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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

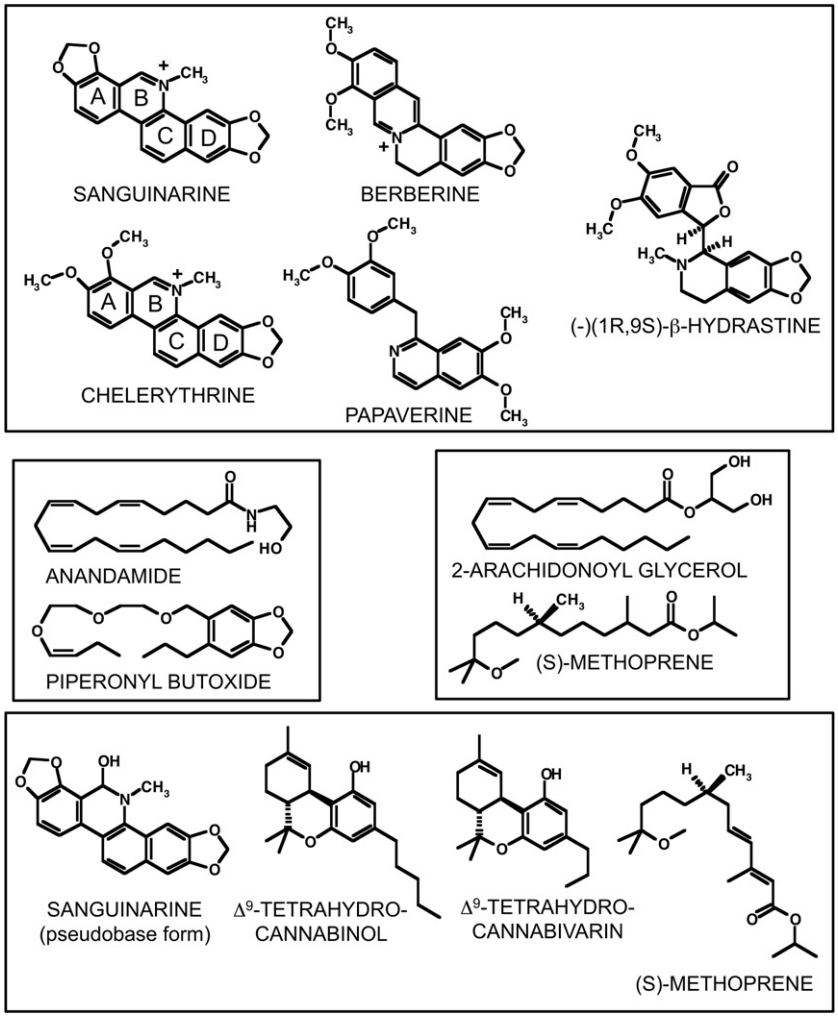

Fig. 1. *Upper panel:* The structures of sanguinarine, chelerythrine, berberine, papaverine and (−)(1R,9S)-β-hydrastine. *Middle panels:* Comparisons of possible conformations of piperonyl butoxide and (S)-methoprene with anandamide and 2-arachidonoyl glycerol respectively. *Lower panel:* Comparison of the pseudobase form of sanguinarine and a possible conformation of (S)-methoprene with Δ^9-tetrahydrocannabinol and Δ^9-tetrahydrocannabivarin.

terminated by the addition of ice-cold wash buffer (2 ml; Trisma base: HCl; pH 7.4) and rapid filtration under vacuum through pre-soaked Whatman GF/B filters. This was quickly followed by three 4 ml washes of the membranes on the filter. Membrane-bound ^{35}S was quantitated as described in Section 2.3. Assay tubes (borosilicate glass culture tubes; 13 × 100 mm) were siliconized with Sigmacote (Sigma-Aldrich Canada) 24 h prior to assay. All assays were conducted in triplicate. Basal binding, defined as the binding occurring in the absence of agonist (CP-55940) minus non-specific binding (measured with 100 μM unlabelled GTPγS present) averaged 95.4 ± 1.4%. 100 nM CP-55940 increased the basal binding of [^{35}S]GTPγS by 65.6 ± 2.8%.

2.5. Data analysis

Results are given as the mean ± S.E.M. Curve fitting by non-linear regression analysis and estimation of IC_{50} (concentration of study compound producing 50% inhibition) was carried out using Prism 4 (GraphPad Software Inc., San Diego, CA). K_i values for study compounds were determined using the Cheng-Prusoff equation with 0.35 nM as the K_d for [^{3}H]CP-55940.

3. Results

Fig. 2 shows the concentration-dependent inhibition of [^{3}H]CP-55940 binding to mouse brain CB_1 receptors by benzophenanthridines under equilibrium conditions. Sanguinarine and chelerythrine exhibited inhibitory potencies as estimated from IC_{50}s of 1.10 μM (95% CI = 0.62–1.93 μM) and 2.20 μM (95% CI = 1.55–3.13 μM) respectively. At higher

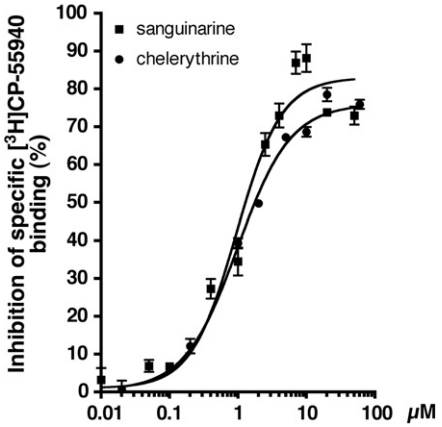

Fig. 2. Concentration-dependent inhibition of [^{3}H]CP-55940 binding to mouse brain CB_1 receptors by sanguinairine and chelerythrine. Values represent mean ± S.E.M. of at least 3 independent experiments each performed in duplicate. K_i values were 0.38 μM (sanguinarine) and 0.57 μM (chelerythrine).

Table 1
Inhibition of specific [^{3}H]CP-55940 binding to mouse brain membranes by isoquinoline type compounds and PMSF. Isoquinolines were present in the assay at 30 μM and PMSF was present at 0.5 mM. Data represent mean ± S.E.M. of 3 independent experiments.

Compound	Inhibition (%)
Berberine	12.05 ± 2.2
1R, 9S-(−)-β-Hydrastine	4.09 ± 1.64
Papaverine	17.56 ± 0.5
PMSF	1.27 ± 3.12

concentrations both approached full inhibition of [^{3}H] radioligand binding, with sanguinarine slightly more effacacious than chelerythrine.

The isoquinoline alkaloids berberine and papaverine, as well as the phthalide isoquinoline (−)-β-hydrastine individually achieved no greater than 17.6 % inhibition of [^{3}H] radioligand binding at 30 μM (Table 1). PMSF (0.5 mM) also had no effect on [^{3}H]CP-55940 binding (Table 1).

Chelerythrine and sanguinarine inhibited both agonist- (CP-55940-) stimulated and basal binding of [^{35}S]GTPγS binding to mouse brain membranes (Figs. 3 and 4). In the agonist-stimulated [^{35}S]GTPγS binding assays (Figs. 3a and 4a), chelerythrine produced an additional 54.92 ± 2.54% and 67.52 ± 3.40% encroachment into the basal signal at 20 μM and 50 μM respectively and sanguinarine caused an additional 50.12 ± 2.84%, 68.28 ± 0.56% and 74.76 ± 1.05% encroachment into the basal signal at 4 μM, 10 μM and 50 μM respectively. The IC_{50}s for agonist stimulation were: chelerythrine 2.09 μM (95% CI = 1.73–2.44 μM) and sanguinarine 1.22 μM (95% CI = 1.05–1.50 μM). The IC_{50}s for basal binding were: chelerythrine 10.06 μM (95% CI = 7.18–15.54 μM) and sanguinarine 5.19 μM (95% CI = 4.59–5.89 μM). Under identical conditions, AM251 inhibited 100 nM CP-55940-stimulated [^{35}S]GTPγS binding by 59.59% and 98.93% at 0.01 μM and 1 μM respectively (Table 2), confirming that the G-protein under investigation is coupled to CB_1 receptors. However, at 20 μM AM251 produced minimal (*circa* 10%) inhibition of basal [^{35}S]GTPγS binding (Table 2). No significant inhibitory effects of berberine, papaverine, (−)-β-hydrastine (all at 40 μM) on CP-55940-stimulated or basal [^{35}S]GTPγS binding were detected (Table 3).

The effects of piperonyl butoxide and (S)-methoprene on [^{3}H]CP-55940 binding and agonist-stimulated [^{35}S]GTPγS binding are shown in Fig. 5a and b. Piperonyl butoxide and (S)-methoprene inhibited [^{3}H]CP-55940 binding to CB_1 receptors [IC_{50}s: piperonyl butoxide 8.2 μM (95% CI = 7.08–9.32 μM) and (S)-methoprene 16.4 μM (95% CI = 13.7–19.06 μM)]. In parallel experiments, 4 μM (S)-methoprene inhibited [^{3}H]CP-55940 binding by 27.32 ± 4.11% and 28.16 ± 4.17% in the absence and presence of 50 μM PMSF respectively, showing that esterases were not limiting the inhibitory potency of this compound. Both compounds also blocked CP-55940-stimulated binding of [^{35}S]GTPγS to the G-protein [IC_{50}s: piperonyl butoxide 22.5 μM (95% CI = 18.98–36.02 μM) and (S)-methoprene 19.31 μM (95% CI = 17.01–21.61 μM)]. No effects of piperonyl butoxide or (S)-methoprene on basal binding of [^{35}S]GTPγS were observed (Table 4).

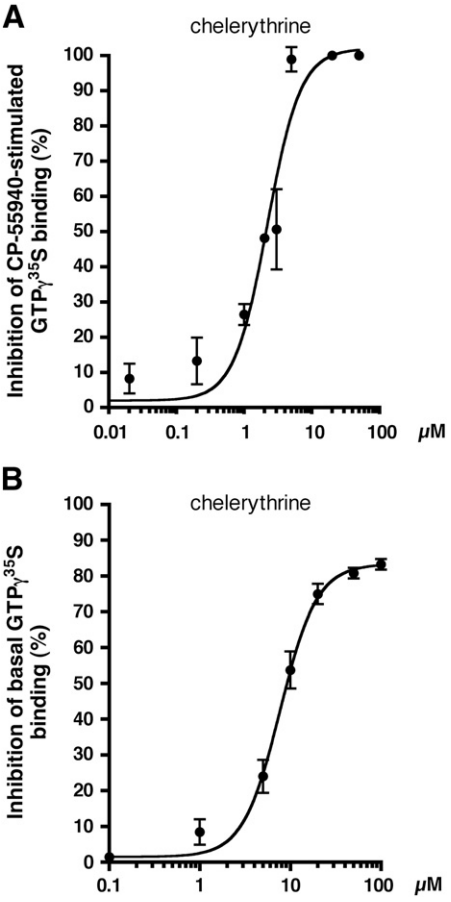

Fig. 3. Inhibition of A) CP-55940-stimulated and B) basal binding of [^{35}S]GTPγS to mouse brain membranes by chelerythrine. Values represent mean ± S.E.M. of 3 independent experiments each performed in triplicate.

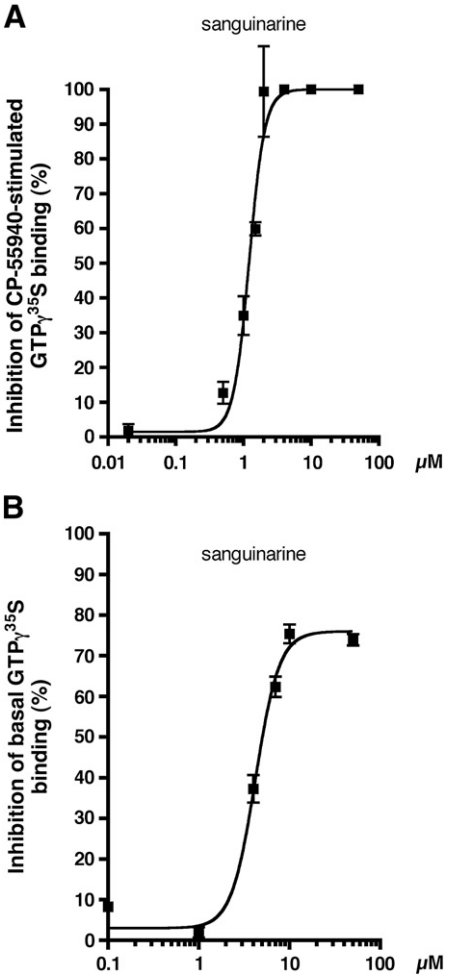

Fig. 4. Inhibition of A) CP-55940-stimulated and B) basal binding of [^{35}S]GTPγS to mouse brain membranes by sanguinarine. Values represent mean ± S.E.M. of 3 independent experiments each performed in triplicate.

Table 2
Inhibition of 100 nM CP-55940-stimulated and basal [^{35}S]GTPγS binding to mouse brain membranes by AM251. Data represent mean ± S.E.M. of 3 independent experiments. ND = not determined.

AM251 (μM)	Inhibition of CP-55940-stimulated [^{35}S]GTPγS binding (%)	Inhibition of basal [^{35}S]GTPγS binding (%)
0.010	59.59 ± 2.66	ND
1.0	98.93 ± 1.62	ND
10.0	100	7.61 + 6.32
20.0	ND	10.01 ± 1.37

9.21 ± 0.76% encroachment of AM251 on the basal component of [^{35}S]GTPγS binding was observed in experiments involving 10 μM CP-55940 agonist, as observed by others with the closely related analog SR141716A (Selley et al., 1996; Petitet et al., 1997).

4. Discussion

The results of this investigation demonstrate that benzophenanthridine alkaloids, piperonyl butoxide and (S)-methoprene inhibit the G-protein-coupled CB_1 receptor of mammalian brain, and suggest a clear functional difference between the actions of the natural product alkaloids and the synthetic compounds at this complex.

The IC_{50}s of sanguinarine and chelerythrine in the [^{3}H]CP-55940 binding assay lie in the 1–2 μM range, which places them very similar in potency to cannabidiol, virodhamine, various Δ^8tetrahydrocannabinol derivatives and certain bicyclic resorcinols (Devane et al., 1988; Compton et al., 1993; Steffens et al., 2005; Wiley et al., 2002), however, these benzophenanthridines are considerably less potent than Δ^9tetrahydrocannabinol and Δ^9tetrahydrocannabivarin, which inhibit the binding of [^{3}H]CP-55940 at low nanomolar concentrations (Devane et al., 1988; Thomas et al., 2005).

Antagonist-like actions for sanguinarine and chelerythrine at the G-protein-coupled CB_1 were strongly indicated since both alkaloids produce inhibition of CP-55940-stimulated [^{35}S]GTPγS binding at IC_{50}s identical to those for displacement of [^{3}H]CP-55940 binding to mouse brain membranes. The effect of these benzophenanthridines in the agonist-stimulated [^{35}S]GTPγS assay is qualitatively similar to that of the CB_1 receptor-selective antagonist AM251. However, at greater than maximum effect concentrations, and in marked contrast to AM251, sanguinarine and chelerythrine showed considerable encroachment into the basal component of [^{35}S]GTPγS binding with CP-55940 present. Again unlike AM251, chelerythrine and sanguinarine strongly inhibited basal [^{35}S]GTPγS binding. On the basis of these G-protein modulatory profiles, chelerythrine and sanguinarine may be more reasonably classified as inverse agonists at the G-protein-coupled CB_1 receptor. The slightly higher inhibitory potency of sanguinarine compared to chelerythrine in the [^{3}H]CP-55940 and agonist stimulated [^{35}S]GTPγS binding assays was likely due to the methylenedioxy moiety in place of the two methoxy groups on ring A. We cannot say whether the cationic (quaternary ammonium) form or the hydroxide adduct (pseudobase; which is formed at physiological pH; Slalinova et al., 2001) binds to the G-protein-coupled CB_1 receptor. However, the existence of an equilibrium state raises the possibility that that lower concentrations of an active species may cause the effects described here. The failure of berberine (which can also form the pseudobase), papaverine and (−)-β-hydrastine to interact with the G-protein-coupled CB_1 receptor in these assays indicates that aromatic ring stacking and position of the nitrogen atom are important for inhibitory activity.

Our original hypothesis that the benzophenanthridines may bind in a similar way to phytocannabinoids at the CB_1 receptor seems improbable. However, since it is known that 1) GTP and non-hydrolysable GTP analogs including GTPγS and guanyl-5′-yl imidodiphosphate allosterically dissociate [^{3}H]CP-55940 from the CB_1 receptor (Devane et al., 1988; Houston and Howlett, 1993) and that 2) low micromolar concentrations of sanguinarine and chelerythrine inhibit the binding of a fluorescent GTP probe to the GTP binding protein Rac1b in a competitive fashion (Beausoleil et al., 2009), we think it most likely that chelerythrine and sanguinarine exert retrograde allosteric inhibition of agonist binding to the CB_1 receptor by targeting the guanine nucleotide recognition site on the associated G-protein. In theory, a drug that acts selectively in this fashion could offer an alternative mechanism to the diarylpyrazoles for downregulating endocannabinoid-mediated signaling in the CNS, and therefore may have potential in weight reduction and the treatment of various metabolic disorders in humans. It is also possible that the level of inhibition of endocannabinoid activation of CB_1 receptors *in vivo* may be more readily managed with moderate potency drugs that are selective for the G-protein component of this complex, potentially reducing psychiatric side effects. Some evidence exists for selectivity of benzophenanthridines, since it is known that chelerythrine and sanguinarine bind more avidly to certain GTP binding proteins than members of the berberine series (Beausoleil et al., 2009). In addition, our data on basal and agonist-stimulated [^{35}S]GTPγS binding, which reflect respectively the sum of binding to *all* GTP binding sites in the membrane fraction versus *only those* specifically activated by CP-55940, indicate that the latter response is 4–5 fold more sensitive to blockade by chelerythrine and sanguinarine. Benzophenanthridines may therefore offer a novel area of chemistry for development of drugs that negatively regulate the endocannabinoid system, but achieving selectivity at CB_1 over CB_2 receptors through this mechanism may be difficult. It is important to note that chelerythrine was reported originally as a potent and specific protein kinase C inhibitor (Herbert et al., 1990), although considerable doubt now exists (Lee et al., 1998), and studies by Garcia et al. (1998) found that stimulation of protein kinase C with phorbol 12-myristate 13-acetate phosphorylates the CB_1 receptor which in turn suppresses both cannabinoid-induced activation of an inwardly rectifying K^+ current and depression of P/Q type Ca^{++} channel activity. If, in our experiments, the benzophenanthridines were inhibiting protein kinase C through this particular mechanism, we would predict they would not block (or possibly facilitate) CP-55940-mediated activation of the G-protein. Consistent with the results presented here, chelerythrine has been reported to inhibit desacetyllevonantradol-dependent activation of the G-protein-coupled CB_1 receptor in N18TG2 neuroblastoma cells, however, this observation was interpreted as supporting other direct evidence for modulation of a downstream protein kinase C by the CB_1 receptor (Rubovitch et al., 2004).

We also considered the possibility that our study compounds may act on CB_1 receptors indirectly by increasing the levels of anandamide and/or 2-AG through inhibition of endocannabinoid degrading enzymes (e.g. FAAH or MAGL). Such a mechanism is involved in the action of organophosphorus compounds (Nomura et al., 2008). However, since these endocannabinoids are CB_1 receptor agonists, our study compounds would be expected to significantly increase the

Table 3
Lack of effect of isoquinoline type compounds on CP-55940-stimulated and basal [^{35}S]GTPγS binding to mouse brain membranes. Study compounds were present in the assay at 40 μM. Data represent mean ± S.E.M. of 3 independent experiments.

Compound	Inhibition of CP-55940-stimulated [^{35}S]GTPγS binding (%)	Effect on basal [^{35}S]GTPγS binding (+ = % increase; − = % decrease)
Berberine	2.82 ± 0.89[a]	1.98 ± 0.35
1R, 9S-(−)-β-Hydrastine	2.23 ± 2.23	−0.50 ± 5.77
Papaverine	5.79 ± 1.77	3.90 ± 2.32

[a] represents a % increase in CP-55940-induced stimulation.

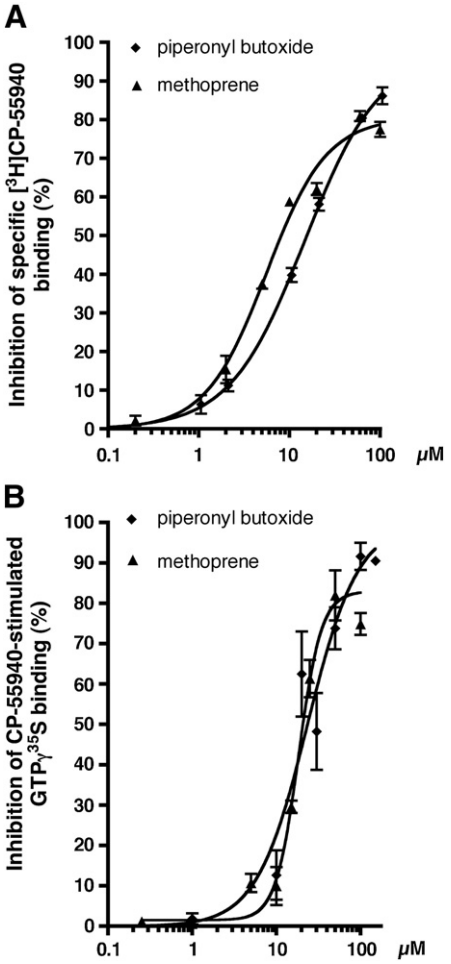

Fig. 5. A) Concentration-dependent inhibition of [^{3}H]CP-55940 binding to mouse brain CB_1 receptors by (S)-methoprene and piperonyl butoxide. K_i values were 2.13 μM (methoprene) and 4.25 μM (piperonyl butoxide). B) Inhibition of CP-55940-stimulated binding of [^{35}S]GTPγS to mouse brain membranes by (S)-methoprene and piperonyl butoxide. Values represent mean ± S.E.M. of 3 independent experiments each performed in triplicate.

basal [^{35}S]GTPγS binding signal (as we show with CP-55940). This is clearly not the case since the benzophenanthridines strongly inhibit, and methoprene and piperonyl butoxide fail to influence basal [^{35}S]GTPγS binding. Furthermore, 0.5 mM PMSF (a concentration which would be expected to fully inhibit FAAH and provide about 50% inhibition of MAGL) has no influence on the binding of [^{3}H]CP55940 to CB_1 receptors in our *in vitro* system, offering another line of evidence that our study compounds could not act by this mechanism.

Piperonyl butoxide is widely used as a synergist for insecticides (Jones, 1998), while methoprene exerts growth regulatory effects in insects rather than direct toxicity (Henrick, 2007). Both exhibit low mammalian toxicity [acute oral LD_{50}s in the rat: 7500–10,000 mg/kg (piperonyl butoxide) and >34,600 mg/kg (methoprene); Siddall, 1976; Hawkins et al., 1977]. Our results indicate that in contrast to the benzophenanthridines, piperonyl butoxide and S-methoprene act as neutral antagonists of the CB_1 receptor, giving some support to our hypothesis that they act as structural mimics of phytocannabinoids or endocannabinoids. However, based on displacement of [^{3}H]CP-55940 binding, their potencies are approximately 4–10 fold lower than the CB_1 receptor inhibitor virodhamine (Steffens et al., 2005) and 2-hydroxyphenyl arachidonamide (Edgemond et al., 1995), 12–24 fold lower than LH-21 (Chen et al., 2008) and 130–260-fold lower than Δ^9-tetrahydrocannabivarin (Thomas et al., 2005). While a variety of hydroxylated analogs of anandamide have been evaluated at CB_1 receptors (Van der Stelt et al., 2002), we are not aware of attempts to optimize alkylalkoxymethylenedioxyphenyl systems, or methoprene. Such compounds may offer alternative starting templates for optimization of novel CB_1 receptor modulators. Studies are underway to profile the action of the study compounds in more detail and explore their actions at CB_2 receptors.

Table 4
Lack of effect of piperonyl butoxide and (S)-methoprene on the basal binding of [^{35}S]GTPγS to mouse brain membranes. Values represent mean ± S.E.M. of 3 independent experiments.

Compound	Concentration	(% increase)
Piperonyl butoxide	20 μM	8.93 ± 6.20
	30 μM	7.21 ± 1.61
	40 μM	1.47 ± 0.47
(S)-Methoprene	25 μM	4.33 ± 6.32
	40 μM	2.80 ± 0.80
	50 μM	0.03 ± 5.59

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