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The effects of endomorphins and diprotin A on striatal dopamine release induced by electrical stimulation—An in vitro superfusion study in rats

Zsolt Bagosi [a], Miklós Jászberényi [a], Erika Bujdosó [b], Gyula Szabó [a], Gyula Telegdy [b,*]

[a] *Department of Pathophysiology, University of Szeged, Hungary*
[b] *Neurohumoral Research Group, Hungarian Academy of Sciences, Szeged, Hungary*

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Abstract

The endomorphins (EM1: Tyr-Pro-Trp-Phe-NH_2, and EM2: Tyr-Pro-Phe-Phe-NH_2) are recently discovered endogenous ligands for mu-opioid receptors (MORs) with role of neurotransmitters or neuromodulators in mammals. Cessation of their physiological action may be effected through rapid enzymatic degradation by the dipeptidyl-peptidase IV (DPPIV) found in the brain synaptic membranes.

An in vitro superfusion system was utilized to investigate the actions of EM1, EM2 and specific DPPIV inhibitor diprotin A on the striatal release of dopamine (DA) induced by electrical stimulation in rats. The involvement of the different MORs (MOR1 and MOR2) in this process was studied by pretreatment with MOR antagonists β-funaltrexamine (a MOR1 and MOR2 antagonist) and naloxonazine (a MOR1 antagonist).

EM1 significantly increased the tritium-labelled dopamine DA release induced by electrical stimulation. EM2 was effective only when the slices were pretreated with diprotin A. β-Funaltrexamine antagonized the stimulatory effects of both EM1 and EM2. The administration of naloxonazine did not appreciably influence the action of EM1, but blocked the action of EM2, at least when the slices were pretreated with diprotin A.

These data suggest that both EM1 and EM2 increase DA release from the striatum and, though diprotin A does not affect the action of EM1, it inhibits the enzymatic degradation of EM2. The DA-stimulating action induced by EM1 seems to be mediated by MOR2, while that evoked by EM2 appears to be transmitted by MOR1.
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Keywords: Endomorphin; Diprotin A; Striatal dopamine; Superfusion

1. Introduction

The endomorphins (EM1: Tyr-Pro-Trp-Phe-NH_2, and EM2: Tyr-Pro-Phe-Phe-NH_2) are recently discovered endogenous ligands for mu-opioid receptors (MORs) with role of neurotransmitters or neuromodulators in mammals (Zadina et al., 1997). Antinociception, the main physiological action of these two neuropeptides, may be prevented through rapid enzymatic degradation in the synapse, as for other neuropeptides (Horvath, 2000). The principal enzyme responsible for this process seems to be dipeptidyl-peptidase IV (DPPIV) found in the brain synaptic membranes (Mentlein, 1999).

The neuroanatomical distribution of the EMs suggests that they may also participate in behavioral and endocrine processes (Martin-Schild et al., 1999). Recent behavioral studies suggest that the EMs induce locomotor hyperactivity through the mediation of corticotropin-releasing factor (CRF) and the secretion of dopamine (DA) either in the nigrostriatal or mesolimbic DA-ergic system (Bujdoso et al., 2001a,b).

Several publications demonstrated that microdialysis (Bednar et al., 2004; Okutsu et al., 2006) and superfusion (Bujdoso et al., 2003) studies are eligible methods for assessing the tritiated DA release from mesolimbic or nigrostriatal DA-ergic structures. The direct action of EM1 on striatal DA release was

* Corresponding author at: University of Szeged, Department of Pathophysiology, P.O. Box 427, Semmelweis u. 1, H-6725 Szeged, Hungary. Tel.: +36 62 545797; fax: +36 62 545710.
E-mail address: telegdy@patph.szote.u-szeged.hu (G. Telegdy).

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earlier investigated in an in vitro superfusion system. Although EM1 did not influence the basal release of tritiated DA, it significantly enhanced the transmitter release evoked by electric impulses with no significant difference between the various concentrations. Pretreatment with the nitric oxide (NO) synthetase inhibitor *N*-nitro-L-arginine antagonized this action, indicating that NO may transmit the DA-stimulating effect of EM1 (Bujdoso et al., 2003).

The superfusion system was also used 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 in the presence of a specific DPPIV inhibitor, diprotin A (Al-Khrasani et al., 2003).

In the present study an in vitro superfusion system was used to investigate the actions of EM1, EM2 and diprotin A on the striatal release of DA induced by electrical stimulation in rats. We used rat striatal slices, sectioning the nigrostriatal DA-ergic pathway through the basal ganglia. The involvement of the different MORs (MOR1 and MOR2) in this process was studied by pretreatment with MOR antagonists β-funaltrexamine (a MOR1 and MOR2 antagonist) and naloxonazine (a MOR1 antagonist).

2. Experimental procedures

Male Wistar rats weighing 180–260 g were decapitated and their brains were rapidly removed. All efforts were made to minimize animal suffering and to reduce the number of animals used. The striata were prepared in a Petri dish filled with ice-cold Krebs solution according to the rat brain atlas by Pellegrino et al. The left and right hemisphere were dissected in the frontal plane at the anterior (approximately 4 mm from the Bregma) and the posterior (approximately 1 mm from the Bregma) borders of the striatum. Then the basal ganglia (caudate nucleus and the putamen) were decapsulated from the surrounding white matter and the dissected tissue was cut with a McIlwain tissue chopper and slices of 300 μm were produced.

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. The slices were labelled with [^{3}H]DA (Amersham Ltd.) during the preincubation; the medium was supplemented with 0.15 mM [^{3}H]DA (specific activity: 14 Ci/mmol).

The superfusion system consisted of four cylindrical perspex chambers (Experimetria Ltd., Budapest, Hungary), each formed of two halves, which enclosed a compartment of about 150 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. To minimalize the dilution of the radioactive compounds we selected the least possible superfusion rate suggested by the literature (Korpi and Oja, 1984). After 30 min, the superfusates were collected in Eppendorf tubes by means of a multichannel fraction collector (Gilson FC 203B) and thus 16× 2 min fraction samples were obtained from each of the four channels. Two minutes after the start of sample collection, one electrical stimulation was delivered to all the four chambers. The stimuli consisted of square-wave impulses (duration, 2 min; voltage, 100 V; pulse length, 5 ms; frequency, 10 Hz). In the end, the remaining slices were solubilized in 200 μl of Krebs solution, using an ultrasonic homogenizer (Branson, Sonifier 250).

Equimolar doses (10 μM) of EM1 (Bachem Ltd.) or EM2 (Bachem Ltd.) were added 12 min before the electrical stimulation. Since earlier publications (Al-Khrasani et al., 2003; Bujdoso et al., 2003) demonstrated the EMs most effective in the concentration of 10 μmol on striatal DA and epinephrine release, we relied on these studies while selecting the minimal effective concentrations of EMs. When DPPIV inhibition was intended, the slices were treated with 0.1 mM diprotin A (Bachem Ltd.) 16 min before the administration of EM1 or EM2. In the antagonist studies equimolar doses (10 μM) of naloxonazine (Sigma–Aldrich Ltd.) or β-funaltrexamine (Sigma–Aldrich Ltd.) were incubated 22 min before the electrical stimulation to allow covalent association with MORs selectively. All the EMs, the MOR antagonists and diprotin A were present in the medium until the end of the experiment.

The radioactivity in the fractions and the homogenized tissue samples was measured with a liquid scintillation spectrometer (Tri-carb 2100TR, Packard) 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.

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.

3. Results

EM1 increased significantly the striatal [^{3}H]DA release induced by electrical stimulation [$F_{14\text{min}}(1, 12) = 76.42$; $p < 0.001$]. The DPPIV inhibitor, diprotin A did not potentiate this effect (Fig. 1). EM2 was effective only when the slices were pretreated with diprotin A [$F_{14\text{min}}(1, 14) = 28.82$; $p < 0.001$] (Fig. 2). The stimulatory effect of EM1 was antagonized by β-funaltrexamine [$F_{14\text{min}}(1, 12) = 16.56$; $p = 0.001$], but not by naloxonazine (Fig. 3). The stimulatory effect of EM2 was antagonized by both MOR antagonists β-funaltrexamine [$F_{14\text{min}}(1, 12) = 24.99$; $p < 0.001$] and naloxonazine [$F_{14\text{min}}(1, 14) = 30.21$; $p < 0.001$], at least when the slices were pretreated with diprotin A (Fig. 4). Further increase of the concentration of EMs (25–50–100 μmol) did not result in considerable increase in electric impulse evoked DA release.

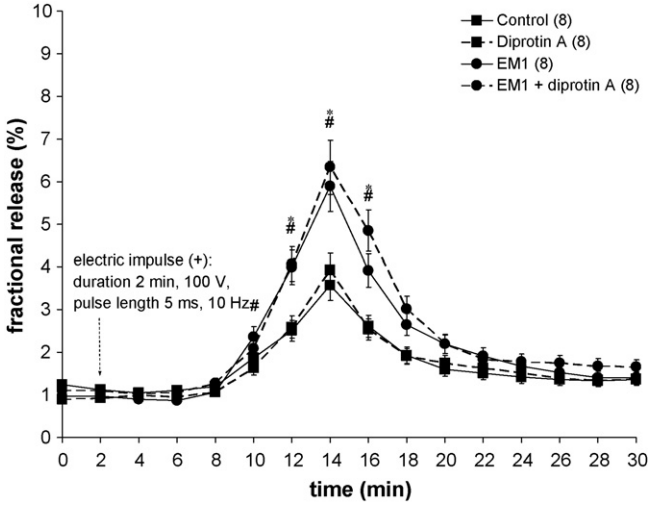

Fig. 1. The effects of endomorphin-1 (EM1) and diprotin A on the striatal dopamine release induced by electrical stimulation. About 10 μM of EM1 and 0.1 mM diprotin A were used. The numbers in brackets represent the number of samples. $^{\#}p < 0.05$ EM1 vs. control; $^{*}p < 0.05$ EM1 + diprotin A vs. control.

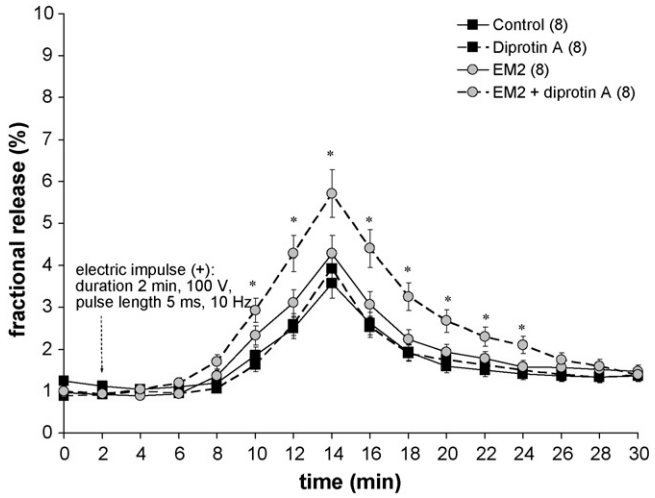

Fig. 2. The effects of endomorphin-2 (EM2) and diprotin A on the striatal dopamine release induced by electrical stimulation. About 10 μM of EM2 and 0.1 mM diprotin A were used. The numbers in brackets represent the number of samples. $^{*}p < 0.05$ EM2 + diprotin A vs. control.

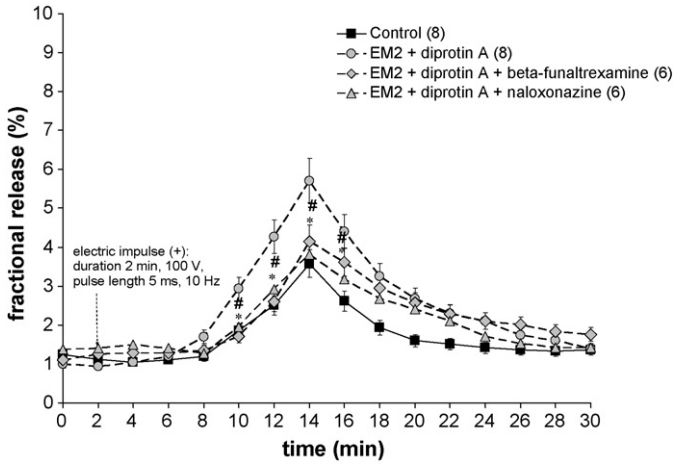

Fig. 4. The effects of the selective mu-opioid receptor (MOR) antagonists on the striatal dopamine release induced by electrical stimulation and enhanced by endomorphin-2 (EM2). Slices were pretreated with 0.1 mM diprotin A. Equimolar doses (10 μM) of EM2, β-funaltrexamine and naloxonazine were used. The numbers in brackets represent the number of samples. $^{\#}p < 0.05$ EM2 + diprotin A + β-funaltrexamine vs. EM2 + diprotin A; $^{*}p < 0.05$ EM2 + diprotin A + naloxonazine vs. EM2 + diprotin A.

Besides, higher concentrations of EMs would not bind selectively to the different subtypes of MORs. Diprotin A, β-funaltrexamine and naloxonazine alone did not affect the striatal [^{3}H]DA release.

4. Discussion

The present experiments clearly demonstrate that both EMs evokes a prominent increase in the striatal DA release. However considerable difference could be detected between the sensitivity of those responses to diprotin-A pretreatment. While EM1 proved to be effective alone, EM2 had a significant impact on DA release only in the presence of the DPPIV inhibitor.

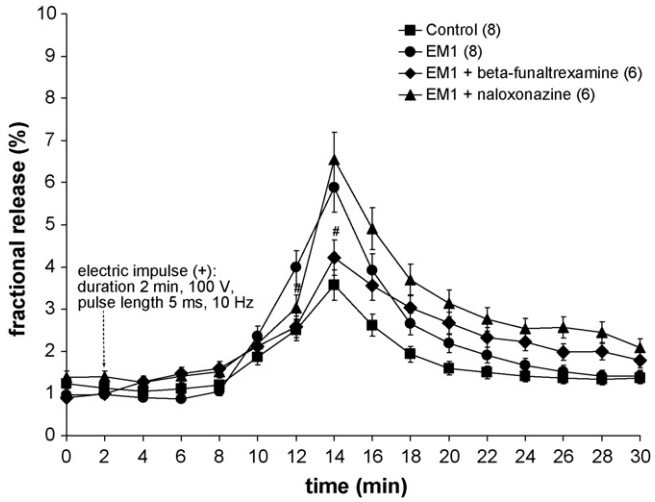

Fig. 3. The effects of the selective mu-opioid receptor (MOR) antagonists on the striatal dopamine release induced by electrical stimulation and enhanced by endomorphin-1 (EM1). Equimolar doses (10 μM) of EM1, β-funaltrexamine and naloxonazine were used. The numbers in brackets represent the number of samples. $^{\#}p < 0.05$ EM1 + β-funaltrexamine vs. EM1.

The catabolism of EM1 and EM2 in a rat brain homogenate was investigated earlier. The half-lives of EMs in crude membrane preparations were found to lie in the range of 15–20 min. Recent publications has revealed that metalloproteases, aminopeptidases (Tomboly et al., 2002) and carboxypeptidases (Wu et al., 2002) might be involved in the degradation of the EMs.

Diprotin A has been demonstrated to cause inhibition of the proteolysis of EM2 and the accumulation of its major metabolites in synaptic membrane preparations from the mouse brain. The diprotin-A evoked potentiation of the antinociceptive action of EM2 can be attributed to the inhibition of the enzymatic degradation by DPPIV at the Pro(2)-Phe(3) cleavage site (Sakurada et al., 2003). On the other hand the enzyme inhibitor neither potentiated nor prolonged the antinociceptive effect of EM1 (Ronai et al., 1999).

Our results unveiled that EM1 acts through the activation of MOR2. Since the specific MOR2 inhibitor did not abolish the action of EM2, while the non-selective MOR antagonist proved to be effective this finding implies that the action of EM2 is mediated by MOR1. These data, concerning receptor specificity, are reinforced by the results of tail flick, tail pressure, formalin tests and hot plate tests (Hao et al., 2000; Tseng et al., 2000; Sakurada et al., 1999, 2000, 2001, 2002) which demonstrated that EM1- and EM2-induced antinociception is mediated by different subtypes of MOR. Nevertheless, in other brain regions non-opioid mechanism has also been demonstrated to mediate the actions of the EMs on DA release (Okutsu et al., 2006).

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