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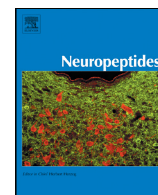

Investigation of receptor binding and functional characteristics of hemopressin(1–7)

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ABSTRACT

The orally active, α-hemoglobin derived hemopressin (PVNFKFLSH, Hp(1–9)) and its truncated (PVNFKFL, Hp(1–7) and PVNFKF, Hp(1–6)) and extended ((R)VDPVNFKFLSH, VD-Hp(1–9) and RVD-Hp(1–9)) derivatives have been postulated to be the endogenous peptide ligands of the cannabinoid receptor type 1 (CB1). In an attempt to create a versatile peptidic research tool for the direct study of the CB1 receptor–peptide ligand interactions, Hp(1–7) was radiolabeled and *in vitro* characterized in rat and CB1 knockout mouse brain membrane homogenates. In saturation and competition radioligand binding studies, [^{3}H]Hp(1–7) labeled membrane receptors with high densities and displayed specific binding to a receptor protein, but seemingly not to the cannabinoid type 1, in comparison the results with the prototypic JWH-018, AM251, rimonabant, Hp(1–9) and RVD-Hp(1–9) (pepcan 12) ligands in both rat brain and CB1 knockout mouse brain homogenates. Furthermore, functional [^{35}S]GTP γS binding studies revealed that Hp(1–7) and Hp(1–9) only weakly activated G-proteins in both brain membrane homogenates. Based on our findings and the latest literature data, we assume that the Hp(1–7) peptide fragment may be an allosteric ligand or indirect regulator of the endocannabinoid system rather than an endogenous ligand of the CB1 receptor.

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1. Introduction

The endogenous, phyto- and synthetic cannabinoids exert their pharmacological effects through the activation of cannabinoid receptors. To date, the cannabinoid type 1 (CB1) and cannabinoid type 2 (CB2) receptors have been cloned that belong to the superfamily of G_i/G_o G-protein coupled receptors (Begg et al., 2005; Pertwee, 1997). The CB1 receptors are primarily expressed in regions of the central nervous system (Herkenham et al., 1990; Matsuda et al., 1990) while CB2 receptors proved to be localized mainly in immune cells of the periphery (Munro et al., 1993), though recent studies reported the presence of CB2 receptors in the brain stem and spinal cord as well (Van Sickle et al., 2005; Zhang et al., 2003).

Lipid endocannabinoids are the best characterized endogenous ligands of the cannabinoid receptors and their physiological effects are primarily mediated through the CB1 receptors (Di Marzo and Petrosino, 2007; Boyd, 2006). The activation of the CB1 receptor is thought to be responsible for the mediation of antinociception, hypothermia, hypotension, sedation and inhibition of locomotor activity (Manzanares et al., 1999, Massi et al., 2001). Consequently, drugs acting on the CB1 receptor and on the entire endocannabinoid system may have therapeutic potential in a number of pathological conditions such as obesity, metabolic syndromes, mood and anxiety disorders, neuropathic pain, inflammation, multiple sclerosis, spinal cord injuries, myocardial infarction, stroke, hypertension, cancer and osteoporosis (Pacher et al., 2006).

Over the past decades, the lipid derived endocannabinoids were believed to be the sole endogenous agonists of the cannabinoid receptors. However, as a result of the pioneering works of Heimann et al. (2007) and Rioli et al. (2003), hemopressin (PVNFKFLSH, Hp(1–9)) was identified as a putative inverse agonist peptide ligand of the CB1 receptor. This peptide is a metabolic product of the hemoglobin α-chain and it was demonstrated to exert non-opioid antinociceptive effects, similar to those of the endo-, phyto- and synthetic cannabinoids (Heimann et al., 2007; Hama and Sagen, 2011). In an *in vivo* model of arthritic pain Hp(1–9) failed to mitigate mechanical allodynia (Petrovszki et al., 2012), however, in other studies, it could prevent carrageen- and bradykinin-induced hyperalgesia (Dale et al., 2005) and chronic constriction injury-induced mechanical hyperalgesia, a model of neuropathic pain (Toniolo et al., 2014a, 2014b). Hp(1–9) was also reported

Abbreviations: AM251, 1-(2,4-dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-(1-piperidyl)pyrazo-le-3-carboxamide; BSA, bovine serum albumin; DAMGO, [D-Ala^{2}, *N*-MePhe^{4}, Gly-ol]-enkephalin; DIEA, diisopropylethylamine; DMF, dimethylformamide; EGTA, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; EtOH, ethanol; GDP, guanosine 5′-diphosphate sodium salt, type I; GTPγS, guanosine 5′-[γ-thio]triphosphate tetralithium salt; Hp, hemopressin; HPLC, high performance liquid chromatography; JWH-018, naphthalen-1-yl(1-pentyl-1*H*-indol-3-yl)methanone; *i*PrOH, 2-propanol; Rimonabant, 5-(4-chlorophenyl)-1-(2,4-dichloro-phenyl)-4-methyl-*N*-(piperidin-1-yl)-1*H*-pyrazole-3-carboxamide; TFA, trifluoroacetic acid; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate.

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to induce weak, but dose-dependent hypotensive effects and to reduce food intake in rodents *via* a CB1 receptor-dependent manner (Blais et al., 2005; Rioli et al., 2003; Dodd et al., 2010, 2013). Very recently, Hp(1–9) was suggested promoting oligodendrocytic differentiation and maturation of subventricular zone progenitor cells, of which processes have significance in myelination abnormalities (Xapelli et al., 2014).

Soon after the discovery and pharmacological characterization of Hp(1–9), the RVD- and VD-extended RVD-Hp(1–9) and VD-Hp(1–9) (Gomes et al., 2009), and the *C*-terminally truncated Hp(1–6) and Hp(1–7) peptides were identified as potent cannabinoid ligands (Dale et al., 2005). RVD-Hp(1–9) and VD-Hp(1–9) were suggested being agonist ligands of the CB1 receptor. *In vivo* data for the *C*-terminally truncated hemopressins demonstrated that Hp(1–9) was not essential for antinociceptive activity, because Hp(1–6) and Hp(1–7) exerted as effective antihyperalgesic effects as the *N*-terminally extended peptides. Further *C*-terminal truncation, however, led to the loss of biological activity (Bomar and Galande, 2012). VD- and RVD-Hps exhibited hypotensive, hypothermic and hypoactive effects at antinociceptive doses, and inhibited bombesin-induced central activation of the adrenomedullary outflow in rats (Tanaka et al., 2014; Han et al., 2014). In addition, central administration of VD-Hp resulted in tolerance to antinociception and stimulated food consumption in a CB1-dependent manner (Han et al., 2014; Pan et al., 2014). The signaling characteristics and regulation of receptor endocytosis of the *N*-terminally extended peptide fragments were found to be distinct, in part, from those of the classical cannabinoid agonists (Gomes et al., 2009).

Circular dichroism, NMR spectroscopy and molecular docking studies on the Hp(1–9) and Hp(1–6) peptides showed that regular turn structures in the central portion of the peptides were essential for an interaction with the receptor, and similarly to the inverse agonist rimonabant the peptides stabilized receptor structures *via* H-bonds (Scrima et al., 2010). This interaction was assumed to be important for the stabilization of the inactive state of CB1 receptor and provides structural basis for the explanation of the activity of hemopressin peptides as agonist.

These observations suggest that hemopressins are novel endogenous peptide ligands of the CB1 receptor, and may have potential for the development of peptide-based research tools or therapeutic agents for the study of the endocannabinoid system or the treatment of cannabinoid-related diseases. In the present study, we report on the synthesis and radiolabeling of the *C*-terminally truncated hemopressin peptide Hp(1–7) and the direct *in vitro* pharmacological characterization of the novel radioligand $[^3H]$Hp(1–7) in brain membrane homogenates of rat and CB1 knockout mouse. Our results suggest that the hemoglobin fragment Hp(1–7) may be a regulator of the endocannabinoid system and that $[^3H]$Hp(1–7) can label either a CB receptor binding site different from the classical cannabinoid ligand binding site or another membrane protein.

2. Materials and methods

The peptides Hp(1–7) (H-Pro-Val-Asn-Phe-Lys-Phe-Leu-OH), ΔPro[1]-Hp(1–7) (H-ΔPro-Val-Asn-Phe-Lys-Phe-Leu-OH), Hp(1–9) (H-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-OH) and RVD-Hp(1–9) (H-Arg-Val-Asp-Pro-Val-Asn-Phe-Lys-Leu-Leu-Ser-His-OH) were synthesized and purified in our laboratory. The peptide synthesis resins, protected amino acids and the coupling reagent TBTU were purchased from Bachem. Hydrogen fluoride used for the cleavage of the peptides was obtained from PRAXAIR N.V. (Oevel, Belgium). Naloxone and rimonabant were kind gifts of Dr. Sándor Hosztafi (Department of Pharmaceutical Chemistry, Semmelweis University, Budapest, Hungary) and Dr. Sándor Benyhe (Hungarian Academy of Sciences, Biological Research Centre, Institute of Biochemistry, Szeged, Hungary). Analytical grade AM251 was obtained from Cayman Chemicals. TFA and BSA were purchased from Fisher Scientific. Protease inhibitor (cat#: P2714), GDP, GTPγS, anisole, ninhydrin, magnesium chloride, EGTA and Bradford reagent were purchased from Sigma-Aldrich Kft. (Budapest, Hungary). Other reagents were obtained from Molar Chemicals Kft. (Budapest, Hungary) or Merck Kft. (Budapest, Hungary). Tritium gas was obtained from Technobexport (Moscow, Russia). Tritium labeling was carried out in a self-designed vacuum manifold and radioactivity was measured with a Packard Tri-Carb 2100 TR liquid scintillation analyzer using Hionic-Fluor scintillation cocktail of PerkinElmer. Radio-HPLC was performed on a Jasco HPLC system equipped with a Packard Radiomatic 505 TR Flow Scintillation Analyser.

2.1. Preparation of hemopressins

The peptide synthesis was carried out manually in a silanized glass reaction vessel. N^{α}-Boc-Leu-or N^{α}-Boc-His(Tos)-PAM resin (0.15 mmol) was swollen for 30 min in DMF. After Boc-deprotection with neat TFA and subsequent washings (three times with DMF and *i*PrOH), TBTU activated N^{α}-Boc-protected amino acids (0.45 mmol) were added for chain elongation in DMF and the unreacted resin-bound peptides were end-capped with an excess of Ac_2O in the presence of DIEA in DMF. Couplings were monitored with the Kaiser-test (Kaiser et al., 1970). After removal of the *N*-terminal protecting group, peptides were cleaved from the resin with HF in the presence of anisole. The crude peptide — resin mixtures were washed with diethylether, then the peptides were dissolved in aqueous TFA and lyophilized. The resulting crude peptides were dissolved in aqueous TFA, and introduced onto an analytical Vydac 218TP54 column and eluted using a linear gradient of 1.5% per min of acetonitrile in water containing 0.1% TFA, starting from 15% acetonitrile at a flow rate of 1 mL/min, $\lambda = 215$ nm. The same elution conditions were used for the purification of the peptides on a semipreparative Vydac 218TP1010 column at a flow rate of 4 mL/min; isolated yields 56% (Hp(1–7)), 74% (ΔPro[1]-Hp(1–7)), 38% (Hp(1–9)) and 42% (RVD-Hp(1–9)). Molecular weights of the peptides were confirmed by MALDI-TOF mass spectrometry (Hp(1–7) $[M + H]^+$ 864.42; ΔPro[1]-Hp(1–7) $[M + H]^+$ 862.63; Hp(1–9) $[M + H]^+$ 1089.26; RVD-Hp(1–9) $[M + H]^+$ 1424.80).

2.2. Preparation of $[^3H]$Hp(1–7)

The precursor peptide ΔPro[1]-Hp(1–7) (2 mg, 2.32 μmol) was dissolved in DMF and 3 mg Pd/$BaSO_4$ catalyst was added to the solution. The reaction mixture was degassed prior to tritium reduction by a freeze–thaw cycle. Then it was stirred under 0.4 bar of tritium gas for 1 h at ambient temperature, followed by the filtration of the catalyst through a Whatman GF/C glass fiber filter. The filtrate was evaporated and labile tritium was removed by repeated evaporations from aqueous EtOH solution. Finally 2.85 GBq of crude $[^3H]$Hp(1–7) was obtained that was purified by HPLC. Quantitative analyses of the concentration and radioactivity of $[^3H]$Hp(1–7) were performed by RP-HPLC *via* UV and radioactivity detection using a calibration curve made by Hp(1–7), and the specific activity of $[^3H]$Hp(1–7) was found to be 1.04 TBq/mmol (28 Ci/mmol). The radioligand was aliquoted as ethanolic solutions and stored in liquid nitrogen until application.

2.3. Preparation of brain membrane homogenates

Wistar rats (male, 180–220 g) were housed locally *ad libitum* and handled according to the European Communities Council Directives (86/609/ECC) and to the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. Section 32). Crude membrane fractions were prepared from the brain without cerebellum. Brains were quickly removed from the euthanized rats and directly put in ice-cold 50 mM Tris/HCl (pH 7.4) buffer. The collected tissue was then homogenized in 30 volumes (v/w) of ice-cold buffer with a Teflon-glass Braun

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