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Reciprocal influences of CB_1 cannabinoid receptor agonists on ERK and JNK signalling in N1E-115 cells

Barbara Bosier[a], Didier M. Lambert[a,1], Emmanuel Hermans[b,*,1]

[a] *Unité de Chimie Pharmaceutique et de Radiopharmacie (UCL 7340), Université catholique de Louvain, 73 40, Av E. Mounier, B-1200 Brussels, Belgium*

[b] *Laboratoire de Pharmacologie expérimentale (UCL 5410), Université catholique de Louvain, 54 10, Av Hippocrate, B-1200 Brussels, Belgium*

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Abstract **Agonists acting at the CB_1 cannabinoid receptor in N1E-115 neuroblastoma cells were found to activate MAPK family members with reciprocal efficacies. Thus, HU 210 robustly increased phosphorylation of ERK1/2 whereas CP 55,940 was more effective in activating JNK. The use of selected kinase inhibitors confirmed that distinct signalling cascades were involved in these responses. This reciprocal control of MAPK activity was correlated with the observation that HU 210- and CP 55,940-mediated regulations of tyrosine hydroxylase gene expression were respectively impaired by MEK and JNK inhibitors. These data indicate that complex interactions of the CB_1 receptor with intracellular signalling partners controlling MAPK activities may explain the apparent disparities in cellular responses to functional selective agonists.**

Keywords: Functional selectivity; Cannabinoid receptor; Tyrosine hydroxylase; MAPK; Drug efficacy; G Protein coupled receptor

1. Introduction

In the central nervous system, the CB_1 cannabinoid (CB_1) receptor mediates most of the behavioural effects of Δ^9-THC, the major psychoactive component of *Cannabis sativa,* through activation of $G_{i/o}$-type G proteins. Accordingly, inhibition of adenylyl cyclase is the predominant short-term response characterised for Δ^9-THC and other ligands activating this receptor. This predominant coupling also supports the inhibition of voltage-gated Ca^{2+}-channels as well as the increase in G protein-coupled inwardly-rectifying K^+ currents. Analysis of downstream signallings also evidenced the recruitment of mitogen activated protein kinases (MAPK) by cannabinoid agonists (see [1] for review). Among the different members of the MAPK family, extracellular signal-regulated kinase 1 and 2 (ERK1/2) [2], c-Jun N-terminal kinase (JNK) and p38 MAPK [3] appear to be regulated by CB_1 receptor through several signalling pathways. Yet, the complexity of cellular responses is also influenced by the model where the receptor is studied. Thus, variables such as the nature of adenylyl cyclase isoforms that are expressed [4], the existence of constitutive effector activity and the subtype of $G_{i/o}$-type G proteins that couple with CB_1 receptor [5,6] may explain paradoxical responses such as increased cAMP accumulation [7], activation of calcium channel [8] or inhibition of ERK1/2 [9].

Considering the multiplicity of signals triggered by cannabinoid agonists, the CB_1 receptor represents an attractive model to study the biochemical mechanisms influencing the pharmacodynamic properties of distinct ligands acting at single G protein-coupled receptors. As different conformations presumably support distinct couplings of the receptor with specific G proteins and intracellular effectors, unrelated agonists may ultimately trigger multiple responses with independent efficacies [10]. Indeed, both HU 210 and CP 55,940 were described as partial agonists in regulating transient intracellular Ca^{2+} mobilisation [11] whereas in guanylyl nucleotide binding and adenylyl cyclase assays, these compounds are commonly considered as full agonists. In addition CP 55,940 was also reported as a partial agonist for inhibition of $[Ca^{2+}]_i$ spiking [12]. Consistent with the low efficacy sometimes reported for CP 55,940, we recently evidenced that the AP-1-dependent modulation of gene transcription by HU 210 was antagonised by CP 55,940 [13]. In the present study, we further characterised the pharmacodynamic profiles of HU 210 and CP 55,940 by measuring the activation of different MAPK family members in N1E-115 neuroblastoma cells. Our data indicate that HU 210 robustly phosphorylates ERK1/2 while CP 55,940 was more effective in activating JNK, suggesting the existence of specific signalling pathway efficacies for these potent agonists of the CB_1 receptor.

2. Materials and methods

2.1. Materials

The gene reporter plasmid pTH250-Luc in which the firefly luciferase sequence is cloned under the control of the TH gene promoter was provided by Dr. M. Najimi [14]. pRL 138 encoding renilla luciferase and used for standardisation of transfection procedures was obtained from Dr. E. Pierreux [15]. The CB_1 receptor inverse agonist/antagonist SR 141716A was generously given by Dr. Barth (Sanofi-Synthélabo Research, Montpellier, France). HU 210 and CP 55,940 were purchased from Tocris Cookson (Bristol, UK) and all kinase inhibitor tested were from Sigma (Boornem, Belgium).

2.2. Cell culture

All cell culture media and supplements were obtained from Invitrogen (Merelbeke, Belgium). Mouse neuroblastoma N1E-115 cells were grown in Dulbecco's MEM/NUT mix F-12 medium supplemented with 10% foetal calf serum, 100 UI/ml penicillin, 100 μg/ml

*Corresponding author. Fax: +32 2 764 5460.
E-mail address: emmanuel.hermans@uclouvain.be (E. Hermans).

[1]D.M. Lambert and E. Hermans contributed equally as senior investigators in this study.

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streptomycin and 2 mM L-glutamine. All drug treatments were conducted in the same culture medium. Cells were cultured at 37 °C in an atmosphere of humidified air and 5% CO_2.

2.3. Immunoblotting studies

At the end of incubation with drugs, cells cultured in 6-well plates were washed with phosphate buffer saline (PBS, Invitrogen), scrapped and collected by brief centrifugation. Pelleted cells were resuspended in lysis buffer containing 50 mM HEPES, 50 mM KF, 1 mM Na_3PO_4, 1 mM EDTA, 1 mM EGTA, 0.5% 2-mercaptoethanol, 5 mM β-glycerolphosphate, 5 mM $Na_4P_2O_7$, 1 mM phenylmethylsulphonyl fluoride, 100 μM Na_3VO_4, 1% Triton X-100, protease inhibitor complete mini EDTA (Roche, Vilvoorde, Belgium) and phosphatase inhibitor cocktail (Sigma). After 30 min incubation, cell lysate was centrifuged to remove cell debris and protein content of the supernatant was determined. All these procedures were carried out at 0–4 °C. Protein extracts were diluted in 5× loading buffer (250 mM Tris–HCl, 500 mM dithioerythritol, 10% sodium dodecylsulfate (SDS), 50% glycerol, 0.5% bromophenol blue). Samples (respectively, 20 or 40 μg for ERK1/2 and JNK detection) were boiled for 5 min, separated on a 12% SDS–polyacrylamide gel and transferred to nitrocellulose membrane. The blots were blocked for 1 h in TBST buffer (25 mM Tris–HCl, pH 7.4, 125 mM NaCl, and 0.5% Tween-20) containing 5% non-fat milk and then incubated with ERK1/2, JNK-1, or p38 MAPK phospho-specific antibodies (phospho-MAPK family antibody sampler kit from Cell Signalling Technology, Bioké, Leiden, The Netherland), overnight at 4 °C in the same buffer. The blots were washed in TBST buffer, and specific immunoreactive proteins revealed with a peroxydase linked anti-rabbit IgG were detected using the SuperSignal West Pico Chemiluminescent Substrate System (Pierce, Erembodegem, Belgium). The blots were then stripped and re-probed with antibodies that recognise both the phosphorylated and non phosphorylated forms of ERK1/2, JNK-1, or p38 MAPK (same kit as above, from Cell Signalling Technology). Relative amounts of protein were quantified by scanning densitometry using the software Image Master (Pharmacia Biotech Benelux, Roosendael, The Netherlands).

2.4. Transient transfection and luciferase assay

N1E-115 cells were cotransfected with pTH250-Luc and pRL 138, using the phosphate co-precipitation method as previously described [16]. For drug treatments, transfected cells were washed with PBS and incubated with 100 nM HU 210 or 100 nM CP 55,940 for 5 h in fresh medium. When indicated, SR 141716A was added 5 min prior the agonists. When testing the influence of kinase inhibitors, these were added 1 h beforehand at concentrations achieving maximal efficacy and target selectivity [17–23].

At the end of the 5 h incubation period, cells were lysed by addition of 100 μl of passive lysis buffer supplied in the Dual Luciferase Reporter Assay System (Promega, Leiden, The Netherlands). Firefly luciferase reporter activity was normalised for the renilla luciferase activity. Respective measurement of light emission was determined according to the manufacturer's instructions with a TD20/20 luminometer (Turner design, Sunnyvale, CA, USA).

2.5. Data analysis

Unless otherwise stated, data presented in the text and figures were expressed as mean percentages ± S.E.M. of the corresponding values obtained with cells treated with vehicle alone (DMSO diluted in culture

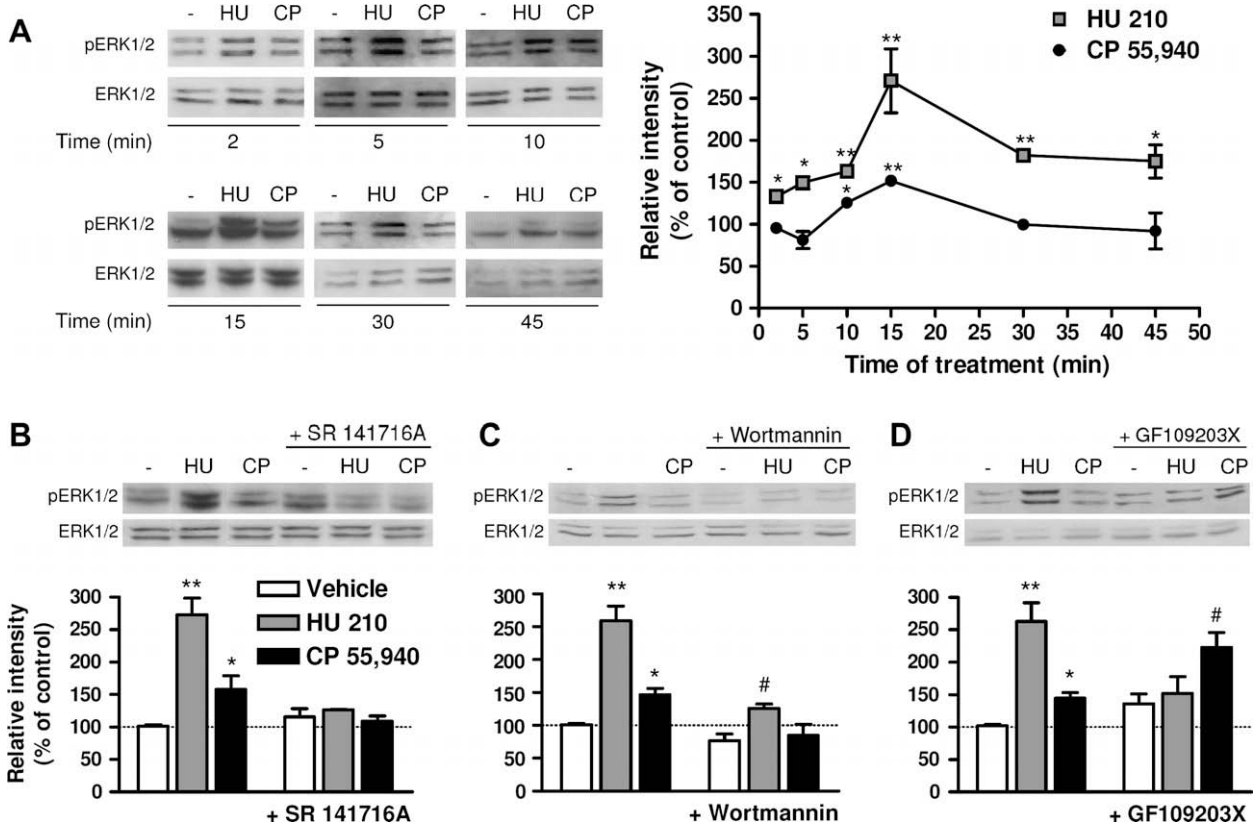

Fig. 1. HU 210 and CP 55,940 increase ERK1/2 phosphorylation in N1E-115 cells. Typical immunoblotting analyses of cells extracts using antibodies recognising either specific residues of phosphorylated ERK1/2 or total ERK1/2. A shows the time-course of ERK1/2 phosphorylation induced by the agonists (100 nM). B, C and D, respectively illustrate the influence of the CB_1 selective antagonist/inverse agonist SR 141716A (1 μM), the PI3K inhibitor wortmannin (0.2 μM) and the PKC inhibitor GF109203X (0.25 μM) on ERK1/2 phosphorylation induced by 15 min exposure of the cells to HU 210 and CP 55,940 (both at 100 nM). Responses were quantified by densitometric analysis of phospho-ERK1/2 immunoreactive bands from at least three independent experiments and normalised for the signal corresponding to total ERK1/2. Data shown are mean values with SEM expressed in percentage of control (vehicle treated cells). $^{*}P < 0.05$, $^{**}P < 0.01$, relative to vehicle treated cells, $^{\#}P < 0.05$ relative to cells treated with the inhibitor only.

medium). Immunoreactive signal for phosphorylated kinases were quantified using densitometry and normalized against signal corresponding to the total amount of the selected kinase. Mean percentages were obtained from at least three independent experiments. One-way analyses of variance (ANOVA) with repeated measures were performed using Graph Pad prism software (San Diego, CA). Post hoc analysis for pair-wise comparisons was carried out by Tukey's test.

3. Results

3.1. Cannabinoid-mediated ERK1/2 phosphorylation

Semi-quantitative analysis of ERK1/2 activation in N1E-115 cells was specifically examined by comparing immunoreactive signals detected with antibodies recognising either total ERK 1/2 or the phosphorylated, activated form of ERK1/2. Immunoblotting studies revealed that the exposure of N1E-115 neuroblastoma cells to either HU 210 (100 nM) or CP 55,940 (100 nM) resulted in a transient phosphorylation of ERK1/2 with a peak response after 15 min treatment for both agonists (Fig. 1A). Inductions measured at this time point revealed concentration-dependent responses (Table 1). When both agonists were used at maximal effective concentrations, the amplitude of the response was markedly different and the levels of phospho-ERK1/2 appeared significantly higher when using HU 210 as compared to CP 55,940. The selective inverse agonist/antagonist SR 141716A (1 μM) totally inhibited the responses to both agonists, demonstrating that ERK1/2 phosphorylation was strictly dependent on the activation of the CB_1 receptor (Fig. 1B). To characterise the signalling pathways involved in CB_1 receptor mediated ERK1/2 activation, N1E-115 cells were pre-incubated for 1 h with different kinase inhibitors (Fig. 1C–D). HU 210-induced ERK1/2 phosphorylation was significantly reduced using the phosphatidyl inositol 3-kinase (PI3 K) inhibitor wortmaninn (0.2 μM) whereas the PKC inhibitor GF109203X abolished this response (0.25 μM). The opposite was observed when examining the response to CP 55,940 as wortmaninn totally suppressed ERK1/2 phosphorylation induced by this agonist whereas GF109203X was ineffective. Hence, inhibiting PKC tended to amplify the response to CP 55,940.

3.2. Cannabinoid-induced JNK phosphorylation

The influence of the cannabinoid agonists on JNK phosphorylation was examined using antibodies specifically raised against phosphorylated p46 and p54 JNK. Both HU 210 (100 nM) and CP 55,940 (100 nM) increased the level of phospho-JNK (Fig. 2A). As maximal responses were measured after 5 min of exposure to the agonists we further evaluated the concentration-dependent effects of either HU 210 or CP 55,940 on cells treated during 5 min (Table 1). At variance with results referring to ERK1/2 phosphorylation, the influence of HU 210 on JNK phosphorylation appeared modest as compared to CP 55,940.Nevertheless, both responses were suppressed in the presence of SR 141716A (Fig. 2B). In the presence of wortmaninn, the responses were totally inhibited (Fig. 2C) suggesting the involvement of PI3K in both HU 210 and CP 55,940-mediated JNK phosphorylation. Noteworthy, the basal level of phospho-JNK was also reduced in cells exposed to wortmaninn.

3.3. Lack of cannabinoid-mediated p38 MAPK phosphorylation

Contrasting with previous studies reporting on the cannabinoid-mediated activation of p38 MAPK [3], neither HU 210 nor CP 55,940 were found to alter phospho-p38 MAPK levels in N1E-115 cells (Fig. 3A). Yet, hydrogen peroxide (5 mM), used as a positive control robustly increased p38 MAPK phosphorylation (Fig. 3B).

3.4. Involvement of MAPK dependent signallings in the modulation of gene transcription by cannabinoid agonists

We previously reported on the reciprocal regulation of tyrosine hydroxylase (TH) gene expression by the cannabinoid agonists HU 210 and CP 55,940. Since these agonists differentially influenced the activity of MAPK and considering that related signallings are frequently involved in the modulation of gene transcription, we evaluated influence of different MAPK inhibitors on the regulation of TH expression by these cannabinoid ligands. Using a luciferase reporter assay, we observed that the MAP/ERK kinase (MEK1/2) inhibitors, U0126 (5 μM) and PD98059 (25 μM) reduced basal activity of the TH gene promoter while SP600125 (10 μM), an inhibitor of JNK, significantly increased this basal activity.

In agreement with its strong influence on ERK1/2, the regulation of the TH gene promoter by HU 210, was inhibited by U0126 and PD98059, as a consequence of the indirect blockade of ERK1/2 by these MEK1/2 inhibitors. In contrast, SP600125 and SB202190 (respectively inhibiting JNK and p38 MAPK, 10 μM) failed to suppress HU 210-mediated responses (Fig. 4). On the other hand, U0126 and PD98059 only slightly reduced CP 55,940-mediated activation of the TH gene promoter. Yet, this response to CP 55,940 was completely abolished with SP600125, suggesting that the regulation of the TH gene promoter activity in N1E-115 cells involves both ERK1/2- and JNK-dependent signalling. The specific

Table 1
Concentration-dependency of cannabinoid-mediated MAPK phorsphorylation.

	0.1 nM	3.16 nM	100 nM	1 μM
ERK1/2				
HU 210	130.8 ± 15.3	202.4 ± 11.6	270.8 ± 38.1**	300.6 ± 25.0**
CP 55,940	91.75 ± 4.2	122.3 ± 6.3	151.6 ± 6.2**	150.6 ± 6.9**
JNK				
HU 210	100.5 ± 15.1	132.6 ± 10.5	176.3 ± 19.5*	154.9 ± 16.6
CP 55,940	148.5 ± 20.4	187.7 ± 35.5	439.3 ± 79.4**	375.2 ± 77.8*

Cells were exposed for 15 min (ERK1/2) or 5 min (JNK) to vehicle or increasing concentrations of cannabinoid agonists before extraction, immunoblotting analyses for phospho-MAPK and densitometric quantifications. Responses (mean values with S.E.M. from three independent experiments) were normalised for the signal corresponding to total MAPK and expressed in percentages relative to control values (vehicle treated cells) arbitrarily set at 100%. *$P < 0.05$, **$P < 0.01$, relative to vehicle treated cells.

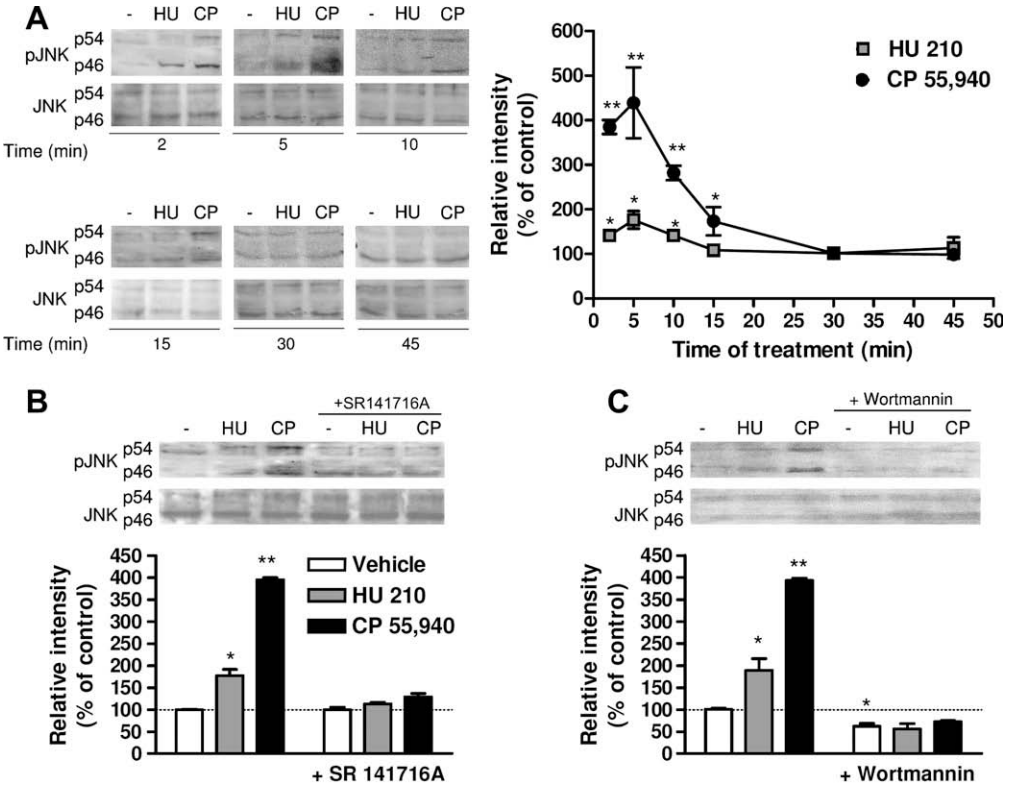

Fig. 2. HU 210 and CP 55,940 increase JNK phosphorylation in N1E-115 cells. Typical immunoblotting analyses of cells extracts using antibodies recognising either specific residues of phosphorylated JNK or total JNK. A shows the time-course of JNK phosphorylation induced by the agonists (100 nM). B and C, respectively, illustrate the influence of the CB_1 selective antagonist/inverse agonist SR 141716A (1 μM) and the PI3K inhibitor wortmannin (0.2 μM) on JNK phosphorylation induced by 15 min exposure of the cells to HU 210 and CP 55,940 (both at 100 nM). Responses were quantified by densitometric analysis of phospho-JNK immunoreactive bands from at least three independent experiments and normalised for the signal corresponding to total JNK. Data shown are mean values with SEM expressed in percentage of control (vehicle treated cells). $^{*}P < 0.05$, $^{**}P < 0.01$, relative to vehicle treated cells, $^{\#}$ <0.05 relative to cells treated with the inhibitor only.

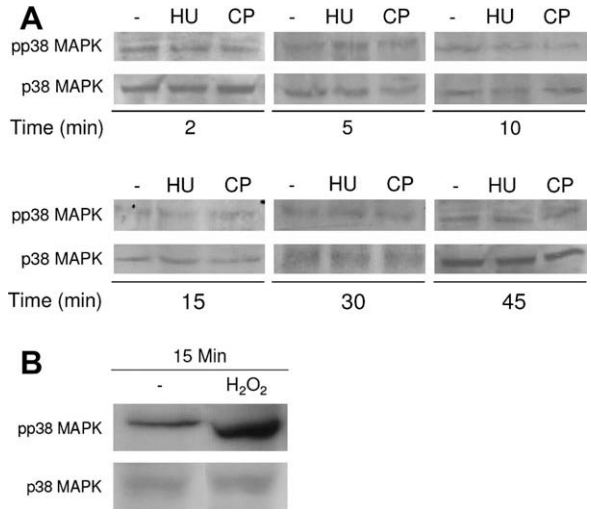

Fig. 3. HU 210 and CP 55,940 do not induce p38 MAPK phosphorylation in N1E-115 cells. Immunoblotting analysis performed using antibodies recognising either specific residues of phosphorylated p38 MAPK or total p38 MAPK. (A) The blots show the lack of p38 MAPK phosphorylation after exposure to the agonists (100 nM) for up to 45 min. (B) The assay was validated using H_2O_2 as positive control for p38 MAPK phosphorylation. The experiments were repeated three times independently with similar results.

responses to both agonists were reduced (partial and total inhibition for HU 210 and CP 55,940, respectively) when blocking PI3 K activation using either wortmannin or LY294002 (25 μM). Finally, confirming the absence of p38 MAPK regulation by cannabinoids in these cells, the response to HU 210 and CP 55,940 was not changed in the presence of SB202190.

4. Discussion

The classical MAPK pathway was originally described as a triple protein kinase cascade (Raf/MEK/MAPK) which is indirectly activated by tyrosine kinase receptors through their interaction with small monomeric G proteins. Presently, evidence is accumulating which indicates that G protein-coupled receptors also recruit MAPK through an intricate signalling network. With respect to cannabinoid receptors, PI3 K was frequently described as a key signalling intermediate in the activation of MAPK [3,9,24]. Yet, in N1E-115 cells, activation of the constitutively expressed CB_1 receptor by WIN 55,212-2 induced ERK1/2 phosphorylation through $G_{i/o}$-dependent inhibition of PKA pathway [25]. Unexpectedly, our preliminary experiments evidenced that in the same cell line, PKA inhibition failed to prevent HU 210 and CP 55,940 mediated increases in phospho-ERK1/2 level (data not shown). Besides, wortmannin was effective in reducing cannabinoid-mediated

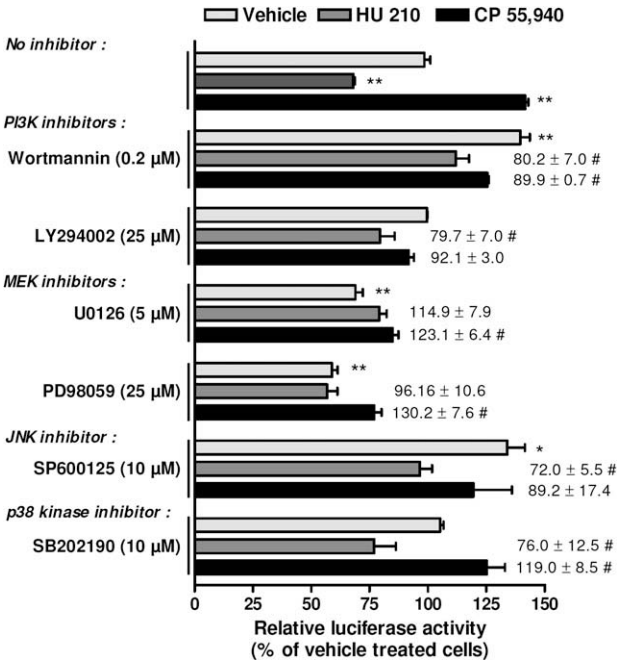

Fig. 4. Characterisation of cell signallings involved in cannabinoid-mediated regulation of TH gene promoter. Luciferase activity was examined in N1E-115 cells carrying pTH250-Luc, pre-treated for 1 h with vehicle or with kinase inhibitors and subsequently exposed for 5 h to HU 210 or CP 55,940 (both at 100 nM). Results (mean values with S.E.M. from three independent experiments performed in triplicate) are given as the percentages of relative luciferase activity (firefly luciferase relative to Renilla luciferase) relative to control cells. Numbers indicated next to each bar correspond to the same data expressed as percentage of activity when testing the inhibitor alone. $^{*}P < 0.05$, $^{**}P < 0.01$ relative to vehicle treated cells, $^{\#}P < 0.05$ relative to cells treated with the inhibitor alone.

ERK1/2 phosphorylation, indicating the recruitment of PI3K to activate ERK1/2 cascade in these cells. Nevertheless, a first set of key observations is that HU 210 increased phosphorylation of ERK1/2 much more efficiently than CP 55,940, and that the response to HU 210 was only partially suppressed with wortmannin, suggesting the involvement of different signalling intermediates. Indeed, inhibition of PKC completely abolished HU 210- but not CP 55,940-mediated increase in phospho-ERK1/2 levels. These data corroborate our previous studies demonstrating that unrelated ligands stimulate distinct transduction pathways upon interaction with the CB_1 receptor. Consistent with a PI3K-dependent activation of JNK described by Rueda [3], we observed a cannabinoid-induced increase in JNK phosphorylation that was inhibited by wortmannin. Noteworthy, PKA and PKC inhibitors failed to reduce JNK phosphorylation mediated by both cannabinoid agonists (data not shown). Finally, while several studies have evidenced a cannabinoid-mediated activation of p38 MAPK [3,26,27,28], neither HU 210 nor CP 55,940 appeared to significantly increase phospho-p38 MAPK level in N1E-115 neuroblastoma cells.

Frequently used as reference agonists for cannabinoid receptors, HU 210 and CP 55,940 are generally characterised as full CB_1 receptor agonists in guanylyl nucleotide binding [29–31] and cAMP accumulation [31–33] assays, displaying similar potencies and efficacies. At variance, focusing on ERK1/2 and JNK pathways, we herein evidence that these agonists show distinct and reciprocal influences on MAPK activation. Concentration-dependent characterisations of ERK and JNK phosphorylation clearly suggest an inverse rank order of efficacy for HU 210 and CP 55,940 when examining the different signalling pathways. In addition, the responses were systematically antagonised using SR 141716A, confirming that the difference in efficacy purely reflects the concept of agonist-selective trafficking (Kenakin 1995), currently also referred as functional selectivity [10]. Noteworthy, while up to now this concept was poorly illustrated at the level of CB_1 signalling, several previous studies already reported agonist-selective coupling of the CB_1 receptor [1,7,34]. Furthermore, it was recently demonstrated that distinct agonist-bound CB_1 receptor conformations exhibit different affinities and efficacies towards the G_{i1} type G protein [35]. This is consistent with the hypothesis that functional selectivity arises though agonist-selective stabilisation of different receptor conformations and subsequent selective interactions with different G proteins.

Activated MAPK participate in the regulation of gene expression through the phosphorylation of transactivating domains of several transcription factors. Thereby, endogenous cannabinoids influence target genes involved in regulation of cell proliferation, differentiation and death [36,37]. In particular, there is emerging evidence that cannabinoids may represent neuroprotective agents through regulation of PI3K/Akt survival pathways [38,39]. Supporting a role of ERK signalling in the survival responses, connections between PI3K/Akt and Raf/ERK pathways were previously suggested [24]. Furthermore, inhibition of ERK activity was shown to participate in cannabinoid-mediated cell apoptosis [40,41]. On the other hand, several studies reported that cannabinoids induce apoptosis through activation of JNK-dependent pathways [42–44]. In this respect, one could propose that different cannabinoid ligands may exhibit opposite influences on cell death/survival signallings through preferential activation of different MAPK family members.

Besides, the present data indicate that exogenous cannabinoid agonists could distinctly regulate gene transcription, leading to unexpected outcomes. Indeed we previously evidenced that the CB_1 receptor operates a complex control of TH expression through an agonist-selective regulation of intracellular signallings [16]. Accordingly, a second key finding of this work is the demonstration that HU 210-mediated reduction of TH gene promoter activity is regulated through ERK1/2 dependent signalling whereas CP 55,940-induced increased in the activity of the same promoter is supported by the JNK cascade. In addition, inhibition of PI3K which acts upstream of both ERK1/2 and JNK reduced the cannabinoid-mediated regulation of TH gene expression. As activation of MAPK family members is commonly reported to up-regulate the expression of TH, the reduced gene transcription obtained with HU 210 remains enigmatic. Likewise, decreased transcriptional activity induced by CB_1 receptor agonists was recently reported and assigned to novel mechanism involving a MAPK-dependent methylation of specific DNA sequences [28]. Noteworthy, the effect of HU 210 on the TH gene promoter was only partially blocked with wortmannin, confirming that the activation of ERK1/2 involved the modulation of additional effectors. Also consistent, inhibition of PI3K with wortmaninn substantially increased the basal activity of the promoter, indicating that a constitutive activity of PI3K exert a negative influence on TH gene transcription.

In conclusion, this study provides compelling evidence that HU 210 and CP 55,940 exhibit reversed order of efficacy in the activation of distinct members of the MAPK family regulated by the CB_1 receptor. In addition, focusing on the regulation of TH which constitutes a putative target of cannabinoids in adrenergic cells, this study reveals that the differential activation of effectors may selectively influence the regulation of downstream responses. Together these findings emphasize on the importance to systematically delineate the complex signalling cascades associated with a given G protein-coupled receptor to explain overall cellular responses to interacting ligands.

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