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The actions of benzophenanthridine alkaloids, piperonyl butoxide and (S)-methoprene at the G-protein coupled cannabinoid CB_1 receptor *in vitro*

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ABSTRACT

This investigation focused primarily on the interaction of two benzophenanthridine alkaloids (chelerythrine and sanguinarine), piperonyl butoxide and (S)-methoprene with G-protein-coupled cannabinoid CB_1 receptors of mouse brain *in vitro*.

Chelerythrine and sanguinarine inhibited the binding of the CB_1 receptor agonist [^{3}H]CP-55940 to mouse whole brain membranes at low micromolar concentrations (IC_{50}s: chelerythrine 2.20 μM; sanguinarine 1.10 μM). The structurally related isoquinoline alkaloids (berberine and papaverine) and the phthalide isoquinoline ((−)-β-hydrastine) were either inactive or considerably below IC_{50} at 30 μM. Chelerythrine and sanguinarine antagonized CP-55940-stimulated binding of [^{35}S] GTPγS to the G-protein (IC_{50}s: chelerythrine 2.09 μM; sanguinarine 1.22 μM). In contrast to AM251, both compounds strongly inhibited basal binding of [^{35}S]GTPγS (IC_{50}s: chelerythrine 10.06 μM; sanguinarine 5.19 μM).

Piperonyl butoxide and S-methoprene inhibited the binding of [^{3}H]CP-55940 (IC_{50}s: piperonyl butoxide 8.2 μM; methoprene 16.4 μM), and also inhibited agonist-stimulated (but not basal) binding of [^{35}S]GTPγS to brain membranes (IC_{50}s: piperonyl butoxide 22.5 μM; (S)-methoprene 19.31 μM). PMSF did not modify the inhibitory effect of (S)-methoprene on [^{3}H]CP-55940 binding.

Our data suggest that chelerythrine and sanguinarine are effacacious antagonists of G-protein-coupled CB_1 receptors. They exhibit lower potencies compared to many conventional CB_1 receptor blockers but act differently to AM251. Reverse modulation of CB_1 receptor agonist binding resulting from benzophenanthridines engaging with the G-protein component may explain this difference. Piperonyl butoxide and (S)-methoprene are effacacious, low potency, neutral antagonists of CB_1 receptors. Certain of the study compounds may represent useful starting structures for development of novel/more potent G-protein-coupled CB_1 receptor blocking drugs.

1. Introduction

Cannabinoid CB_1 receptors are widely distributed in mammalian brain and occur at high density in the cerebral cortex, hippocampus, cerebellum and basal ganglia (Herkenham et al., 1991; Tsou et al, 1998). CB_1 receptors are predominantly presynaptic and interface directly with G-proteins in the neuronal membrane, forming the initial presynaptic element of a negative feedback mechanism regulating transmitter exocytosis (Howlett et al., 1986; Katona et al., 1999; Kawamura et al., 2006). During heightened synaptic activity, postsynaptic neurons generate endocannabinoids which translocate retrogradely to activate presynaptic CB_1 receptors. Activation of the coupled G-protein leads to inhibition of voltage-sensitive Ca^{++} channels (Mackie and Hille, 1992; Twitchell et al., 1997; Kushmerick et al., 2004), negative modulation of adenylate cyclase (Howlett and Fleming, 1984; Howlett, 1985) and activation of K^+ currents (Deadwyler et al., 1993; Mackie et al., 1995. The net effect is a downward adjustment of transmitter release from the nerve ending (Chevaleyre et al., 2006; Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001; Howlett, et al., 2002).

In addition to endocannabinoids, various other natural and synthetic compounds including Δ^9-tetrahydrocannabinol, CP-55940 and WIN55212-2 exert agonist effects at brain cannabinoid receptors (Devane et al., 1988; Compton et al., 1992). Selective CB_1 receptor antagonists such as the diarylpyrazoles AM251 and SR141716A, and the phytocannabinoid Δ^9-tetrahydrocannabivarin have also been discovered (Rinaldi-Carmona et al., 1994; Lan et al., 1999; Thomas et al., 2005). These CB_1 receptor modulators exert potent effects *in vitro*, acting in the nanomolar range. Despite unfavorable psychiatric side effects associated with the first group of CB_1 antagonists/inverse agonists developed to treat obesity, compounds with this pharmacological profile remain of substantial interest (Szabo et al., 2009; Wu et al., 2009; Riedel et al., 2009).

Chelerythrine and sanguinarine are quaternary benzophenanthridine alkaloids of plant origin. We considered that the pseudobase

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forms of chelerythrine and sanguinarine might engage CB_1 receptors in a similar way to Δ^9-tetrahydrocannabinol or Δ^9-tetrahydrocannabivarin (see Fig. 1) based on preliminary findings that both natural products displace the binding of [^{3}H]CP-55940 to mouse brain membranes. In other exploratory experiments, binding inhibition was noted for two synthetic chemicals used in insect pest management: piperonyl butoxide, which we hypothesized may adopt an endocannabinoid-like conformation; and (S)-methoprene, which may represent a highly flexible analog of Δ^9-tetrahydrocannabinol or Δ^9-tetrahydrocannabivarin or perhaps mimic 2-AG (see Fig. 1).

The aim of the present work was to investigate the *in vitro* effects of these study compounds on the G-protein coupled CB_1 receptor in mouse brain in more depth. Interactions with this signaling complex were evaluated on the basis of ability to 1) displace the binding of [^{3}H] CP-55940, a radioligand that binds to a region of the CB_1 receptor shared with the recognition sites for endocannabinoids, classical cannabinoids, aminoalkylindoles and diarylpyrazoles (Devane et al., 1988; Song and Bonner, 1996; McAllister et al., 2003) and 2) modify the binding of [^{35}S]GTPγS to brain G-proteins in the presence and absence of agonist, an assay which determines functional coupling of the CB_1 receptor to its G-protein (Selley et al., 1996; Petitet et al., 1997).

2. Materials and methods

2.1. Radioligands, drugs and study compounds

[^{3}H]CP-55940 [(1*R*,3*R*,4*R*)-3-[2-hydroxy-4-(1,1-dimethylheptyl) phenyl]-4-(3-hydroxy-propyl)cyclohexan-1-ol; side chain-2,3,4-[^{3}H]; sp. act. 139.6 and 174.6Ci/mmol) and guanosine 5′-*O*-(γ−[^{35}S]thio)-triphosphate ([^{35}S]GTPγS; sp. act. 1250Ci/mmol) were obtained from Perkin Elmer Life and Analytical Sciences, Canada. Chelerythrine, berberine, sanguinarine, (as chloride or hydrochloride salts), papaverine, (−)-β-hydrastine, CP-55940, *N*-piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251), 2,3-dihydro-5-methyl-3-[(4-morpholinyl) methyl] pyrrolo-[1,2,3-*de*]-1,4-benzoxazin-6-yl](1-naphthyl)-methanone (WIN55,212-2), phenylmethanesulfonylfluoride (PMSF) and piperonyl butoxide were purchased from Sigma-Aldrich, Canada. (S)-Methoprene (98.5% purity) was kindly supplied by Doug Vangundy, Director of Speciality Product Development, Wellmark International (Dallas, Texas).

2.2. Animals

Male CD1 mice (20–25 g) obtained from Charles River Laboratories, (Saint-Constant, Quebec, Canada) were used for all experiments. Animals were maintained on a 12 h light:dark cycle with food and water provided *ad libitum*. All procedures using mice adhered to the Canadian Council on Animal Care standards regarding the use of animals in research and had approval of the Simon Fraser University Animal Care Committee.

2.3. Determination of the effects of study compounds on the binding of [^{3}H]CP-55940 to CB_1 receptors in mouse brain membranes

We evaluated several published procedures for the measurement of specific binding of [^{3}H]CP-55,940 to CB_1 receptors. The method described by Quistad et al. (2002) was adopted with minor modifications for the present investigation. Mice were euthanized by rapid cervical dislocation and all isolation procedures were carried out at 0–4 °C. Mouse whole brains were homogenized (10 up/down strokes) in ice-cold buffer (Trisma base (100 mM), EDTA (1 mM) adjusted to pH 9 with HCl; 1 brain/10 ml buffer) using a motor driven homogenizer (pestle rotation approx. 1500 rpm). Homogenates were centrifuged in a Beckman J2HS centrifuge at 900×*g* for 10 min in a JA20 rotor. The supernatant containing the neuronal membranes was centrifuged at 11,500×*g* for 20 min. Pellets were thoroughly resuspended to a protein concentration of close to 6.5 mg/ml in storage buffer (Trisma base (50 mM), EDTA (1 mM) and $MgCl_2 \cdot 6H_2O$ (3 mM), adjusted to pH 7.4 with HCl) and stored in aliquots at −80 °C. When required for experiments, membranes were thawed on ice, taken up in a 5 ml syringe and thoroughly resuspended by moving the suspension out and in (6 times) through an 18 g needle (with its square cut tip held close to the base of the tube) and then vortexed. For assay, compounds (in DMSO; 5 µl) were added to borosilicate glass culture tubes (13×100 mm; Kimble-Chase; no siliconization), followed by binding buffer (500 µl; Trisma base (50 mM), EDTA (1 mM), $MgCl_2 \cdot 6H_2O$ (3 mM), BSA (fatty acid free; 3 mg/ml) adjusted to pH 7.4 with HCl). Membranes (154.3±3.5 µg protein) were then added to each tube and the mixture vortexed and incubated for 15 min at room temperature. Following addition of [^{3}H]CP-55940 (added in 10 µl DMSO; final radioligand concentration 1.0 nM), the tube contents were thoroughly mixed and incubations run for 90 min at 30 °C with gentle shaking. Binding reactions were stopped by adding ice-cold wash buffer (0.9% NaCl containing 2 mg/ml BSA; 1 ml) and membranes were collected by rapid vacuum filtration on pre-soaked Whatman GF/C filters. Membranes trapped on the filter were immediately washed (3×4 ml) with ice-cold wash buffer. Filters were thoroughly air dried before adding scintillant (4 ml; BCS, Amersham Bioscience UK) and radioactivity was quantitated using liquid scintillation counting. Non-specific binding, measured in the presence of unlabeled CP-55,940 or WIN55,212-2 (both at 10 µM), was subtracted from total binding to yield the specific binding signal which averaged 80.9±4.7 % and 80.7=3.1% respectively. In each experiment, binding in the absence and presence of unlabeled CP-55,940 or WIN55212-2 was performed in triplicate and test compounds were assayed in duplicate. A minimum of three experiments were conducted for every treatment. All protein measurements were carried out as described by Peterson (1977).

2.4. Determination of the effects of study compounds on basal and CP-55940-stimulated [^{35}S]GTPγS binding to mouse brain membranes

The procedure for isolating brain membranes and measuring the effects of study compounds on basal and agonist-stimulated [^{35}S] GTPγS binding was adapted from that of Breivogel and Childers (2000). The isolation of brain membranes was carried out at 0–4 °C. Immediately following the cervical dislocation procedure, whole brains were removed from two mice and homogenized (Polytron Kinematica GmBH; speed setting 6 for 15 s) in isolation buffer (Trisma base (50 mM), $MgCl_2 \cdot 6\,H_2O$ (3 mM), EGTA (0.2 mM), NaCl (100 mM) with pH adjusted to 7.4 with HCl). The homogenate was centrifuged in a Beckman J2HS centrifuge (JA20 rotor) at 24,000×*g* for 25 min, and the resulting pellet was then resuspended in isolation buffer and re-centrifuged. The final membrane pellet was thoroughly homogenized in isolation buffer, the protein concentration adjusted to 7 mg/ml and aliquots transfered to the −80 °C freezer. After removal from storage at −80 °C, brain membranes were thawed on ice and thoroughly dispersed as described in Section 2.3. [^{35}S]GTPγS binding experiments were performed as follows. The test compound (in DMSO; 5 µl) or DMSO control, as appropriate, was placed in the tube first followed by assay buffer (500 µl; isolation buffer (pH 7.4) containing, bovine serum albumin (fatty-acid free; 1 mg/ml), guanosine diphosphate (GDP; 100 µM), dithiothreitol (20 µM), [^{35}S]GTPγS (0.14 nM final concentration) and adenosine deaminase (0.004 units/ml). The brain membranes (70.1±4.2 µg protein) were then added and after thorough vortexing, a 15 min preincubation at room temperature was carried out. Following this, CP-55940 (100 nM final concentration; in 5 µl DMSO) or DMSO control was added (total assay volume=535 µl), the samples were mixed thoroughly and the incubation continued at 30 °C for 90 min with gentle shaking. Where effects on basal binding were investigated, no agonist addition was made after the preincubation. The incubation was