A new peptide, 5-HT-moduline, isolated and purified from mammalian brain specifically interacts with 5-HT$_{1B/1D}$ receptors

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Abstract

5-HT-Moduline (Leu-Ser-Ala-Leu) is a new endogenous peptide purified from rat brain which interacts specifically with 5-HT$_{1B/1D}$ receptors. The binding interaction of 5-HT-Moduline with 5-HT$_{1B/1D}$ receptors appeared to be a non-competitive process, since the B$_{max}$ value of $[^{125}$I]cyanopindolol binding on rat brain cortical membranes was decreased without modification of the K$_D$. This interaction was conserved on NIH 3T3 cells expressing the 5-HT$_{1A}$ receptor (IC$_{50}$=10$^{-11}$M) suggesting that the binding site for 5-HT-Moduline is localized on the 5-HT$_{1B}$ receptor protein. The observed interaction may lead to functional alterations of 5-HT$_{1B/1D}$ receptors known to play an important role in regulating the release of 5-HT from serotonergic nerve terminals (autoreceptors) as well as the release of other neurotransmitters (heteroreceptors).

Keywords: Serotonin (5-HT); 5-HT$_{1A}$ receptor; Peptide; 5-HT$_{1B/1D}$ receptor; 5-HT release; 5-HT moduline

1. Introduction

The neuronal release of 5-HT is regulated by various mechanisms including the inhibitory effect exerted by 5-HT$_{1B/1D}$ Receptor activation. The latter receptors have been cloned in rat (5-HT$_{1B}$) [1–3] and in human (5-HT$_{1D}$) [4–7] and previous pharmacological experiments had shown their existence in these species [8–11]. Their functional role was determined as their ability to inhibit the release of neurotransmitters either that of 5-HT itself (autoreceptors) or that of non-serotonergic transmitters (heteroreceptors) [12–16]. Therefore, the serotonergic activity is, at least partly, controlled via 5-HT$_{1B/1D}$ receptors. Functional consequences are related to this mechanism i.e. transgenic mice lacking 5-HT$_{1B}$ receptors exhibit an increased aggressivity [17]. The present work describes a peptide which was isolated and purified from rat brain and shown to interact specifically with 5-HT$_{1B/1D}$ receptors.

2. Materials and methods

2.1. Brain Tissue

Brain tissue (usually from 60 rats) were lyophilized, homogenized using an Ultraturrax apparatus (Ikka Werk) in 10 V (W/V) of H$_2$O containing 2 mM EDTA, 5 IU/l aprotinin and 0.1 mM PMSF (phenylmethylsulfonylfluoride) and centrifugated (17,500 g/40 min/+ 4°C). The resulting supernatant was then submitted to acidic (1 M acetic acid) and organic (75% acetone) extractions. After evaporation, the dried extract was resuspended in H$_2$O and ultracentrifugated (120 000 g/60 min/+ 25°C). The supernatant was lyophilized and store at -70°C until use.

2.2. 5-HT-moduline isolation procedure

2.2.1. Localization of biological activity

At the completion of each following chromatographic step, an aliquot of each collected fraction (1/100$^p$) was tested for its ability to modify the binding of $[^{3}$H]5-HT to the 5-HT$_{1B/1D}$ binding sites. Rat brain cortical membranes were incubated in a 50 mM Tris-HCl buffer pH 7.4, containing 0.1% ascorbic acid, 0.1% BSA, 4 mM CaCl$_2$, 1 μM Pargyline, 5 nM $[^{3}$H]5-HT, 0.1 μM 8-OH-DPAT (2-(N,N-di[2,3-(n)-propylamino]-7-hydroxy)-1,2,3,4-tetrahydro napththalene), in the presence or the absence of the various fractions separated by chromatographic procedure. Incubation was carried out for 15 min at 37°C. Non specific binding was determined in the presence of 10 μM of 5-HT. Active fractions were pooled and lyophilized before the next step of their purification.
2.2.2. Isolation procedure

The crude extract of rat brains were dissolved in 5 ml of 50 mM ammonium acetate buffer pH 5, loaded onto a TSK HW 40 S column (700 x 26 mm) equilibrated in the same buffer and eluted at a flow rate of 2 ml/min. UV absorption was measured at 280 nm and 60 fractions of 5 min each were collected.

Fractions 12–24 (F1 fraction) of the TSK column were pooled, lyophilized and resuspended in 5 ml of 50 mM ammonium acetate buffer pH 5. Aliquots (1 ml) were injected into a C18 Ultrabase reverse phase column (250 x 10 mm), equilibrated in the same buffer. The elution was performed at a flow rate of 4 ml/min with a linear gradient of acetonitrile (0–12%) followed by a 5-min step gradient (50% acetonitrile). The detection wavelength was 240 nm. Thirty fractions of 1 min were collected and the active fractions pooled and lyophilized.

The active sample obtained from C18 Ultrabase chromatography was dissolved in 2 ml of a 10 mM ammonium acetate buffer pH 5 and loaded onto a Sephadex G25 column. Elution was performed at a flow rate of 0.3 ml/min with the same buffer. 50 fractions of 20 min were followed by UV absorption at 230 nm and collected. A reverse phase HPLC column (C18 Ultrabase, 250 x 10 mm) was then used. The eluting buffer consisted in a mixture of ammonium acetate 50 mM pH 5 and acetonitrile (85:15). The elution was performed with a 15-min linear gradient of acetonitrile (15–25%) followed by a 5-min step gradient at 50% acetonitrile at a flow rate of 4 ml/min. UV absorption was determined at 230 nm and 20 fractions of 1 min were collected. After lyophilization, the active fraction was injected into a carbon column (Hypercarb, 100 x 3 mm) equilibrated with a 50 mM ammonium
acetate buffer pH 5. A 30-min linear gradient of acetonitrile (0–30%) was used. The flow rate was 1 ml/min and 40 fractions of 1 min were collected. UV absorption was measured at 240 nm. After lyophilization, the active fraction was reinjected into the same column equilibrated with the same buffer. Elution was performed with a 30-min linear gradient of acetonitrile (0–20%). The flow rate was 1 ml/min and 30 fractions of 1 min were collected. UV absorption was measured at 240 nm.

Final purification consisted of reverse phase chromatography using a C18 Ultrabase column (150 × 4 mm) under isocratic elution condition. This column was equilibrated and eluted in a mixture of 0.5% trifluoroacetic acid pH 2.5 and acetonitrile (83:17) at 1 ml/min. The active fraction was collected under UV absorption monitoring at 215 nm. The active fraction was then analyzed either by aminoacid analysis (Beckman 6300 analyser) or NMR (Varian 500 MHz) and sequenced on an Applied Biosystem 473 apparatus.

2.3. Pharmacological studies

Rat brain cortical membranes (200 µg) were incubated 30 min at 25°C with various radiolabelled specific ligands in the presence or absence of 1 nM of the peptide.

The different binding conditions used for serotonergic receptors were those previously described [18]. For other bindings, the incubation medium consisted of 50 mM Tris-HCl pH 7.4 containing 120 mM NaCl and 50 mM KCl (D2 dopaminergic, muscarinic: 2 nM [3H]spiroperidol, muscarinic: 3 nM [3H]quinuclidinylbenzylate, opiates: 2 nM [3H]naloxone) or 4 mM CaCl2 and 4 mM MgCl2 (benzodiazepine: 3 nM [3H]flunitrazepam) or 120 mM NaCl, 5 mM KCl, 2.5 mM CaCl2 and 1 mM MgSO4 (z-adrenergic: 2 nM [3H]prazosin) or 90 mM NaCl (β-adrenergic: 3 nM [3H]dihydroalprenolol).

3. Results and discussion

The isolation and purification procedure was essentially carried out using high performance liquid chromatography. The criterium of separation was based on the ability of one purified fraction to inhibit the binding of [3H]5-HT to 5-HT1 receptors. The various steps of purification led to the purification to homogeneity of a compound characterized by that binding interaction (Fig. 1). Further characterization using NMR analysis, aminoacid analysis and protein sequencing allowed to determine the peptidic nature of this compound and to show it was a tetrapeptide (Leu-Ser-Ala-Leu).

The peptide inhibited the binding of [125I]cyanopindolol to 5-HT1 receptors. Fig. 2 illustrates saturation curves of [125I]cyanopindolol on 5-HT1 receptors in the presence or absence of 5-HT moduline. Rat brain cortical membranes (25 µg of protein) were incubated in 200 µl of a Tris-HCl buffer (10 mM Tris, 155 mM NaCl), pH 7.4, for 60 min at 37°C in the presence of 30 µM isoproterenol and increasing concentrations of [125I]cyanopindolol (0.06–0.4 nM) without (●) or with 5-HT moduline at 0.1 (□) or 1 nM (○), in a 96-well filtration plate (MultiScreen Filtration System, 0.65 µm Hydrophilic membrane, Millipore). Non-specific binding was determined in the presence of 10 µM 5-HT. At the end of the incubation period, free and bound radioactivities were separated by filtration under vacuum. Each well was washed twice with 320 µl of ice-cold incubation buffer. The amount of radioactivity was measured in a gamma counter (Packard, Crystal multi detector RIA system). Specific 5-HT1 binding was 60–70% of the total binding. Each point corresponded to the mean ± S.E.M. of three independent determinations. B: scatchard plots of the curves shown in A.
Fig. 3. Dose response curves of 5-HT-Moduline on 5-HT<sub>1B</sub> receptors. Rat brain cortical or NIH 3T3 cell membranes (25 μg of prot.) were incubated with 0.3 nM of [³²P] cyanopindolol and increasing concentrations of 5-HT-Moduline (10⁻¹³M to 10⁻⁷ M) as described in Fig. 2. Each point is the mean ± SEM of four independent determinations.

Fig. 4. Pharmacological properties of 5-HT-Moduline. Rat brain cortical membranes were incubated 30 min at 25°C with various radiolabelled specific ligands in the presence or absence of 1 nM of the peptide as described in material and methods. Each bar corresponds to the mean ± S.E.M. of five independent determinations. This experiment was repeated twice.

somal membranes prepared from rat striatum or rat hippocampus (not shown).

The fact that Leu-Ser-Ala-Leu actually interacts with 5-HT<sub>1B</sub> receptors was further supported by the results obtained using cells transfected with the 5-HT<sub>1B</sub> receptor gene and expressing specifically this receptor. Indeed, displacement curves of the radioligand also supported the existence of a non-competitive interaction (not shown), furthermore the corresponding dose-effect curve showed an IC<sub>50</sub> value (3·10⁻¹¹ M) very similar to that observed using rat brain synaptosomal membranes (Fig. 3).

To study the pharmacological properties of Leu-Ser-Ala-Leu, its potential interactions were studied on other receptors. The binding of specific radioligands to dopamine ([³H]spiroperidol), α-adrenergic ([³H]prazosin), β-adrenergic ([³H]dihydroalprenolol), muscarinic ([³H]quinuclidylenzylate), benzodiazepine ([³H]flunitrazepam) and opiate receptors ([³H]naloxone) were not significantly affected by concentrations of the peptide which had a maximal inhibiting effect on 5-HT<sub>1B</sub> receptors. Furthermore, other 5-HT receptors specifically radiolabelled as 5-HT<sub>1A</sub> ([³H]8-OH-DPAT), 5-HT<sub>2</sub> ([³H]ketanserin and [³H]DOB) and 5-HT<sub>3</sub> receptors ([³H]BRL 43694) were not affected by the peptide (Fig. 4). These results strongly suggest that Leu-Ser-Ala-Leu specifically interacts with 5-HT<sub>1B</sub> receptors.

In conclusion, the herein presented results demonstrate the existence of a peptide Leu-Ser-Ala-Leu isolated and purified from rat brain tissue. This peptide was shown to non-competitively inhibit the binding of [³²P]cyanopindolol to 5-HT<sub>1B</sub> receptors with an IC<sub>50</sub> expressing a very high affinity for the site recognizing the peptide. The observed interaction may lead to functional alterations of these receptors known to play an important role in regulating the 5-HT release from serotonergic nerve terminals as autoreceptors as well as regulating other neurotransmissions as heteroreceptors. Thus, the potential effect of Leu-Ser-Ala-Leu as a modulator of the serotoninergic activity via its interaction with 5-HT<sub>1B</sub> receptors suggested its provisional name as 5-HT-moduline. Experiments are currently in progress to further study the functional consequences of the effects of this peptide.

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References


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