

# In Vitro Effects of Beta-Endorphin on Testicular Release of Androgens in the Lizard *Podarcis sicula* Raf

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**ABSTRACT** The effects of the proopiomelanocortin-derived opioid peptide, beta-endorphin ( $\beta$ -EP), and of the opioid antagonist, naloxone (NAL), on both basal and pituitary-stimulated androgen secretion from superfused quiescent and active testes were assessed in the adult lizard, *Podarcis sicula*. In the absence of the homologous pituitary, in vitro treatment with  $\beta$ -EP and/or NAL did not affect basal secretion of androgens from quiescent and active testes. Conversely, in the presence of the homologous pituitary, treatment with  $\beta$ -EP brought about a decrease in androgen secretion in active testes, but no effect on quiescent ones. Naloxone counteracted the inhibitory effect of  $\beta$ -EP in active testes, and enhanced maximal pituitary-stimulated secretion of androgens in quiescent but not in active testes. The effects produced by  $\beta$ -endorphin and naloxone were reversible. These results suggest that, in this lizard, opioids might be involved in the control of androgen release. The lack of effect of  $\beta$ -EP and naloxone when added directly to the testes seems to suggest that the opioid agonist and antagonist act on androgen release by modulating pituitary gonadotrophin output.

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**Key Words:**  $\beta$ -endorphin, Testicular activity, Androgens, Lizard

## INTRODUCTION

In mammals, an endogenous opioid system has been shown to modulate reproductive processes by acting at both hypothalamus-pituitary and gonadal levels (Leadem and Kalra, 1985; Fabbri et al., 1989; Kalra and Kalra, 1991; Rivier and Rivest, 1991; Bonavera et al., 1993, 1994).

Opioid peptides have been isolated from the testes of many species (Tsong et al., 1982; Margioris et al., 1983, 1985). Leydig cells appear to be the sole source of testicular opioid peptides (Shaha et al., 1984; Douglass et al., 1987), while Sertoli and possibly peritubular cells are the only intratesticular cells having opiate receptors (Fabbri et al., 1985). The functional role of  $\beta$ -endorphin ( $\beta$ -EP) in the testis is not known. However, evidence has been accumulating about the role of  $\beta$ -EP in the regulation of testosterone secretion, although the mechanism by which  $\beta$ -EP action takes place is not clear

(Gerendai et al., 1984, 1986; Margioris et al., 1989; Chandrashekar and Bartke, 1992).

In the seasonally-breeding lizard, *Podarcis sicula*, the physiological mechanisms responsible for timing the onset and cessation of gonadal function have not been well-understood. These changes are characterized by dramatic alterations in steroidogenesis and gametogenesis (Angelini et al., 1976; Ciarcia, 1993). In this lizard,  $\beta$ -EP immunoreactivity has been detected in the testis by both radioimmunoassay (RIA) and immunocytochemistry; it has shown seasonal variations, which are most pronounced in the testicular interstitial cells of sexually quiescent lizards (Ciarcia et al., 1994).

The aim of the present study was to establish whether  $\beta$ -EP is involved in the regulation of testosterone production in lizard testes, and whether it has a role in modulating testicular activity in a seasonal breeding species. The striking functional difference between the quiescent (winter period) and the active (spring period) testis in the adult lizard, *Podarcis sicula*, provides a unique experimental model for evaluating the role of  $\beta$ -EP in the regulation of testicular functions.

An in vitro superfusion system was used to assess the effects of  $\beta$ -EP and naloxone, given separately or combined, on androgen release from quiescent and active testes of this lizard. In a first series of experiments,  $\beta$ -EP and naloxone were added either separately or combined to a superfusion system where only testes were present. In another series of experiments,  $\beta$ -EP and naloxone were added either separately or together to a superfusion system where testes and homologous pituitaries were present.

This approach may allow clarification of whether the two drugs act directly on the testes (basal androgen secretion) or via the pituitary (pituitary-stimulated androgen secretion).

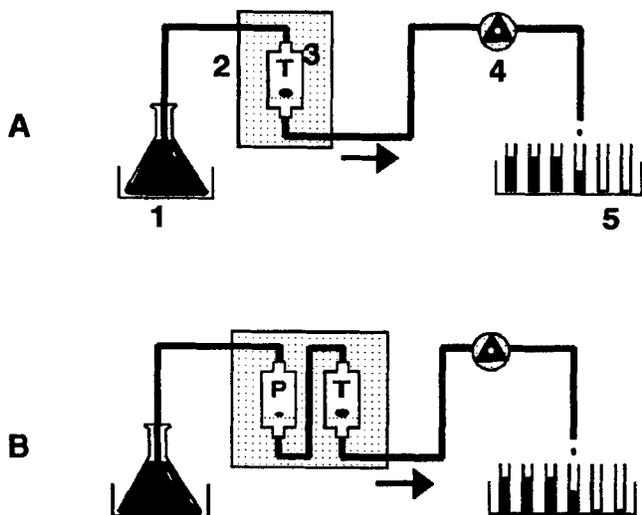
## MATERIALS AND METHODS

### Animals

Adult males of *Podarcis sicula* Raf. were captured in the neighborhood of Naples (Arzano) during different

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**Fig. 1.** Schematic representation of superfusion apparatus. 1, flask with superfusion fluid; 2, thermostatic box; 3, organ glass chamber; 4, peristaltic pump; 5, fraction collector; P, pituitary gland; T, testis.

periods of the gonadal cycle, i.e., 1) nonreproductive period (quiescent testes), and 2) breeding period (active testes). The animals ranged from 12–14 g in weight, and were about 7–8 cm in length (snout–vent). For each experiment, testes and pituitary glands were immediately removed from the animals, plunged in cool superfusion fluid (4°C), washed gently several times, and placed individually in superfusion chambers as described below.

#### In Vitro Superfusion System

The apparatus utilized for superfusion studies consisted of a series of glass chambers (1.5 × 3 cm) provided, at the bottom, with a glass wool filter and connected through Teflon capillary tubes to a superfusion fluid reservoir. A testis was laid on the incubation vessel filter and just covered with superfusion fluid (1.5 ml) (Fig. 1A). When combined superfusions of the pituitary and testis were carried out, they were put in individual incubation chambers arranged in cascade in order to deliver the pituitary effluents to the testis (Fig. 1B).

The superfusion fluid consisted of Dulbecco's modified Eagle's medium, (DME), supplemented with penicillin, streptomycin (100 µg/ml), and amphotericin B (10 µg/ml) (Gibco, UK). The medium was pushed upwards (i.e., by countergravity) by four-channel peristaltic pumps (Gilson, France). The superfusion chambers were immersed vertically in a recirculating water bath at a temperature of 28°C, at which point optimal responsiveness was observed (Ciarcia, 1993).

The superfusion flow rate was very low (5 µl/min at first), and then rose gradually (within 20 min) to a final flow of 33 µl/min. After the 90 min required to equilibrate the system and evaluate hormone content at the beginning of the experiment (time, 0 min), the incubations were performed for an additional 480 min. Each fraction (1 ml) was collected for 30 min, and stored at –80°C.

Experimental treatment of basal and pituitary-stimu-

lated testosterone secretion from quiescent and active superfused testes included assessment of human β-EP and/or naloxone effects.

Human β-endorphin (β-EP, Peninsula Laboratories, UK) and naloxone (NAL, Sigma Chemical Co., St. Louis, MO) were added, separately and/or combined, to DME and infused constantly in each experiment, which lasted 480 min. Different concentrations (10<sup>-5</sup>, 10<sup>-6</sup>, and 10<sup>-7</sup>) of β-EP or NAL were used in preliminary tests to assess effect on testosterone secretion. The dose of 10<sup>-5</sup> M of β-EP and of NAL was thereafter used, since it resulted in the most effective alteration of androgen secretion. Each experiment was carried out in triplicate.

In some experiments, incubation was carried out for 270–390 min, and the superfusion fluid contained both β-EP and NAL. In other experiments, incubation with β-EP was carried out for 240 min. β-EP was then removed, and the incubation was prolonged up to 480 min.

At the end of each incubation, the pituitary gland and the testis were removed from the superfusion system and plunged into Bouin's fixative fluid, for use as histological control.

#### Determination of Androgens

Androgen content in the media was measured by radioimmunoassay (RIA), as previously described for this species (Ciarcia et al., 1986). Since the antiserum used (courtesy of Dr. G.F. Bolelli, Bologna, Italy) was cospecific for testosterone and 5-dihydrotestosterone, results are expressed as androgens. Sensitivity was 2 pmol/l in test tubes. Intra- and interassay coefficient of variation (CV) were 6% and 12%, respectively.

#### Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA), followed by the multiple-range Duncan's test.

### RESULTS

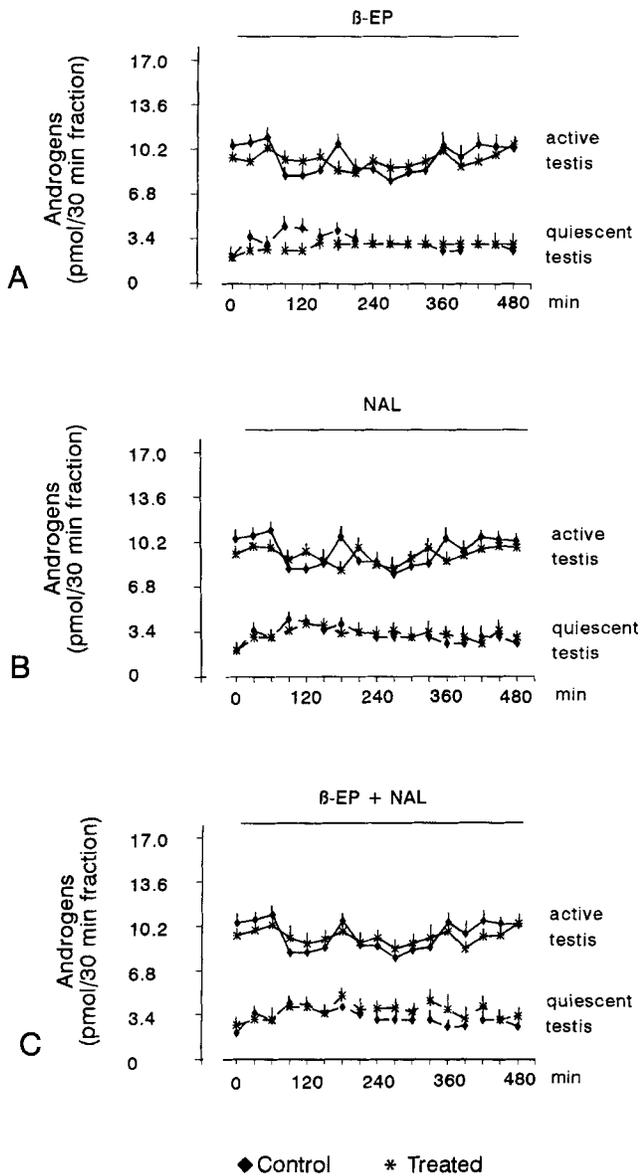
#### Effect of β-Endorphin (β-EP) and/or Naloxone (NAL) on Basal Androgen Secretion From Superfused Testis

Figure 2 describes the effects of β-EP and NAL, added separately (Fig. 2A,B) or together (Fig. 2C), on basal androgen secretion. Figure 2 shows that androgen release is much higher from active control testes (superfused only with medium) than from control quiescent testes. The two components (β-EP and NAL), given separately or combined, had no effect on androgen release from both quiescent and active testes.

#### Effects of β-Endorphin (β-EP) and/or Naloxone (NAL) on Pituitary-Stimulated Androgen Secretion From Superfused Testis

In control tests, the addition of a homologous pituitary gland to the active testis resulted in a significant increase of androgen output in the superfusion fluid. This effect was not observed when a quiescent testis was used. These findings are consistent with previous observations carried out on the same species (Ciarcia, 1993).

The addition of β-EP to systems containing a pituitary

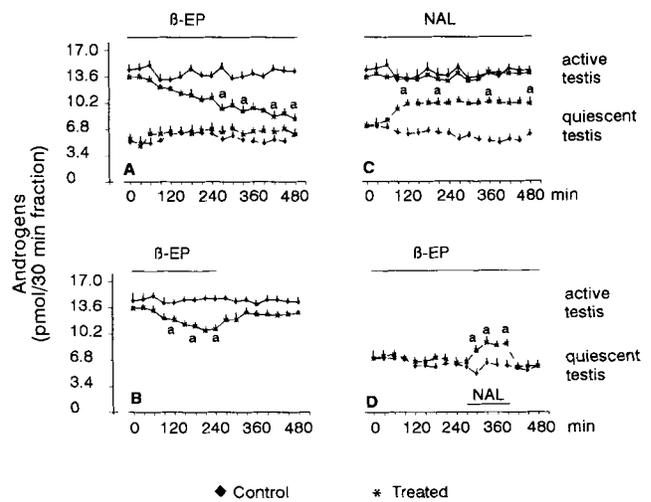


**Fig. 2.** Effects of human  $\beta$ -endorphin ( $\beta$ -EP) and naloxone (NAL) on basal secretion of androgens from both active (—) and quiescent (---) superfused testes of *Podarcis sicula*. In the medium,  $\beta$ -EP, NAL, or  $\beta$ -EP plus NAL were added from the beginning of superfusion (see horizontal black line). **A:** Continuous infusion of  $\beta$ -EP for 480 min. **B:** Continuous infusion of NAL for 480 min. **C:** Combined effect of continuous infusion of  $\beta$ -EP and NAL for 480 min. Each point represents mean ( $\pm$ SD) of three androgen assays relative to independent experiments.

and an active testis resulted in a decrease of androgen output ( $P < 0.01$ ) (Fig. 3A). This effect was reversible, since the original hormone secretion was restored after  $\beta$ -EP was removed from the superfusion system (Fig. 3B).

Inhibition of androgen output could not be observed when  $\beta$ -EP was added to the pituitary-quiescent testis system, since, in this case, androgen secretion was generally relatively low.

The addition of NAL to the pituitary-active testis sys-



**Fig. 3.** Effects of human  $\beta$ -endorphin ( $\beta$ -EP) or naloxone (NAL) on androgens output from a homologous pituitary-testis superfusion system: (—) active and (---) quiescent testis of *Podarcis sicula*. **A:** Continuous infusion of  $\beta$ -EP for 480 min (see horizontal black line). **B:** Infusion of  $\beta$ -EP for to 240 min (see horizontal black line). **C:** Continuous infusion of NAL for 480 min (see horizontal black line). **D:** Continuous infusion of  $\beta$ -EP for 480 min combined with addition of NAL from 270–390 min (see horizontal black lines). Each point represents mean ( $\pm$ SD) of three androgen assays relative to independent experiments. \* $P < 0.01$  compared with control value.

tem did not alter androgen production. However, this substance induced a significant increase ( $P < 0.01$ ) in androgen output in the pituitary-quiescent testis association (Fig. 3C). When NAL was added to a pituitary-quiescent testis system, superfused with  $\beta$ -EP, an increase in androgen output was observed. This effect proved reversible (Fig. 3D).

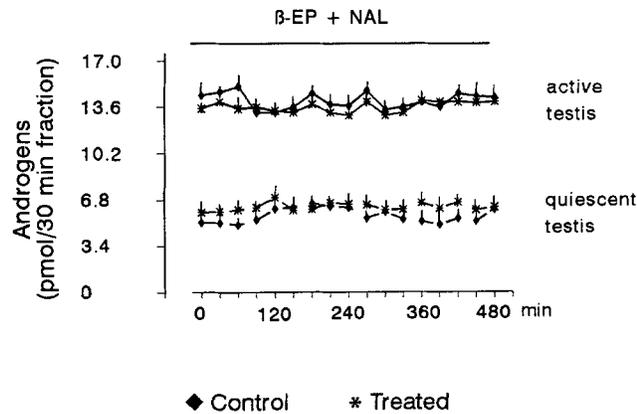
Treatment of pituitary-stimulated testis with  $\beta$ -EP plus naloxone, added to the medium from the beginning of superfusion, did not significantly affect androgen secretion in both active and quiescent testes (Fig. 4).

## DISCUSSION

Our results provide further evidence that  $\beta$ -EP influences the sexual function not only in mammals (Cheng et al., 1985; Lebouille et al., 1986; Fabbri et al., 1989; Rivier and Rivest, 1991) but also in a nonmammalian vertebrate such as the lizard.

$\beta$ -EP and NAL affected androgen release from lizard testes only when homologous pituitaries were present. On the contrary, the two drugs were devoid of any effect when added directly to isolated testes. These data corroborate the recently advanced hypothesis that  $\beta$ -EP regulates androgen secretion in the lizard by inhibiting pituitary gonadotropin release (Ciarcia et al., 1994). The effects produced by  $\beta$ -EP and NAL proved to be reversible, since the removal of  $\beta$ -EP and NAL from the superfusion restored the original androgen output.

The results here show that exogenous  $\beta$ -EP could considerably reduce androgen output when added to pituitary-testis systems derived from lizards in the active phase of the reproductive cycle; this effect was not ob-



**Fig. 4.** Effects of continuous infusion for 480 min of human β-endorphin plus naloxone (β-EP + NAL) on pituitary-stimulated androgen secretion from both active (—) and quiescent (---) superfused testes of *Podarcis sicula*. Each point represents mean (±SD) of three androgen assays relative to independent experiments. \**P* < 0.01 compared with control value.

served when lizards in the quiescent phase were used. These results seem to be consistent with data previously obtained in this laboratory; in fact, it has been reported that, in the lizard, the pituitary content of β-EP is maximal during the quiescent phase of the reproductive cycle and is significantly reduced throughout the active phase (Ciarcia et al., 1994). One may infer that when the pituitary-gonadal axis is active, exogenous β-EP may decrease gonadotropin secretion, and consequently androgen output, while during the quiescent phase, this opioid cannot further inhibit the pituitary-gonadal axis already depressed by high levels of pituitary endogenous β-EP.

The observation that naloxone is effective in pituitary-testis complexes derived from lizards in the quiescent phase suggests that naloxone may act on a system at a low functional level. On the contrary, the opioid antagonist cannot further stimulate a pituitary-testis system already maximally stimulated by the series of events taking place during the active phase of the reproductive cycle. This interpretation seems to be supported by experiments performed in mammals: reportedly, naloxone stimulates gonadotropin release in all phases of the ovulatory cycle in rats, monkeys, and women; however, the drug has little or no effect when applied during the rise of the preovulatory gonadotropin surge, i.e., when the pituitary is maximally stimulated by endogenous hypothalamic luteinizing hormone-releasing hormone (LHRH) (Kalra, 1993). The lack of effect of β-EP and NAL when added together to pituitary-testis systems may indicate a reciprocal inhibition of the opioid agonist and of its antagonist. This observation and the absence of “escape” phenomena suggest that the two drugs might act specifically via opioid receptors.

The lack of effect of β-EP and naloxone on testes superfused in the absence of the homologous pituitaries may depend on the absence of opioid receptors in lizard Leydig cells.

Previous findings from this laboratory have shown

that β-EP is present in lizard testes, and that its content fluctuates according to different phases of the reproductive cycle (Ciarcia et al., 1994); however, this does not necessarily mean that testicular β-EP is involved in the local control of androgen release in this species. The observation that β-EP is also present on spermatogonia and spermatocytes (Ciarcia et al., 1994) may rather indicate that testicular β-EP is connected with the processes leading to the maturation of spermatozoa. Further studies are needed to clarify this issue; in particular, analysis of the distribution of opioid receptors on the different components of the testis may be helpful. Opioid receptors have been found in Leydig cells of Amphibia, and it has been possible to demonstrate that, in this class of vertebrates, β-EP exerts a local control on androgen output (Facchinetti et al., 1992, 1993).

On the other hand, in mammals such as rats and mice, an intratesticular effect of opioids on androgen release has been reported by some groups (Knotts and Glass, 1988; Chandrashekar and Bartke, 1992), but not demonstrated by others (Margioris et al., 1989). These conflicting results may be explained by the fact that opioid receptors have not been detected so far on the plasma membranes of mammalian Leydig cells (Fabbri et al., 1989).

Probably, androgen opioid peptides act at multiple levels in regulating male fertility, as demonstrated by the presence of opioid receptors in mature sea urchins and human sperm, and in both of these organisms they function in regulating sperm motility (Cariello et al., 1986; Ram Sastry et al., 1991). Furthermore, proenkephalin products, stored in the sperm acrosome of several mammals, are depleted from sperm following the acrosome reaction, suggesting that they may affect the egg during fertilization (Kew et al., 1990).

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