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European Journal of Pharmacology 404 (2000) 137–143

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EP 60761 and EP 50885, two hexarelin analogues, induce penile erection in rats

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Received 14 February 2000; received in revised form 4 July 2000; accepted 7 July 2000

Abstract

The effect of hexarelin and four related peptide analogues, EP 40904, EP 40737, EP 50885 and EP 60761, injected into the paraventricular nucleus of the hypothalamus of male rats in doses between 2 and 2000 ng on spontaneous penile erection was studied. Of these peptides, EP 60761 and EP 50885, but not hexarelin, EP 40904 or EP 40737, increased dose-dependently the number of spontaneous penile erections. EP 60761 was active already at the dose of 20 ng, which induced the sexual response in 70% of the treated rats. The maximal response was induced by 200 ng of the peptide. EP 50885 was less potent than EP 60761, with 1000 ng being the minimal effective dose and 2000 ng as the dose required to induce the maximal response. At the doses used, both peptides also increased slightly the number of spontaneous yawning episodes. EP 60761- and EP 50885-induced penile erection was prevented by the oxytocin receptor antagonist [d(CH_2)$_5$Tyr(Me)^2-Orn^8]vasotocin (0.1–1 μg) given intracerebroventricularly (i.c.v.), but not into the paraventricular nucleus (0.1–1μg), by the competitive nitric oxide (NO) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) given either into the paraventricular nucleus (10–20 μg) or i.c.v. (75–150 μg), by the N-type Ca^{2+} channel blocker ω-conotoxin-GVIA (2–5 ng) or by the opiate morphine (1–10 μg), but not by the dopamine receptor antagonist (*Z*)-4-[3-[2-(trifluoromethyl)-9*H*-thioxanthen-9-ylidene]propyl]-1-piperazine-ethanol (*cis*-flupenthixol) (10 μg) or by the *N*-methyl-D-aspartic acid (NMDA) receptor antagonist (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine ((+)-MK-801) (1 μg), all given into the paraventricular nucleus before either peptide. The present results show that EP 60761 and EP 50885 induced penile erection by increasing central oxytocin transmission, possibly by activating NO synthase in the cell bodies of oxytocinergic neurons located in the paraventricular nucleus that control penile erection.

Keywords: Penile erection; Hexarelin; EP 60761; EP 50885; Oxytocin; Nitric oxide (NO); Paraventricular nucleus; (Rat)

1. Introduction

Several neurotransmitters and neuropeptides are involved at the central level in the control of penile erection. Among these, the best known are adrenocorticotropin, oxytocin, opioid peptides, dopamine, serotonin, excitatory amino acids and nitric oxide (NO). These compounds influence this sexual response by acting in different brain areas, namely the medial preoptic area, the paraventricular nucleus of the hypothalamus, the bed nucleus of the stria terminalis, the amygdala, the hippocampus, the medulla oblongata and the spinal cord (for a review on the central control of penile erection, see Meisel and Sachs, 1994; Argiolas and Melis, 1995; Andersson and Wagner, 1995; Argiolas, 1999). Recent studies suggest that oxytocinergic neurons, originating in the paraventricular nucleus and projecting to extrahypothalamic brain areas (i.e. the hippocampus, the ventral medulla and the spinal cord), are involved in the control of penile erection in in copula and ex copula contexts in the male rat (see Argiolas and Melis, 1995; Melis et al., 1999a,b; Veronneau-Longueville et al., 1999). Accordingly, the activation of these neurons by dopamine, excitatory amino acids and oxytocin itself, or by the presence of an inaccessible receptive female rat facilitates penile erection, while their inhibition by morphine and, possibly, by opioid peptides impairs this sexual

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00481-7

response. The activation and the inhibition of these oxytocinergic neurons, together with the facilitation and the inhibition of penile erection, is apparently mediated by an increased and a decreased production of NO in the paraventricular nucleus, respectively, as determined by in vivo microdialysis (Melis et al., 1996, 1997a,b,c,d, 1998, 1999a,b).

Some of the above neurotransmitters exert an opposite role on sexual function and eating. The most clear examples are those of oxytocin, which induces penile erection and facilitates sexual behaviour (see above) and decreases eating (see Arletti et al., 1989), and of opioid peptides, which impair penile erection and sexual behaviour (see above) and increase eating (see Morley, 1987). As the paraventricular nucleus is also involved in the control of feeding (see Morley, 1987), this raises the possibility that the two behaviours might be controlled in an opposite fashion by some of these neurotransmitters at the paraventricular level. Recently, a new class of peptide molecules that release growth hormone (GH) in experimental animals and humans with a efficacy higher than that of the endogenous GH-releasing hormone (GHRH) has been characterised (see Deghenghi, 1996; Muller et al., 1999 and references therein). These GH-releasing peptides, like GHRH, also increase eating in laboratory animals when injected intracerebroventricularly (i.c.v.) (Locke et al., 1995; Okada et al., 1996; Torsello et al., 1998). The eating effect is not strictly related to the ability of these peptides to release GH (Torsello et al., 1998), since they act on receptors different from those activated by GHRH (Codd et al., 1989; Pong et al., 1986; Howard et al., 1996) and are linked to intracellular messenger pathways different from those utilised by GHRH (Cheng et al., 1989; Akman et al., 1993). Here, we report that EP 60761 and EP 50885, two analogues of the GH-releasing peptide hexarelin (Deghenghi et al., 1994), induce in rats penile erection episodes indistinguishable from those elicited by dopamine receptor agonists, oxytocin or *N*-methyl-D-aspartic acid (NMDA), when injected into the paraventricular nucleus and irrespective of their effect on GH release and eating. A possible mechanism underlying the action of the two peptides on penile erection is also reported.

2. Material and methods

2.1. Animals

Male Sprague–Dawley rats (200–220 g) (Charles River, Como, Italy) were used in all the experiments. The animals were caged in groups of 4–6 at 24°C, humidity 60%, lights on from 0700 to 1900 h with water and standard laboratory food ad libitum. The experiments were performed between 0900 and 1300 h.

2.2. Drugs and peptides

Hexarelin, EP 60761, EP 50885, EP 40904 and EP 40737 (see Table 1 for structures) were synthesised by one of us (RD) by conventional solid-phase synthesis. [d(CH_2)$_5$Tyr(Me)^2-Orn^8]vasotocin and ω-conotoxin-GVIA were purchased from Peninsula Eur. (St. Helen/Merseyside, UK), N^G-nitro-L-arginine methyl ester (L-NAME)) from Sigma (St. Louis, MO, USA), (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate ((+)-MK-801) and (*Z*)-4-[3-[2-(trifluoromethyl)-9*H*-thioxanthen-9-ylidene]propyl]-1-piperazine-ethanol dihydrochloride (*cis*-(*Z*)-flupenthixol-2HCl) from Research Biochemicals (Natick, MA, USA) and morphine–HCl from SALARS (Como, Italy). All the other reagents were of the highest available purity.

2.3. Microinjections into the lateral ventricle (i.c.v.) and into the paraventricular nucleus of the hypothalamus

Stainless-steel guide cannulas (22 gauge, 0.71 mm) aimed unilaterally at the paraventricular nucleus were stereotaxically implanted (David Kopf Instruments, USA) under chloral hydrate anaesthesia 2 days before the experiments (coordinates: 0.2 mm anterior to bregma, 0.4 mm lateral to midline and 2.0 mm ventral to dura) (Pellegrino and Cushman, 1971). Each rat was used only once. The same guide cannula was used for i.c.v. and paraventricular injections. For i.c.v. injections, all substances were injected in a volume of 10 μl of saline in 1 min, via an internal cannula (28 gauge) which extended 1 mm below the tip of the guide cannula and which was connected by polyethylene tubing to a 10-μl Hamilton syringe driven by a micrometric screw. Controls received 10 μl of saline. After injection, the tip of the cannula was left in the injection site for 30 s to allow the spread of the injected solution. For paraventricular injections, all substances were injected in a volume of 0.3 μl of saline, in 2 min through an internal cannula that extended 5.3 mm below the tip of the guide cannula and which was connected by polyethylene tubing to a 10-μl Hamilton syringe driven by a Stoelting microinfusion pump. Controls received 0.3 μl of saline in the paraventricular nucleus. After microinjection,

Table 1
Structure of hexarelin and analogues used in this study

Hexarelin	His-D-Trp(2-Me)-Ala-Trp-D-Phe-LysNH_2
EP 40904	Thr-D-Trp(2-Me)-Ala-Trp-D-Phe-LysNH_2
EP 40737	D-Thr-D-Trp(2-Me)-Ala-Trp-D-Phe-LysNH_2
EP 50885	GAB-D-Trp(2-Me)-D-βNal-Phe-LysNH_2
EP 60761	GAB-D-Trp(2-Me)-D-Trp(2-Me)-D-Trp(2-Me)-LysNH_2

Abbreviations: GAB = γ-aminobutyryl; βNal = β-(2-naphthyl)alanine.

the tip of the cannula was left in the injection site for 30 s to allow the spread of the injected solution.

2.4. Behavioural studies

When dose-curves were made, each peptide or saline alone was microinjected over a 2-min period into the paraventricular nucleus of rats implanted with chronic guide cannulas. Saline, ω-conotoxin, morphine, [d(CH_2)$_5$-Tyr(Me)^2-Orn^8]vasotocin, L-NAME, (+)-MK-801 or *cis*-flupenthixol was injected over a 2-min period into the paraventricular nucleus of rats implanted with chronic guide cannulas, 10 min before saline or one of the peptides. [d(CH_2)$_5$Tyr(Me)^2-Orn^8]vasotocin or L-NAME was injected i.c.v. over a 1-min period, 10 min before saline or one of the peptides. Shortly after treatment, the animals were placed individually into Plexiglas cages (30 × 30 × 30 cm) and observed for 60 min, during which penile erection episodes were counted by an observer who was not aware of the treatments to eliminate subjective evaluation.

2.5. Histology

At the end of the experiments, the animals were killed by decapitation, and the brains were immediately removed and stored in 2% aqueous formaldehyde for 10–12 days. To localise the injection site, 50-μm transverse brain sections were prepared by means of a freezing microtome, stained with Neutral Red and inspected on a phase-contrast microscope. The injection site was localised by following the internal cannula tract through a series of brain sections. Only data from those animals found to have the internal cannula tip positioned correctly i.c.v. or in the paraventricular nucleus were included for the statistical evaluation of the results.

2.6. Statistics

Statistical evaluation of the results was performed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test for the comparison of differences among multiple groups. Student's *t*-test was used to compare differences between two groups. A $P < 0.025$ was considered significant.

3. Results

3.1. Effect of hexarelin peptide analogues injected into the paraventricular nucleus on spontaneous penile erection

Of the five peptides tested, hexarelin, EP 60761, EP 50885, EP 40904 and EP 40737, when injected into the paraventricular nucleus in doses between 2 and 2000 ng, only EP 60761 and EP 50885 were found capable of increasing dose-dependently the number of spontaneous penile erection episodes in male rats. The minimal effective dose of EP 60761 was 20 ng, which induced the response in more than 70% of the treated animals. The maximal effect was found with 200 ng, a dose that was active in more than 90% of the rats. EP 50885 was also active, but only at doses higher than 500 ng, and a dose of 2000 ng was required to induce the response in more than 90% of the animals (Fig. 1). After an active dose of EP 60761 or EP 50885, the response began 5–8 min later and lasted for 50–60 min. Each episode of penile erection lasted 0.5–2 min and was associated with or followed by genital grooming. At the above doses, EP 60761 and EP 50885 also increased the number of spontaneous yawning episodes, although this effect was not so marked as that on penile erection and, in this instance, clear dose dependence was not found (Fig. 1).

3.2. Effect of [d(CH_2)$_5$Tyr(Me)^2-Orn^8]vasotocin and L-NAME on EP 60761- and EP 50885-induced penile erection

[d(CH_2)$_5$Tyr(Me)^2-Orn^8]-vasotocin, an oxytocin receptor antagonist, (0.1 and 1 μg) or L-NAME (75 and 150 μg), an inhibitor of NO synthase, injected i.c.v. 10 min before EP 60761 (200 ng) or EP50885 (2 μg) into the paraventricular nucleus reduced dose-dependently the in-

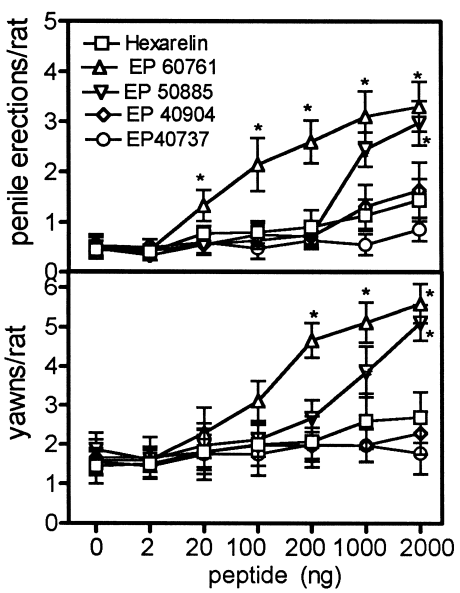

Fig. 1. Effect of hexarelin peptide analogues injected into the paraventricular nucleus on spontaneous penile erection and yawning episodes: dose–response curves. The peptides were dissolved in saline and injected into the PVN in a volume of 0.3 μl. After treatment, the rats were put individually into Plexiglas cages and observed for 60 min, to count penile erection and yawning episodes. Values are means ± S.E.M. for eight rats per group. $^*P < 0.01$ with respect to saline-treated rats (peptide = 0; one-way ANOVA, followed by Duncan's multiple range test).

crease in the number of penile erection episodes induced by the two peptides. A similar effect was observed when L-NAME (10 and 20 μg) was injected into the paraventricular nucleus 10 min before EP 60761 or EP50885. In contrast, the oxytocin receptor antagonist at the same doses given i.c.v. was ineffective when injected into the paraventricular nucleus 10 min before EP 60761 or EP50885 (Fig. 2).

3.3. Effect of ω-conotoxin-GVIA, morphine, (+)-MK-801 or cis-flupenthixol on EP 60761- and EP 50885-induced penile erection

The N-type Ca^{2+} channel blocker ω-conotoxin-GVIA (2 and 5 ng) or morphine (1, 5 and 10 μg) injected into the paraventricular nucleus 10 min before EP 60761 (200 ng) or EP 50885 (2 μg) into the paraventricular nucleus reduced the increase in penile erection induced by the two peptides in a dose-dependent manner (Fig. 3). In contrast, the NMDA excitatory amino acid receptor antagonist (+)-MK-801 (1 μg) and the dopamine receptor antagonist *cis*-flupenthixol (10 μg) injected into the paraventricular nucleus were ineffective (Fig. 3).

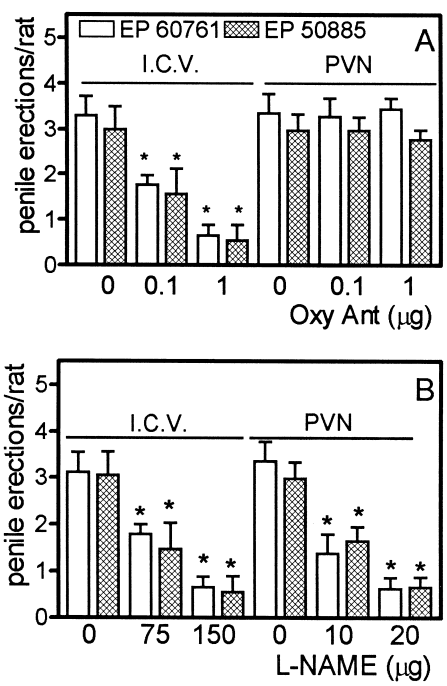

Fig. 2. Effect of [d(CH$_2$)$_5$Tyr(Me)^2-Orn^8]vasotocin (A) and L-NAME (B) on EP 60761- and EP 50885-induced penile erection. [d(CH$_2$)$_5$Tyr(Me)^2-Orn^8]vasotocin or L-NAME was injected i.c.v. in 10 μl of saline or into the paraventricular nucleus in 0.3 μl of saline 10 min before EP 60761 (200 ng; empty bars) or before EP 50885 (2000 ng; crosshatched bars). After treatment, the rats were put individually into Plexiglas cages and observed for 60 min, to count penile erection episodes. Values are means ± S.E.M. for eight rats per group. $^*P < 0.01$ with respect to the corresponding group treated with EP 60761 or EP 50885 alone ([d(CH$_2$)$_5$Tyr(Me)^2-Orn^8]vasotocin or L-NAME = 0; one-way ANOVA, followed by Duncan's multiple range test).

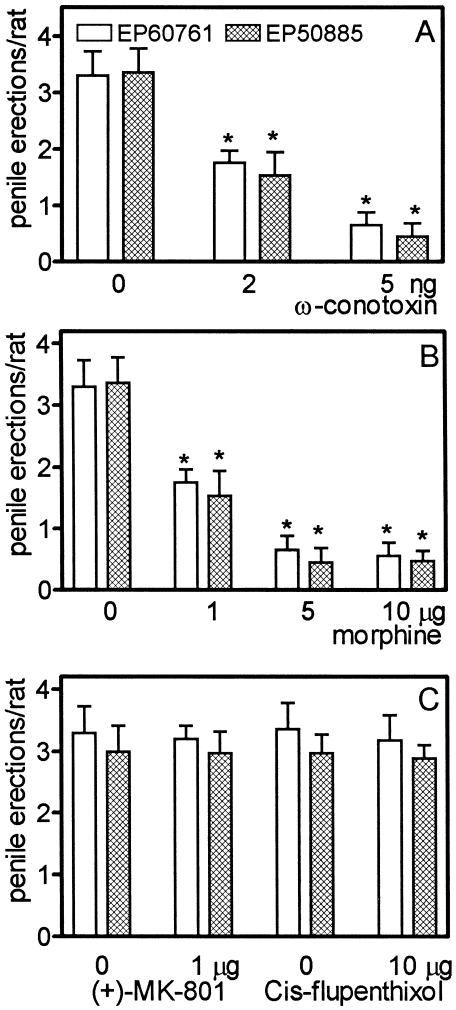

Fig. 3. Effect of ω-conotoxin-GVIA (A), morphine (B), (+)-MK-801 and *cis*-flupenthixol (C) on EP 60761- and EP 50885-induced penile erection. ω-conotoxin-GVIA (2 and 5 ng), morphine (1, 5 and 10 μg), (+)-MK-801 (1μg) or *cis*-flupenthixol (10 μg) was injected into the paraventricular nucleus in a volume of 0.3 μl 10 min before EP 60761 (200 ng; empty bars) or EP 50885 (2000 ng; crosshatched bars). After treatment, the rats were put individually into Plexiglas cages and observed for 60 min, to count penile erection episodes. Values are means ± S.E.M. for eight rats per group. $^*P < 0.01$ with respect to the corresponding group treated with EP 60761 or EP 50885 alone (ω-conotoxin, morphine, (+)-MK-801 or *cis*-flupenthixol = 0; one-way ANOVA, followed by Duncan's multiple range test).

4. Discussion

The present results show that the hexarelin analogues EP 60761 and, with a lower potency, EP 50885, injected into the paraventricular nucleus induced penile erection episodes indistinguishable from those induced by oxytocin, dopamine receptor agonists and NMDA, which act in this

hypothalamic nucleus to elicit this male sexual response. The facilitative effect of EP 60761 and of EP 50885 on penile erection was prevented by [d(CH_2)$_5$Tyr(Me)^2-Orn^8]vasotocin, a potent and selective oxytocin receptor antagonist (Bankowski et al., 1980), by L-NAME, a potent NO synthase inhibitor (Rees et al., 1990), and by ω-conotoxin-GVIA, a potent blocker of Ca^{2+} channels of the N-type (McCleskey et al., 1987). Hence, it is likely that EP 60761 and EP 50885 induce this sexual response by acting in the paraventricular nucleus with a mechanism similar to that suggested for oxytocin, dopamine receptor agonists and NMDA, e.g. by stimulating oxytocinergic neurons that originate in the paraventricular nucleus and project to extra-hypothalamic brain areas (see Argiolas and Melis, 1995). In agreement with this hypothesis, penile erection induced either by EP 60761 or by EP 50885 was prevented by [d(CH_2)$_5$Tyr(Me)^2-Orn^8]vasotocin when given i.c.v., but not into the paraventricular nucleus. This finding suggests that EP 60761 and EP 50885 induce penile erection by releasing oxytocin at sites distant from the paraventricular nucleus. A similar explanation was provided for the ability of [d(CH_2)$_5$Tyr(Me)^2-Orn^8]vasotocin, when given i.c.v. but not into the paraventricular nucleus, to prevent dopamine receptor agonist- and NMDA-induced penile erection (Melis et al., 1992, 1994). The ability of L-NAME given either i.c.v. or into the paraventricular nucleus to prevent EP 60761- and EP 50885-induced penile erection suggests that the activation of oxytocinergic transmission by EP 60761 and EP 50885 is apparently mediated by an increased NO production in the paraventricular nucleus, as found for oxytocin-, dopamine receptor agonist- and NMDA-induced penile erection (Melis et al., 1996, 1997b,c). Activation of NO synthase in the paraventricular nucleus by EP 60761 or EP50885 might be secondary to an increased Ca^{2+} influx, possibly in the cell bodies of oxytocinergic neurons mediating penile erection, because EP 60761- or EP 50885-induced penile erection was prevented by the blockade of N-type Ca^{2+} channels in the paraventricular nucleus by ω-conotoxin-GVIA. In line with this hypothesis, ω-conotoxin-GVIA injected into the paraventricular nucleus prevents dopamine receptor agonist- and oxytocin-induced penile erection (Argiolas et al., 1990; Succu et al., 1998). Likewise, NMDA-induced penile erection is prevented by (+)-MK-801 (Melis et al., 1994; Succu et al., 1998), which blocks NMDA receptor-coupled Ca^{2+} channels (Monaghan et al., 1989). Interestingly, an increased Ca^{2+} influx has been shown to modulate the GH release induced by GH-releasing peptides, including hexarelin, in the pituitary gland (Akman et al., 1993; Sartor et al., 1985). Finally, favouring the view that EP 60761 and EP 50885 induce penile erection via oxytocinergic transmission was the ability of morphine to prevent this response when injected into the paraventricular nucleus. Indeed, morphine reduces oxytocin-, dopamine receptor agonist- and NMDA-induced penile erection, apparently by preventing the activation of NO synthase induced by these substances at the paraventricular level (Melis et al., 1997a,d).

The molecular mechanism(s) subserving EP 60761 and EP 50885 activation of oxytocinergic neurons in the paraventricular nucleus is unknown at present. It may be hypothesised that the two peptides stimulate specific receptors, possibly located in the cell bodies of oxytocinergic neurons and coupled to Ca^{2+} influx. In this regard, it is pertinent to recall that specific receptors for GH-releasing peptides have been identified in the pituitary gland, the hypothalamus and other brain regions (Codd et al., 1989; Pong et al., 1986; Howard et al., 1996, Muccioli et al., 1998). Perhaps more relevant to this work, activation of these receptors triggers GH release from the pituitary by increasing Ca^{2+} influx (Akman et al., 1993; Sartor et al., 1985). If this hypothesis is correct, since hexarelin was inactive, whereas EP 50885 and EP 60761 did induce penile erection when injected into the paraventricular nucleus, the existence of a GH-releasing peptide receptor mediating penile erection with an affinity for EP 60761 and EP 50885 higher than that for hexarelin may be envisaged. Along this line, it is noteworthy that EP 50885 and hexarelin are both capable of releasing GH and of increasing eating behaviour, EP 40737 is active on GH release but is inactive on eating, EP 40904 is instead inactive on GH release but active on eating, and EP 60761 has no activity either on GH release or eating (Torsello et al., 1998). From the foregoing, the GH-releasing peptide receptors mediating penile erection would be different from those mediating GH release or eating behaviour. This would also explain the surprising inability of hexarelin to induce penile erection. Indeed, this finding might simply indicate that receptors mediating penile erection have binding properties for GH-releasing peptides different from those mediating GH release or eating behaviour. The existence of a specific receptor mediating a distinct effect of these peptides, i.e. penile erection, would not be so speculative, since previous experimental evidence supports the existence of different sub-populations of GH-releasing peptide receptors. First, the effects of hexarelin and its analogues on GH release are divorced from the eating effects (Torsello et al., 1998). Second, receptors for hexarelin and other GH-releasing peptides, whose activation induces effects independent of the GH-releasing properties of the peptides, have been detected in other tissues, e.g. in the heart (Bisi et al., 1999; Bodart et al., 1999; Locatelli et al., 1999). Finally, cloning studies have revealed the existence of several forms of receptors for GH-releasing peptides (Smith et al., 1999). Alternatively, other possible mechanisms of actions for EP 60761 and EP 50885 cannot be ruled out at present. For instance, these peptides might induce penile erection by releasing neurotransmitters that influence penile erection in the paraventricular nucleus, e.g. dopamine, oxytocin and/or excitatory amino acids. However, this hypothesis is unlikely, since (+)-MK-801, an antagonist of NMDA receptors,

cis-flupenthixol, which blocks dopamine receptors, and [d(CH_2)$_5$Tyr(Me)^2-Orn^8]vasotocin, an antagonist of oxytocin receptors, failed to prevent EP 60761- and EP 50885-induced penile erection when injected into the paraventricular nucleus. This finding suggests that the erectile response induced by the two peptides is not mediated by the release of dopamine or excitatory amino acids or oxytocin in the paraventricular nucleus. If this was the case, the above compounds would have been capable of preventing EP 60761- and EP 50885-induced penile erection, as was found for dopamine receptor agonist-, NMDA- or oxytocin-induced penile erection (Argiolas and Melis, 1995).

In conclusion, EP 60761 and EP 50885, two peptides related to the GH-releasing peptide hexarelin, induced penile erection when injected into the paraventricular nucleus, apparently via pathways unrelated to those subserving the release of GH or increase of eating behaviour. Further studies are mandatory to clarify the molecular mechanism by which EP 60761 and EP 50885 induce this sexual response. However, the finding that these peptides induce penile erection when injected into the paraventricular nucleus, a kind of integrative centre between the central and peripheral nervous system (Swanson and Sawchensko, 1983) that plays an important role in the control of sexual function (see Argiolas and Melis, 1995), suggests that peptide molecules of this kind may be useful for the treatment of erectile dysfunction of central origin in men.

Acknowledgements

This work was supported partially by MURST 40% to AA and EEM, and RAS grants to AA.

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