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Signaling pathway associated with stimulation of CB2 peripheral cannabinoid receptor

Involvement of both mitogen-activated protein kinase and induction of Krox-24 expression

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Cannabinoids, known for their psychoactive effects, also possess immunomodulatory properties. The recent isolation and cloning of the G-protein-coupled peripheral cannabinoid receptor (CB2), mainly expressed in immune tissues, have provided molecular tools to determine how cannabinoid compounds may mediate immunomodulation. We here investigated the CB2 signaling properties using stably transfected Chinese hamster ovary cells expressing human CB2. First, we showed that stimulation by a cannabinoid agonist activated mitogen-activated protein (MAP) kinase in time- and dose-dependent manners. The rank order of potency for MAP kinase activation of cannabinoid agonists correlated well with their binding capacities. Second, we demonstrated that, following MAP kinase activation, cannabinoids induced the expression of the growth-related gene Krox-24, also known as NGFI-A, *zif*/268, and *egr*-1. Pertussis toxin completely prevented both MAP kinase activation and Krox-24 induction, even more these responses appeared to be dependent of specific proteine kinase C isoforms and independent of inhibition of adenylyl cyclase. A similar coupling of CB2 to a mitogenic pathway and to the regulation of Krox-24 expression was also observed in human promyelocytic cells HL60. Taken together, these findings provide evidence for a functional role of the CB2 receptor in gene induction mediated by the MAP kinase network.

Keywords: peripheral cannabinoid receptor; cannabinoid receptor CB2; mitogen-activated protein kinase; Krox-24; cannabinoid.

Δ^9-Tetrahydrocannabinol, the major active component of marijuana as well as other cannabinoids, is known to exert a wide range of physiological effects: drowsiness, alterations in cognition and memory, analgesia, as well as anti-inflammatory and immunomodulatory effects [1, 2]. Many studies have been conducted to decipher the complexity of the cannabinoid system. First attributed to nonspecific cell membrane alterations, the cannabinoid effects are now known to be mediated through cannabinoid receptors. Two proteins with seven transmembrane-spanning domains typical of G-protein-coupled receptors have been identified as tetrahydrocannabinol receptors and referred to as CB1 and CB2.

The CB1 receptor is predominantly expressed in the brain [3, 4] and could account for the psychoactive effects of cannabinoids. This receptor is also found in the periphery but at a much lower abundance [5−7]. Several signaling pathways triggered by the stimulation of CB1 have already been described, all being sensitive to pertussis toxin (PTX). Activation of CB1 inhibits adenylyl cyclase activity [8] as well as voltage-dependant N-type calcium channel activity in neurons [9]. More recently, we have described an induction of immediate-early genes after cannabinoid receptor stimulation: treatment by the agonist CP-55940 of human astrocytoma cells U373 MG as well as Chinese hamster ovary (CHO) cells transfected with human CB1 led to the expression of the growth-related gene *Krox*-24 [10]. Similar effects were observed *in vivo* in rat forebrain [11] and striosomes [12]. Although the molecular mechanisms located downstream from the G protein and leading to Krox-24 activation by cannabinoids remain to be identified, we have provided evidence that the central cannabinoid receptor CB1 is functionally coupled to the MAP kinase cascade [13], a finding which raises the possibility of a signal transduction pathway linking CB1 to the regulation of the Krox-24 gene.

The CB2 receptor, identified by Munro et al. [14] in the marginal zone of rat spleen, exhibits 44 % identity with the entire CB1 amino-acid sequence, whereas the similarity increases to 68 % when considering the transmembrane residues supposed to confer ligand specificities. CB2 is not expressed in the brain but only in the periphery [14]. We have analyzed more precisely its expression by performing quantitative reverse transcriptase (RT) PCR analyses on leukocyte subsets [7] and confirmed the anatomical distribution of the CB2 in immune tissues with a prominent expression in B lymphocytes, natural killer cells, and monocytes. The distribution pattern of CB2 expression strongly suggests that the immunomodulation effects induced by cannabinoids are mediated through the CB2 receptor [7].

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Abbreviations. MAP, mitogen-activated protein; MBP, myelin basic protein; CHO, Chinese hamster ovary; CB1, central cannabinoid receptor; CB2, peripheral cannabinoid receptor; Br^8cAMP, 8-bromoadenosine 3′,5′-monophosphate; Bt_2cAMP, N^6,2′-dibutyryl-adenosine 3′,5′-monophosphate; EMSA, electrophoretic mobility shift assay; RT, reverse transcriptase; PTX, pertussis toxin; PKC, protein kinase C.

With the exception of the inhibition of cAMP synthesis [15], nothing is known about the CB2 signaling pathway. In the present paper, we describe biological functions associated with this receptor by using cell lines expressing either endogenous CB2 receptor or a transfected one.

MATERIALS AND METHODS

Reagents. CP-55940 was obtained from Pfizer. [^{3}H]CP-55940 was purchased from New England Nuclear Corporation. Δ^9-Tetrahydrocannabinol, 8-bromoadenosine 3′,5′-monophosphate (Br^{8}cAMP), bovine myelin basic protein (MBP), leupeptin, aprotinin, phenylmethylsulfonyl fluoride ($PhMeSO_2F$), benzamidine and sodium orthovanadate were from Sigma. WIN 55212−2 and pertussis toxin (PTX) were obtained from Research Biochemicals Inc. Phorbol 12-myristate 13-acetate (phorbol ester) and herbymicin A were from Gibco BRL. The bisindolylmaleimide (GF 109203X) was from Calbiochem. N^6,2′-dibutyryladenosine 3′,5′-monophosphate (Bt_2cAMP) and 3-isobutyl-1-methylxanthine were from Boehringer. SR 141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride] was synthesized at the Chemistry Department of Sanofi Recherche (Montpellier, France) as described [15]. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham International. Anti-p44 (C-16, anti-ERK-1), anti-p42 (C-14, anti-ERK-2) rabbit polyclonal antibodies (ERK ≡ external signal regulated kinase) and anti-Krox-24 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz CA). Oligonucleotides were synthetized on a Biosearch 8750 automated synthesizer (Millipore).

Expression of human CB2 receptor in CHO cells. CB2 cDNA was obtained by screening a cDNA library from the human promonocytic cell line U937 with a horseradish-peroxidase-labeled CB2-specific oligonucleotide (5-CTCACACACTTCTTCCAGTG), followed by detection using the ECL system (Amersham). Human CB2 coding region was amplified by PCR using the primer pair 5′-CCACACAAGCTTGCCACCATGGAGGAATGCTGGGTG and 5′-CCACTCGGATCCTCAGCAATCAGAGAGGTCTAG and inserted into the p658 plasmid, an expression vector derived from p7055 [16] in which the IL-2 coding sequence was replaced by a polylinker. The sequence of the insert in the expression vector was verified before transfection into CHO dihydrofolate-reductase-negative cells by a modified $Ca_3(PO_4)_2$ precipitation method [17].

Cell lines and culture conditions. The human B lymphoblastoid cell line Daudi, the human promyelomonocytic cell line HL60 and the human astrocytoma cell line U373 MG were purchased from ATCC. Cell lines were maintained at 37 °C in humidified 5 % CO_2/95 % air atmosphere in RPMI 1640 medium (Gibco-BRL) supplemented with 10 % heat-inactivated fetal calf serum, 2 mM glutamine, streptomycin (100 μg/ml) and penicillin (100 U/ml). CHO cells stably transfected with CB1 [10] or CB2 were grown as monolayers in minimal-essential medium supplemented with 10 % dialysed fetal calf serum, 2 mM glutamine, 40 μg/ml l-proline, 1 % anti-PPLO agent, 1 mM sodium pyruvate, and 5 μg/ml gentamicin. The wild-type CHO cells were grown in the same medium except it contained 10 % fetal calf serum. Wild-type and CHO cells transfected with CB1 or CB2 are referred to as CHO-wt, CHO-CB1 and CHO-CB2 cells, respectively. Cells were maintained in 0.5 % fetal calf serum medium for 24 h before cannabinoid treament.

Radioligand-binding assays. For binding experiments, CHO-CB2 cells were seeded in 225-cm^2 culture flasks and grown to confluence. Cells were collected by versene treatment, spun at 1000×*g* for 10 min at 25 °C. The cell pellet was then resuspended in phosphate-buffered saline ($NaCl/P_i$). Cells (10^6 cells) were incubated at 30 °C with [^{3}H]CP-55940 in 0.5 ml binding buffer ($NaCl/P_i$ supplemented with 0.5 % BSA) in polypropylene tubes for 1 h. A rapid filtration using a 48-well filtration apparatus (Brandel) and Whatman GF/C filters treated with 0.5 % poly(ethyleneimine) was used to harvest and rinse labelled cells. Three consecutive washes were performed with $NaCl/P_i$ containing 0.1 % BSA. Filter-bound radioactivity was measured with Biofluor liquid scintillator. Nonspecific binding was determined in the presence of 1 μM CP-55940. In competition experiments, the drug concentrations producing 50 % inhibition (IC_{50}) of radioligand binding values were determined from Hill plots of log(*B*/*B*o-*B*) vs log (concentration) of test drug, where *B*o and *B* are specific bindings in the absence and presence of competitor, respectively. Inhibition constant (K_i) values were calculated from IC_{50} values using the Cheng and Prusoff equation [18].

cAMP analysis. Cells were grown to confluence, washed twice in $NaCl/P_i$ and incubated for 30 min in serum-free medium containing Ro-201724 (0.25 mM), isobutylmethylxanthine (0.1 mM) and forskolin (5 μM), supplemented or not with cannabinoids. The reaction was ended by the addition of 0.1 M HCl. cAMP levels were determined by radioimmunoassay according to the manufacturer's instructions (Pharmacia). Each data point is the mean of triplicates and experiments were repeated twice.

MAP kinase immunocomplex analysis. MAP kinase activities were measured as previously described [19]. Cells grown to 80 % confluence in 60-mm Petri dishes were maintained in medium containing 0.5 % fetal calf serum for 24 h prior to the application of ligands. After treatment with cannabinoids, cells were washed twice in buffer A (50 mM Hepes pH 7.5, 150 mM NaCl, 10 mM $Na_4P_2O_7$, 100 mM NaF, 10 mM EDTA, 20 mM glycerol 2-phosphate, 1 mM EGTA, 2 mM Na_3VO_4) and lysed for 15 min in buffer A supplemented with 1 % Triton X-100, 100 units/ml aprotinin, 20 μM leupeptin and 0.2 mg/ml $PhMeSO_2F$. The solubilized cell extracts were clarified by centrifugation at 14 000×*g* for 15 min and then incubated for 3 h with agarose-coupled antibodies anti-ERK_1 (C-16) and anti-ERK_2 (C-14). Following immunoprecipitation, pellets were washed three times with solubilization buffer and twice with buffer B (50 mM Hepes, 150 mM NaCl, 10 % glycerol, 0.1 % Triton X-100, 0.2 mM Na_3VO_4), then air-dried and resuspended in 50 μl buffer B supplemented with 0.2 mM Na_3VO_4, 100 units/ml aprotinin, 20 μM leupeptin and 0.2 mg/ml $PhMeSO_2F$. Phosphorylation of MBP was initiated by adding 10 μl sixfold concentrated mixture consisting of 150 μg/ml MBP, 10 mM magnesium acetate, 1 mM dithiothreitol and 5 μM [γ-^{32}P]ATP (33 Ci/mmol). The phosphorylation reaction was performed for 30 min at 30 °C (linear assay conditions) and ended by spotting P-81 Whatman filters which were then dropped into 0.1 % orthophosphoric acid. The papers were washed in this solution, rinsed with ethanol, air-dried, and the radioactivity incorporated in MBP was determined by liquid scintillation counting. In some experiments, cells were cultured in complete medium in the presence of PTX for 18 h prior to cannabinoid treatment.

Analysis of MAP kinase phosphorylations. Phosphorylations of p42 (ERK2) and p44 (ERK1) mitogen-activated protein kinases were determined by the electrophoretic mobility shift assay [20, 21]. After exposure to cannabinoid ligands for various times, cells were washed once in ice-cold buffer consisting of 50 mM Hepes pH 7.4 and 0.2 mM sodium orthovanadate, then directly lysed in Laemmli's loading buffer containing 6 M urea. Proteins were run on 10 % polyacrylamide gel [22] before being blotted onto nitrocellulose filters. Nonspecific binding of antibodies was prevented by incubating filters in 10 % dried milk powder in buffer C (10 mM Tris pH 7.6, 150 mM NaCl, 0.05 % Tween). Buffer C was also used for all incubation and washing

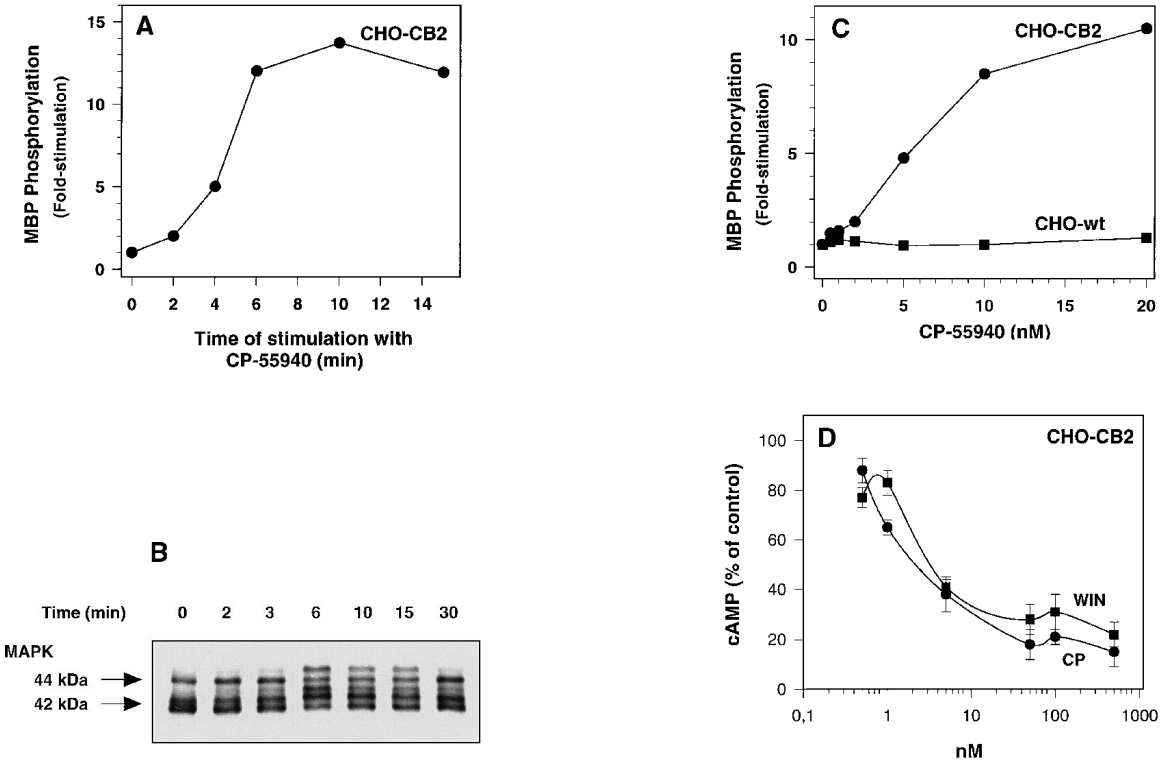

Fig. 1. Effect of cannabinoids on MAP kinase activity in CHO-CB2 cells. (A) Activation of MAP kinases by cannabinoids. Growth-arrested CHO-CB2 cells were treated with 10 nM CP-55940 at 37 °C for the indicated time periods. MAP kinase activities were measured in cell lysates using MBP as substrate as described under Materials and Methods. (B) Western blot analysis of MAP kinases in growth-arrested CHO-CB2 cells treated with 10 nM CP-55940 for the indicated time periods. Reaction was ended by the addition of Laemmli's SDS buffer and protein extracts were processed as detailed under Materials and Methods. (C) Dose-dependent effects of cannabinoids on MAP kinases. Growth-arrested CHO-CB2 (●) or wild-type CHO (■) cells were treated for 10 min with the indicated concentration of CP-55940. MAP kinase activities were measured as described above. Results are representative of one experiment performed five times. (D) Cannabinoid-induced inhibition of forskolin-stimulated cAMP production in CHO-CB2 cells. CHO-CB2 cells were treated with 5 μM forskolin and various concentrations (indicated on the abscissa) of CP-55940 (●) or WIN 55212-2 (■) for 30 min. cAMP levels were determined as described in Materials and Methods. Data represent the average of cAMP accumulation ± SE of two independent determinations performed in triplicate.

steps. p42 and p44 MAP kinases were immunostained for 1 h at room temperature using purified anti-p42 and anti-p44 antiserum (0.25 μg/ml). Following extensive washings, the blots were subsequently incubated for 45 min at room temperature with a peroxidase-labeled anti-rabbit-IgG antibody. After washing, immunostained MAP kinases were visualized using the enhanced chemiluminescence detection (ECL) system according to the supplier's instructions (Amersham).

Electrophoretic mobility shift assay (EMSA). CHO-CB1 and CHO-CB2 cells were stimulated for 90 min with the various agents, washed with ice-cold NaCl/P_i, and the nuclei isolated by treatment with 0.1 % NP-40. Nuclear extracts were prepared as described by Dignam et al. [23] with minor modifications [24]. Briefly, a synthetic oligonucleotide containing a single copy of the Krox-24 binding site (5′-CCCGGCGCGGGGGGCGATTTC-GAGTCA-3′) was 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (3000 Ci/mmol). The binding reaction was performed by incubating 10 μg nuclear extract with 0.8 μg poly[(dI-dC) · (dI-dC) Pharmacia-LKB) for 10 min on ice in a buffer containing 20 mM Hepes pH 7.9, 70 mM KCI, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 2 mM dithiothreitol and 12 % glycerol. Then 0.5 ng of the labeled probe was added to the mixture containing the nuclear extract and the mixture incubated for 30 min at room temperature. The samples were loaded on a 6 % polyacrylamide gel in 22.25 μM Tris borate, 22.25 μM boric acid, 0.5 mM EDTA and the gel run at 120 V at 4 °C. For competition experiments, an excess of unlabeled Krox-24 binding oligonucleotides was added to the reaction mixture 10 min prior to the addition of the radiolabeled probe. Similarly, in some experiments, 0.5 μg of anti-Krox-24 antibody was added to the reaction mixture and incubated for 60 min before the addition of the radiolabeled probe.

Western-blot analysis. Following stimulation, cells were washed and lysed. Proteins were extracted, run on SDS/PAGE and blotted onto nitrocellulose filters as described above (Analysis of MAP kinase phosphorylations). The blots were incubated with the anti-Krox-24 antibody for 3 h at room temperature. After washing, Krox-24 proteins were detected using anti-(rabbit IgG)−peroxidase conjugate and ECL system.

Extraction of cellular RNA, RT-PCR and Northern blot analysis. Total RNA was isolated from cells by lysis in guanidinium isothiocyanate and purified by CsCl gradient ultracentrifugation. The integrity of RNAs was assessed by analysing 18S and 28S rRNA contents on denaturing 1 % agarose gel. For RT-PCR analysis, total RNA was treated with DNase I for 30 min, purified by two phenol/chloroform extraction steps, then ethanol-precipitated. RNAs were resuspended in reverse transcriptase buffer with $(dT)_{12-18}$ primers and MMLV reverse transcriptase Superscript II (Gibco-BRL) and incubated for 60 min at 45 °C. PCR amplification was performed using the primer pairs: 5′-TATATTCTCTGGAAGGCTCACAGCC and 5′-GAG-CATACTGCAGAATGCAAACACC (for amplification of a 270-bp product for human CB1) or 5′-TTTCCCACTGATCCC-CAATG and 5′-TTTCACGGTGTGGACTCC (for CB2, 333-bp

Table 1. Effect of various receptor cannabinoid agonists on MAP kinase activation in CHO-CB2 cells. K_i values are concentrations needed to displace [^{3}H]CP-55940 binding to CHO-CB2. EC_{50} values for MAP kinase activation were determined as described under Materials and Methods. EC_{50} values for cAMP accumulation were determined in forskolin-stimulated CHO-CB2 cells and derived from Fig. 1D. All values are given ± SEM.

Drug	K_i for displacement of [^{3}H]CP.55940	EC_{50} for	
		MAP kinase activation	cAMP accumulation
	nM		
CP-55940	4 ± 0.7	8 ± 2	2 ± 0.5
WIN 55212.2	22 ± 1.6	12 ± 4	3 ± 0.5

product) or 5′-CCAGCAGAGAATGGAAAGTC and 5′-GATGCTGCTTACATGTCTCG (for amplification of a 268-bp product for β_2-microglobulin).

The steps of amplification were 95 °C, 20 s; 60 °C, 30 s; 75 °C, 40 s/cycle and performed with a 9600 cycler according to the manufacturer's instructions (Perkin Elmer). No PCR product was detected when reverse transcriptase was omitted indicating that the PCR products derived specifically from cDNA. Northern blot analysis was performed following standard procedures [25]. The Krox-24 and β_2-microglobulin radiolabeled probes were prepared from PCR products obtained using the primers specific for Krox-24 and β_2-microglobulin, as previously described [10].

RESULTS

Stimulation of cannabinoid CB2 receptor activates MAP kinases. To investigate the functional coupling of the cannabinoid CB2 receptor, we used CHO cells stably transfected with the human CB2 cDNA, which exhibit specific binding for the tritiated cannabinoid receptor ligand CP-55940. Scatchard plot analysis revealed that the dissociation constant (K_d) and the maximal binding (B_{max}) were 0.9 ± 0.06 nM and 152 000 ± 35 000 sites/cell, respectively. As MAP kinases can be stimulated through G-protein-coupled receptors [26, 27, 19], we investigated whether stimulation through the CB2 could result in a similar activation. We thus immunoprecipitated MAP kinases and the immune complexes were assayed for kinase activity towards myelin basic protein (MBP) as substrate, as described under Materials and Methods. As shown in Fig. 1A, resting CHO-CB2 cells displayed a slight constitutive MAP kinase activity, which was significantly enhanced following treatment with CP-55940. This effect was measurable after 2 min of stimulation, maximum between 6−15 min, then slowly returned to baseline level after 30 min (Fig. 1A and data not shown). CP-55940 activation of MAP kinases was dose-dependent, with a noticeable effect at a concentration of 2 nM and a maximal effect at 20 nM (Fig. 1C). The specific involvement of CB2 in these experiments was assessed by the absence of MAP kinase activation in wild-type CHO cells treated with CP-55940 (Fig. 1C).

We found that the cannabinoid agonist WIN 55212.2 was very close to CP-55940 in its potency to stimulate MAP kinases, a result which was in fairly good agreement with the concentrations of these product required to displace [^{3}H]CP-55940 binding and to inhibit adenylyl cyclase (Table 1 and Fig. 1D).

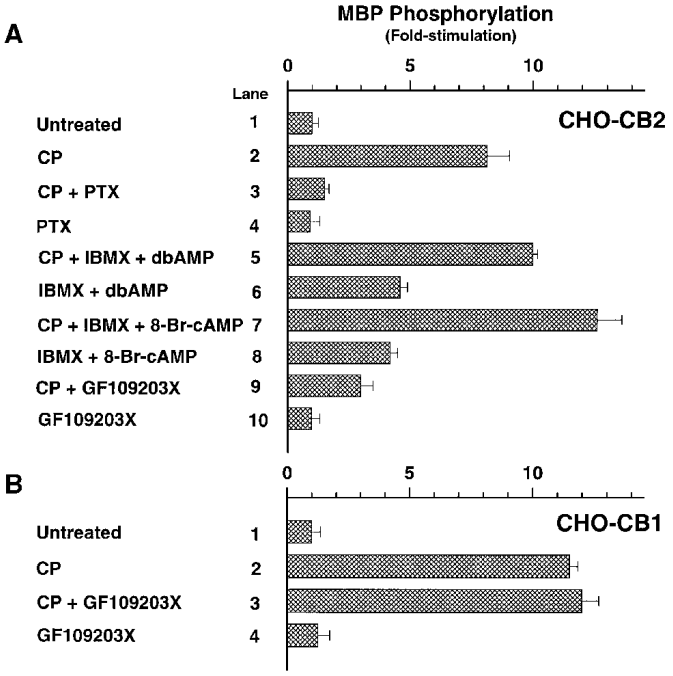

Fig. 2. Effect of PTX, cAMP and PKC inhibitor on CP-55940-induced MAP kinase activity in CHO-CB2 cells. (A) CHO-CB2 cells were treated as indicated for 10 min before determining MAP kinase activity. Lane 1, untreated cells; lane 2, 10 nM CP-55940; lane 3, 10 nM CP-55940 + 100 ng · ml^{-1} PTX; lane 4, 100 ng · ml^{-1} PTX; lane 5, 10 nM CP-55940 + 0.1 mM isobutylmethylxanthine (IBMX) + 1 mM Bt_2cAMP; lane 6, 0.1 mM IBMX + 1 mM Bt_2cAMP; lane 7, 10 nM CP-55940 + 0.1 mM IBMX + 0.5 mM Br^{8}cAMP; lane 8, 0.1 mM IBMX + 0.5 mM Br^{8}cAMP; lane 9, 10 nM CP-55940 + 5 μM GF 109203X; lane 10, 5 μM GF 109203X. (B) CHO-CB1 cells were treated as indicated for 10 min before determining MAP kinase activity. Lane 1, untreated cells; lane 2, 10 nM CP-55940; lane 3, 10 nM CP-55940 + 5 μM GF 109203X; lane 4, 5 μM GF 109203X. Treatment with GF 109203X or PTX was performed 3 h or 24 h before stimulation, respectively. Results are representative of one experiment performed three times.

MAP kinases are readily detectable on immunoblots where the activated forms, which are phosphorylated on both Tyr and Thr residues [28], display a lower electrophoretic mobility than the non-phosphorylated inactive forms [20]. Fig. 1B shows that CP-55940 treatment of CHO-CB2 cells induced a shift which was time-dependent and fitted with the activation pattern depicted in Fig. 1A. It is noteworthy that, in addition to p42 and p44, another protein of the MAP kinase family with a molecular mass of approximately 40 kDa was also stimulated following CP-55940 treatment.

CP-55940 induces MAP kinase activation in CHO-CB2 via a G_i/G_o-protein-dependent, cAMP-independent transduction pathway. To further investigate the effector pathway responsible for MAP kinase activation in CHO-CB2, we first examined the effect of PTX. As shown in Fig. 2, the stimulation of MAP kinase activity by CP-55940 was reduced to the basal level by initial cell treatment with 100 ng/ml PTX, a result which is consistent with the selective coupling of CB2 and G_i proteins.

It has been described that the elevation of intracellular cAMP by using either Bt_2cAMP or Br^{8}cAMP could prevent MAP kinase activation [29]. Surprisingly, in CHO-CB2 cells, these agents alone significantly increased the basal MAP kinase level (Fig. 2A). In combination with cAMP, CP-55940 acted in an additive manner on MAP kinase activity (Fig. 2A) suggesting that these two agents involved different transduction pathways.

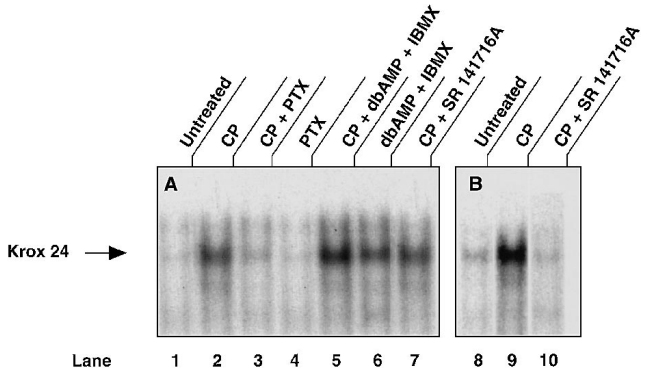

Fig. 3. Induction by CP-55940 of Krox-24 DNA binding activity. Nuclear extract from cells was incubated with a ^{32}P-labeled oligonucleotide probe containing the consensus binding site for Krox-24 and submitted to EMSA as described under Materials and Methods. Treatment with 100 ng/ml PTX was performed 24 h before stimulation. Results are representative of one experiment performed three times. (A) CHO-CB2 cells were incubated as indicated for 90 min before the preparation of cell extracts. Lane 1, untreated cells; lane 2, 10 nM CP-55940; lane 3, 10 nM CP-55940 + 100 ng · ml^{-1} PTX; lane 4, 100 ng · ml^{-1} PTX; lane 5, 10 nM CP-55940 + 0.1 mM isobutylmethylxanthine (IBMX) + 1 mM Bt_2cAMP; lane 6, 0.1 mM IBMX + 1 mM Bt_2cAMP; lane 7, 10 nM CP-55940 + 500 nM SR 141716A. (B) CHO-CB1 cells were incubated as indicated for 90 min before the preparation of cell extracts. Lane 8, untreated cells; lane 9, 10 nM CP-55940; lane 10, 10 nM CP-55940 + 500 nM SR 141716A.

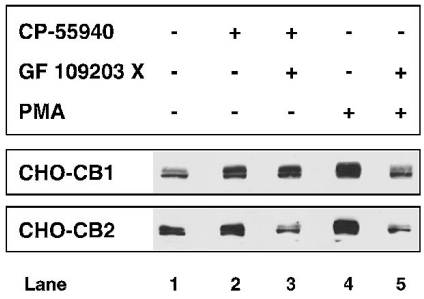

Fig. 4. Induction by CP-55940 of Krox-24 expression. Quiescent CHO-CB1 or CHO-CB2 cells were stimulated with 10 nM CP-55940 at 37°C for 90 min. Reaction was stopped by addition of Laemmli's SDS buffer and proteins fractionated by PAGE. Blots were incubated with the anti-Krox-24 antibody. Lane 1, untreated cells; lane 2, 10 nM CP-55940; lane 3, 10 nM CP-55940 + 5 μM GF 109203X; lane 4, 20 ng/ml phorbol ester (PMA); lane 5, 20 ng/ml PMA + 5 μM GF 109203X. Treatment with GF 109203X was performed 3 h before stimulation.

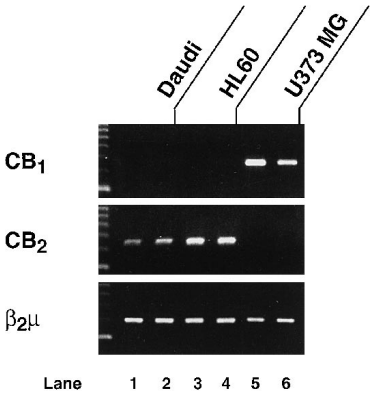

Fig. 5. RT-PCR analyses of CB1 and CB2 gene expression. cDNAs (1 ng) were amplified with primers specific for CB1, CB2 and β_2-microglubulin ($\beta_2\mu$) as described under Materials and Methods. The PCR products were analysed after 30 amplification cycles (for CB1 and CB2) and 14 amplification cycles (for $\beta_2\mu$) and visualized by ethidium bromide staining. Each reaction was performed in duplicate. Daudi cell line (lanes 1, 2), HL60 cell line (lanes 3, 4), U373 MG cell line (lanes 5, 6).

The protein kinase C (PKC) inhibitor, GF 109203X [30], significantly prevented CP-55940-stimulated MAP kinase activity in CHO-CB2 cells, suggesting that this mechanism is PKC-dependent. In contrast, similar experiments on CHO-CB1 cells showed that GF 109203X had no effect, excluding the involvement of PKC (Fig. 2B).

Cannabinoid induces Krox-24 in CHO-CB2 cells via a G_i/G_o-protein-dependent, cAMP-independent transduction pathway. We have already demonstrated that the stimulation of CB1 by cannabinoid agonist induces the immediate-early gene Krox-24 expression both at the mRNA and protein levels in the human astrocytoma cell line U373 MG as well as in CHO-CB1 cells [10]. Krox-24 is a nuclear phosphoprotein containing three zinc finger motifs of the Cys_2-His_2 subclass, which binds to a specific G+C-rich consensus DNA sequence (CGCCCCCCGC) in a zinc-dependent manner [31]. We have evaluated the ability of the CB2 to elicit a similar functional response by quantifying Krox-24 DNA binding activity by EMSA on nuclear extracts of CHO-CB2 cells. As shown in Fig. 3A, the stimulation of CB2 by CP-55940 strongly induced a retarded band. This band reflects the binding of specific factors since its formation is prevented by the presence of an excess of unlabeled Krox-24 oligonucleotides and in addition can be supershifted by an anti-Krox-24 antibody (data not shown). Krox-24 induction was blocked by incubation with PTX but not by hydrolysis-resistant cAMP analogues (Fig. 3A), indicating the involvement of a PTX-sensitive heterotrimeric G_i protein which also bypasses the adenylyl cyclase pathway. As already described, a similar response was obtained with CHO-CB1 cells (Fig. 3B).

We further examined the effect of the cannabinoid receptor antagonist SR 141716A which is selective of CB1 but not of CB2. As expected, SR 141716A was able to block the cannabinoid-induced activation of Krox-24 in CHO-CB1 cells but not in CHO-CB2 cells (Fig. 3). The specificity of the involvement of cannabinoid receptors was also demonstrated by using CHO$^-$ cells in which CP-55940 had no effect on Krox-24 expression (data not shown).

The induction of Krox-24 in CHO-CB2 cells by CP-55940 was confirmed by Western blot analysis using anti-Krox-24 antibodies. As shown in Fig. 4, CP-55940 treatment resulted in an enhancement of Krox-24 protein which appeared as two bands corresponding to the p82 and p88 Krox-24 previously described [31]. While this induction was comparable in intensity to that observed in CHO-CB1 cells, GF 109203X inhibited CP-55940-induced Krox-24 only in CHO-CB2 cells. As expected, GF 109203X prevented Krox-24 expression when phorbol ester was used as inducer (Fig. 4).

Cannabinoid induces MAP kinases and Krox-24 gene expression in HL60 cells. The above data were obtained from a cell line overexpressing the CB2 receptor. We next examined whether they could be extended to human cell lines expressing endogenous CB2 receptors. Two different types of cell lines were employed: cells expressing CB2 mRNA (the human promyelomonocytic cell line HL60 and the human B lymphoblastoid line Daudi) and cells only expressing CB1 (the human astrocytoma cell line U373 MG) used as positive control for Krox-24 induction (Fig. 5). The ability of the CB2 receptor to elicit

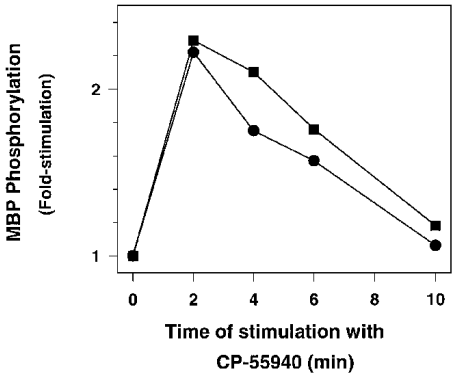

Fig. 6. Cannabinoid induces MAP kinase activation in HL60 cells. Growth-arrested HL60 cells were treated with 10 nM CP-55940 for the indicated periods of time and MAP kinase was measured as described in Fig. 1. The two curves represent two independent experiments.

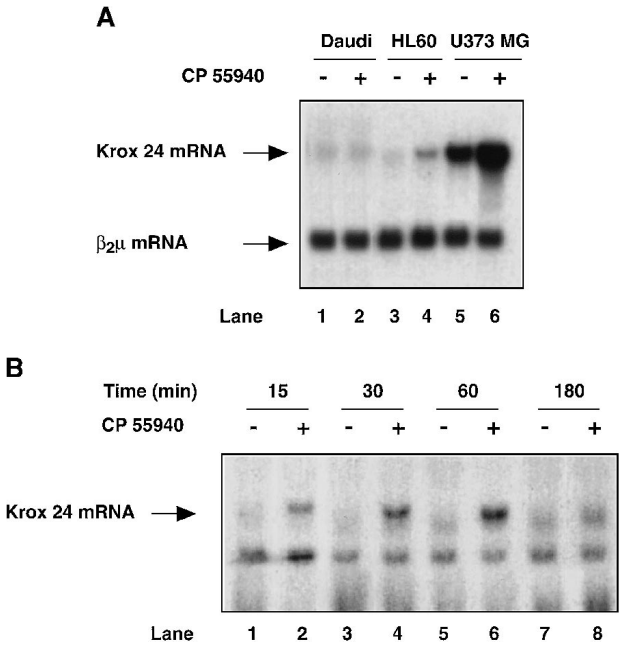

Fig. 7. Krox-24 gene activation in HL60 cells. RNA (10 μg) extracted from cells treated as indicated were fractionated on 1% agarose gel and blotted onto nitrocellulose and the filters hybridized with ^{32}P-labeled Krox-24 and β_2-microglobulin ($\beta_2\mu$). (A) Effect of CP-55940 on Krox-24 gene expression. Cells were maintained in culture media containing 0.5% fetal calf serum for 48 h and then incubated for 1 h at 37°C with or without 10 nM CP-55940. Untreated (lanes 1, 3, 5) or CP-55940-treated (lanes 2, 4, 6) cells from Daudi (lanes 1, 2), HL60 (lanes 3, 4) or U373 MG (lanes 5, 6). (B) Kinetics of induction of Krox-24 in HL60 cells. Cells were incubated with (lanes 2, 4, 6, 8) or without (lanes 1, 3, 5, 7) 10 nM CP-55940 for 15, 30, 60, or 180 min (lanes 1 and 2, lanes 3 and 4, lanes 5 and 6, lanes 7 and 8, respectively).

functional response was first investigated on adenylyl cyclase. Except for the U373 MG cells, none of these cells could be modulated for cAMP either assessed for activation or inhibition (data not shown). In contrast, both MAP kinase stimulation and Krox-24 mRNA increase were observed in HL60 cells after cannabinoid stimulation. As shown in Fig. 6, the agonist activation of HL60 cells resulted in a rapid and transient stimulation of MAP kinases which was maximal at 2 min. Fig. 7 shows the kinetics of induction of Krox-24 gene as analyzed by Northern blot analysis. However, these two responses, which were observed in the human astrocytoma cells U373 MG as previously reported, were not detectable on Daudi cells (Fig. 7 A). Taken together, these results provide evidence that the couplings induced by cannabinoid in CHO-CB2 cells may also be relevant in some cells that endogenously express the CB2 receptor.

DISCUSSION

The object of the present study was to characterize biochemical signals transduced by the peripheral cannabinoid receptor CB2. We here report evidence that this receptor is functionally coupled to several biological functions.

Using CHO cells stably transfected with the human CB2 receptor cDNA, we showed that stimulation of this receptor by the cannabinoid agonist CP-55940, potently activates MAP kinases in a dose- and time-dependent manner. This activation was accompanied by phosphorylation of the isoforms, which is part of their mechanisms of activation (reviewed by Nishida and Gotoh [32]). The inhibitory effect of PTX on cannabinoid-induced signal indicated that MAP kinase activation was regulated by PTX-sensitive G protein(s).

In accordance with previous observations, we showed that the stimulation of CB2 also inhibits adenylyl cyclase [15]. Numerous studies have described that the modulation of cAMP was associated with a decreased activation of MAP kinases, suggesting that the activation of cAMP-dependent protein kinases might represent a mechanism for antagonising MAP activation [29]. The situation in CHO-CB2 cells was different since (a) cAMP-stimulated MAP kinases, and (b) the costimulation by both cannabinoid and cAMP resulted in an enhanced signal which corresponded to additive effects of the two stimuli. This argues against the possibility that MAP kinase activation is secondary to a G_i-mediated inhibition of basal cAMP levels, and suggests that CP-55940 simultaneously inhibits cAMP production and induces MAP kinases through independent pathways. This observation may have biological relevance since it raises the possibility that the MAP kinase cascade may integrate and amplify signals deriving from receptors employing cAMP and CB2.

Although the molecular mechanisms located downstream from the G protein and leading to MAP kinase activation are currently under study, we showed that GF 109203X abolished this activation suggesting that a PKC lies on the route between G_i and MAP kinase. Interestingly, we observed that GF 109203X was without any effect on cAMP inhibition induced by CP-55940 (data not shown), a further confirmation that cAMP and MAP kinase pathways are different. We examined the consequences of down-regulation of PKC, by chronic treatment of CHO-CB2 cells with phorbol esters, on MAP kinase activation by cannabinoids. Surprisingly, PKC-depleted cells still respond to CB2 receptor stimulation (data not shown). This result is interesting in the light of previous observations which showed that not all PKC isoforms are equally sensitive to PKC depletion. The PKC-ε and ζ are particularly resistant to the chronic treatment of phorbol ester [33]. These results therefore suggest that specific PKC isoforms could be involved in CB2 but not in CB1 signal transduction.

MAP kinase plays a role in the control of gene transcription by phosphorylating transcription factors which in turn modulate the expression of target genes such as Krox-24 [34]. We thus explored whether the activation of CB2 was associated with such gene induction. Indeed we showed that CP-55940 treatment of CHO-CB2 cells resulted in an increase of Krox-24 levels by measuring the Krox-24 DNA-binding activity by EMSA. These results were confirmed by measuring the amount of Krox-24 by Western blot analysis (data not shown). We propose that the coupling of CB2 to the Krox-24 induction may be explained by

the activation of the mitogenic signal pathway for the following reasons: (a) both MAP kinase and Krox-24 inductions are completely PTX-sensitive and independent of adenylyl cyclase; (b) in combination with cannabinoid, cAMP acts in additive manner on both MAP kinase activity and Krox-24 enhancement; (c) the protein kinase inhibitor GF 109203X, which abolished the MAP kinase activation, blocks the Krox-24 gene induction as well; and (d) MAP kinase has been shown to phosphorylate a member of the ETS family, p62TCF, [35] which binds with high affinity to specific binding sites located in three copies in the *Krox*-24 promoter, two of them being necessary for transcriptional activity [36, 37].

We next examined whether the functional coupling observed in CHO-CB2 cells can be extended to cell lines naturally expressing the CB2 receptor. We showed that the application of cannabinoid-induced MAP kinase activity and enhanced Krox-24 mRNA in HL60 cells. These effects were not associated with any modulation of adenylyl cyclase supporting the above notion that they bypass the metabolism of cAMP. We observed that both modulation of MAP kinase and Krox-24 in HL60 cells by cannabinoids were blocked by PTX-treated cells, but not by the CB1 antagonist SR 141716A, consistent with the hypothesis of a CB2 signal transduction mediated by a G_i protein (data not shown). On the other hand, cannabinoids had no effect on the other cell line expressing CB2 mRNA (Daudi); this could be related to the lower expression level of CB2-mRNA in these cells when compared to HL-60 cells (Fig. 5). However, our results first established in CB2-transfected cells and then extended to a naturally expressing CB2 cell line, indicate that the CB2-induced biological response could be of general relevance.

The absence of any modulation by cannabinoids of adenylyl cyclase in HL60 cells contrasts with the observation that cannabinoid agonists inhibit adenylyl cyclase on mouse spleen cells which express CB2 receptor [38, 39]. However, the concentration of cannabinoids needed for this activity did not correlate with the pharmacological profile of these molecules as demonstrated in binding assay, thus questioning whether these effects were receptor-mediated processes.

Comparison of CB1 and CB2 is stricking. These two receptors share a number of properties although their sequence similarity is relatively low. Radioligand binding analysis demonstrated that the cannabinoid compounds displayed similar affinities for CB2 and CB1 although some differences have been raised. For instance, cannabinol has a higher affinity for CB2 [14] whereas the anandamide, a candidate endogenous ligand for CB1, displays an affinity for CB2 30-fold less than for CB1 [14]. In the present paper, we also showed that WIN 55212.2 bound more efficiently to CB2 than to CB1.

A stricking parallel was also observed in biological responses mediated by CB1 and CB2 in CHO cells since both involve cAMP metabolism, MAP kinase activation and Krox-24 gene induction. However, our experiments with CHO-CB2 cells underline two important differences. First, the intensity of the response (e.g. MAP kinase) is similar although the expression level of receptor is 40-fold higher for CB2 than for CB1, which suggests a possible higher potency of CB1. Second, the CB2-mediated cannnabinoid response was blocked by a PKC inhibitor, an effect which was not observed for the CB1-mediated one. MAP kinases have been found to be activated by a wide variety of agents via a cascade of kinase/effector molecules which include PKC and/or $p21^{ras}$, Raf-1, and MEK (extracellular signal-regulated protein kinase kinase) [40]. We show here for the first time a different mechanism in MAP kinase activation through cannabinoid receptors of both subtypes.

Concluding remarks. Cannabinoids widely modulate immune functions, effects which have to be paralleled with the preferential expression of CB2 in immune tissues. Understanding that MAP kinases are activated by stimulation of the cannabinoid receptor subtype CB2 strengthens the notion that this receptor might play an important role in the regulation of macrophages and lymphocytes once activated either by cannabinoids or by putative natural endogenous ligand. We recently showed that cannabinoids, at nanomolar concentration, can promote cell growth of human B lymphocytes costimulated with anti-Igs, an effect very likely mediated through the CB2 receptor [2]. Interestingly, MAP kinases were found to be activated in B lymphocytes through antigen receptor to induce the expression of immediate-early gene *Krox*-24 and cell proliferation [37]. Given the pivotal role of the MAP kinase network in growth factor functions, we propose that this pathway may be responsible, at least in part, for the mitogenic activity of cannabinoid agonists in B lymphocytes.

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REFERENCES

1. Dewey, W. L. (1986) Cannabinoid pharmacology, *Pharmacol. Rev. 38*, 151−178.
2. Derocq, J.-M., Ségui, M., Marchand, J., Le Fur, G. & Casellas P. (1995) Cannabinoids enhance human B-cell growth at low nanomolar concentrations, *FEBS Lett. 369*, 177−182.
3. Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C. & Bonner, T. I. (1990) Structure of a cannabinoid receptor and functional expression of the cloned cDNA, *Nature 346*, 561−564.
4. Gérard, C. M., Mollereau, U. C., Vassart, G. & Parmentier, M. (1991) Molecular cloning of a human cannabinoid receptor which is also expressed in testis, *Biochem. J. 279*, 129−134.
5. Kaminski, N. E., Abood, M. Y., Kessler, F. K., Martin, B. R. & Schatz, A. R. (1992) Identification of a functionally relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation, *Mol. Pharmacol. 42*, 736−742.
6. Bouaboula, M., Rinaldi-Carmona, M., Carayon, P., Carillon, C., Delpech, B., Shire, D., Le Fur, G. & Casellas, P. (1993) Cannabinoid receptor expression in human leukocytes, *Eur. J. Biochem. 214*, 173−180.
7. Galiègue, S., Mary, S., Marchand, J., Dussossoy, D., Carrière, 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.
8. Howlett, A. C. & Fleming, R. M. (1984) Cannabinoid inhibition of adenylate cyclase. Pharmacology of the response in neuroblastoma cell membranes, *Mol. Pharmacol. 26*, 532−538.
9. Mackie, K. & Hille, B. (1992) Cannabinoids inhibit N-type calcium channels in neuroblastoma-glioma cells, *Proc. Natl Acad. Sci. USA 89*, 3825−3829.
10. Bouaboula, M., Bourrié, B., Rinaldi-Carmona, M., Shire, D., Le Fur, G. & Casellas, P. (1995a) Stimulation of cannabinoid receptor CB1 induces Krox-24 expression in human astrocytoma cells, *J. Biol. Chem. 270*, 13973−13980.
11. Mailleux, P., Verslype, M., Preud'homme, X. & Vanderhaeghen, J. J. (1994) Activation of multiple transcription factor genes by tetrahydrocannabinol in rat forebrain, *Neuroreport 5*, 1265−1268.
12. Glass, M. & Dragunow, M. (1995) Induction of the Krox 24 transcription factor in striosomes by a cannabinoid agonist, *Neuroreport 6*, 241−244.
13. Bouaboula, M., Poinot-Chazel, C., Bourrié, B., Canat, X., Calandra, B., Le Fur, G. & Casellas, P. (1995b) Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1, *Biochem. J. 312*, 637−641.
14. Munro, S., Thomas, K. L. & Abu-Shaar, M. (1993) Molecular characterization of a peripheral receptor for cannabinoids, *Nature 365*, 61−65.

15. Rinaldi-Carmona, M., Barth, F., Héaulme, M., Shire, D., Calandra, B., Congy, C., Martinez, S., Maruani, J., Néliat, G., Caput, D., Ferrara, P., Soubrié, P., Brelière, J.-C. & Le Fur, G. (1994) A potent and selective antagonist of the brain cannabinoid receptor, *FEBS Lett. 350*, 240−244.
16. Miloux, B. & Lupker, J. H. (1994) Rapid isolation of highly productive recombinant Chinese hamster ovary cell lines, *Gene 149*, 341−344.
17. Graham, F. L. & Van der Eb, A. J. (1973) Transformation of rat cells by DNA of human adenovirus 5, *Virology 54*, 536−539.
18. Cheng, Y. C. & Prusoff, W. H. (1973) Relationship between the inhibition constant and the concentration of inhibitor which causes 50 % inhibition of an enzymatic reaction, *Biochem. Pharmacol. 22*, 3099−3108.
19. Frödin, M., Peraldi, P. & Van Obberghen, E. (1994) Cyclic AMP activates the mitogen-activated protein kinase cascade in PC12 cells, *J. Biol. Chem. 269*, 6207−6214.
20. Leevers, S. J. & Marshall, C. J. (1992) Activation of extracellular signal-regulated kinase, ERK2, by p21^{ras} oncoprotein, *EMBO J. 11*, 569−574.
21. de Vries-Smits, A. M. M., Burgering, B. M. T., Leevers, S. J., Marshall, C. J. & Bos, J. L. (1992) Involvement of p21^{ras} in activation of extracellular signal-regulated kinase 2, *Nature 357*, 602−604.
22. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4, *Nature 227*, 680−685.
23. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei, *Nucleic Acids Res. 11*, 1475−1489.
24. Cao, X., Mahendran, R., Guy, G. R. & Tan, Y. H. (1993) Detection and characterization of cellular EGR-1 binding to its recognition site, *J. Biol. Chem. 268*, 16949−16957.
25. Church, G. M. & Gilbert, W. (1984) Genomic sequencing, *Proc. Natl Acad. Sci. USA 81*, 1991−1995.
26. Cobb, M. H., Boulton, T. G. & Robbins, D. J. (1991) Extracellular signal-regulated kinases: ERKs in progress, *Cell Regul. 2*, 965−978.
27. Kahan, C., Seuwen, K., Meloche, S. & Pouysségur, J. (1992) Coordinate, biphasic activation of p44 mitogen-activated protein kinase and S6 kinase by growth factors in hamster fibroblasts. Evidence for thrombin-induced signals different from phosphoinositide turnover and adenylylcyclase inhibition, *J. Biol. Chem. 267*, 13369−13375.
28. Anderson, N. G., Maller, J. L., Tonks, N. K. & Sturgill, T. W. (1990) Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase, *Nature 343*, 651−653.
29. Sevetson, B. R., Kong, X. & Lawrence, J. C. Jr (1993) Increasing cAMP attenuates activation of mitogen-activated protein kinase, *Proc. Natl Acad. Sci. USA 90*, 10305−10309.
30. Toullec, D., Pianetti, P., Coste, H., Bellevergue, P., Grand-Perret, T., Ajakane, M., Baudet, V., Boissin, P., Boursier, E., Lorriole, F., Duhamel, L., Charon, D. & Kirilovsky, J. (1991) The bisindolylmaleimide GF 109203X is a potent and selective inhibitor of protein kinase C, *J. Biol. Chem. 266*, 15771−15781.
31. Lemaire, P., Vesque, C., Schmitt, J., Stunnenberg, H., Frank, R. & Charnay P. (1990) The serum-inducible mouse gene Krox 24 encodes a sequence-specific transcriptional activator, *Mol. Cell. Biol. 10*, 3456−3467.
32. Nishida, E. & Gotoh, Y. (1993) The MAP kinase cascade is essential for diverse signal transduction pathways, *Trends Biochem. Sci. 18*, 128−131.
33. Chen, C. C. (1993) Protein kinase C α, δ, ε, and ζ in C6 glioma cells. TPA induces translocation and down-regulation of conventional and new PKC isoforms but not atypical PKC ζ, *FEBS Lett. 332*, 169−173.
34. Seth, A., Alvarez, E., Gupta, S. & Davis, R. J. (1991) A phosphorylation site located in the NH2-terminal domain of c-Myc increases transactivation of gene expression, *J. Biol. Chem. 266*, 23521−23524.
35. Treisman, R. (1994) Ternary complex factors: growth factor regulated transcriptional activators, *Curr. Opin. Genet. Dev. 4*, 96−101.
36. Tsai-Morris, C. H., Cao, X. M. & Sukhatme, V. P. (1988) 5′ flanking sequence and genomic structure of Egr-1, a murine mitogen inducible zinc finger encoding gene, *Nucleic Acids Res. 15*, 8835−8846.
37. McMahon, S. B. & Monroe, J. G. (1995) Activation of the p21^{ras} pathway couples antigen receptor stimulation to induction of the primary response gene egr-1 in B lymphocytes, *J. Exp. Med 181*, 417−422.
38. Kaminski, N. E. (1993) Evidence for a cannabinoid receptor in immunomodulation by cannabinoid compounds, *Adv. Exp. Med. Biol. 335*, 115−120.
39. Kaminski, N. E., Koh, W. S., Yang, K. H., Lee, M. & Kessler, F. K. (1994) Suppression of the humoral immune response by cannabinoids is partially mediated through inhibition of adenylate cyclase by a pertussis toxin-sensitive G-protein coupled mechanism, *Biochem. Pharmacol. 48*, 1899−1908.
40. Marshall, C. J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase, *Curr. Opin. Genet. Dev. 4*, 82−89.