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Isolation of new ligands for orphan receptor MRGPRX1—hemorphins LVV-H7 and VV-H7

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ABSTRACT

The human MAS-related G protein-coupled receptor X1 (MRGPRX1) is a member of the GPCR family. The receptor is primate specific and expressed in the sensory neurons of dorsal root ganglion and trigeminal ganglion, where it is considered to be involved in the pain perception. The MRGPRX1 has unusual binding mechanism, as it is activated by several different ligands as well as several different fragments of precursor proteins. Thus, we hypothesize that it is activated by several unknown compounds as well since the receptor is still classified as orphan. Here, we describe the isolation of two novel endogenous ligands for the MRGPRX1 from human platelet preparation. The isolated ligands are hemoglobin β-chain fragments, known members of the hemorphin family.

1. Introduction

MAS-related G protein-coupled receptors (MRGPR) are members of the G protein-coupled receptor (GPCR) family, previously known as the Mas-related gene receptors or the sensory neuron-specific GPCRs [12,23]. The MRGPRs can be found throughout the tetrapods, but there are significant differences between species [3]. Mice and rats have over 30 different genes from subfamilies *Mrgpra-h*, whereas humans have only four *MRGPRX* genes, *X1–X4* and one member of each subfamily *MRGPRD-G* [12,37]. Even though the human and murine receptors are significantly different, the amino acid sequences in primate receptors are highly conserved [8,41,43].

The MAS-related G protein-coupled receptor X1 (MRGPRX1) is considered to be an orphan primate specific receptor although several ligands have already been described [10,43]. The MRGPRX1 is expressed in sensory neurons of dorsal root ganglion (DRG) and trigeminal ganglion (TG) and in low levels in tryptase and chymase expressing mast cells (MC_{TC}) [21,38–40]. The receptor has been described to have a role in the sensory neurons in the perception of pain and itch [12,23,36]. In mast cells the receptor has been reported to be responsible for IgE-independent degranulation as well as stimulation of chemokine release [38–40]. The MRGPRX1 differs from other more typical GPCRs, as it has multiple different ligands and it is activated by several fragments of the same precursor protein, such as proenkephalin derived bovine adrenal medulla (BAM) fragments BAM(1–22), BAM (1–20), BAM(2–22), BAM(6–22), BAM(8–22), BAM(13–22) and BAM (8–25) [16,23,25,40]. Due to the primate specificity of the receptor and the variety of ligands, the function of MRGPRX1 is not well understood. We speculated that the receptor is rather promiscuous and more ligands are still to be discovered.

Thus, the purpose of this study was to isolate novel ligands to the MRGPRX1 from human platelets. For the isolation process human

Abbreviations: BAM, bovine adrenal medulla; GPCR, G protein-coupled receptor; LVV-H5, LVV-hemorphin-5; LVV-H7, LVV-hemorphin-7; MRGPR, MAS-related G protein-coupled receptors; VV-H7, VV-hemorphin-7

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material was required due to the human specificity of the receptor and platelets were chosen because if their high peptide content and availability. Here, we report the isolation of two new endogenous ligands for the MRGPRX1. The isolated ligands were determined as fragments of the hemoglobin β-chain and are members of the hemorphin family, LVV-hemorphin-7 (LVV-H7) and VV-hemorphin-7 (VV-H7).

2. Materials and methods

2.1. Preparation of the HEK 293 cells stably expressing MRGPRX1

The DNA fragment of human MRGPRX1 was amplified by PCR from cDNAs of HMC1 cell line, and cloned into EcoRI site of pEF1/myc-HisA (Invitrogen), followed by sequencing to confirm no change of the amino acid sequence of MRGPRX1. HEK 293 cells were transfected with MRGPRX1-pEF1/myc-HisA using Lipofectamine (Invitrogen) and the single cells stably expressing MRGPRX1 were obtained by G418 selection.

Further, the cell clone which showed the highest calcium mobilization induced by MRGPRX1 agonist (BAM8-22) was selected by using fluorescence imaging system (Victor 2, PerkinElmer).

2.2. Measurements of intracellular calcium

HEK 293 cells stably expressing MRGPRX1 were incubated with the culture medium containing 2 μM Fluo-4AM and 0.02% Pluronic F127 (Invitrogen) for 1 h at 37 °C. The cells were washed thrice with calcium buffer (20 mM HEPES, 115 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 13.8 mM glucose, and 0.2% BSA, pH 7.4) containing 0.02% Pluronic F127 and 2.5 mM Probenecid (Invitrogen) and suspended to final cell concentration (2×10^6 cells/ml). Ligand induced intracellular calcium mobilization was determined by injecting 100 μl of cell suspension onto 100 μl of samples in calcium buffer on 96 well plate and measuring fluorescence (excitation at 485 nm and emission at 535 nm) in real time for 30 s with a spectrofluorometer. Samples were determined as positive, if more than 5% increase in the emission from the baseline was detected.

2.3. Peptide purification by ultrafiltration and HPLC

A human platelet preparation (50 ml/bag, Finnish Red Cross) was used as starting material. One unit of platelets was diluted with 200 ml H_2O and acidified with 12.5 ml of 10% TFA. The diluted platelet preparation was ultra-filtrated under N_2 with 10 kDa filter (PBGC, Millipore) over 24 h at 4 °C. 250 ml of the platelets preparations (5 units) was treated to yield approximately 0.9 liters of the filtrate. The various HPLC separation steps are described in the result section.

2.4. Determination of EC_{50}

To determine the EC_{50} values of the hemoglobin fragments the MRGPRX1 expressing HEK 293 cells were subjected to calcium mobilization assay with serial dilutions (1 nM to 100 μM) of the test compounds. For each sample the signal ratio was compared to maximum activation with 100 nM BAM(8–22). 4-parameter logistic equation was used to calculate the EC_{50} values. GraphPad Prism 6 statistical software (GraphPad Software) was used for the calculations.

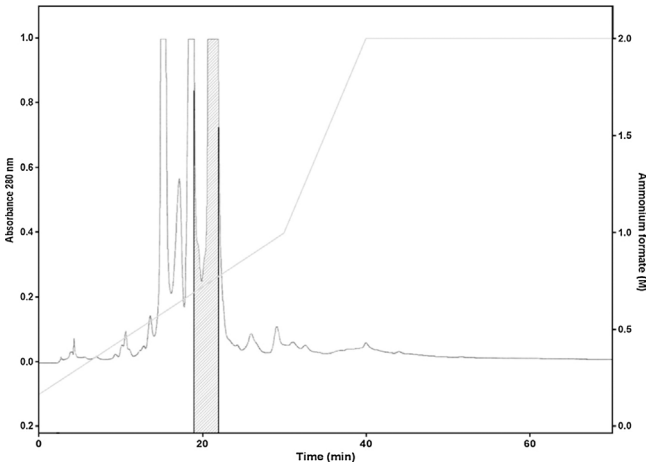

Fig. 1. Purification of the endogenous MRGPRX1 ligands from human platelets with ion exchange HPLC with a two-step linear gradient of ammonium formate, from 200 mM to 800 mM in 30 min and then to 2 M (pH 3.8) in 10 min, flow rate 1 ml/min, absorbance 280 nm. The MRGPRX1 activity containing area is marked (shaded).

3. Results

3.1. Purification of the endogenous ligands for MRGPRX1 via HPLC

As a first HPLC purification step of the filtrated platelet preparation a reversed-phase HPLC with a C18 column (UltraSphere ODS, 5 μm, 80 Å, 10 × 250 mm, Beckman Coulter Inc) and a gradient of acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) was used. For monitoring the purification process, the fractions obtained after each step were tested with MRGPRX1 expressing HEK 293 cells with calcium assay. The next step, HPLC purification from the activity-containing fractions, was performed with an ion-exchange HPLC using a TSK gel SP-2SW column (Tosoh) with an ammonium formate gradient (Fig. 1). The active fractions were purified further with an analytical C18 HPLC (Vydac 218TP, 5 μm, 300 Å, 4.6 × 250 mm, Alltech Associates Inc) with an ACN gradient in 0.1% TFA (Fig. 2). The last HPLC purification was performed with a C8 column (Capcell Pak C8-DD 5 μm, 80 Å, 4.6 × 250 mm, Shiseido)

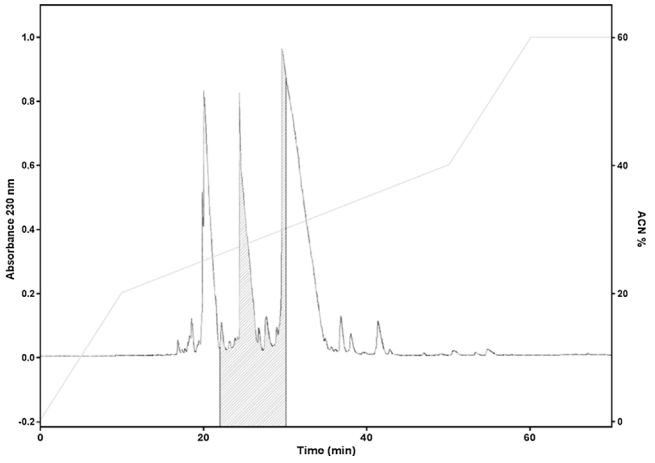

Fig. 2. Reversed phase HPLC on a C18 column with a three-step linear gradient of acetonitrile in 0.1% TFA, from 0 to 20% in 10 min, then to 40% in 40 min and finally to 60% in 10 min, flow rate 1 ml/min, absorbance 230 nm. The MRGPRX1 activity containing area is marked (shaded).