Activation of mitogen-activated protein kinases by stimulation of the central cannabinoid receptor CB1

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The G-protein-coupled central cannabinoid receptor (CB1) has been shown to be functionally associated with several biological responses including inhibition of adenylate cyclase, modulation of ion channels and induction of the immediate-early gene *Krox-24*. Using stably transfected Chinese Hamster Ovary cells expressing human CB1 we show here that cannabinoid treatment induces both phosphorylation and activation of mitogen-activated protein (MAP) kinases, and that these effects are inhibited by SR 141716A, a selective CB1 antagonist. The two p42 and p44 kDa MAP kinases are activated in a time- and dose-dependent manner. The rank order of potency for the activation of MAP kinases with various cannabinoid agonists is CP-55940 > Δ^9-tetrahydrocannabinol > WIN 55212.2, in agreement with the pharmacological profile of CB1. The activation of MAP kinases is blocked by pertussis toxin but not by treatment with hydrolysis-resistant cyclic AMP analogues. This suggests that the signal transduction pathway between CB1 and MAP kinases involves a pertussis-toxin-sensitive GTP-binding protein and is independent of cyclic AMP metabolism. This coupling of CB1 subtype and mitogenic signal pathway, also observed in the human astrocytoma cell line U373 MG, may explain the mechanism of action underlying cannabinoid-induced *Krox-24* induction.

INTRODUCTION

The central cannabinoid receptor (CB1) belongs to the G-protein-coupled receptor superfamily [1,2]. Several signalling pathways triggered by the activation of this receptor have already been described, including modulation of adenylate cyclase [3] or N-type calcium channels [4]. More recently, induction of immediate-early genes has been observed 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 leads to the expression of the growth-related gene *Krox-24*, also known as *NGFI-A*, *zif*/268 and *egr*-1 [5]. Furthermore a similar effect was observed *in vivo*, in rat forebrain [6] and striosomes [7]. Although the molecular mechanisms located downstream of the G protein and leading to *Krox-24* activation by cannabinoid remain to be identified, some elements are already known. We have recently shown that the activation of *Krox-24*, which is blocked by pertussis toxin (PTX) treatment, cannot be ascribed to known PTX-sensitive G-protein pathways: adenylate cyclase, phospholipase C and ion channel modulation [5]. Besides, although activation by cannabinoid agonists of phospholipase A_2 had been reported [8,9], Felder et al. [10,11] established that this effect was not receptor mediated. Using U373 MG cells and CB1-transfected CHO cells, we did not observe any cannabinoid-induced arachidonate release, which was not in favour of the involvement of phospholipase A_2. By contrast, we showed that *Krox-24* induction was inhibited by the tyrosine kinase inhibitor herbimycin A, suggesting that a protein tyrosine kinase may lie on the route between G_i and *Krox-24* [5].

The mitogen-activated protein (MAP) kinases, which can be blocked by tyrosine kinase inhibitors [12], can be stimulated through G-protein-mediated mechanisms [13–15] and might activate transcription factors such as c-Jun or c-Myc that, in turn, modulate the expression of target genes [16]. For these reasons, we here selectively studied the ability of CB1-mediated signal transduction pathways to activate the MAP kinase regulatory network.

We here provide evidence that CB1 is functionally coupled to the MAP kinase cascade. These findings raise the possibility of a signal transduction pathway linking CB1 to the regulation of the immediate-early gene *Krox-24*.

MATERIALS AND METHODS

Reagents

CP-55940 was obtained from Pfizer. [^{3}H]CP-55940 was purchased from New England Nuclear Corporation. Δ^9-Tetrahydrocannabinol (Δ^9-THC), 8-bromoadenosine 3′,5′-cyclic monophosphate, bovine myelin basic protein (MBP), leupeptin, aprotinin, PMSF and sodium orthovanadate were from Sigma Chemicals. WIN 55212-2 and PTX were obtained from Research Biochemicals Inc. Phorbol 12-myristate 13-acetate (PMA) and herbimycin A were from Gibco BRL. Dibutyryl-cyclic AMP and 3-isobutyl-1-methylxanthine (IBMX) were from Boehringer. SR 141716A [*N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride] was synthesized at the Chemistry Department, Sanofi Recherche as described previously [17]. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham Corp. Horseradish peroxidase-coupled

Abbreviations used: CHO, Chinese Hamster Ovary; CB1, central cannabinoid receptor; IBMX, 3-isobutyl-1-methylxanthine; MAP, mitogen-activated protein; MBP, myelin basic protein; ERK, external-signal-regulated kinase; Δ^9-THC, Δ^9-tetrahydrocannabinol; PMA, phorbol 12-myristate 13-acetate; PTX, pertussis toxin.

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anti-phosphotyrosine antibodies (PY20) were bought from ICN. Anti-p44 (C-16, anti-ERK-1) and anti-p42 (C-14, anti-ERK-2) rabbit polyclonal antibodies were bought from Santa Cruz Biotechnology Inc.

Obtention of CHO cells transfected with human CB1

CB1 cDNA from IM-9 was amplified with a sense primer bearing a *Hin*dIII site, a Kozak consensus sequence (5'-CCACACAAGCTTGCCACCATGGAGGAATGCTGGGTG) and an antisense primer bearing an *Eco*R1 site (5'-CCACTCGGATCCTCAGCAATCAGAGAGGTCTAG). The amplicon was digested with *Hin*dIII/*Eco*R1 and inserted into p658, an expression plasmid derived from p7055 [18] in which the IL-2 coding sequence was replaced by a polylinker. The CB1 expression vector was transfected into CHO dihydrofolate reductase $(DHFR)^-$ cells by a modified $Ca_3(PO_4)_2$ precipitation method [19]. Wild-type and transfected CHO cells are referred to as CHO-wt and CHO-CB1 cells respectively.

Cell lines

The human astrocytoma cell line U373 MG from ATCC was grown as a monolayer in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), 1% vitamins and 1 mM sodium pyruvate. CHO cells stably transfected with CB1 were grown as monolayers in Dulbecco's modified Eagle's medium supplemented with 10% dialysed fetal calf serum, 2 mM glutamine, 40 g/ml L-proline, 1% anti-PPLO (anti-mycoplasm) agent, 1 mM sodium pyruvate and 5 g/ml gentamycin. Wild-type CHO cells were grown in the same medium supplemented with 10% fetal calf serum. Cells were maintained in 0.5% fetal calf serum medium for 24 h before treatment.

Radioligand-binding assays

Binding experiments using CHO-CB1 were performed with [^{3}H]CP-55940 as described previously [20]. Membranes were isolated from CHO-CB1 cells after centrifugation of the total cell homogenate (15 min, 2000 *g*) and the supernatant was centrifuged for 1 h at 105000 *g*. Protein concentration was measured on the pellet, and membranes were stored at −80 °C until use. In competition experiments, the drug concentrations producing 50% inhibition (IC_{50}) of radioligand binding were determined from Hill plots of $\log(B/B_0-B)$ versus log[test drug], where B_0 and B were specific binding 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 [21].

Cyclic AMP analysis

Cells were grown to confluence, washed twice in PBS and incubated for 30 min in serum-free medium containing Ro-201724 (0.25 mM), IBMX (0.1 mM) and forskolin (5 M), supplemented or not with cannabinoids. The reaction was terminated by the addition of 0.1 M HCl. Determination of cyclic AMP levels was performed by radioimmunoassay according to the manufacturer's instructions (Pharmacia). Each data point is the mean of triplicate samples, and experiments were repeated twice.

Immunoblotting of tyrosine-phosphorylated proteins

After exposure of CHO-CB1 cells to cannabinoid ligands for various time periods, cells were washed once in ice-cold 50 mM Hepes, pH 7.4, 0.2 mM sodium orthovanadate, then directly lysed in Laemmli's loading buffer containing 6 M urea and run on PAGE [22]. Proteins were blotted on to nitrocellulose filters, and phosphotyrosine-containing proteins were detected by the use of horseradish peroxidase-coupled antiphosphotyrosine antibodies (PY20) and the enhanced chemiluminescence Western-blotting detection system (Amersham), as described previously [23].

MAP kinase phosphorylation

Phosphorylation of p42 (ERK2) and p44 (ERK1) MAP kinases was determined by the electrophoretic mobility shift assay [24,25]. Stimulated cells were treated as above, and proteins were separated on 10% polyacrylamide gels before transfer to nitrocellulose filters. Non-specific binding of antibodies was prevented by incubating filters in 10% dried milk powder in TBST buffer [10 mM Tris (pH 7.6)/150 mM NaCl/0.05% Tween; this buffer was also used for all incubation and washing steps]. Immunostaining of p42 or p44 MAP kinases was carried out using purified anti-p42 and anti-p44 antiserum (0.25 μg/ml) and a horseradish peroxidase-coupled anti-rabbit IgG antibody and revealed with the Amersham enhanced chemiluminescence detection system.

MAP kinase immunocomplex

MAP kinase activity was measured as described by Frödin et al. [15]. 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 glycerophosphate/1 mM EGTA/2 mM Na_3VO_4] and lysed for 15 min in buffer A containing 1% (v/v) Triton X-100, 100 units/ml aprotinin, 20 μM leupeptin and 0.2 mg/ml PMSF. The solubilized cell extracts were clarified by centrifugation at 14000 *g* for 15 min and then incubated for 3 h with the 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% (v/v) glycerol/0.1% (v/v) Triton X-100/0.2 mM Na_3VO_4], then air-dried and resuspended in 50 μl of buffer B supplemented with 100 units/ml aprotinin, 20 μM leupeptin and 0.2 mg/ml PMSF. Phosphorylation of MBP was initiated by addition of 10 μl of a 6-fold concentrated mixture consisting of 150 μg/ml MBP, 10 mM magnesium acetate, 1 mM dithiothreitol and [γ-^{32}P]ATP (5 μM, 33 Ci/mmol). The phosphorylation reaction was performed for 30 min at 30 °C (linear assay conditions) and was stopped by spotting on Whatman P-81 filter papers which were then dropped into 0.1% (v/v) orthophosphoric acid. The papers were washed in this solution, rinsed with ethanol and air-dried, and the radioactivity incorporated in MBP was determined by liquid-scintillation counting.

RESULTS AND DISCUSSION

Cannabinoid induces tyrosine phosphorylation of p42/44 MAP kinases in CHO-CB1 cells

In this study, experiments on cannabinoid signal transduction were performed on CHO cells transfected with the human CB1 cDNA. These cells exhibit specific binding properties for the cannabinoid receptor ligand [^{3}H]CP-55940 with a K_d of 0.4 ± 0.09 nM and a $B_{max.}$ of 200 ± 21.6 fmol/mg of protein. From our previous observation of a potent inhibition of CP-

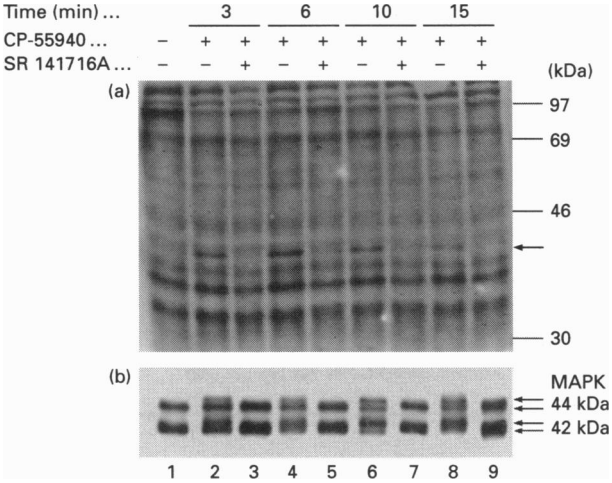

Figure 1 Time course of tyrosine phosphorylation of MAP kinase in CHO-CB1 by CP-55940

Growth-arrested CB1-transfected CHO cells were treated with 10 nM CP-55940 in the presence or not of 100 nM SR 141716A at 37 °C for the indicated time periods. Reaction was stopped by the addition of Laemmli SDS buffer, and protein extracts were processed as detailed in the Materials and methods section. (**a**) Tyrosine-phosphorylated immunoblot analysis with the monoclonal anti-phosphotyrosine antibody PY20. A polypeptide of 42/44 kDa immunoreactive with the antibody is indicated by an arrow. (**b**) MAP kinases were detected by Western blotting using specific anti-p42 and anti-p44 MAP kinase rabbit antibodies. Lane 1, untreated cells; lanes 2, 4, 6 and 8, cells incubated with 10 nM CP-55940 for 3, 6, 10 and 15 min respectively; lanes 3, 5, 7 and 9, cells incubated with 10 nM CP-55940 + 100 nM SR 141716A for 3, 6, 10 and 15 min respectively.

55940-mediated *Krox-24* induction by herbimycin A (an inhibitor of tyrosine kinase) [5], we first examined whether the CB1 activation was followed by tyrosine phosphorylation. CHO-CB1 cells were treated with the cannabinoid agonist CP-55940 for various time periods, before proteins from cell lysates were separated by SDS/PAGE and immunoblotted with a specific anti-phosphotyrosine antibody. Results presented in Figure 1(a) show a significant increase in phosphotyrosine-containing proteins, and mainly in proteins with a molecular mass close to that of MAP kinase (p42–p44 kDa). This effect was transient, with a maximum level observed at 6 min, and was specific for CB1 stimulation, since it was completely abrogated by the CB1 antagonist SR 141716A.

We next tested whether this phosphotyrosine-containing protein was a member of the MAP kinase family by immunoblot analysis using specific anti-p42 and anti-p44 kDa MAP kinase antibodies. Figure 1(b) shows that CP-55940 treatment induced a shift to lower electrophoretic mobility of p42 and p44 MAP kinase proteins in a time-dependent manner, which is consistent with the modification of electrophoretic mobility of the activated phosphorylated form as compared with the non-phosphorylated inactive form [24]. The specific involvement of CB1 was demonstrated by experiments including co-exposure to SR 141716A. These findings indicated that MAP kinases became tyrosine-phosphorylated in cannabinoid-treated cells.

Cannabinoid activates MAP kinases in CHO-CB1 cells

Activation of MAP kinases requires phosphorylation on both tyrosine and threonine residues [26]. To determine if, in addition to tyrosine phosphorylation, cell treatment with CP-55940 induced activation of MAP kinases, MAP kinases immunoprecipitated with anti-MAP kinase antibodies were assayed for

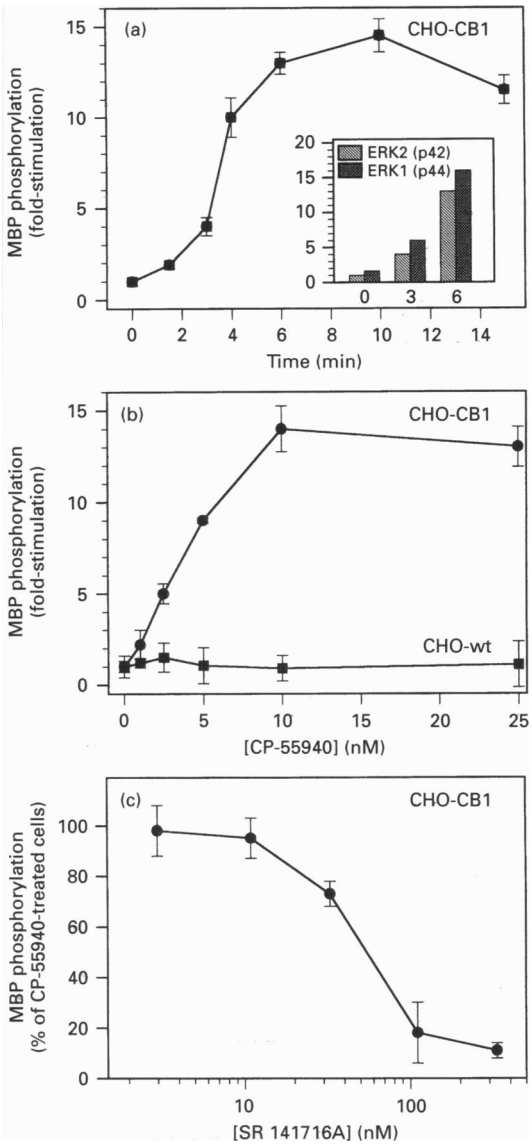

Figure 2 Effect of cannabinoid on MAP kinase activity

P42/44 MAP kinase activity was measured in cell lysates using MBP as a substrate as described in the Materials and methods section. Results are representative of one experiment performed five times. (**a**) Kinetics of cannabinoid activation of MAP kinases. Growth-arrested CHO-CB1 (●) cells were treated with 10 nM CP-55940 at 37 °C for the indicated time periods. Insert represents p42 and p44 kDa MAP kinase activity measured independently. (**b**) Dose-dependent effect of cannabinoid on MAP kinases. Growth-arrested CHO-CB1 cells (●) or wild-type CHO cells (■) were treated for 10 min with the indicated concentrations of CP-55940. (**c**) Effect of the CB1 antagonist SR 141716A on cannabinoid-induced MAP kinases. Growth-arrested CHO-CB1 cells (●) were treated with various concentrations of SR 141716A in the presence of 10 nM CP-55940 for 10 min before analysis.

kinase activity towards MBP as a substrate [27]. Resting CHO-CB1 cells displayed a slight constitutive activity that was markedly enhanced upon exposure to CP-55940 (Figure 2a). MAP kinase activity was measurable after 1 min of stimulation, peaked between 6 and 10 min, remained elevated until 15 min then slowly declined and returned to the baseline value after 30 min (results not shown). When the MBP phosphorylation was measured using either anti p42- or p44-kDa MAP kinase antibodies, a similar stimulation profile was observed, indicating

Table 1 Effect of cannabinoid agonists on MAP kinase activation in CHO-CB1 cells

Results are means ± S.E.M.

Drugs	Displacement of [^{3}H]CP-55940 K_i (nM)	MAP kinase activation IC_{50} (nM)	Cyclic AMP accumulation IC_{50} (nM)
CP-55940	0.68 ± 0.1	3.4 ± 0.5	1.2 ± 0.07
Δ^9-THC	3.89 ± 0.6	15 ± 5.2	10 ± 0.2
WIN 55212.2	485 ± 63	40 ± 15	14 ± 8

Table 2 Effect of PTX, cyclic AMP modulation and tyrosine kinase inhibitor on CP-55940-induced MAP kinase activity in CHO-CB1 cells

Cells were untreated (−) or treated with 10 nM CP-55940 (+). Results are means ± S.E.M.

Drugs	MAP activity (fold-increase)*	
	−	+
None	1 ± 0.28	13.5 ± 2.6
PTX	0.95 ± 0.3	1.7 ± 0.4
IBMX + dibutyryl cyclic AMP	3.4 ± 0.85	24 ± 3.1
IBMX + 8-bromo-cyclic AMP	5.8 ± 1.9	26 ± 2.7
Herbimycin A	0.92 ± 0.7	2.9 ± 0.5

** MAP kinase activity in untreated cells was taken as unity.

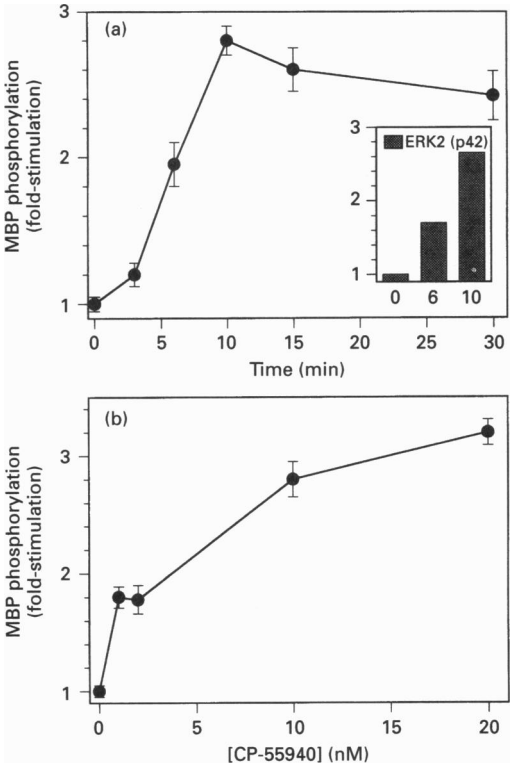

Figure 3 Cannabinoid induces MAP kinase activation in U373 MG cells

(**a**) Growth-arrested U373 MG cells were treated with 10 nM CP-55940 for the indicated periods of time before analysis for p42/44 MAP kinase activity. Insert represents p42 kDa MAP kinase activity as measured independently; p44 kDa MAP kinase activity was not detectable. (**b**) U373 MG cells were treated with the indicated concentration of CP-55940 for 10 min before analysis. Results are representative of one experiment performed three times.

the activation of both kinases (Figure 2a insert), which is in accordance with the gel mobility shift of activated MAP kinases depicted in Figure 1(b). CP-55940 activated MAP kinases in CHO-CB1 in a dose-dependent manner, with a noticeable effect at a concentration of 1 nM and a maximal effect at 10 nM (Figure 2b). Using different cannabinoid agonists we found that the order of potency for MAP kinase activation was CP-55940 > Δ^9-THC > WIN 55212.2 (Table 1). These results were in fairly good agreement with the concentration of these products required to displace [^{3}H]CP-55940 binding or to inhibit adenylate cyclase (Table 1). Furthermore, the specific involvement of CB1 was assessed (i) by the absence of MAP kinase activation in wild-type CHO cells (Figure 2b) and (ii) by the abrogation of activation by SR 141716A in CHO-CB1 cells (Figure 2c).

MAP kinase activation in U373 MG cells

Because the signalling pathway, particularly the coupling to G-proteins, might be defined by the cell line used for transfection, we next examined whether the above data obtained using an overexpressed receptor system could be extended to the human astrocytoma cell line U373 MG naturally expressing CB1 [5]. A similar time- and dose-dependent MAP kinase stimulation was observed in U373 MG cells. In contrast to CHO-CB1 cells, the p42 kinase protein accounted for the whole MBP phosphorylation, since no p44 kinase activity was detected (Figure 3 insert). This is also confirmed by the lack of expression of p44 kinase protein in U373 MG cells observed in immunoblot analysis (results not shown). The lower magnitude in MAP kinase activation in U373 MG cells as compared with CHO-CB1 cells could be explained by a 10-fold lower level in the CB1 expression (results not shown). Taken together these results provide evidence that cannabinoids must be added to the list of ligands that activate MAP kinases and that this mechanism of coupling is not restricted to transfected cells.

CP-55940 induces MAP kinases in CHO-CB1 cells via a G_i/G_o-protein-dependent, cyclic AMP-independent transduction pathway

To further investigate the effector pathway responsible for MAP kinase activation in CHO-CB1, we examined the effect of PTX. As shown in Table 2, stimulation of MAP kinase activity by CB1 was reduced to the basal level by cell pretreatment with PTX (100 ng/ml), consistent with the selective coupling of CB1 and G_i proteins, which are substrates for PTX.

In Table 1 we showed that cannabinoids potently inhibited adenylate cyclase, suggesting that MAP kinase activation might be secondary to the inhibition of adenylate cyclase. Thus increasing cyclic AMP levels should prevent MAP kinase activation. To explore this possibility, intracellular cyclic AMP levels were raised using IBMX in combination with either dibutyryl cyclic AMP or 8-bromo-cyclic AMP. These agents alone significantly increased the basal MAP kinase level. Surprisingly, the addition of CP-55940 under these conditions led to an enhanced signal corresponding to additive effects of the two stimuli, as would be expected for agents acting on different transduction pathways (Table 2). From these results, we conclude

that cannabinoid activation of MAP is not secondary to the inhibition of adenylate cyclase and consequently that the CB1 transduction pathway can trigger these two signals independently.

We have previously demonstrated that stimulation of CB1 by cannabinoid agonists induces the expression of the immediate-early gene *Krox-24* through a PTX-sensitive heterotrimeric G_i protein that also by-passes the adenylate cyclase pathway [5]. This suggests that MAP kinase, whose activation precedes *Krox-24* expression, could be a candidate for regulation of this latter effect. This notion can be supported by the observation that cells pretreated with the tyrosine kinase inhibitor herbimycin A fail to respond to CP-55940 in the enhancement of both MAP kinase activity (Table 2) and *Krox-24* expression [5]. In agreement with such a possibility, McMahon and Monroe recently demonstrated that the MAP kinase pathway was responsible for *Krox-24* induction in B cells after cross-linking of the antigen receptor [28].

In the central nervous system, MAP kinases were found to be activated in hippocampal neurons through *N*-methyl-D-aspartic acid receptors to induce the expression of immediate-early genes such as c-*fos* [29]. We here provide data for the induction of *Krox-24* by MAP kinase-mediated CB1 stimulation. The molecular mechanisms located downstream of the G protein and leading to MAP kinase activation still remain to be established and are currently under study. An interesting possibility would be that $\beta\gamma$ dimers rather than αi subunits are directly involved in G_i–MAP coupling [30,31].

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REFERENCES

1 Matsuda, L. A., Lolait, S. J., Brownstein, M. J., Young, A. C. and Bonnet, T. I. (1990) Nature (London) **346**, 561–564
2 Gérard, C. M., Mollereau, C., Vassart, G. and Parmentier, M. (1991) Biochem. J. **279**, 129–134
3 Howlett, A. C. (1985) Mol. Pharmacol. **27**, 429–436
4 Mackie, K. and Hille, B. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 3825–3829
5 Bouaboula, M., Bourrié, B., Rinaldi-Carmona, M., Shire, D., Le Fur, G. and Casellas, P. (1995) J. Biol. Chem. **270**, 13973–13980
6 Mailleux, P., Verslype, M., Preud'homme, X. and Vanderharghen, J. J. (1994) Neuroreport **5**, 1265–1268
7 Glass, M. and Dragunow, M. (1995) Neuroreport **6**, 241–244
8 Burstein, S., Budrow, J., Debatis, M., Hunter, S. A. and Subramanian, A. (1994) Biochem. Pharmacol. **48**, 1253–1264
9 Wartmann, M., Campbell, D., Subramanian, A., Burstein, S. H. and Davis, R. (1995) FEBS Lett. **359**, 133–136
10 Felder, C. C., Veluz, J. S., Williams, H. L., Briley, E. M. and Matsuda, L. A. (1992) Mol. Pharmacol. **42**, 838–845
11 Felder, C. C., Briley, E. M., Axelrod, J., Simpson, J. T., Mackie, K. and Devane, W. A. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 7656–7660
12 Winitz, S., Russel, M., Qian, N., Gardner, A., Dwyers, L. and Johnson, G. L. (1993) J. Biol. Chem. **268**, 19196–19199
13 Cobb, M. H., Boulton, T. G. and Robbins, D. J. (1991) Cell Regul. **2**, 965–978
14 Kahan, C., Seuwen, K., Meloche, S. and Pouysségur, J. (1992) J. Biol. Chem. **267**, 13369–13375
15 Frödin, M., Prealdi, P. and Van Obberghen, E. (1994) J. Biol. Chem. **269**, 6207–6214
16 Seth, A., Alvarez, E., Gupta, S. and Davis, R. J. (1991) J. Biol. Chem. **266**, 23521–23524
17 Rinaldi-Carmona, M., Barth, F., Héaulme, M. et al. (1994) FEBS Lett. **350**, 240–244
18 Miloux, B. and Lupker, J. B. (1994) Gene **149**, 341–344
19 Graham, F. L. and Van der Eb, A. J. (1973) FEBS Lett. **54**, 536–539
20 Bouaboula, M., Rinaldi, M., Carayon, P. et al. (1993) Eur. J. Biochem. **214**, 173–180
21 Cheng, Y. and Prusoff, W. (1973) Biochem. Pharmacol. **22**, 3099–3108
22 Laemmli, U.K. (1970) Nature (London) **227**, 680–685
23 Offermanns, S., Seifert, R., Metzger, J. W. et al. (1992) Biochem. J. **282**, 551–557
24 Leevers, S. J. and Marshall, C. J. (1992) EMBO J. **11**, 569–574
25 De Vries-Smith, A. M. M., Burgering, B. M. T., Leevers, S. J., Marshall, C. J. and Bos, J. L. (1992) Nature (London) **357**, 602–604
26 Anderson, N. G., Maller, J. L., Tonks, N. K. and Sturgill, T. W. (1990) Nature (London) **343**, 651–653
27 Ahn, N. G., Weiel, J. E., Chan, C. P. and Krebs, E. G. (1990) J. Biol. Chem. **265**, 11487–11494
28 McMahon, S. B. and Monroe, J. G. (1995) J. Exp. Med. **181**, 417–422
29 Bading, H., Ginty, D. D. and Greenberg, M. E. (1993) Science **260**, 181–186
30 Birnbaumer, L. (1992) Cell **71**, 1069–1072
31 Koch, W. J., Hawes, B. E., Allen, L. F. and Lefkowitz, R. J. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 12706–12710

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