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Identification of melanin concentrating hormone (MCH) as the natural ligand for the orphan somatostatin-like receptor 1 (SLC-1)

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Abstract To identify possible ligands of the orphan somatostatin-like receptor 1 (SLC-1), rat brain extracts were analyzed by using the functional expression system of *Xenopus* oocytes injected with cRNAs encoding SLC-1 and G protein-gated inwardly rectifying potassium channels (GIRK). A strong inward current was observed with crude rat brain extracts which upon further purification by cation exchange chromatography and high performance liquid chromatography (HPLC) yielded two peptides with a high agonist activity. Mass spectrometry and partial peptide sequencing revealed that one peptide is identical with the neuropeptide melanin concentrating hormone (MCH), the other represents a truncated version of MCH lacking the three N-terminal amino acid residues. *Xenopus* oocytes expressing the MCH receptor responded to nM concentrations of synthetic MCH not only by the activation of GIRK-mediated currents but also by the induction of Ca^{2+} dependent chloride currents mediated by phospholipase C. This indicates that the MCH receptor can couple either to the G_i- or G_q-mediated signal transduction pathway, suggesting that MCH may serve for a number of distinct brain functions including food uptake behavior.

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Key words: Somatostatin; Melanin concentrating hormone receptor; Reverse physiology; Neuropeptide; Food intake behavior

1. Introduction

Many neurotransmitter receptors occur in numerous different subtypes. This is particularly true for the G protein-coupled receptors (GPCRs) of the somatostatin and opioid receptor family [1]. Five somatostatin receptors (SSTR1–5) and four opioid receptors (OR-μ, OR-δ, OR-κ and OR-FQ) have been identified to date. In addition, a somatostatin-like receptor, SLC-1, was identified by its homology to members of the somatostatin receptor family [2] and subsequently cloned from rat brain cDNA [3]. Although the SLC-1 receptor shares 40% identity with the SSTRs in the TM domains, it was neither activated by somatostatin-14 (SST14) nor somatostatin-28 (SST28). Here, we report the identification of the cognate ligand for SLC-1 by a 'reverse physiology' (also called 'reverse pharmacology') strategy using rat brain extracts and a sensitive screening system in which SLC-1 is functionally co-expressed with G protein-gated inwardly rectifying potassium channels (GIRKs) in *Xenopus* oocytes. Upon several purification steps, followed by mass spectrometric analysis and peptide sequencing, the ligand was identified as melanin concentrating hormone (MCH), revealing that the orphan SLC-1 is the MCH receptor.

2. Materials and methods

2.1. Expression in Xenopus oocytes

For functional expression in *Xenopus* oocytes, rat SLC-1 cDNA was subcloned in the *Xenopus* expression vector pGEMHE which contains *Xenopus* β-globin 5′- and 3′-untranslated regions [4]. cRNA was in vitro transcribed using T7-RNA-polymerase and a *Nhe*I-linearized plasmid and co-injected with GIRK1 cRNA essentially as described previously [5]. For recording, oocytes were superfused with ND-96 medium (96 mM NaCl, 2 mM KCl, 2.5 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM HEPES, pH 7.5) and whole cell-clamped at −60 mV. For agonist measurements, the medium was changed to high K+ medium (hK, ND-96 with 96 mM KCl and 2 mM NaCl). After the initial inward current had reached a plateau, agonists were applied in the same medium. Agonist treatment was terminated by wash-out with hK and a subsequent switch to ND-96 medium.

2.2. Extraction of rat brain peptides

For the purification of the native ligand of the SLC-1 receptor, 51 rat brains (67 g) were homogenized with an Ultra-Turrax in a solution with 0.5 M acetic acid, 10 mM ascorbic acid, 1 mM EDTA (10 ml/brain) and incubated for 10 min at 95°C to precipitate proteins. After 30 min centrifugation at 14 000×*g* at 4°C, the supernatant fraction was applied to C18-SepPak cartridges (Waters, Eschborn, Germany), washed with 5 ml 0.1% trifluoroacetic acid (TFA) and eluted with 80% methanol, 0.1% TFA. Peptides were lyophilized, dissolved in 0.1 M acetic acid and size-fractionated using a Sephadex G-25 column. Aliquots of fractions were concentrated with a vacuum centrifugator (SpeedVac), dissolved in hK medium and tested for SLC-1 agonist activity. Active fractions were further separated with a MonoS cation exchange column (Pharmacia, Freiburg, Germany) linked to a LKB high performance liquid chromatography (HPLC) system. Peptides were eluted at a linear gradient from 10 mM to 1 M ammonium formiate (pH 4.0). Active fractions were further separated with a C18 reverse phase HPLC column (Spherisorb OD52, Higgins Analytical, Mountain View, USA) and two runs on a C4 reverse phase HPLC column (Vydac, The Separations Group, Hesperia, CA, USA).

2.3. Peptide analysis

The HPLC-purified peptides were analyzed by matrix-assisted laser desorption/ionization mass spectrometry (Reflex, Bruker Daltonics, Bremen, Germany) with α-cyano-4-hydroxy-cinnamic acid as matrix. The sequences of the peptides were determined by automated Edman degradation (model 473, PE Biosystems, Foster City, USA). Synthetic human/rat MCH and ^{13}Phe, ^{19}Tyr-MCH were obtained by the Amer-

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Abbreviations: MCH, melanin concentrating hormone; SLC-1, somatostatin-like receptor 1; GPCR, G protein-coupled receptor; GIRK, G protein-gated inwardly rectifying potassium channel; HPLC, high performance liquid chromatography; hK, high K^+ medium; SST14, somatostatin-14; SST28, somatostatin-28

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ican Peptide Company (Sunnyvale, CA, USA) and Bachem (Hannover, Germany), respectively.

3. Results and discussion

In order to identify the cognate ligand for the orphan SLC-1, the latter was co-expressed together with GIRK in *Xenopus* oocytes. Fractions of the crude rat brain extract induced a strong GIRK-mediated inward current in oocytes expressing the rat SLC-1 receptor. No response was detected in control oocytes injected with GIRK cRNA only (Fig. 1). The crude rat brain extract was purified by Sephadex G-25 chromatography and cation exchange chromatography. The active fractions were combined and run on a C18 reverse phase HPLC yielding two active fractions, F72 and F84 (Fig. 2A). Both fractions were purified to homogeneity after two additional runs on a C4 reverse phase HPLC (Fig. 2B and C). Mass spectrometry revealed single masses with main peaks of 2009.08 and 2386.2 Da, respectively (Fig. 2D). Sequence analysis of the two peptides was performed using an automated amino acid sequencer. 13 Amino acid residues were identified matching the N-terminus of MCH (Table 1). Together with the mass spectrometric analysis, the data indicate that the peptide of fraction 72 consists of NH_2-Met-Leu-Arg-Xaa-Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-Xaa-Xaa-Gln-Xaa and

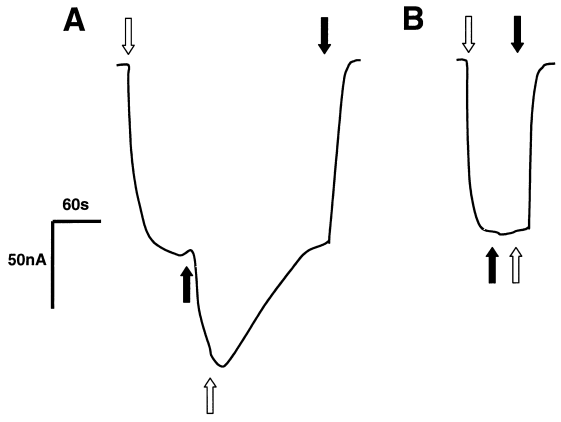

Fig. 1. Currents induced by rat brain extract, passed through a Sephadex G-25 column, recorded from *Xenopus* oocytes expressing SLC-1 and GIRK1 (A) and GIRK1 only (B). Downward open arrows, switch from ND-96 to hK medium; upward filled arrows, fraction application (10 μl aliquot of 4 ml total G-25 fraction 8, concentrated and dissolved in 2 ml hK, application of 1 ml to each oocyte); upward open arrows, wash-out with hK; downward filled arrows, switch to ND-96.

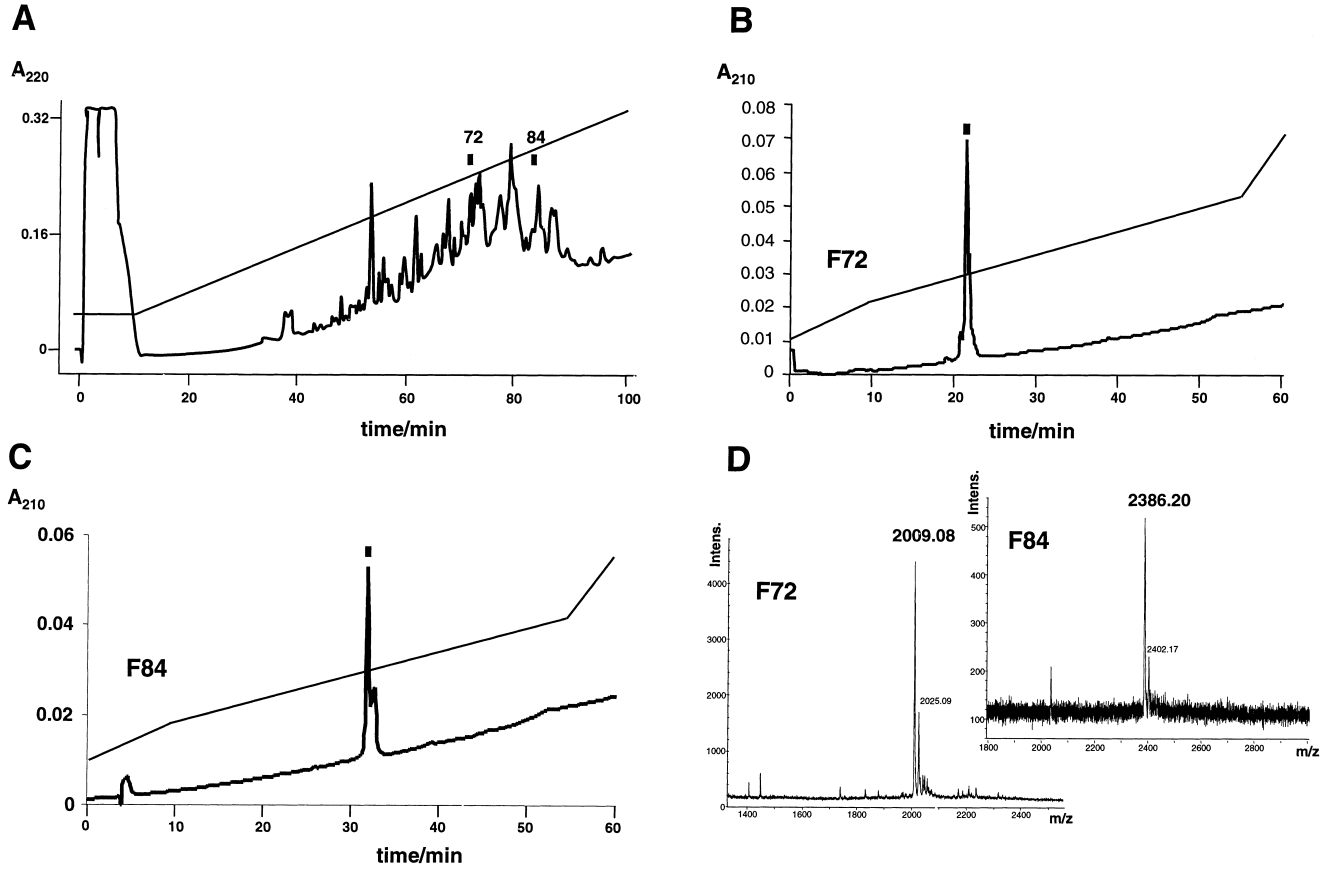

Fig. 2. Purification of the cognate ligand of SLC-1 using rat brain peptide extracts. Active fractions from the MonoS cation exchange column were applied to a reverse phase HPLC (C18) and eluted with a linear gradient from 0 to 70% acetonitrile, 0.1% TFA (1 ml/min, 10–100 min). Activity was detected in two fractions, F72 and F84 (A). Both fractions were purified to homogeneity after two more reverse phase HPLC runs on a C4 column, final purification was achieved with a step wise gradient (0–10 min/14–21% acetonitrile, 0.1% TFA; 10–55 min/21–53% acetonitrile, 0.1% TFA; 55–60 min/53–70% acetonitrile, 0.1% TFA). With this gradient, the activity of F72 elutes with 30% acetonitrile (B) and of F84 with 37.5% acetonitrile (C). Activities of respective fractions are indicated by a bar. (D) Purified peptides were analyzed with mass spectrometry and relative masses of 2009.08 Da for F72 and 2386.2 Da for F84 were detected.

Table 1
Alignment of the isolated brain peptides with native and modified MCH

	1																		19
F72:					M	L	R	X	M	L	G	R	V	Y	R	P	X	X	Q	X
F84:	D	F	D	M	L	R	X	M	L	G	R	V	Y	R	P	X	X	X	X	
MCH (hum/rat):	D	F	D	M	L	R	C	M	L	G	R	V	Y	R	P	C	W	Q	V	
MCH (^{13}F, ^{19}Y):	D	F	D	M	L	R	C	M	L	G	R	V	**F**	R	P	C	W	Q	**Y**	

the peptide of fraction 84 of NH_2-Asp-Phe-Asp-Met-Leu-Arg-Xaa-Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-Xaa-Xaa-Xaa-Xaa. Position 4 of the peptide of fraction 72 and 7 of the peptide of fraction 84 could not be determined, suggestive for a Cys residue at the respective positions. As indicated in Table 1, the peptide of fraction 84 is reminiscent to the 19 amino acid peptide MCH, whereas that of fraction 72 is apparently a truncated version lacking the three N-terminal amino acid residues. Both peptides show a similar ligand activity when tested in the frog oocyte expression system, suggesting that the N-terminal Asp-Phe-Asp of MCH is not essential for the GIRK-mediated inward potassium current. Whether or not the shorter MCH version is of biological relevance or just a degradation product obtained during purification remains to be seen.

To verify the purification results, the synthetic peptides MCH and a modified MCH peptide (^{13}Phe, ^{19}Tyr) were analyzed in the frog expression system. Dose-response curves revealed an EC_{50} value of 2.3 nM for the native MCH peptide and of 5 nM for the modified MCH peptide, respectively (Fig. 3), strongly suggesting that the orphan SCL-1 is the MCH receptor.

The activation of GIRK currents in the *Xenopus* oocyte system is suggestive for a G_i-coupled signal transduction pathway for the MCH receptor. When the MCH receptor was expressed in oocytes, alone high nM concentrations of MCH were also able to activate Ca^{2+}-induced chloride currents (not shown), suggestive for activation of the phospholipase C pathway. Thus, the MCH receptor may couple to G_i or G_q protein.

The cyclic peptide MCH was first isolated from salmon pituitary glands on the basis of its telling paling property of teleost melanophore. More recently, MCH became of particular interest due to its proposed role in the regulation of food intake behavior and associated pathologies such as obesity [6–9]. The MCH receptor described here will certainly help to unravel the precise function of MCH as neurotransmitter in various brain circuits.

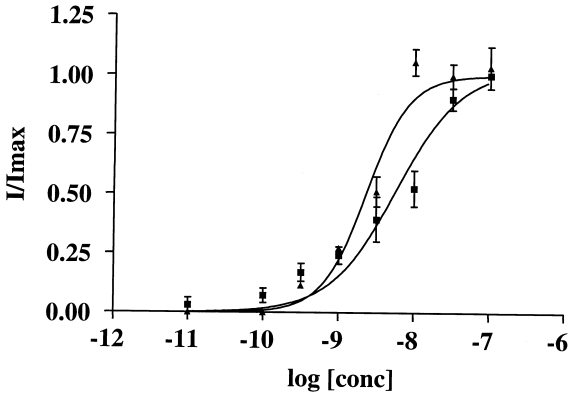

Fig. 3. Dose-response curves for the native mammalian MCH and the modified MCH. Agonist-activated GIRK currents were measured as described for Fig. 1, peak currents were normalized against maximum currents obtained for each oocyte. Four oocytes were independently measured for each agonist. The dose-response data were then subjected to non-linear regression analysis using the GraphPad Prism software (GraphPad, San Diego, CA, USA). EC_{50} values were 2.3 nM for native MCH (triangles) and 5 nM for the modified MCH (squares), respectively.

During submission of this manuscript, the identification of MCH as cognate ligand for the SLC-1 receptor was published independently by two other groups both using transfected mammalian cells [10,11].

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