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Short communication

Cannabinoid receptor antagonism and inverse agonism in response to SR141716A on cAMP production in human and rat brain

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Abstract

The effects of cannabinoid drugs on cAMP production were examined in mammalian brain. The cannabinoid receptor agonist (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3,-*d*,*e*-1,4-benzoxazin-6-yl]-(1-naphthalenyl) methanone (WIN55, 212-2) decreased forskolin-induced cAMP accumulation in a concentration-dependent manner (10^{-8}–10^{-5} M) in membranes from several rat and human brain regions, this effect being antagonized by 10^{-5} M *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (SR141716A). Furthermore, high micromolar concentrations of SR141716A evoked a dose-dependent increase in basal cAMP in rat cerebellum and cortex, as well as in human frontal cortex. This effect was antagonized by WIN55,212-2 and abolished by *N*-ethylmaleimide, consistent with the involvement of cannabinoid CB_1 receptors through the activation of $G_{i/o}$ proteins. These results suggest a ligand-independent activity for cannabinoid CB_1 receptor signaling cascade in mammalian brain.

Keywords: SR141716A; Inverse agonism; Cannabinoid system; Adenylate cyclase

1. Introduction

In the central nervous system, the responses to cannabimimetic drugs such as (*R*)-(+)-[2,3-dihydro-5-methyl-3-[(4-morpholinyl)methyl]pyrrolo[1,2,3,-*d*,*e*-1,4-benzoxazin-6-yl]-(1-naphthalenyl) methanone (WIN55,212-2) are mediated by the cannabinoid CB_1 receptor. Cannabinoid CB_1 receptors are coupled to the $G_{i/o}$ class of G-proteins, which mediate inhibition of adenylate cyclase activity (for review, see Pertwee, 1997).

In the last few years, a variety of compounds have been reported to behave as inverse agonists on overexpressed receptors in heterologous systems. The putative therapeutic potential of these inverse agonists largely depends on the existence of endogenous constitutively active receptors. Several lines of evidence suggest that the aryl pyrazole, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (SR141716A), initially characterized as the first potent and selective cannabinoid CB_1 receptor antagonist (Rinaldi-Carmona et al., 1994), also presents inverse agonist properties. These evidences mainly come from biochemical studies regarding adenylate cyclase and mitogen-activated protein kinase (MAPK) activity in heterologous expression systems (Bouaboula et al., 1997), as well as from [^{35}S]GTPγS binding studies and in situ biological assays (Gifford and Ashby, 1996; Pertwee and Fernando, 1996; Coutts et al., 2000; Meschler et al., 2000; Sim-Selley et al., 2001). In vivo injection of SR141716A stimulates motor activity in mice when administered at high doses (Compton et al., 1996).

Antagonism of cannabinoid effects by SR141716A has not been clearly reported for human tissue. On the other hand, inverse agonism properties have been suggested from adenylate cyclase studies in N18TG2 neuroblastoma cells (Meschler et al., 2000), but there is no evidence for SR141716A promoting an increase in cAMP levels in mammalian brain membranes, including human tissue. This would directly support the functional relevance of the existence of constitutively active cannabinoid CB_1 receptors. This study was conducted in order to assess both the ability of SR141716A to antagonize cannabinoid-induced

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modifications of adenylate cyclase activity in human brain and the existence of constitutive activity of cannabinoid CB_1 receptors in rat and human brain, by studying its profile as putative inverse agonist.

2. Materials and methods

Rats (male Wistar–Hannover, 200–250 g) were sacrificed by decapitation and the brains dissected on ice and stored at −70 °C until assay. Human brain samples were obtained from three subjects who had died by sudden and/or violent causes, without evidence of neurological disorders. Brain regions were dissected at the time of autopsy, and stored at −70 °C until assay.

Cerebral samples (~100 mg) were homogenized in 60 volumes of homogenization buffer (20 mM Tris–HCl, 1 mM EGTA, 5 mM EDTA, 300 mM sucrose, 5 mM Dithiothreitol, 25 μg/ml leupeptin, pH = 7.4). The crude homogenates were immediately centrifuged (1500 × *g*) for 5 min at 4 °C, and the supernatants were then centrifuged (13,000 × *g*) for 15 min at 4 °C. The resultant pellets were resuspended in 60 volumes of ice cold homogenization buffer. In the experiments carried out in the presence of *N*-ethylmaleimide, the membranes were incubated with the compound for 60 min on ice. The reaction was stopped by 20 mM Dithiothreitol, and the membranes were washed three times with homogenization buffer and resuspended 1:60 in this buffer.

Adenylate cyclase assays were performed by preincubation of freshly prepared membrane aliquots (50 μl in a final volume of 250 μl) for 5 min at 37 °C in assay buffer (80 mM Tris–HCl, 2 mM $MgCl_2$, 0.2 mM EGTA, 1 mM EDTA, 60 mM sucrose, 10 μM GTP, 1 mM Dithiothreitol, 0.5% bovine serum albumin, 0.5 mM 3-isobutylmethylxanthine, 5 mM phosphocreatine, 50 U/ml creatine phosphokinase and 5 U/ml myokinase, pH = 7.4) and the indicated concentrations of each drug. The adenylate cyclase activity was started by addition of 0.2 mM ATP. Tubes were incubated for 10 min at 37 °C, and the samples boiled for 5 min at 100 °C in order to stop the reaction. After 3 min centrifugation at 13,000 × *g* at 4 °C, cAMP accumulation was quantified in 50 μl supernatant aliquots by using a [^{3}H]cAMP commercial kit, based on the competition of a fixed amount of [^{3}H]cAMP and the unlabelled form of cAMP for a specific protein, achieving the separation of protein-bound nucleotide by adsorption on coated charcoal. (TRK 432, Amersham Pharmacia Biotech U.K. Limited, Buckinghamshire, England). The assays involving WIN55,212-2 as agonistas well as in the presence of SR141716A as antagonist were carried out with 100 mM NaCl and 10^{-5} M forskolin, while those analyzing SR141716A as inverse agonist were carried out in the absence of sodium and forskolin.

The data were analyzed using GraphPad Prism data analysis program (GraphPad Software San Diego, CA, USA). Curves were analyzed by a non-linear regression analysis. Student's *t*-test was used for the comparison of statistical significance between pEC_{50} or E_{max} values.

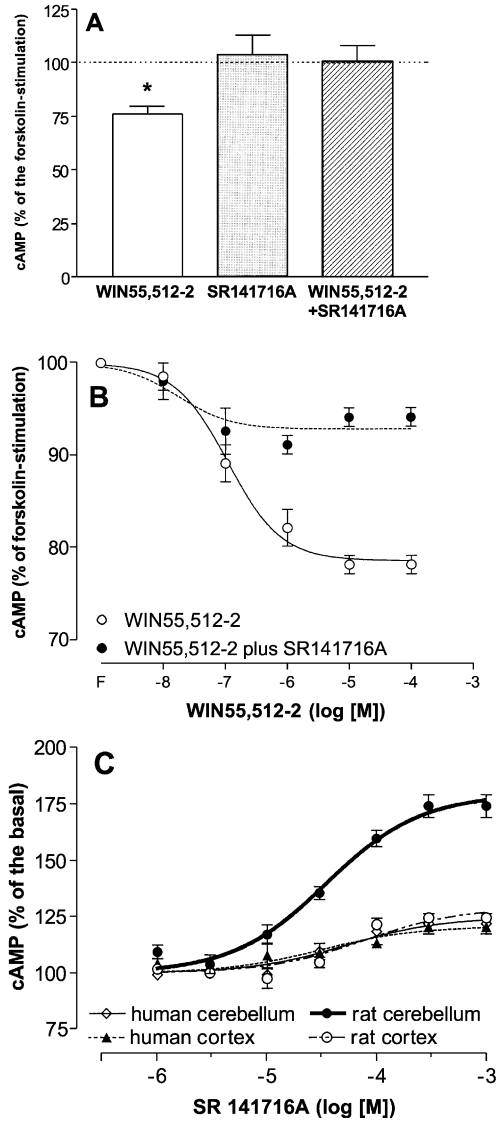

Fig. 1. (A) Effect of WIN55,512-2 (10^{-5} M), SR141716A (10^{-5} M) and WIN55,512-2 (10^{-5} M) plus SR141716A (10^{-5} M) in crude membranes from human frontal cortex ($n = 3$) on adenylate cyclase activity expressed as percentage of cAMP inhibition over the forskolin induced increase; * $p < 0.05$ vs. forskolin induced cAMP increase (100%). (B) Effect of increasing doses of WIN55,512-2 (10^{-8}–10^{-4} M) alone (○) and in the presence of SR141716A (10^{-5} M; ●) on adenylate cyclase activity expressed as percentage of cAMP inhibition over the forskolin induced increase (F) in crude membranes from human striatum ($n = 3$). In (A) and (B), the experiments were carried out in the presence of 100 mM of NaCl and 10^{-5} M of forskolin. (C) Effect of increasing concentrations of SR141716A on cAMP levels in crude membranes from rat cerebellum ($n = 5$), rat cortex ($n = 3$) human cerebellum ($n = 3$) and human frontal cortex ($n = 3$). These experiments were carried out in the absence of NaCl and forskolin. All the data represent mean ± S.E.M. Samples from each subject were independently assayed.

3. Results

The cannabinoid CB_1 receptor agonist WIN55,212-2 (10^{-8}–10^{-4}) decreased forskolin-induced cAMP accumulation in a concentration-dependent manner in rat cortex (pEC_{50}: 6.9 ± 0.2; E_{max}: 24.0 ± 2.2%) and in several human brain regions frontal cortex (pEC_{50}: 6.4 ± 0.3; E_{max}: 26.7 ± 7.1%), striatum (pEC_{50}: 7.0 ± 0.2; E_{max}: 21.6 ± 1.2%), hippocampus (pEC_{50}: 7.2 ± 1.1; E_{max}: 22.2 ± 0.7%), cerebellum (pEC_{50}: 5.5 ± 0.2; E_{max}: 15.5 ± 1.8%). SR141716A (10^{-5} M) fully antagonized this WIN55,212-2-induced inhibitory response (Fig. 1A and B).

The direct effects of SR141716A on adenylate cyclase basal activity in rat and human brain membranes were then evaluated. As the presence of Na^+ facilitates the uncoupled state of G protein-dependent receptors, unmasking the identification of inverse agonism responses (Tian and Deth, 1993; Meschler et al., 2000), these studies were carried out in the absence of Na^+. As shown in Fig. 1C, SR141716A (10^{-6}–10^{-3} M) increased the production of basal cAMP, behaving as an inverse agonist. The highest increase (E_{max}) in cAMP accumulation was found in rat cerebellum (79.6 ± 23.7%). Lower increases were observed in rat cortex (28.8 ± 2.8%), human frontal cortex (20.6 ± 1.9%) and human cerebellum (24.3 ± 3.3) (Fig. 1C). pEC_{50} values were in the high micromolar range (rat cerebellum: 4.5 ± 0.3; rat cortex: 4.2 ± 0.1; human frontal cortex: 4.5 ± 0.7 and human cerebellum 4.3 ± 0.2).

In order to obtain some information on the possible participation of G_s-mediated activation of adenylate cyclase in these effects, we repeated the studies with *N*-ethylmaleimide, an inhibitor of the activation of $G_{i/o}$ proteins (Asano and Ogasawara, 1986). Preincubation with *N*-ethylmaleimide dose-dependently inhibited the SR141716A-induced increase of cAMP accumulation. The presence of 50 μM and 100 μM *N*-ethylmaleimide reduced the maximum increase in rat cerebellum from 79.6 ± 23.7% to 60.2 ± 10.6% and 10.0 ± 1.6%, respectively. Preincubation with 1 mM *N*-ethylmaleimide completely abolished the response.

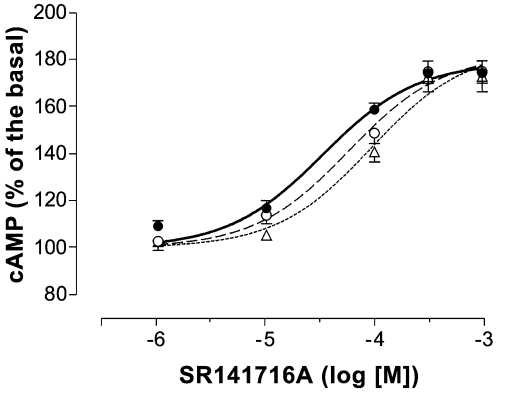

Fig. 2. Effect of the coincubation with WIN55,212-2 on SR141716A-induced increase in basal cAMP in crude membranes from rat cerebellum. SR141716A alone (●, $n=5$), SR141716A in the presence of 10^{-5} M (○; $n=4$) and 10^{-4} M (△, $n=3$) WIN55,212-2. The experiments were carried out in the absence of NaCl and forskolin Data represent mean ± S.E.M. Samples from each subject were independently assayed.

The presence in the incubation medium of the positive cannabinoid CB_1 receptor agonist WIN55,212-2 resulted in a concentration-dependent displacement to the right of the dose–response curve of SR141716A in rat cerebellum (Fig. 2). Coincubation with 10^{-5} and 10^{-4} M WIN55,212-2 resulted in a reduction of the pEC_{50} value for SR141716A from 4.5 ± 0.3 to 4.2 ± 0.4 and to 4.1 ± 0.1, respectively. This difference reached statistical significance for the 10^{-4} M WIN55,212-2 plus SR141716A group ($p<0.01$). However, the maximum increase of cAMP by SR141617A was not significantly modified by WIN55,212-2 (Fig. 2).

4. Discussion

The results of this study demonstrate the ability of WIN55,212-2 to decrease cAMP production in human brain tissue, as well as the antagonism of this response by SR141716A. These data provide one of the first evidences of a pharmacological effect, of a cannabinoid nature, linked to adenylyl cyclase activity in human brain.

On the other hand, our results clearly show that SR141716A stimulates cAMP production in the mammalian brain, providing the evidence for an inverse agonist effect of SR141716A with functional relevance in the central nervous system, including human tissue. Furthermore, the competitive antagonism of this effect shown by WIN55,212-2 supports the mediation of the cannabinoid CB_1 receptor in the stimulation of adenylate cyclase activity. Taking together, all these data support an interpretation of our results from *N*-ethylmaleimide experiments in terms of a possible mediation of a $G_{i/o}$ protein coupling mechanism. The anatomical pattern of response was in parallel with the distribution of CB_1 receptors: in the rat, their density in the cerebellum is much higher than in the cortex (Herkenham et al., 1991), while in the human brain this difference is much less marked (Glass et al., 1997). Our results confirm the requirement of high concentrations of SR141716A to produce this inverse agonist response, already reported in previous studies (Compton et al., 1996; McAllister et al., 1999; Sim-Selley et al., 2001). Several explanations have been proposed for the difference in the potency of SR141716A as an antagonist of cannabinoid CB_1 receptor agonist-induced effects and that shown when behaving as inverse agonist. The existence of two different loci of interaction with the receptor for this compound could explain this apparent mismatch (see Sim-Selley et al., 2001).

The adenylate cyclase stimulatory response induced by SR141716A here reported cannot be explained in terms of antagonism of the activity of endogenous cannabinoids present in the tissue. First, any endogenous ligand present in the tissue was subject to strong metabolism during the

assay and additionally discarded during the washing procedure. And second, competitive antagonism of endogenous cannabinoids activity should require only nanomolar concentrations of SR141716A. As above mentioned, antagonist vs. inverse agonist effects of this drug can be differentiated on the basis of potency. The possibility of these changes being mediated by a new CB receptor subtype recently proposed (Breivogel et al., 2001) cannot be fully discarded, although the predominant response found in the cerebellum in the present study is not in agreement with the distribution reported for this new site.

In conclusion, the quantitation of adenylyl cyclase activity allows the demonstration, from the functional point of view, of the existence of a ligand-independent activity for cannabinoid CB_1 receptors in native rat and human brain tissue. It also confirms the behaviour of SR141716A as an inverse cannabinoid CB_1 receptor agonist, previously reported by several in vivo and in vitro studies in the rat (Compton et al., 1996; Landsman et al., 1997; McAllister et al., 1999; Sim-Selley et al., 2001), showing that it is also present in human brain samples. The relevant implications of this constitutive activity of receptors from the point of view of therapeutic potential have to be explored.

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