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AM-251 and rimonabant act as direct antagonists at mu-opioid receptors: Implications for opioid/cannabinoid interaction studies

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ABSTRACT

Mu-opioid and CB1-cannabinoid agonists produce analgesia; however, adverse effects limit use of drugs in both classes. Additive or synergistic effects resulting from concurrent administration of low doses of mu- and CB1-agonists may produce analgesia with fewer side effects. Synergism potentially results from interaction between mu-opioid receptors (MORs) and CB1 receptors (CB1Rs). AM-251 and rimonabant are CB1R antagonist/inverse agonists employed to validate opioid–cannabinoid interactions, presumed to act selectively at CB1Rs. Therefore, the potential for direct action of these antagonists at MORs is rarely considered. This study determined if AM-251 and/or rimonabant directly bind and modulate the function of MORs. Surprisingly, AM-251 and rimonabant, but not a third CB1R inverse agonist AM-281, bind with mid-nanomolar affinity to human MORs with a rank order of affinity (K_i) of AM-251 (251 nM) > rimonabant (652 nM) > AM281 (2135 nM). AM-251 and rimonabant, but not AM-281, also competitively antagonize morphine induced G-protein activation in CHO-hMOR cell homogenates (K_b = 719 or 1310 nM, respectively). AM-251 and rimonabant block morphine inhibition of cAMP production, while only AM-251 elicits cAMP rebound in CHO-hMOR cells chronically exposed to morphine. AM-251 and rimonabant (10 mg/kg) attenuate morphine analgesia, whereas the same dose of AM-281 produces little effect. Therefore, in addition to high CB1R affinity, AM-251 and rimonabant bind to MORs with mid-nanomolar affinity and at higher doses may affect morphine analgesia via direct antagonism at MORs. Such CB1-independent actions of these antagonists may contribute to reported inconsistencies when CB1/MOR interactions are examined via pharmacological methods in CB1-knockout versus wild-type mice.

1. Introduction

Two cannabinoid receptors have been well characterized: CB1 receptors (CB1Rs), one of the most abundantly expressed G-protein coupled receptors in the central nervous system, and CB2 receptors (CB2Rs) which are localized primarily on immune cells (Howlett et al., 2004). Both CB1Rs and CB2Rs are coupled to the $G_{i/o}$-subtype of G-proteins that inhibit the activity of adenylyl cyclase (Kaminski, 1996) and activate mitogen-activated protein (MAP) kinases (Bouaboula et al., 1995, 1996). CB1Rs (Mackie and Hille, 1992) and CB2Rs (Atwood et al., 2012) additionally inhibit the function of voltage gated calcium channels, while only CB1Rs have been shown to modulate the activity of potassium channels (Hampson et al., 1995). CB1R agonists produce a characteristic tetrad of *in vivo* effects in rodents, defined by a decline in locomotor activity, hypothermia, catalepsy, and analgesia (McLaughlin et al., 2005). Since compounds that activate CB1Rs produce analgesia, selective CB1R agonists are being investigated as potential novel

Abbreviations: AM-251, (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide); AM-281, (1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide); ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphate; CHO-hCB2 cells, Chinese hamster ovary cells stably expressing human cannabinoid 2 receptors; CHO-hMOR cells, Chinese hamster ovary cells stably expressing human mu-opioid receptors; CP-55,950, [(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol]; DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; DMEM, Dulbecco's modified Eagle's medium; GTPγS, guanosine 5′-O-[gamma-thio]triphosphate; hCB2Rs, human cannabinoid 2 receptors; hMOR, human mu-opioid receptors; mCB1Rs, mouse cannabinoid 1 receptors; mMORs, mouse mu-opioid receptors; O-2050, ((6aR,10aR)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran).

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analgesic agents (Jhaveri et al., 2007). CB2R agonists also appear to modulate inflammatory hyperalgesia and neuropathic pain (Fox and Bevan, 2005; Manzanares et al., 2006). Although CB1Rs may represent a novel therapeutic option for treatment of acute and chronic pain, psychoactive side effects associated with CB1R activation limits the potential usefulness of drugs in this class (Hosking and Zajicek, 2008).

It is well known that activation of mu-opioid receptors (MORs) by agonists such as morphine produces potent and efficacious analgesia (Trescot et al., 2008). Similar to CB1Rs, mu-opioid receptors (MORs) couple to pertussis toxin-sensitive $G_{i/o}$-subtype of G-proteins to inhibit cAMP production, activate MAP-kinase activity, close voltage gated calcium channels, and open inwardly rectifying potassium channels (Waldhoer et al., 2004). Unfortunately, also like CB1R agonists, both acute and prolonged use of MOR-analgesics is associated with several therapeutically limiting adverse effects such as euphoria, tolerance and dependence (Waldhoer et al., 2004). It has long been observed that concurrent administration of MOR and CB1R agonists produces additive or synergistic analgesic effects (Welch and Eads, 1999). Therefore, combining lower doses of drugs from both classes might represent a means to reduce adverse effects while maintaining adequate analgesia (Cichewicz, 2004). A common mechanism proposed to explain the synergism of analgesia observed when opioids and cannabinoids are co-administered is a direct interaction between MORs and CB1Rs (Schoffelmeer et al., 2006).

Interactions between CB1Rs and MORs might be predicted based on observations that MORs are frequently co-localized with CB1Rs on neurons (Rodriguez et al., 2001; Salio et al., 2001), and both receptors form functional heterodimers (Hojo et al., 2008) and utilize the same pool of G-proteins (Shapira et al., 2000). Indeed, recent *in vitro* studies demonstrate that the constitutive activity of the CB1Rs negatively regulates MOR function (Canals and Milligan, 2008). For example, the neutral CB1R antagonist O-2020 produces no effect on MOR activity, but the CB1R inverse agonist SR-141716A (*e.g.*, rimonabant) enhances MOR function. Interactions between CB1Rs and MORs have also been predicted to occur *in vivo*. For example, da Fonseca Pacheco *et al.* reported that AM-251, a selective CB1R inverse agonist, antagonizes peripheral analgesia produced by morphine (da Fonseca Pacheco et al., 2008). Co-administration of AM-251 with morphine also decreases the development of tolerance and dependence in chronically treated mice (Trang et al., 2007). Lastly, CB1 agonists intensify morphine analgesia (Reche et al., 1996; Vaysse et al., 1987; Williams et al., 2008). Importantly, these studies collectively suggest that co-administration with CB1R ligands might not only lower doses of chronically administered MOR agonists required to achieve adequate analgesia, but also delay or prevent development of opioid tolerance and/or dependence as well. Although these and additional pharmacologically based studies in wild-type mice do provide sufficient evidence for a role for CB1Rs in mediating MOR analgesia, curiously, these results are often not validated when examined by similar studies employing CB1-knockout mice (Miller et al., 2011; Raffa and Ward, 2012).

Many studies utilize the high affinity, "selective" CB1 antagonist/inverse agonists AM-251 (da Fonseca Pacheco et al., 2008; Haghparast et al., 2009; Pacheco Dda et al., 2009; Trang et al., 2007) or rimonabant (Fattore et al., 2005; Kirkham and Williams, 2001; Miller et al., 2011; Schoffelmeer et al., 2006) to investigate potential interactions between CB1Rs and MORs. AM-251 has been reported to exhibit approximately 306-fold selectively for CB1Rs over CB2Rs, with a K_i value of 0.23–7.49 nM for CB1Rs (Gatley et al., 1997; Lan et al., 1999). However, AM-251 appears to also act as an agonist at the putative cannabinoid receptor GPR55 in the low to mid-nanomolar range (Sharir and Abood, 2010). Rimonabant similarly binds with high affinity to CB1Rs (*e.g.*, K_i = 1.98–11.8 nM), displaying roughly 82-fold selectivity when compared to CB2Rs (Felder et al., 1995; Rinaldi-Carmona et al., 1994). For most interaction studies, it is thus assumed that both antagonists bind selectively to CB1Rs and not to other receptors, most importantly to MORs. As such, few studies have actually examined whether AM-251 or rimonabant might also interact directly with MORs. The purpose of this study was to explore the possibility that AM-251 and/or rimonabant might indeed bind to and modulate the activity of MORs. Therefore, we tested the hypothesis that the frequently reported effects of these antagonists on MOR function are not entirely mediated by indirect action at CB1Rs, but also by via direct binding to MORs. If correct, this could help to explain the reported inconsistencies when CB1/MOR interactions are examined via pharmacological methods in CB1-knockout versus wild-type mice (Miller et al., 2011). We report that both AM-251 and rimonabant bind to MORs with mid-nanomolar affinity and function as antagonists. In addition, both antagonists (10 mg/kg, i.p.) attenuate morphine analgesia, whereas the same dose of AM-281, a third high affinity CB1 antagonist with much lower MOR affinity, does not.

2. Materials and methods

2.1. Drugs

AM-251 (N-(Piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide) and AM-281 (1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide) are both CB1R antagonist/inverse agonists (Pertwee, 2005) and were purchased from Tocris Bioscience (Ellisville, MO). O-2050 ((6aR,10aR)-3-(1-Methanesulfonylamino-4-hexyn-6-yl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6H-dibenzo[b,d]pyran) is a neutral antagonist at CB1Rs (Gardner and Mallet, 2006) and was purchased from Tocris Bioscience (Ellisville, MO). The CB1R selective antagonist/inverse agonist rimonabant (originally reported as SR141715A) (Rinaldi-Carmona et al., 1994) was obtained from Cayman Chemical Company (Ann Arbor, MI). Naloxone is a non-selective antagonist at mu, delta and kappa-opioid receptors (Spivak et al., 1997) and was purchased from Sigma-Aldrich (St. Louis, MO). Morphine sulfate was obtained through Cardinal Health (Dublin, OH) and acts as an agonist at MORs (Trescot et al., 2008). The highly selective MOR peptide agonist [^{3}H]DAMGO (Spivak et al., 1997), CB1R/CB2R agonist [^{3}H]CP-55,940 (Hojo et al., 2008) and [^{35}S]GTPγS used in G-protein activation studies, were all purchased from Perkin-Elmer (Waltham, MA). Non-radiolabeled CP-55,940 and DAMGO were obtained from Tocris Bioscience (Ellisville, MO). Non-radioactive GTPγS was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Mice

The University of Arkansas for Medical Sciences IACUC committee approved animal use protocols employed in this study. All efforts were made to minimize animal suffering and to reduce the number of animals used. B6SJL mice were obtained from an in-house breeding colony and C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animals were maintained on a 12 h light/dark cycle with free access to food and water. Following anesthesia with isoflurane, B6SJL mice were sacrificed and brains were harvested by decapitation and snap frozen employing liquid nitrogen.

2.3. Cell culture

Chinese hamster ovary (CHO-K1) cells were stably transfected in our laboratory with either the human CB2 receptor (CNR2; CHO-hCB2) (Shoemaker et al., 2005) or the human mu-opioid receptor (hMOR) containing an N-terminal cMyc epitope tag (OPRM1; CHO-hMOR) (described following). All cells were cultured in DMEM (Mediatech Inc., Manassas, VA) containing 10% fetal calf serum (Gemini Bioproducts, Sacramento, CA), 0.05 IU/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Carlsbad, CA), and 250 μg/ml of Geneticin (G418; Sigma-Aldrich, St. Louis, MO). Cells were maintained in a humidified chamber at 37 °C with 5% CO_2, harvested weekly and only cells from passages 5–15 were used in all experiments.

CHO-K1 cells stably expressing hMOR receptors (CHO-hMOR) were created using the cationic-lipid lipofectin as described previously for production of CHO-hCB2 cells (Shoemaker et al., 2005). Briefly, CHO cells were cultured to 80% confluence (3×10^6 cells in 100-mm dishes) and incubated for 6 h with 5 μg of pcDNA3.1 plasmids containing the cDNA encoding for the human mu-opioid receptor (OPRM1) containing an N-terminal cMyc epitope tag, and 15 μg of lipofectin reagent in serum-free Opti-MEM. Selection antibiotic (1 mg/ml geneticin)