells. (C) Effects of Δ^9-THC (30 μM), HU210 (30 μM), 18α-glycyrrhetinic acid (30 μM, aGA) and palmitoic acid (PA, 10 μM) on the transfer of Lucifer Yellow between cultured human umbilical vein endothelial cells. The data shown represent results obtained in at least five independent experiments, compounds were applied 15 min prior to the determination of intercellular coupling, $^{**}P<0.01$, $^{***}P<0.001$ (by ANOVA).

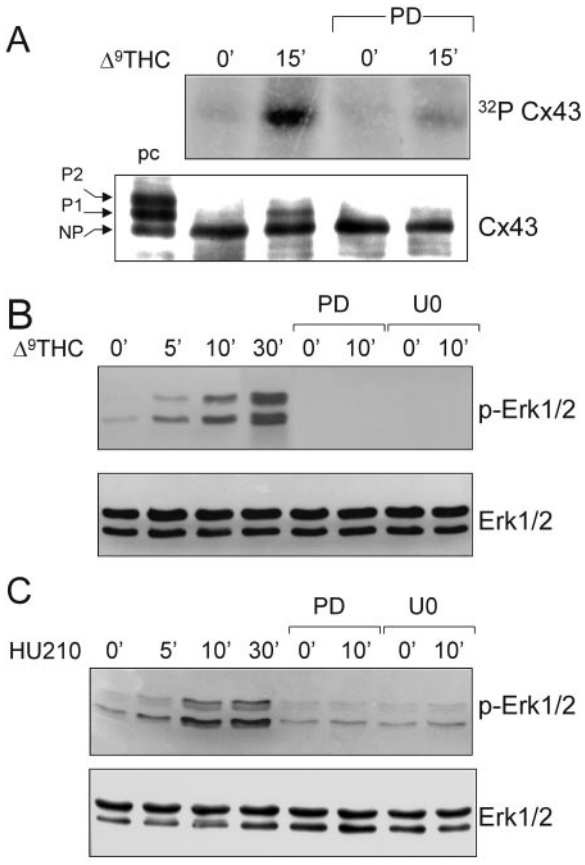

Figure 2 Effect of cannabinoids on the phosphorylation of connexin 43 and ERK 1/2 in human endothelial cells. (A) Autoradiography of connexin 43 (Cx43) immunoprecipitates (top) and Western blot (bottom) showing changes in the incorporation of ^{32}P into Cx43 following stimulation with Δ^9-THC (10 μM, 15 min) in the absence and presence of PD98059. (B and C) Western blot analysis showing the time course of the ERK1/2 phosphorylation elicited by either Δ^9-THC (10 μM, B) or HU210 (10 μM, C). Experiments were performed in the absence and presence of PD98059 (PD, 50 μM) and U0126 (U0, 10 μM); pc denotes positive control for phosphorylated Cx43. Similar results were obtained in three additional experiments.

of ERK1/2 was completely prevented by U0126 (10 μM) as well as by PD98059 (50 μM, Figure 2B,C).

Effects of protein kinase inhibitors on the cannabinoid-induced inhibition of cell coupling

To determine whether or not the ERK1/2-mediated phosphorylation of connexin 43 was responsible for the Δ^9-THC-induced inhibition of gap junctional communication, cell coupling experiments were performed in the presence of PD98059 and U0126. Both substances prevented the Δ^9-THC-induced inhibition of gap junctional communication (Figure 3), whereas inhibition of p38 MAP kinase with SB203580 (20 μM), and blockade of protein kinase C using Ro31-8220 (0.3 μM) had no effect (data not shown). cAMP released by flash photolysis of a cage compound, increased gap junction coupling ($+90.2\pm7.05\%$; $P<0{,}05$, $n=6$). However, following pretreatment with Δ^9-THC, cAMP failed to restore or improve gap junction coupling ($-21.4\pm8\%$, $P=$ ns, $n=5$).

Effects of ERK1/2 inhibition on cannabinoid-induced inhibition of EDHF-mediated responses

In the presence of U46619, diclofenac and L-NA, Δ^9-THC significantly inhibited the bradykinin-induced, EDHF-

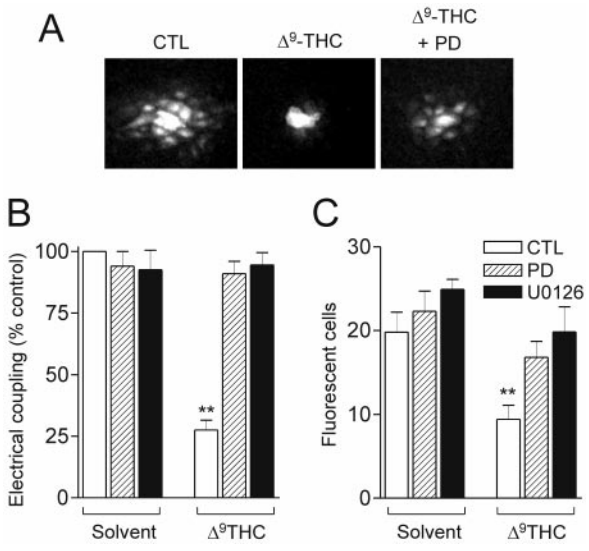

Figure 3 Involvement of ERK1/2 in the Δ^9-THC-mediated inhibition of gap junctional communication. (A) Original recordings showing the effect of PD98059 (50 μM) on the Δ^9-THC-induced inhibition of dye coupling in human endothelial cells. (B and C) Statistical summary of the effects of PD98089 or U0126 on gap junctional communication assessed as either changes in electrical capacity in porcine coronary endothelial cells (B) or changes in dye transfer between human endothelial cells (C). Experiments were performed in the presence and absence of Δ^9-THC (10 μM). The data shown represent results obtained in at least five independent experiments, $^{**}P<0.01$ (by ANOVA).

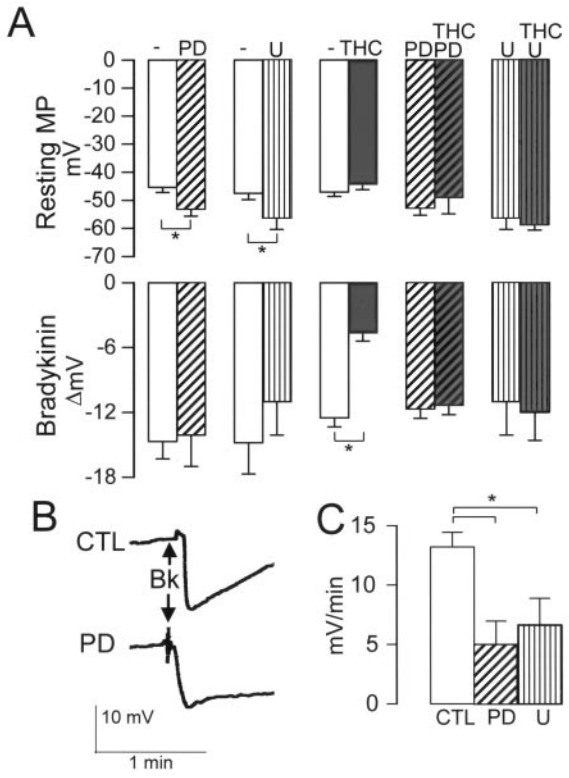

Figure 4 Effects of Δ^9-THC on the resting membrane potential and the bradykinin-induced hyperpolarization of porcine coronary arteries. (A) The resting membrane potential (resting MP, upper panel) and the bradykinin (1 μM)-induced hyperpolarization (ΔmV, lower panel) of porcine coronary smooth muscle cells in the continuous presence of U46619 (0.1 μM), diclofenac (10 μM) and N^{ω}-nitro-L-arginine (300 μM). Experiments were performed under control conditions (CTL) and in the presence or absence of Δ^9-THC (10 μM), PD98059 (PD, 50 μM) and U0126 (U, 10 μM) (B and C) Original tracing (B) and statistical analysis (C), showing the effect of preventing MAP kinase activation on the repolarization speed of vascular smooth muscle cells following the bolus application of bradykinin (Bk, 1 μM). The data shown summarize results obtained in five independent experiments, $^{*}P<0.05$ (t-test).

mediated hyperpolarization of porcine coronary arteries. When either PD98059 or U0126 were included, Δ^9-THC failed to inhibit the EDHF-mediated hyperpolarization of vascular smooth muscle cells (Figure 4A). The resting membrane potential was not affected by Δ^9-THC on its own. PD98059 and U0126 both induced a significant decrease in the resting membrane potential but did not affect the magnitude of the bradykinin-induced hyperpolarization.

EDHF-mediated hyperpolarizations and relaxations are usually transient. We therefore determined whether or not inhibition of ERK1/2 affected the time course of EDHF-induced effects. In porcine coronary arteries, PD98059 as well as U0126 significantly prolonged the repolarization phase of the smooth muscle response to the bolus application of bradykinin (Figure 4B,C).

The bradykinin-induced, EDHF-mediated relaxation of porcine coronary artery rings was attenuated by Δ^9-THC (Figure 5A), as described previously (Fleming *et al*., 1999), but was unaffected by the adenylyl cyclase inhibitor 2′-5′-dideoxyadenosine (Figure 5D). In the absence of L-NA and diclofenac, Δ^9-THC had no effect on either the bradykinin-induced NO-mediated relaxation or the relaxation induced by cromakalim (data not shown). The inhibition of EDHF-mediated relaxation by Δ^9-THC was prevented by pretreatment with PD98059 and U0126. In the presence of L-NA but absence of Δ^9-THC, PD98059 (Figure 5A) and U0126 (Figure 5B) induced a significant leftward shift in the concentration–relaxation curve to bradykinin. In the absence of L-NA, however, neither compound affected the bradykinin-induced relaxation (Figure 5C).

Discussion

The results of the present study demonstrate that the cannabinoids, Δ^9-THC and HU210, are potent inhibitors of interendothelial gap junctional communication as well as the EDHF-mediated hyperpolarization and relaxation of porcine coronary arteries. The molecular mechanism underlying cannabinoid-mediated inhibition of gap junctional communication appears to involve the phosphorylation of connexins, such as connexin 43, by ERK1/2.

Gap junctions are aqueous pores connecting adjacent cells, and are formed by the association of connexin proteins in a classical pentalaminar structure. Of the gap junctions in a gap junctional plaque, about 80% are thought to be 'open', thus facilitating the transfer of electrical and chemical signals (molecular mass <1 kDa) between adjacent cells (Dhein, 1998). Numerous intracellular events such as changes in intracellular pH, membrane potential, and connexin phosphorylation can however enhance or decrease the passage of ions and small molecules through the connexon pore (permselectivity of connexons) (Christ *et al*., 1996). Several different

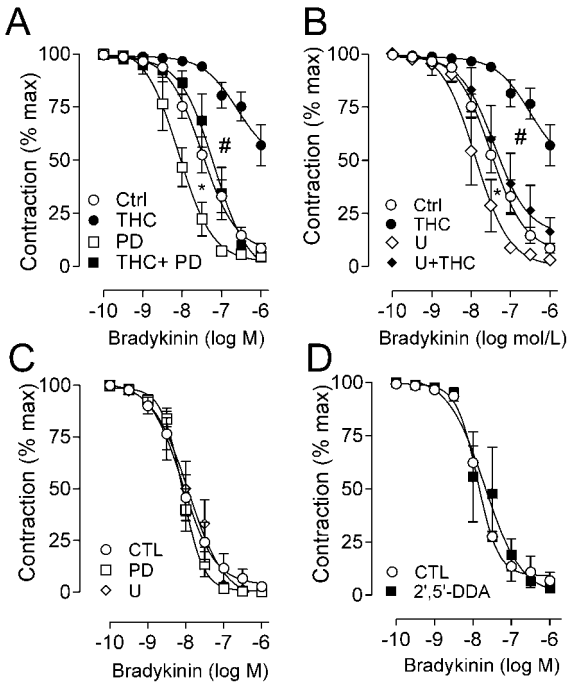

Figure 5 Effects of Δ^9-THC and 2′,5′-dideoxyadenosine on the EDHF- and NO-mediated relaxation of pre-contracted rings of porcine coronary artery. Concentration–relaxation curves to bradykinin (A) in the absence and presence of Δ^9-THC (30 μM) and in the absence and presence of PD 98059 (50 μM), (B) in the absence and presence of Δ^9-THC (30 μM) and in the absence and presence of U0126 (10 μM) (C) in the absence and presence of PD98059 and U0126 and (D) in the absence and presence of 2′,5′-dideoxyadenosine (50 μM). Experiments were performed in the continuous presence of diclofenac (10 μM) and in the absence (C) and presence (A, B and D) of N^{ω}-nitro-L-arginine (300 μM). The data shown summarize results obtained in 8–10 independent experiments, $\#P<0.05$ THC *vs* THC+inhibitor of ERK1/2 activation; $*P<0.05$ CTL *vs* CTL+inhibitor of ERK1/2 activation; ANOVA for repeated measurements.

kinases have been shown to phosphorylate connexin protein (Lampe & Lau, 2000). Protein kinase (PK) A has been suggested to phosphorylate connexin 43 and to increase gap junction permeability to dyes such as Lucifer Yellow *via* translocation of the connexin to the plasma membrane (Lampe & Lau, 2000). Phosphorylation of specific amino acid residues by Src (Tyr^{247} and Tyr^{265}) (Lin *et al*., 2001), PKC (Ser^{268}) (Lampe *et al*., 2000), and ERK1/2 (Ser^{279}/Ser^{282}) (Zhou *et al*., 1999), on the other hand, prevent dye transfer and are thought to promote gap junction uncoupling.

Homocellular gap junctional communication between endothelial cells and smooth muscle cells and heterocellular communication between endothelial and smooth muscle cells has been suggested to play an important role in the regulation of vascular tone, especially in the phenomena of ascending dilatation (de Wit *et al*., 2000; Emerson & Segal, 2000) and NO/PGI_2-independent, or EDHF-mediated, relaxation (Chaytor *et al*., 1998; Brandes *et al*., 2000). The role of gap junctions in establishing an electrical syncytium between endothelial and smooth muscle cells has been demonstrated most conclusively in small resistance arteries and arterioles (de Wit *et al*., 2000; Coleman *et al*., 2001). However, in some large arteries, gap junctions seem to play an important role in the transfer of hyperpolarization between smooth muscle cells if not from endothelial to smooth muscle cells (Edwards *et al*., 1999). The fact that the NO/PGI_2-independent relaxation elicited by receptor-dependent agonists such as acetylcholine or bradykinin was reported to be sensitive to gap junction blockers as well as small peptides (gap peptides) which disrupt the assembly of gap junctional plaques (Chaytor *et al*., 1998), led to the proposal that gap junctions are involved in the phenomenon of EDHF-mediated responses.

In the present study, we observed that the cannabinoids Δ^9-THC and HU210 inhibited electrical communication as well as the transfer of Lucifer Yellow between endothelial cells. The effects of Δ^9-THC were reversible upon washout, but could only be partially prevented by the CB1 receptor antagonist SR141716A, suggesting that the Δ^9-THC-mediated inhibition of gap junctional communication was at least in part mediated *via* CB1 receptor-independent effects. Indeed, the inhibition of EDHF-mediated responses in several different arteries by Δ^9-THC and HU210 was observed only when high concentrations (greater than 10 μM), were employed i.e., much higher than those reported to selectively activate CB receptors. Many of the effects observed in response to cellular stimulation with cannabinoids are reportedly receptor-independent. For example, high concentrations of CB agonists inhibit inositol trisphosphate formation (Nah *et al*., 1993), and elicit the release of intracellular calcium (Felder *et al*., 1992; 1993) as well as the liberation of arachidonic acid (Howlett, 1995). One proposed explanation for these receptor-independent effects is that the cannabinoids incorporate into the membrane and directly modulate the activity of integrated membrane proteins such as $G\alpha_i$ (Bayewitch *et al*., 1995). Independently of whether or not the effects of Δ^9-THC and HU210 on gap junctional communication occurred *via* a mechanism other than CB1 receptor activation, these effects were selectively and completely prevented by substances which inhibit MAP kinase kinase (MEK) and therefore the activation of the MAP kinases such as ERK1/2, but were unaffected by inhibitors of PKC or the p38 MAP kinase. Therefore, Δ^9-THC-induced activation of ERK1/2 appears to directly mediate the Δ^9-THC-induced inhibition of interendothelial communication. However, it is currently not possible to rule out a role for ERK5 in the phosphorylation of endothelial cell connexins and the regulation of gap junctional coupling.

Cannabinoids have previously been reported to activate ERK1/2 in various different cell types (Bouaboula *et al*., 1996; Wartmann *et al*., 1995), and in the present study we observed that the time course of the cannabinoid-induced inhibition of gap junction coupling was mirrored by the phosphorylation of Cx43, which was the most predominant connexin expressed in the endothelial cells studied. Both the incorporation of ^{32}P into Cx43 as well as its mobility shift in SDS–PAGE were prevented by MEK inhibitors which also prevent the cannabinoid-induced activation of ERK1/2.

The role, if any, played by gap junctions in the regulation of coronary artery tone is unclear. For example, although 'classic' gap junction inhibitors do not affect the EDHF-mediated relaxation of coronary artery rings (Beny & Schaad, 2000), the agonist-induced hyperpolarization of coronary artery smooth muscle is reported to be disrupted by interfering with gap junctional communication (Edwards *et al*., 1999). The results of the present investigation clearly

show that Δ^9-THC inhibits the bradykinin-induced, EDHF-mediated hyperpolarization and relaxation of coronary artery rings. Because this Δ^9-THC-mediated inhibition of EDHF responses was also sensitive to agents which prevent the activation of ERK1/2, it is tempting to speculate that the gap junction uncoupling actions of Δ^9-THC can also account for these phenomena. In the absence of Δ^9-THC, PD98059 and U0126 improved the bradykinin-induced EDHF-mediated relaxation of porcine coronary arteries. This observation can most likely be attributed to the fact that bradykinin activates ERK1/2 in endothelial cells (Fleming *et al*., 1995) and implies that the agonist-induced activation of this kinase functionally antagonizes EDHF-mediated relaxation. There was however a significant difference in relaxations observed in rings pretreated with PD98059/U0126 alone or PD98059/U0126 together with Δ^9-THC. Although neither inhibitor affected the EDHF-mediated relaxation in response to substance P which is a weak stimulator of ERK1/2 phosphorylation (Fleming, unpublished observation), we cannot rule out the possibility that Δ^9-THC attenuates EDHF-mediated relaxation by an additional, as yet unidentified MAP kinase-independent mechanism.

The signalling events targeted by Δ^9-THC are apparently specific to EDHF since neither the NO-mediated relaxation nor that elicited by cromakalim were affected by Δ^9-THC, PD98059 or U0126. Such observations would tend to rule out interference between Δ^9-THC and the activation of the B_2 kinin receptor and the subsequent release of intracellular calcium. Moreover, inhibition of cAMP production as a consequence of CB1 receptor activation (Howlett *et al*., 1986; Bouaboula *et al*., 1995; 1996) is unlikely to explain the actions of Δ^9-THC on EDHF-mediated changes in membrane potential and tone since the adenylyl cyclase inhibitor 2′,5′-dideoxyadenosine had no effect on the bradykinin-induced EDHF-mediated relaxation. It has been suggested that cannabinoids elicit the release of EDHF from rat mesenteric arteries (Jarai *et al*., 1999). However, as reported previously (Fleming *et al*., 1999), the Δ^9-THC and HU210-induced relaxation in rabbit mesenteric and porcine coronary arteries are endothelium-independent. Moreover, in the present study, in the continuous presence of L-NA and diclofenac, we did not observe any direct effects of Δ^9-THC on the membrane potential of endothelium-intact porcine coronary arteries.

Inhibition of ERK1/2 using PD98059 and U0126, induced a significant hyperpolarization of smooth muscle cells in porcine coronary arteries and prolonged the duration of the repolarization phase observed following exposure to bradykinin. This observation suggests that ERK1/2 may be at least partially responsible for the transient nature of the EDHF-induced hyperpolarization, and that the effectors of EDHF-mediated hyperpolarization such as potassium channels and/or the Na-K-ATPase can be modulated by ERK1/2. Whether or not ERK1/2 directly phosphorylates these proteins, as demonstrated for voltage-dependent potassium channels in the brain (Adams *et al*., 2000; Ekinci *et al*., 1999), remains to be determined.

In summary, we have demonstrated that cannabinoids inhibit gap junctional communication in endothelial cells and impair the bradykinin-induced EDHF-mediated hyperpolarization and relaxation of porcine coronary artery rings. These effects are mediated by a cannabinoid-induced activation of ERK1/2 and subsequent phosphorylation of connexins and proteins involved in EDHF-mediated responses. Moreover, the activation of ERK1/2 partially determines the time course of the EDHF-mediated hyperpolarization of porcine coronary artery smooth muscle cells.

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