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Benzophenanthridine alkaloid, piperonyl butoxide and (S)-methoprene action at the cannabinoid-1 receptor (CB_1-receptor) pathway of mouse brain: Interference with [^{3}H]CP55940 and [^{3}H]SR141716A binding and modification of WIN55212-2-dependent inhibition of synaptosomal L-glutamate release

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ABSTRACT

Benzophenanthridine alkaloids (chelerythrine and sanguinarine) inhibited binding of [^{3}H]SR141716A to mouse brain membranes (IC_{50}s: <1 μM). Piperonyl butoxide and (S)-methoprene were less potent (IC_{50}s: 21 and 63 μM respectively). Benzophenanthridines and piperonyl butoxide were more selective towards brain CB_1 receptors versus spleen CB_2 receptors. All compounds reduced B_{max} of [^{3}H]SR141716A binding to CB_1 receptors, but only methoprene and piperonyl butoxide increased K_d (3–5-fold). Benzophenanthridines increased the K_d of [^{3}H]CP55940 binding (6-fold), but did not alter B_{max}. (S)-methoprene increased the K_d of [^{3}H]CP55940 binding (by almost 4-fold) and reduced B_{max} by 60%. Piperonyl butoxide lowered the B_{max} of [^{3}H]CP55940 binding by 50%, but did not influence K_d. All compounds reduced [^{3}H]SR141716A and [^{3}H]CP55940 association with CB_1 receptors. Combined with a saturating concentration of SR141716A, only piperonyl butoxide and (S)-methoprene increased dissociation of [^{3}H]SR141716A above that of SR141716A alone. Only piperonyl butoxide increased dissociation of [^{3}H]CP55940 to a level greater than CP55940 alone. Binding results indicate predominantly allosteric components to the study compounds action. 4-Aminopyridine-(4-AP-) evoked release of L-glutamate from synaptosomes was partially inhibited by WIN55212-2, an effect completely neutralized by AM251, (S)-methoprene and piperonyl butoxide. With WIN55212-2 present, benzophenanthridines enhanced 4-AP-evoked L-glutamate release above 4-AP alone. Modulatory patterns of L-glutamate release (with WIN-55212-2 present) align with previous antagonist/inverse agonist profiling based on [^{35}S]GTPγS binding. Although these compounds exhibit lower potencies compared to many classical CB_1 receptor inhibitors, they may have potential to modify CB1-receptor-dependent behavioral/physiological outcomes in the whole animal.

1. Introduction

Cannabinoid-1(CB_1) receptors are present in numerous regions of mammalian brain and are particularly abundant within the cerebral cortex, hippocampus, cerebellum and basal ganglia (Herkenham et al., 1991; Tsou et al., 1998). CB_1 receptors couple to G-proteins in the plasma membrane of nerve terminals and together they constitute the primary presynaptic element of the endocannabinoid signaling pathway that regulates transmitter release through negative feedback (Howlett et al., 1986; Katona et al., 1999; Kawamura et al., 2006). Endocannabinoids, generated in postsynaptic neuronal cell bodies when synaptic activity intensifies, migrate retrogradely and bind to presynaptic CB_1 receptors. G-protein activation leads to inhibition of voltage-sensitive Ca^{++} channels (Mackie and Hille, 1992; Twitchell et al., 1997; Kushmerick et al., 2004; Guo and Ikeda, 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, Childers and Deadwyler, 1996; Guo and Ikeda, 2004). Since these various signaling mechanisms reduce the ability of action potentials impinging on the nerve ending to depolarize and activate calcium entry, transmitter release is adjusted downwards, thus

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completing the negative feedback loop (Chevaleyre et al., 2006; Kreitzer and Regehr, 2001; Wilson and Nicoll, 2001; Howlett, et al., 2002; Freund et al., 2003).

Certain plant natural products and synthetic drugs mimic endocannabinoid activation of this signaling pathway by exerting potent (nanomolar) agonist actions at CB_1 receptors. Prominent xenocannabinoid agonists include Δ^9-tetrahydrocannabinol, the main psychoactive principle of *Cannabis sativa* (Razdan, 1986), CP55940 (Johnson and Melvin, 1986) and the aminoalkylindole WIN55212-2 (Compton et al., 1992). Selective high potency CB_1 receptor antagonists, notably the phytocannabinoid Δ^9-tetrahydrocannabivarin and the diarylpyrazole antagonist/inverse agonists AM251 and SR141716A, have also been reported (Rinaldi-Carmona et al., 1994; Lan et al., 1999; Thomas et al., 2005).

There is considerable interest in possible therapeutic applications of CB_1 receptor modulators. Agonists and allosteric activators of agonist action have been considered in the relief of pain, muscle spasms, anxiety states and depressive illness, and they can also block emesis, improve sleep and stimulate appetite (Van Sickle et al., 2001; Iversen, 2003, Ligresti et al., 2009; Bradshaw and Walker, 2005; Di Marzo, 2009). On the other hand, CB_1 receptor antagonists/inverse agonists such as the diarylpyrazole rimonabant (SR141716A) have shown effectiveness in reducing body weight through suppression of appetite (Colombo et al., 1998), but rimonabant use in human medicine was curtailed due to adverse psychiatric side effects. Nevertheless, discovery of a CB_1 receptor inhibitor divorced of such unfavorable symptoms clearly remains of considerable interest (Szabo et al., 2009; Wu et al., 2009; Riedel et al., 2009).

Research in our laboratory has focused on other natural products and synthetic environmental chemicals capable of interacting with the endocannabinoid system. Specifically we have demonstrated that at very low to moderate micromolar concentrations, the benzophenanthridine alkaloids (sanguinarine and chelerythrine), the pesticides (piperonyl butoxide and (S)-methoprene), and certain phthalate dialkyl ester plasticizers, inhibit both the binding of [^{3}H]CP55940 to CB_1 receptors, as well as CB_1 receptor agonist-dependent activation of the G-protein (Dhopeshwarkar et al., 2011; Bisset et al., 2011).

Endocannabinoids, the high potency synthetic agonists (e.g. CP55940 and WIN55212-2) and dihydropyrazole antagonists/inverse agonists (e.g. AM251 and SR141716A) engage with a discrete binding pocket on the CB_1 receptor and available evidence suggests individual binding domains are distinct or may have tendency to partially overlap (Shim, 2010; Kapur et al., 2007). The purpose of this phase of our research program was to examine the effects of sanguinarine, chelerythrine, piperonyl butoxide and (S)-methoprene in greater detail with regards their ability to interfere with the equilibrium binding and kinetic properties of radioligands that engage with the binding pocket of CB_1 receptors (specifically [^{3}H]CP55940 and [^{3}H]SR141716A). Moreover, since we predicted that, based on their abilities to modulate basal and CB_1-receptors-agonist- (CP55940-) stimulated binding of [^{35}S]GTPγS to the G protein, the benzophenanthridines were likely inverse agonists and piperonyl butoxide and (S)-methoprene were neutral antagonists of CB_1 receptors (Dhopeshwarkar et al., 2011), it was critical to investigate whether modification of neurotransmitter release (i.e. the ultimate consequence of presynaptic CB_1-R engagement by these compounds) was consistent with this functional profiling.

2. Materials and methods

2.1. Chemicals and supplies

2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl)-cyclohexyl]-5-(2-methyloctan-2-yl)-phenol (CP-55940),5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide (AM251), (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)pyrrolo[1,2,3-*de*]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN55212-2), dimethylsulfoxide (DMSO), sanguinarine, chelerythrine, piperonyl butoxide, ethylene diamine tetraacetic acid (EDTA), ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 4-aminopyridine (4-AP), veratridine (VTD), tetrodotoxin (TTX), acetone, Percoll®, glutamate dehydrogenase (Type II from bovine liver), β-nicotinamide adenine dinucleotide phosphate (sodium salt hydrate; $NADP^+$), bovine serum albumin (BSA; fatty acid free) and all other chemicals required for assay buffers and salines, were purchased from Sigma Aldrich, Canada. 5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3- carboxamide (SR141716A) was obtained from Cayman Chemical and (S)-methoprene (98.5% purity) was supplied by Doug Vangundy, Director of Speciality Product Development, Wellmark International (Dallas, Texas). Radioligands, [^{3}H]CP55940 (side chain-2,3,4-[^{3}H]; specific activities 139.6 and 174.6 Ci/mmol) and [^{3}H]SR141716A (piperidine ring 3,4-[^{3}H]; specific activity 56 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences, Canada.

2.2. Animals

All experiments described in this report were performed with male CD1 mice (20–25 g) purchased from Charles River Laboratories (Saint-Constant, Quebec). Animal orders were placed by Animal Care Services of Simon Fraser University, Burnaby, Canada. Upon receipt, mice were housed under standardized environmental conditions (21 °C; 55% relative humidity; 12 h light/dark cycle) and allowed unlimited access to food and water. Our animal housing, handling and experimental procedures conformed to Canadian Council on Animal Care guidelines and were formally approved by the Simon Fraser University Animal Care Committee prior to embarking on this investigation.

2.3. Isolation of membranes from mouse brain for binding studies

The isolation of brain membranes were carried out at 0–4 °C according to a method published previously (Dhopeshwarkar et al., 2011). Mouse whole brains were homogenized (10 pestle excursions; pestle rotation approx. 1500 rpm) in ice-cold buffer [Trisma base (100 mM), EDTA (1 mM), adjusted to pH 9 with HCl; 1 brain per 10 ml buffer]. The homogenate was centrifuged for 10 min at 900 × *g* in a JA20 rotor of a Beckman J2HS centrifuge. Centrifugation of the supernatant at 11,500 × *g* for 25 min produced a membrane pellet which was resuspended in ice-cold buffer [Trisma base (50 mM), EDTA (1 mM) and $MgCl_2 \cdot 6H_2O$ (3 mM); adjusted to pH 7.4 with HCl] at a protein concentration of approx. 6.5 mg/ml. The membrane preparation was then frozen in aliquots at −80 °C. Just prior to assay, the preparation was thawed on ice and carefully dispersed by slowly moving the membrane suspension in and out of a syringe fitted with 18 gauge needle (6 times) followed by vortexing.

2.4. Inhibition of [3H]SR141716A binding to brain CB1 receptors by benzophenanthridine alkaloids, (S)-methoprene and piperonyl butoxide

On the day of experiment, membranes were thawed on ice and resuspended. Compounds (formulated in DMSO; 5 μl) were added to unsiliconized borosilicate glass tubes (13 × 100 mm) containing binding buffer. (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; 500 μl) and brain membranes (227.09+0.66 μg protein). Binding reactions were initiated by addition of [3H]SR141716A (added in