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ORIGINAL PAPER

The Effects of Endomorphins on Striatal [^{3}H]Gaba Release Induced by Electrical Stimulation: An In vitro Superfusion Study in Rats

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Abstract The endomorphins (EM1 and EM2) are selective endogenous ligands for mu-opioid receptors (MOR1 and MOR2) with neurotransmitter and neuromodulator roles in mammals. In the present study we investigated the potential actions of EMs on striatal GABA release and the implication of different MORs in these processes. Rat striatal slices were preincubated with tritium-labelled GABA ([^{3}H]GABA), pretreated with selective MOR1 and MOR2 antagonist beta-funaltrexamine and selective MOR1 antagonist naloxonazine and then superfused with the selective MOR agonists, EM1 and EM2. EM1 significantly decreased the striatal [^{3}H]GABA release induced by electrical stimulation. Beta-funaltrexamine antagonized the inhibitory action of EM1, but naloxonazine did not affect it considerably. EM2 was ineffective, even in case of specific enzyme inhibitor diprotin A pretreatment. The results demonstrate that EM1 decreases GABA release in the basal ganglia through MOR2, while EM2 does not influence it.

Keywords Endomorphin · Mu-opioid receptor · Striatal GABA · Superfusion

Introduction

The endomorphins (EM1 and EM2) are selective endogenous ligands for mu-opioid receptors (MOR1 and MOR2) with neurotransmitter and neuromodulator roles in mammals [1, 2]. However, their neurophysiological action may be inhibited by rapid enzymatic degradation of the dipeptidyl-peptidase IV (DPPIV) found in the brain synaptic membranes [3, 4].

A recent in vitro superfusion study has demonstrated that EM1 and EM2 stimulate dopamine (DA) release from the rat striatum through MOR2 and MOR1, respectively [5], expressed abundantly in this brain region [6]. In the present study the same superfusion system was utilized to investigate the actions of EM1 and EM2 on the striatal release of tritium-labelled GABA ([^{3}H]GABA) induced by electrical stimulation in rats. The involvement of different receptors in this process was studied by pretreatment with the selective MOR antagonists beta-funaltrexamine (MOR1 and MOR2 antagonist) and naloxonazine (MOR1 antagonist). To potentiate the action of EM2, considered to be more sensitive to enzymatic degradation than EM1, specific DPPIV inhibitor diprotin A (Ile-Pro-Ile) was also added. Diprotin A was used previously in another in vitro superfusion study to demonstrate the inhibitory effect of the EMs on tritium-labelled norepinephrine ([^{3}H]NE) release from the rat nucleus tractus solitarii dorsal motor vagal nucleus complex [7].

Experimental Procedure

Male Wistar rats weighing 180–260 g were decapitated, the brains were rapidly removed and the striata were dissected in a Petri dish filled with ice-cold Krebs solution. The dissected tissue was cut with a McIlwain tissue chopper and slices of 300 μm were produced. The weight of each of the slices was between 15 and 20 mg (wet weight) and was measured by an analytical scale, after the experiments, since the delay caused by the measuring process decreases the viablitity of the slices.

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The slices were preincubated for 30 min in 8 ml of Krebs solution (113 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.5 mM glucose, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, pH = 7.4), submerged in a water bath at 37°C and gassed through a single-use needle (30 G; 0.3 × 13) with carbogen (a mixture of 5% CO_2 and 95% O_2); the pH was maintained at 7.4 and the pretreated with 0.15 mM [^{3}H]GABA (specific activity: 14 Ci/mmol, Amersham Ltd.), during preincubation.

The superfusion system consisted of four cylindrical perspex chambers (Experimetria Ltd., Budapest, Hungary), each formed of two halves which enclosed a cylindrical compartment of about 100 µl (5-mm long and 5-mm in diameter). Gold electrodes were attached to both the upper and the lower halves of the chambers and the electrodes were connected to an ST-02 electric stimulator (Experimetria Ltd.).

After preincubation, the labelled slices were transferred to the superfusion chambers and washed for 30 min, using a multichannel peristaltic pump (Gilson Minipuls 2), to allow tissue equilibrium and to remove the excess radioactivity from the labelled samples. The striatal slices were superfused with Krebs buffer at a rate of 200 µl/min from a reservoir kept at 37°C, and gassed with carbogen.

After 30 min, the superfusates were collected in Eppendorf tubes by means of a multichannel fraction collector (Gilson FC 203B). Two minutes after starting the fraction collector, one electrical stimulation was delivered to each of the four chambers. The stimuli consisted of square-wave impulses (duration, 2 min; voltage, 100 V; pulse length, 5 ms; frequency, 10 Hz). Equimolar doses (10 µM) of EM1 or EM2 (Bachem Ltd.) were added 12 min before the electrical stimulation. When DPPIV inhibition was intended, the slices were pretreated with 0.1 mM diprotin A (Bachem Ltd.) 15 min before the administration of EM2. Equimolar doses (10 µM) of naloxonazine or beta-funaltrexamine were administered 22 min before the electrical stimulation. The concentrations used in the present experiments proved to be the most effective in our previous superfusion studies [5, 8, 9]. In the end, the remaining slices were solubilized in 200 µl of Krebs solution, using an ultrasonic homogenizer (Branson, Sonifier 250).

The radioactivity in the fractions and the homogenized tissue samples was measured with a liquid scintillation spectrometer (Tri-carb 2100TR, Packard) and expressed in CPM (count per minute per scintillation vial) after the addition of 3 ml of appropriate scintillation fluid (Ultima Gold, Packard). The fractional release was calculated as a percentage of the radioactivity present in the slices at the sample collection time, i.e. the radioactivity of a given sample divided by the sum of the radioactivities of the remaining samples and the residual activity of the tissue. This value stands for the overall radioactivity of released GABA and GABA metabolites in the samples.

Statistical analysis of the results was performed by analysis of variance ANOVA (Statistica Software, StatSoft Inc.). Two-way ANOVA with repeated measures was applied and a probability level of 0.05 was accepted as indicating a statistically significant difference.

Results

EM1 inhibited significantly the striatal [^{3}H]GABA release induced by electrical stimulation [$F_{14\ \text{min}}(1,12) = 16.90$; $P < 0.005$]; the inhibitory effect of EM1 was antagonized by beta-funaltrexamine [$F_{14\ \text{min}}(1,12) = 88,75$; $P < 0.001$], but not by naloxonazine (Fig. 1). EM2 did not influence considerably the striatal [^{3}H]GABA release, not even in the case of diprotin A pretreatment, compared to EM1 (Fig. 2). Diprotin A, beta-funaltrexamine and naloxonazine alone did not affect the striatal [^{3}H]GABA release following electrical stimulation. Basal [^{3}H]GABA release was not changed by any of the substances or concentrations tested in previous studies.

Discussion

The results demonstrate that EM1 decreases GABA release in the striatum while EM2 does not influence it. They also suggest that the action of EM1 is mediated by MOR2 and

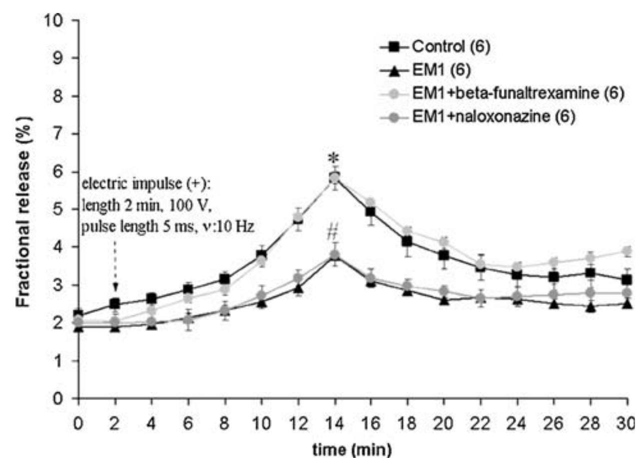

Fig. 1 The actions of endomorphin-1 (EM1) and endomorphin-1 plus naloxonazine or beta-funaltrexamine (EM1 + naloxonazine/ EM1 + beta-funaltrexamine) on the striatal [^{3}H]GABA release induced by electrical stimulation. Data are expressed as mean ± - SEM. Equimolar doses (10 µM) of mu-opioid receptor (MOR) agonist and antagonists were used. The numbers in brackets represent the number of samples. $^{\#}P < 0.05$ EM1 vs. control; $^{*}P < 0.05$ EM1 + beta-funaltrexamine versus EM1

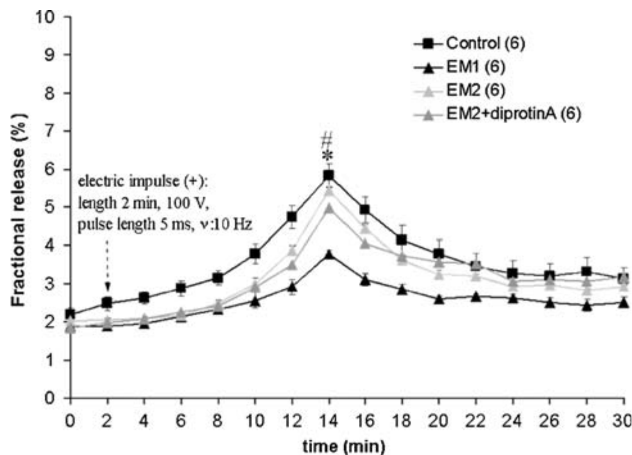

Fig. 2 The actions of endomorphin-2 (EM2) and endomorphin 2 plus diprotin A (EM2 + diprotinA) on the striatal [^{3}H]GABA release induced by electrical stimulation compared to the action of endomorphin-1 (EM1). Data are expressed as mean ± SEM. Equimolar doses (10 µM) of mu-opioid receptor (MOR) agonists were used. The numbers in brackets represent the number of samples. $^{\#}P < 0.05$ EM1 versus EM2, $^{*}P < 0.05$ EM1 versus EM2 + diprotin

that enzymatic degradation of EM2 is not implicated in its inactivity.

The catabolism of EM1 and EM2 was investigated in detail earlier in numerous in vitro and in vivo studies. Some studies suggested that the proteolysis of EM1 involves the participation of carboxypeptidase, not aminopeptidase activity [10, 11]. Other studies have revealed that diprotin A inhibits the enzymatic degradation of EM2 produced by DPPIV at the Pro[2]-Phe[3] cleavage site [12, 13]. However a similar superfusion study concluded that the enzymatic degradation of the EMs might not be a rapid and highly effective process in brain slices [7].

Several neuroendocrine studies indicated that both EM1 and EM2 induce locomotor hyperactivity and stereotype behaviour through the mediation of corticotropin-releasing factor (CRF) and secretion of dopamine (DA) in the nigrostriatal and mesolimbic DA-ergic system [5, 8, 9, 14]. The activation of the mesolimbical DA-ergic system by the EMs is realized by the disinhibition of the GABAergic neurons, through the mediation of MORs highly expressed at this place [4]. We propose that a similar mechanism is involved in the activation of nigrostriatal pathway, concerning EM1, but not EM2. Recent in vivo microdialysis studies have sustained the suspicion that EM1, in contrast with EM2, stimulates DA release by GABA-dependent mechanism at least in the nucleus accumbens, and discussed the different implication of GABA A and GABA B receptors in this phenomenon [15, 16].

Taking all these observations into consideration, we suppose that EM1 may inhibit GABA release within the striatum acting on recurrent axon collaterals of GABAergic projection neurons that synapse on nigrostriatal dopaminergic axon terminals. Direct or indirect or both GABAergic projections neurons could be involved in this process. EM2 influences striatal GABAergic-dopaminergic interaction by an alternative mode: it may act on opioid receptors located directly on dopaminergic axon terminals. Enzymatic degradation of EM2 is not seriously implicated in this process.

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