Background and purpose: Long-term adaptations to pharmacological stimuli frequently originate from modulation of complex intracellular signalling pathways. We previously reported that HU210 and CP55940, two CB1 cannabinoid receptor agonists, induced opposite effects on TH expression. Herein, we characterized their influence on cAMP response element (CRE) and activator protein 1 (AP-1)-mediated regulation of gene transcription.

Experimental approach: The activity of the agonists was examined on transfected N1E-115 cells in which expression of the luciferase reporter gene was controlled by transcription promoters consisting of repeats of either CRE or AP-1 elements. In addition, the implication of classical signalling pathways was investigated using a variety of kinase inhibitors.

Key results: Consistent with the CB1-mediated reduction of cAMP accumulation, both ligands decreased CRE-driven luciferase expression with similar potencies. HU210 also exhibited a concentration-dependent reduction of luciferase activity in cells engineered to examine AP-1-controlled transcription, whereas such response was not obtained with CP55940. Responses were all inhibited by SR141716A and were modified in Pertussis toxin-treated cells, suggesting agonist-selective regulations of distinct G\textsubscript{i/o}-dependent mechanisms through CB1 receptor activation. Finally, PKC inhibitors efficiently inhibited the paradoxical effect of HU210 on AP-1-mediated transcription, indicating selective regulation of PKC-dependent responses.

Conclusions and implications: Together, our results demonstrate that two cannabinoid ligands, commonly used as reference agonists acting on the same receptor with similar affinities, differentially modulate gene transcription through distinct controls of AP-1. This could reflect activation of distinct subsets of G\textsubscript{i/o}-proteins, supporting the concept of functional selectivity at CB1 receptors.

Keywords: luciferase; cannabinoid; agonist-selective trafficking of receptor signalling; transcription; CRE; AP-1; functional selectivity

Abbreviations: AC, adenylyl cyclase; AP-1, activator protein 1; CP55940, (1\text{R},3\text{R},4\text{R})-3-(2-hydroxy-4-(1,1-dimethylheptyl)phenyl)-4-(3-hydroxypropyl)cyclohexan-1-ol; CRE, cAMP response element; CREB, cAMP response element-binding protein; ERK, extracellular-signal regulated kinase; HU210, (6\text{R})-trans-3-(1,1-dimethylheptyl)-6,7,10,11-tetrahydro-6,6-dimethyl-6\text{H}-dibenzo(b,d)pyran-9-methanol; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated kinase kinase; PTx, Pertussis toxin; SR141716A, N-((1\text{S})-endo-1,3,3-trimethylbicyclo(2.2.1)heptan-2-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1\text{H} pyrazole-3-carboxamide hydrochloride; SR144528, N-((1\text{S})-endo-1,3,3-trimethylbicyclo(2.2.1)heptan-2-yl)-5-(4-chloro-3-methylphenyl)-1-(4-methylbenzyl)pyrazole-3-carboxamide; TH, tyrosine hydroxylase

Introduction

Besides radioligand-binding studies, pharmacological characterization of synthetic drugs acting at G-protein-coupled receptors consists of the study of their potency and intrinsic activity in functional assays. Considering the signalling pathways activated by these receptors, these assays are generally focused on the measure of conventional immediate and transient responses, such as production of second messengers, activation of kinases or alteration in ion equilibriums. Although these acute responses provide relevant details regarding the pharmacodynamic properties of the ligand, it is well documented that delayed or long-term modulations of cell functions contribute to the clinical outcome obtained with these ligands.
With respect to cannabinoids that are classically documented to signal through inhibition of adenyl cyclase (AC), several reports indicate that activation of cannabinoid receptors leads to the regulation of several DNA-binding proteins, including activator protein 1 (AP-1) (Porcella et al., 1998) and cAMP response element-binding protein (CREB) (Herring et al., 1998). Little is presently known regarding the nature of cannabinoid-mediated cell signals putatively involved in these delayed responses. Hence, paradoxical results were sometimes observed regarding agonist-mediated alteration of second messenger production and modulation of transcriptional activity. Thus, 2-arachidonoylglycerol was shown to enhance cell transformation and carcinogenesis through the induction of AP-1 DNA binding (Zhao et al., 2005), whereas Δ⁹-tetrahydrocannabinol reduced interleukin-2 transcription through a decrease in nuclear factors binding to the AP-1 site of the interleukin-2 promoter (Condie et al., 1996; Faubert and Kaminski, 2000).

Apart from inhibition of AC via a Pertussis toxin (PTx)-sensitive G_{i/o} protein (Pertwee, 1999; Alexander et al., 2008), cannabinoid-induced accumulation of cAMP has also been reported (Glass and Felder, 1997). This response is likely to reflect a functional coupling with G_{i/o} type G proteins, as it is preserved in recombinant (Bonhaus et al., 1998; Calandra et al., 1999) and native (Maneuf and Brotchie, 1997; Bass et al., 2003) systems after G_{i/o} inactivation by PTx. Even more convincing are studies showing the functional switch of CB₁ receptor coupling from G₂α to G₂β, following the sequestration of G₁α G-protein pool through dopamine D₂ receptor coactivation (Glass and Felder, 1997) or overexpression (Jarrahian et al., 2004). In addition, both CB₁ and CB₂ receptors were reported to stimulate ERK1/2 (extracellular-signal regulated kinase) (Bouaboula et al., 1995) in a G₁α-dependent manner, through the Gβγ subunit. The complexity of the intracellular signalling associated with the CB₁ receptor extends to the activation of members of the mitogen-activated protein kinase (MAPK), including c-Jun N-terminal kinase and p38 MAPK (Rueda et al., 2000) and the modulation of calcium and potassium currents (Mackie and Hille, 1992; Mackie et al., 1995). In addition, CB₁ receptors may induce rapid and transient elevations of intracellular free Ca²⁺ concentrations through Gβγ-mediated activation of PLCβ (Sugiura et al., 1997). Finally, regulation of PLC by CB₁ receptors was proposed to require coupling with different z-subunits of the G₉/₁₁ family (Ho et al., 1999). Hence, the selective coupling to G₉/₁₁ protein in an agonist-specific manner was more recently demonstrated (Lauckner et al., 2005; McIntosh et al., 2007).

We recently reported cannabinoid-mediated regulation of tyrosine hydroxylase (TH) expression in the N1E-115 neuroblastoma cell line constitutively and selectively expressing the CB₁ receptor subtype. Particularly, different classes of full CB₁ agonists, belonging to different chemical families, were shown to induce opposite effects (Bosier et al., 2007), raising the question of functional selectivity at the CB₁ receptor. In this context, the present study aims at further investigating CB₁ receptor-mediated signalling pathways involved in the regulation of gene expression. In view of the essential roles of both CRE and AP-1 cis-enhancer elements in constitutive and induced TH gene expression, CRE- or AP-1-driven luciferase reporter gene assays were carried out to elucidate the CB₁ receptor-mediated, agonist selective regulation of signalling pathways involved in long-term control of gene transcription.

**Methods**

**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⁻¹ penicillin, 100 μg mL⁻¹ streptomycin and 2 mM L-glutamine. All drug treatments were conducted in the same culture medium. At confluence, cells were trypsinized for dilutions. Cells were cultured at 37°C in an atmosphere of humidified air and 5% CO₂.

**cAMP assay**

N1E-115 cells were seeded in 96-well plates (10⁴ cells per well), 24 h before cannabinoid treatments. Cells were pretreated for 30 min with 0.1 mM IBMX (3-isobutyl-1-methyloxanthine). Then, cannabinoid agonists were added for another 30 min incubation treatment in the absence or presence of 1 μM forskolin. When N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H pyrazole-3-carboxamide hydrochloride (SR141716A) and N-((1S)-endo-1,3,3-trimethylbicyclo(2.2.1)heptan-2-yl)-(S)-4-chloro-3-methylphenyl)-1-(4-methylbenzyl)-pyrazole-3-carboxamide (SR144528) were used, cells were pretreated for 5 min with the cannabinoid inverse agonists/antagonists at 1 μM before the addition of the cannabinoid agonist. Where indicated, cells were treated overnight with 100 ng mL⁻¹ PTx. cAMP levels were measured using cAMP Biotrak enzyme immuno-assay system (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), according to the protocol provided by the manufacturer. Cells were incubated at 37°C.

**Plasmids, transfection and dual luciferase assay**

pCRE-Luc and pAP-1-Luc plasmids were purchased from Stratagene (La Jolla, CA, USA) and included 4 and 7 copies of CRE and AP-1 cis-enhancer elements, respectively, fused to the firefly luciferase gene. pRL 138 obtained from Dr E Pierreux (UCL, ICP, Brussels, Belgium) was used as an internal control to normalize for transfection variability. pRL 138 was constructed by the introduction of a 225 bp sequence of the PFK-2 promoter in the pRL null vector from Promega (Mannheim, Germany) encoding for Renilla luciferase, as described previously (Pierreux et al., 1998).

Cells were plated at a density of 10⁵ cells per well in 24-well plates. Culture medium was changed after 24 h, and the cells were co-transfected with a reporter plasmid (3 μg per well and 5 μg per well for pCRE-Luc and pAP-1-Luc, respectively) and the pRL 138 plasmid at 0.25 μg per well. Transfection was performed overnight using the calcium phosphate co-precipitation method. After transfection, cells were washed three times with PBS buffer (137 mM NaCl,
2.7 mM KCl, 8.1 mM Na₂HPO₄, 1.47 mM KH₂PO₄, pH 7.4) and fresh medium was added 24 h before the 5 h treatment with the appropriate amounts of cannabinoid agonists. When indicated, SR141716A and SR144528 were added 5 min before applying the agonist. When PTx was used, cells were treated overnight with a 100 ng mL⁻¹ concentration. To stop the reaction, cells were washed three times with PBS buffer before lysis by addition of 100 μl passive lysis buffer supplied with the Dual Luciferase Reporter Assay System (Promega). Firefly luciferase reporter activity was normalized for Renilla luciferase activity. Respective measurement of light emission was determined according to the manufacturer’s instructions. Luminescence was detected by a TD20/20 luminometer (Turner design, Sunnyvale, CA, USA).

**Data analysis and statistical procedures**

Data presented in the text and in the figures were expressed as percentages of the corresponding values obtained with cells treated with vehicle (dimethyl sulphoxide diluted in culture medium). pEC₅₀ values were determined from at least three separate experiments by nonlinear regression analysis performed using Graph Pad prism software (San Diego, CA, USA). Statistical analysis was performed on the log-transformed value of relative luminescence or on the net cAMP level by ANOVA with repeated measurements or Student’s t-test. A post hoc analysis was performed by Scheffe test, using the SPSS software.

**Drugs, chemical reagents and other materials**

(6,8)-trans-3-(1,1-dimethylheptyl)-6,7,10,10-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo(b,d)pyran-9-methanol (HU210) and (1R,3R,4R)-3-(2-hydroxy-4-(1,1-dimethylheptyl) phenyl)-4-(3-hydroxypropyl)cyclohexan-1-ol (CP55940) were purchased from Tocris Cookson (Bristol, UK). The CB₂ receptor inverse agonist/antagonist, SR141716A, and the CB₁ receptor inverse agonist/antagonist, SR144528, were generously provided by Dr Barth and Dr Mossé, respectively (Sanofi-Synthélabo Research, Montpellier, France). Forskolin, IBMX, phorbol 12-myristate 13-acetate and PTx were purchased from Sigma (Boornem, Belgium). Stock solutions of drugs were prepared in dimethyl sulphoxide at 0.01M and stored in aliquots at –80 °C. The final dimethyl sulphoxide concentration never exceeded 0.1%, which had no significant effect on assays.

**Results**

**Modulation of cAMP accumulation**

In agreement with several studies indicating the functional coupling of the cannabinoid receptors with G protein, both HU210 (0.1 μM) and CP55940 (0.1 μM), two typical cannabinoid full agonists, elicited a significant reduction in cAMP accumulation in N1E-115 neuroblastoma cells (21.8 and 36.5% reduction relative to control cells, respectively) (Figure 1a). To facilitate the detection of Giso-mediated responses, basal AC activity was enhanced by the addition of 1 μM forskolin (leading to a robust 725 ± 56% induction of cAMP accumulation, relative to control). At the tested concentration, maximal response for forskolin is certainly not reached, as the EC₅₀ value of forskolin in mediating cAMP accumulation in N1E-115 cells is close to 10 μM (Stenstrom et al., 1985; Murphy and Byczko, 1989). In these conditions, both HU210 and CP55940 exhibited a more pronounced inhibition of cAMP formation (50.9 and 56.0% reduction relative to forskolin treated cells, respectively). These results are expressed as percentage of responses in control cells (exposed to vehicle or forskolin, which caused a sevenfold increase in basal cAMP accumulation). Data are mean values with s.e.mean from three separate experiments performed in triplicate. † † † P < 0.01, † † * P < 0.05 relative to control condition, † † † † P < 0.01, † † † † * P < 0.05 relative to responses in the presence of PTx.

**Figure 1** Regulation of cAMP accumulation in N1E-115 neuroblastoma cells. Cells were exposed to HU210 or CP55940 (0.1 μM), in the absence (a) or presence (b) of 1 μM forskolin. The responses to both agonists were also measured after overnight treatment of the cells with PTx (100 ng mL⁻¹). Results are expressed as percentage of responses in control cells (exposed to vehicle or forskolin, which caused a sevenfold increase in basal cAMP accumulation). Data are mean values with s.e.mean from three separate experiments performed in triplicate. † † † P < 0.01, † † * P < 0.05 relative to control condition, † † † † P < 0.01, † † † † * P < 0.05 relative to responses in the presence of PTx.
absence of functional $G_{i/o}$ proteins, and is currently attributed to a functional coupling of the CB$_1$ receptor with $G_s$ proteins.

Cannabinoid-mediated regulation of CRE and AP-1-dependent transcriptional activity

To examine the effects of CB$_1$ receptor activation on CRE and AP-1-dependent transcriptional activities, N1E-115 cells were transiently transfected with plasmid constructs containing the luciferase gene under the control of cis-enhancer element repeats of either CRE (pCRE-Luc) or AP-1 (pAP-1-Luc). As expected, cells transfected with pCRE-Luc constructs displayed a robust basal luciferase activity (not shown), confirming a constitutive activity of CRE-dependent transcription in N1E-115 cells. In addition, the strong increase in luciferase activity (up to 850% observed with forskolin 1 $\mu$m) confirmed that the luciferase reporter gene is a reliable indicator of cAMP levels in this model. Both HU210 and CP55940 were found to modestly inhibit CRE-mediated transcriptional activity (estimated pEC$_{50}$ values of 7.21 ± 0.01 and 6.82 ± 0.09, respectively) (Figure 2a). More convincingly, when the agonists were tested in cells exposed to forskolin, both markedly reduced luciferase expression in a concentration-dependent manner, with pEC$_{50}$ values of 7.60 ± 0.01 and 8.07 ± 0.04 for HU210 and CP55940, respectively. In this assay, the analysis of concentration–response curves indicated Hill slopes distinct from unity (0.47 ± 0.04 and 0.41 ± 0.02 for HU210 and CP55940, respectively). Nevertheless, the responses to both agonists were markedly impaired using SR141716A (Figure 2b), suggesting that inhibition of CRE activity was specifically driven through CB$_1$ receptor-dependent signalling pathways.

Experiments were repeated in cells engineered to examine AP-1-controlled luciferase activity. As with the CRE construct, a noticeable constitutive AP-1-driven luciferase activity was detected. The efficiency of this system was validated using 0.08 $\mu$m phorbol 12-myristate 13-acetate, a PKC activator known to regulate AP-1 transcription, which produced a modest but significant increase in luciferase activity (124.8 ± 2.4%, relative to control; $P < 0.01$). In this assay, HU210 elicited a concentration-dependent reduction of AP-1 activity (pEC$_{50}$ of 7.95 ± 0.11) (Figure 3a) and experimental data best fitted with a standard sigmoidal curve (Hill slope close to 1). Again, SR141716A abolished the HU210-mediated response (Figure 3b), confirming that the regulation of AP-1-dependent transcription was operating through CB$_1$ receptor activation.

Surprisingly, no significant influence on AP-1 activity was observed using CP55940, indicating that this commonly used agonist of the CB$_1$ receptor behaves as a neutral ligand on the cell-signalling pathway regulating AP-1-dependent transcriptional activity. The pharmacological profile of CP55940 was further examined by analysing its ability to antagonize cannabinoid-mediated regulation of AP-1 activity. As shown in Figure 4, CP55940 caused a concentration-dependent rightward shift of the sigmoidal curve for HU210-induced luciferase activation. This clearly identifies CP55940 as a competitive antagonist at the CB$_1$ receptor in this functional assay and Schild plot transformation indicated a pA$_2$ value of 7.48 (95% confidence interval of 8.22–8.92).

Signalling pathways involved in CRE and AP-1-dependent transcriptional activity

The cellular mechanisms involved in the regulation of CRE and AP-1-dependent transcriptional activity by cannabinoid agonists were examined using inhibitors of intracellular signalling cascades putatively associated with CB$_1$ receptor functions. As this receptor appears predominantly associated with modulation of cAMP levels, we first studied the effect of the PKA inhibitors, H89 and KT5720, on both CRE and
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AP-1-driven luciferase activities. Also, as cannabinoid responses on CRE activity were more pronounced when the basal activity of AC wasboosted, the effect of inhibitors on CRE-controlled transcription was evaluated in the presence of 1 μM forskolin. As expected, incubation of N1E-115 cells with H89 (10 μM) or KT5720 (5 μM) significantly reduced pCRE-Luc activity in the presence of forskolin (Table 1). In the same conditions, these PKA inhibitors completely abolished the modulation of pCRE-Luc activity caused by the cannabinoid agonists HU210 and CP55940.

The effects of MAPK kinase (MEK) and PI3K inhibitors were also investigated, as cannabinoids are known to regulate MAPK activity, possibly through PI3K activation. The MEK inhibitors U0126 (5 μM) and PD98059 (25 μM) were found to decrease the effect of forskolin on CRE-driven luciferase transcription. Similar reduction in CRE activity was observed in the absence of forskolin (74.5 ± 7.9 and 67.1 ± 2.3%) relative to control cells, for U0126 and PD98059,
Table 2 Biochemical characterization of the signalling pathways involved in the modulation of pAP-1-Luc activity by cannabinoid ligands

<table>
<thead>
<tr>
<th>Treatments</th>
<th>No agonist</th>
<th>HU210</th>
<th>CP55940</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>100.0 ± 0.76</td>
<td>65.3 ± 5.8**</td>
<td>101.1 ± 4.4</td>
</tr>
<tr>
<td>PKA inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H89 (10 μM)</td>
<td>98.5 ± 11.2</td>
<td>71.5 ± 6.5</td>
<td>104.8 ± 7.1</td>
</tr>
<tr>
<td>KT5720 (5 μM)</td>
<td>94.3 ± 1.0</td>
<td>70.1 ± 2.3</td>
<td>96.9 ± 2.5</td>
</tr>
<tr>
<td>MEK/MAPK KK inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>U0126 (5 μM)</td>
<td>94.9 ± 12</td>
<td>58.1 ± 9.2</td>
<td>104.6 ± 9.6</td>
</tr>
<tr>
<td>PD98059 (25 μM)</td>
<td>74.3 ± 1.0**</td>
<td>52.8 ± 1.5</td>
<td>74.3 ± 1.8</td>
</tr>
<tr>
<td>PI3K inhibitor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wortmannin (0.2 μM)</td>
<td>300.4 ± 68.1*</td>
<td>212.7 ± 58.3</td>
<td>315.9 ± 38.3</td>
</tr>
<tr>
<td>PKC inhibitors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chelerythrine (10 μM)</td>
<td>70.1 ± 2.8**</td>
<td>70.9 ± 4.9</td>
<td>72.8 ± 6.9</td>
</tr>
<tr>
<td>GF109203X (0.25 μM)</td>
<td>65.6 ± 11.0**</td>
<td>75.3 ± 25.0</td>
<td>73.6 ± 19.1</td>
</tr>
<tr>
<td>Negative control for PKC inhibition</td>
<td>100.0 ± 1.6</td>
<td>108.7 ± 17.1</td>
<td>113.4 ± 8.6</td>
</tr>
</tbody>
</table>

MEK, mitogen-activated kinase; MAPK, mitogen-activated protein kinase.

Transfected N1E-115 cells carrying pAP-1-Luc construct were pretreated for 1 h in the absence or presence of inhibitors. Thereafter, cells were exposed to a vehicle, HU210 (0.1 μM) or CP55940 (0.1 μM), and the luciferase activity was monitored 5 h later. Results are given as the percentages of luciferase activity compared with control. Numbers in italics show the same data normalized to basal activity of CRE respectively, suggesting that MAPKs are involved in the key role of this kinase in the modulation of AP-1-dependent transcriptional activity, which was confirmed by our finding that none of the inhibitors tested here affected the response to this agonist. When used alone, inhibitors of MEK, PI3K or PKC were also found to affect the basal AP-1-mediated luciferase activity, suggesting that these kinases are involved in the constitutive AP-1-dependent transcriptional activity. Finally, neither MEK nor PI3K inhibitors appeared to influence the response to HU210.

Cannabinoid-mediated regulation of CRE and AP-1-dependent transcriptional activity is directed by G_{i/o} proteins

The role of G_{i/o}-type G proteins in the modulation of gene transcription by cannabinoid agonists was examined by repeating the luciferase reporter assays in cells treated overnight with PTx (100 ng/mL). In cells expressing either the pCRE-Luc or pAP-1-Luc constructs, the basal luciferase activities appeared to be substantially increased after treatment with PTx (17.4 and 12.4% relative to control, respectively), indicating that both transcription promoters were negatively influenced by a constitutive activation of G_{i/o}-type G proteins.

As shown in Figure 2c, the inhibition of CRE-dependent luciferase activity induced by both HU210 and CP55940 in the presence of forskolin was abolished by PTx pretreatment (Figure 2c). In contrast, when the assay was conducted in the absence of forskolin, the effect of cannabinoid agonists was switched from inhibition to induction of CRE-dependent transcription, and concentration–response curves revealed pEC_{50} values of 6.64 ± 0.26 and 6.67 ± 0.27 for HU210 and CP55940, respectively (Figure 5a). These data are consistent with the above-mentioned increase in cAMP levels in PTx-treated cells, demonstrating the dual coupling of the CB1 receptor with the G_{i/o} and G_{s}-type G proteins.

Such reversal of the functional response to HU210 in PTx-treated cells was also observed when studying the modulation of luciferase under the control of AP-1. In these conditions, HU210 concentration-dependently stimulated luciferase activity with pEC_{50} values of 7.66 ± 0.38. More surprisingly, in this model of PTx-treated cells, CP55940 appeared to be effective in inducing AP-1-dependent transcription with a pEC_{50} of 6.48 ± 0.23 (Figure 5b). Together, these data indicate that the regulation of AP-1 activity by cannabinoid agonists was also operating through the activation of members of the G_{i/o} protein family.

Discussion and conclusions

The effects of cannabinoids on CNS activity are frequently related to the CB1 receptor-mediated regulation of signalling cascades, controlling the release of other neurotransmitters. Beside these acute responses, several reports indicate that activation of the CB1 receptor modulates transcription factors, including CREB, nuclear factor kappa B, nuclear factor-activated T cells and AP-1 (Ouyang et al., 1998; Herring et al., 2001). In this study, using N1E-115 neuroblastoma cells that constitutively express CB1 receptors, we report that the cannabinoid agonist HU210 concentration-dependently inhibits both CRE and AP-1-mediated transcriptional activities. In contrast, the unrelated cannabinoid agonist CP55940 fails to influence AP-1-controlled gene expression while efficiently regulating CRE activity. Furthermore, these responses are abolished and/or reversed in the

transfected with either pCRE (CRE and AP-1-dependent transcriptional activities by cannabinoid activity) compared with control. *P luciferase activity (firefly luciferase relative to performed in triplicate and are given as the percentage of relative values with s.e.mean from at least three separate experiments luciferase activity was monitored after 5 h. Results shown are mean exposed to increasing concentrations of HU210 or CP55940 and consistent with our data showing a decreased cAMP facilitated in cells exposed to forskolin, which boosts basal responses by SR141716A. The detection of this response was demonstrated in discrete regions of the rat brain after in CREB activation through ERK-dependent signalling was previously demonstrated (Casu et al., 2005). Likewise, HU210-mediated inhibition of AP-1-driven luciferase activity was efficiently inhibited by PKC inhibitors and antagonized using SR141716A. Although regulation of PKC-related pathways by cannabinoids remains poorly documented, several studies have already suggested a role of this kinase in cannabinoid receptor-mediated reduction of potassium currents (Hampson et al., 2000) and regulation of L-type voltage dependent calcium channels (Rubovitch et al., 2002). In addition, demonstrations that CB1 receptors may functionally interact with Gqi1-type G protein in response to (R)-(+)-(2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)-pyrrolo-(1,2,3-de)-1,4-benzoazin-6-yl)-1-naphthalenylmetha- none mesylate (WIN55212-2) (Lauckner et al., 2005) and efficiently regulate PLC activity (Sugiuira et al., 1996, 1997; Ho et al., 1999) are compelling indications for a CB1 receptor-mediated regulation of PKC.

The absence of CP55940-mediated regulation of AP-1-dependent transcription clearly shows that distinct signalling cascades contribute to the modulation of CRE- and AP-1-dependent activities. In addition, these data support the concept of functional selectivity of agonists acting at the CB1 receptor. Indeed, functional selectivity of receptor signalling is characterized by the ability of distinct ligands.
acting at a single G-protein-coupled receptor to differentially control intracellular signal transduction pathways. Thus, functionally selective ligands activate or inhibit multiple intracellular cascades with different efficacies or potencies (Bosier and Hermans, 2007). Such selectivity at the level of regulation of AP-1-dependent transcriptional activity by cannabinoid agonists is an unprecedented observation. Although the expression of the CB1 receptor in this model of neuroblastoma cells remains poorly characterized, we have accumulated convincing pharmacological data showing that both agonists tested act on a single molecular target identified as the CB1 receptor. It is noteworthy that both agonists promote reduction of CRE-dependent luciferase activity with similar potencies and decrease cAMP. This is also consistent with the data showing the possibility to competitively antagonize the HU210-mediated regulation of AP-1-dependent response by CP55940. Importantly, the pA2 value determined for CP55940 in this competition assay is consistent with its published nanomolar affinity for the CB1 receptor. The detection of signalling specificity with some drugs, as shown here for CP55940, could be purely related to their partial agonist profile. For these drugs, the density of receptors and the availability of defined signalling partners could influence the apparent efficacy in functional assays. However, to our knowledge, the vast majority of pharmacological studies have identified CP55940 as a full agonist of the CB1 receptor. In spite of repeated attempts, we failed to find cannabinoid agonist-induced [35S]-GTPgS binding to G proteins in this model of neuroblastoma cells, suggesting that the expression of the CB1 receptor is not sufficient to allow the detection of significant responses. Therefore, we were unable to characterize the potencies and efficacies of CP55940 and HU210 at the earliest steps of the signalling cascade in this model. Nevertheless, in our hands, both ligands were characterized as full agonists of the CB1 receptor in [35S]-GTPgS-binding assays conducted on mouse cerebellar membrane preparations (pEC50: 7.77 ± 0.72 and 7.80 ± 0.18; Emax: 173 ± 1 and 176 ± 2% stimulation over basal, for HU210 and CP55940, respectively; Bosier et al., unpublished data).

Although functional selectivity at G-protein-coupled receptors is extensively documented in the literature, little is known regarding the CB1 receptor, and most available studies have been focused on coupling with different members of the G10 protein family (Glass and Northup, 1999; Prather et al., 2000; Mukhopadhyay and Howlett, 2005). Nevertheless, the ability of different CB1 receptor agonists to differentially activate G10 or G-dependent signalling pathways was described in recombinant systems (Bonhaus et al., 1998). To our knowledge, this study provides the first evidence for functional selectivity at the native CB1 receptor in a neural cell line, reflected by an agonist-selective response monitored at the transcriptional level. In a closely related study conducted in the same cell line, we recently reported the complex regulation of TH by CB1 receptor agonists (Bosier et al., 2007). Importantly, HU210 or CP55940 was shown to decrease or increase the expression of TH, respectively, raising the question of an agonist-selective trafficking of CB1 receptor signalling. Earlier studies demonstrated that AP-1 and CRE motifs present in the rat TH gene promoter were involved in the pharmacological modulation of transcription (Kim et al., 1993; Najimi et al., 2002; Lewis-Tuffin et al., 2004). Therefore, identifying an agonist-dependent differential regulation of TH expression in a catecholamine-producing cell line, highlights the putative relevance of functional selectivity.

Inhibition or reversal of the responses to the cannabinoid agonists in PTX-treated cells suggests that both CRE and AP-1 activities are regulated through activation of G10-type G proteins, in accordance with the documented predominant coupling of the CB1 receptor. The unexpected reduction of basal cAMP level observed after overnight treatment with PTX could reveal some influence of this toxin on the metabolism or proliferation of N1E-115 cells. However, forskolin was found to induce equivalent increases in cAMP accumulation in cells with or without exposure to PTX (725.08 ± 55.97 and 728.58 ± 41.15% stimulation, relative to control), suggesting that the pharmacological modulation of AC and associated signalling were not affected. Taking advantage of an internal control plasmid (encoding for...
Renilla luciferase) that allowed us to normalize for cell density, an enhanced basal luciferase activity in PTx-treated cells was detected, confirming the stimulation of both CRE and AP-1-dependent transcription by some constitutive activation of G<sub>i/o</sub>-type G proteins in N1E-115 cells. In addition, the inverse agonist, SR141716A, also increased luciferase activity (not shown), indicating that a constitutive coupling of the CB<sub>1</sub> receptor participates in the activation of CRE and AP-1 in the absence of agonist.

The putative interaction of the CB<sub>1</sub> receptor with several isoforms of G<sub>i/o</sub> proteins could certainly hold clues for the diversity in intracellular signalling triggered by cannabinoid ligands and explain the results observed with HU210 and CP55940 (Figure 6). On the other hand, although the aim of the study was not to examine the diversity of couplings with different types of G proteins, our data suggest that in the absence of functional G<sub>i/o</sub> protein, the CB<sub>1</sub> receptor may operate atypical coupling with other G proteins. Indeed, increased cAMP accumulation and stimulation of CRE-driven luciferase activity in PTx-treated cells indicate that CB<sub>1</sub> receptors also exert a positive influence on AC through a putative coupling with G<sub>i</sub>-type G protein. Besides, considering the consequence of PTX pretreatment on HU210 and CP55940-mediated regulation of AP-1 activity, the possibility of coupling with members of the G<sub>i/11</sub> proteins family cannot be excluded.

In conclusion, the present results indicate that two cannabinoid ligands, commonly used as reference agonists at the CB<sub>1</sub> receptor, may differentially influence gene transcription through distinct controls on AP-1 cis-enhancer DNA element. Supporting the concept of functional selectivity, these data suggest that the choice of appropriate ligands acting on a given receptor should help to selectively modulate defined cellular responses. In addition, the present observations clearly indicate that ligands, with similar pharmacodynamic properties and eliciting similar regulation of classical immediate effectors, may trigger divergent delayed responses. This should encourage the systematic study of a wide variety of early and late responses to drugs to better define their therapeutic potential or explain the differences in their clinical profiles.

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Conflict of interest

The authors state no conflict of interest.

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