



# Inhibition by amphetamine of testosterone secretion through a mechanism involving an increase of cyclic AMP production in rat testes

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- 1 The effect of amphetamine on the secretion of testosterone and the production of testicular adenosine 3':5'-cyclic monophosphate (cyclic AMP) in rats was studied.
- 2 A single intravenous injection of amphetamine decreased the basal and human chorionic gonadotropin (hCG)-stimulated levels of plasma testosterone. Plasma LH levels were not altered by the injection of amphetamine.
- 3 Administration of amphetamine *in vitro* resulted in a dose-dependent inhibition of both basal and hCG-stimulated release of testosterone.
- 4 Amphetamine enhanced the basal and hCG-increased levels of cyclic AMP accumulation *in vitro* in rat testes.
- 5 These results suggest that amphetamine inhibits the spontaneous and hCG-stimulated secretion of testosterone from the testes through a mechanism involving an increase in cyclic AMP production.

**Keywords:** Amphetamine; testosterone; human chorionic gonadotropin (hCG); cyclic AMP; rat testes

## Introduction

It has been well documented that amphetamine is a dopamine agonist which stimulates dopamine release (Crowley & Zelman, 1981; Dluzen & Ramirez, 1990a,b; Keiser, 1990), and reduces the thyrotropin-releasing hormone (TRH) content in rat striata (Przegalinski *et al.*, 1991). In humans who chronically abuse amphetamine, sudden abstinence often precipitates an organic mood disorder that mimics many symptoms of major depression.

Following castration or replacement with testosterone propionate (TP) in orchietomized rats, striatal tissue fragments continue to release dopamine in response to amphetamine stimulation (Becker & Ramirez, 1981). In addition, castration increases the amphetamine-induced stereotyped behaviour while TP injection reverses this effect (Beatty *et al.*, 1982). Testing the activity of their mesolimbic dopamine system by ventral striatum microdialysis, amphetamine injections increased dopamine more in castrated than in normal rats, and this exaggerated response was attenuated by TP therapy (Hernandez *et al.*, 1994). These observations suggest a regulatory role of testosterone on amphetamine-increased catecholaminergic activity and behaviour.

Conversely amphetamine may influence testosterone secretion either via stimulating the hypothalamic-pituitary-adrenal (HPA) axis hormones or by altering catecholamine activity within the central nervous system (CNS). Swerdlow *et al.* (1991) has shown that amphetamine exposure and withdrawal in rat modifies the HPA axis endocrine responses and also regional brain catecholamine levels. Corticotropin-releasing hormone (CRH), adrenocorticotrophic hormone (ACTH) and the corticosteroids all influence the hypothalamic-pituitary-gonadal axis (Moberg, 1981) and the catecholamines have multiple and complex effects on gonadotropin release, with

both noradrenaline and dopamine exerting inhibitory or stimulatory effects depending on the endocrine milieu and age of the animal (Barraclough & Wise, 1982; Kalra, 1986; Taleisnik & Sawyer, 1986).

Thus the catecholamines modulate testosterone production via altering gonadotropin release. However, it is clear that, in addition, the catecholamines can have a direct effect on steroidogenesis at the gonadal level. In the ovaries of immature chicks and immature pregnant-mare serum treated rats, isoprenaline (a  $\beta$ -adrenoceptor agonist) increases progesterone accumulation (Gonzalez *et al.*, 1994; Mori *et al.*, 1994), and in women with polycystic ovarian syndrome excess ovarian noradrenaline correlated with a hyperandrogenaemic status (Yoshino *et al.*, 1991). In the immature golden hamster testes, noradrenaline, adrenaline, isoprenaline and phenylephrine (an  $\alpha$ -adrenoceptor agonist) all significantly stimulate testosterone production *in vitro* and the effect of noradrenaline can be blocked by both  $\beta$ - and  $\alpha$ -receptor antagonists (Mayerhofer *et al.*, 1992). As far as we know, no work has been carried out, to date, on the direct effects of amphetamine on the gonads.

In the present study, we examine the effect of amphetamine on the basal and human chorionic gonadotropin (hCG)-stimulated secretion of testosterone both *in vivo* and *in vitro* in male rats. The effect of amphetamine on the production of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in rat testes was also evaluated to determine whether cyclic AMP production is involved in the regulation by amphetamine of testosterone secretion in rats.

## Methods

### Animals

Male rats of the Sprague-Dawley strain weighing 300–350 g were housed in a temperature controlled room ( $22 \pm 1^\circ\text{C}$ ) with 14 h of artificial illumination daily (06 h 00 min–20 h 00 min) and given food and water *ad libitum*.

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### In vivo experiments

Male rats were catheterized via the right jugular vein (Wang *et al.*, 1989; 1994; Hwang *et al.*, 1990). Twenty hours later, they were injected with hCG (5 iu ml<sup>-1</sup> kg<sup>-1</sup>, Sigma), amphetamine (0.4 µg ml<sup>-1</sup> kg<sup>-1</sup>, Sigma) or hCG plus amphetamine via the jugular catheter. Blood samples (0.5 ml each) were collected at 0, 15, 30, 60, 120, 180, 360, 480, and 1440 min after the challenge. An equal volume of heparinized saline was injected immediately after each bleeding.

Plasma was separated by centrifugation at 10000 g for 1 min. The concentration of testosterone and luteinizing hormone (LH) in each plasma sample was measured by radioimmunoassay (RIA) (Wang *et al.*, 1994).

### In vitro experiment

Male rats were decapitated. The testes were decapsulated and cut into eight equal pieces before preincubation for 90 min with Locke solution containing 10 mM glucose, 0.003% bacitracin, and 0.05% HEPES at 34°C (Wang *et al.*, 1994). Each piece was placed in a flask containing 2 ml medium. The medium was aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The testes blocks were then incubated with amphetamine (0–10<sup>-6</sup> M), or hCG plus amphetamine for 1 h. At the end of the incubation, the testicular tissues were weighed. The media were collected, and stored at -20°C until analysed for testosterone by RIA.

For studying the accumulation of cyclic AMP in response to amphetamine, some testicular tissues were primed and then incubated for 1 h with 2 ml medium containing 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma), a phosphodiesterase inhibitor. At the end of the incubation, tissues were mixed with 2 ml of 65% ice-cold ethanol, homogenized by polytron (PT-3000, Kinematic Ag., Switzerland), and centrifuged at 2000 g for 15 min. The supernatants were lyophilized in a vacuum concentrator (Speed Vac, Savant, U.S.A.) and reconstituted with assay buffer (0.05 M acetate buffer with 0.01% sodium azide, pH 6.2) before measuring the concentration of cyclic AMP by the RIA. The protein concentration in tissue extracts was determined by the method of Lowry *et al.* (1951).

### RIA of testosterone and LH

The concentration of plasma and medium testosterone was determined by RIA as described previously (Wang *et al.*, 1994). With anti-testosterone serum No. W8, the sensitivity of testosterone RIA was 2 pg per assay tube. The intra- and interassay coefficients of variation were 4.1% (*n* = 6) and 4.7% (*n* = 10), respectively.

The concentration of plasma LH was determined by RIA as described previously with anti-LH serum PW11-2 (Hwang *et al.*, 1990; Wang *et al.*, 1994). The rat LH-I-6 used for iodination and the rat LH-RP-3 which served as standard preparations were provided by NIAMDD. The sensitivity was 0.1 ng for LH RIA. The intra- and interassay coefficients of variability were 3.8% (*n* = 4), and 6.6% (*n* = 5), respectively.

### RIA of cyclic AMP

The concentration of cyclic AMP in testicular tissues extracted by ethanol was measured by RIA kits provided by Amersham Ltd. In the cyclic AMP RIA, the sensitivity was 13.5 fmol; the cross-reactivities were 0.0005% with cyclic GMP, 0.02% with cyclic IMP, and less than 0.001% with cyclic CMP, AMP, ADP, ATP, EDTA, and theophylline (Wang *et al.*, 1994).

### Materials

Chemicals used in the study included: amphetamine (Sigma); and human chorionic gonadotropin (hCG, Sigma). Chemicals were prepared as stock solutions solubilized in twice deionized H<sub>2</sub>O, and made daily. The doses of drugs are expressed as unit

weight per body weight *in vivo*, e.g. iu ml<sup>-1</sup> or µg ml<sup>-1</sup>; concentrations of drugs for *in vitro* experiment are expressed in their final molar concentrations in the flask.

### Statistical analysis

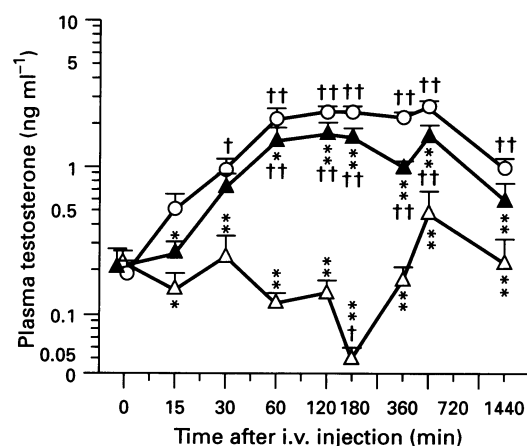
All values are given as the mean ± standard error of the mean (s.e. mean). In some cases, the treatment means were tested for homogeneity by a two-way analysis of variance, and the difference between specific means was tested for significance by Duncan's multiple-range test (Steel & Torrie, 1960). In other cases, Student's *t* test was employed. A difference between two means was considered statistically significant when *P* < 0.05.

## Results

### Effects of a single intravenous injection of amphetamine on testosterone and LH secretion

Apart from the level (0.54 ± 0.11 ng ml<sup>-1</sup>, *n* = 7) of plasma testosterone at 15 min, the post-hCG levels (0.93 ± 0.20 to 2.55 ± 0.18 ng ml<sup>-1</sup>, *n* = 7) of plasma testosterone were significantly (*P* < 0.05 or *P* < 0.01) greater than the value (0.19 ± 0.04 ng ml<sup>-1</sup>, *n* = 7) at 0 min (Figure 1). Intravenous injection of amphetamine did not alter the level of plasma testosterone until 120 min. From 120 to 180 min following amphetamine injection, the mean concentration of plasma testosterone dropped by 76% (0.05 ± 0.01 ng ml<sup>-1</sup> at 180 min, *n* = 7, versus 0.22 ± 0.05 ng ml<sup>-1</sup> at 0 min, *n* = 7, *P* < 0.05). The plasma testosterone returned to and stayed at the basal level 6 h later. The levels of plasma testosterone from 1 to 8 h following coinjection of amphetamine and hCG were significantly (*P* < 0.01) greater than the basal level. Although the plasma testosterone was increased, coinjection of amphetamine and hCG resulted in a significantly lower level of plasma testosterone (0.26 ± 0.05 ng ml<sup>-1</sup>, *n* = 7) at 15 min than that induced by hCG alone (0.54 ± 0.11 ng ml<sup>-1</sup>, *n* = 7, *P* < 0.05) except for the level at 30 min after challenge.

The mean levels of plasma LH at all time points were 4.11 ± 0.39 ng ml<sup>-1</sup> for the hCG-injected group, and



**Figure 1** Effects of amphetamine on the basal and hCG-stimulated concentration of plasma testosterone in male rats. Rats were given a single intravenous injection of amphetamine (Δ), hCG (○), or amphetamine plus hCG (▲) via right jugular vein: amphetamine 0.4 µg ml<sup>-1</sup> kg<sup>-1</sup>, *n* = 6–7; hCG 5 iu ml<sup>-1</sup> kg<sup>-1</sup>, *n* = 7; amphetamine + hCG, *n* = 7. Blood samples were collected via the jugular catheter at the time indicated after injection. Plasma testosterone was extracted by ether before measuring by radioimmunoassay. Each value represents mean ± s.e. mean \**P* < 0.05 and \*\**P* < 0.01 compared with hCG-injected animals; †*P* < 0.05, and ‡*P* < 0.01 compared with the value at 0 min.

$3.80 \pm 0.35$  ng ml<sup>-1</sup> for the amphetamine group and the animals injected with both hCG and amphetamine, respectively. No difference was observed in the plasma LH concentration among these three groups.

#### Effect of amphetamine on testosterone and cyclic AMP production in vitro

As compared with the control group, amphetamine in the range  $10^{-9}$ – $10^{-6}$  M caused a dose-dependent inhibition of testosterone release from rat testes ( $22.69 \pm 2.73$  to  $14.71 \pm 3.51$  pg mg<sup>-1</sup> testis h<sup>-1</sup>,  $n=7$ , versus basal level  $38.31 \pm 3.62$  pg mg<sup>-1</sup> testis h<sup>-1</sup>,  $n=7$ ,  $P < 0.05$  or  $P < 0.01$ ) (Figure 2). Incubation of testis blocks with hCG ( $0.5$  iu ml<sup>-1</sup>) for 60 min increased the level of testosterone secretion (hCG-treated group  $212.57 \pm 32.20$  pg mg<sup>-1</sup> testis h<sup>-1</sup>,  $n=8$  versus basal group,  $P < 0.01$ ). Combination of hCG with amphetamine concentrations of  $10^{-11}$  to  $10^{-6}$  M resulted in a significant inhibition of the hCG-stimulated release of testosterone ( $97.32 \pm 12.71$  to  $136.48 \pm 15.60$  pg mg<sup>-1</sup> testis h<sup>-1</sup>,  $n=8$  versus hCG alone treated group,  $P < 0.01$ ).

Administration of hCG significantly increased the accumulation of cyclic AMP in rat testes (hCG-treated group  $29.35 \pm 1.44$  fmol mg<sup>-1</sup> protein h<sup>-1</sup>,  $n=8$ , versus control group  $10.65 \pm 0.89$  fmol mg<sup>-1</sup> protein h<sup>-1</sup>,  $n=8$ ,  $P < 0.01$ ) (Figure 3). Amphetamine concentrations ranging from  $0.1$  nM to  $1$   $\mu$ M increased the content of cyclic cAMP in rat testes fragments ( $13.38 \pm 0.76$  to  $16.47 \pm 1.04$  fmol mg<sup>-1</sup> protein h<sup>-1</sup>,  $n=8$ , versus control group,  $P < 0.01$ ). Amphetamine of  $10^{-8}$  and  $10^{-6}$  M enhanced the hCG-stimulated cyclic AMP accumulation ( $50.42 \pm 6.76$  and  $46.64 \pm 4.94$  fmol mg<sup>-1</sup> protein h<sup>-1</sup>,  $n=8$ , versus hCG-treated group,  $P < 0.01$ ).

#### Discussion

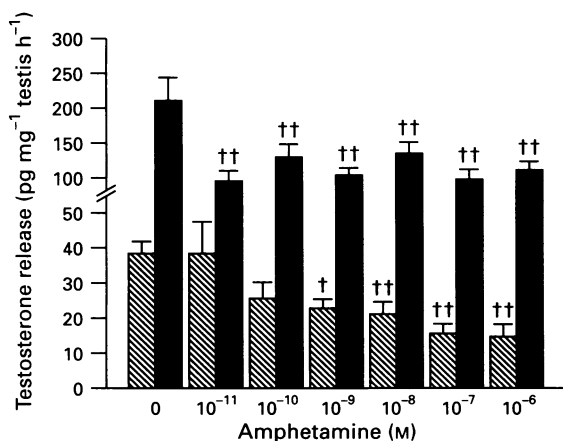
In the present study we found that administration of amphetamine in rats diminished the secretion of testosterone, both *in vivo* and *in vitro*, and increased the generation of testicular cyclic AMP.

The present data provide evidence that amphetamine diminishes the release of rat testosterone by acting directly and dose-dependently on the testicular fragments. The increase in testicular cyclic AMP in response to amphetamine reflects a correlation between activation of adenylate cyclase and inhibition of testosterone production following administration of amphetamine. In addition to the second messenger cyclic

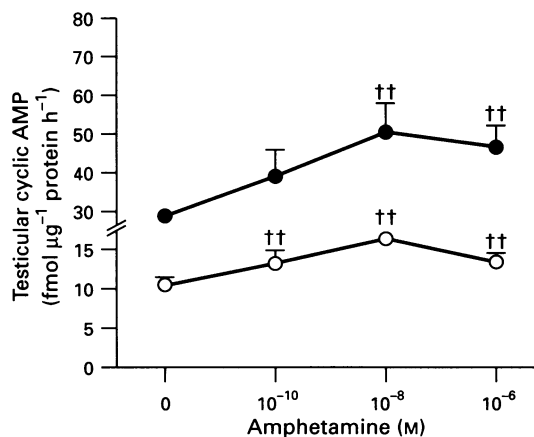
AMP, the Ras pathway, including the protein product of the Ras oncogene has been shown to be an important pathway for transmitting hormones, cytokines, and growth factors (Marx, 1993). Meanwhile, biological 'crosstalk' between cyclic AMP and the Ras pathway has been proposed; in other words, cyclic AMP possibly acting through protein kinase A (PKA) blocks transmission of Ras signals to Raf-1 in some cells, e.g. adipocytes, fibroblasts, and cancer cells, and therefore prevents activation of mitogen-activated protein (MAP) kinase, finally inhibiting many of the cellular responses to hormones, cytokines, or growth factors (Cook & McCormick, 1993; Severson *et al.*, 1993; Wu *et al.*, 1993; Häfner *et al.*, 1994; Gallo *et al.*, 1995). It is possible that in rat Leydig cells, both cyclic AMP and Ras pathways exist which are linked, and activated by amphetamine. The cyclic AMP stimulated by amphetamine acts through PKA to block the Ras pathway and then inhibits the release of testosterone. Whether this hypothesis is acceptable is still open to examination. Since amphetamine did not alter the plasma LH concentration, the mechanism by which amphetamine reduces testosterone secretion appears to be LH-independent.

It has been well established that hCG stimulates testosterone secretion both *in vivo* (Saez & Forest, 1979; Padron *et al.*, 1980; Wang *et al.*, 1994) and *in vitro* (Simpson *et al.*, 1987; Nakhla *et al.*, 1989; Liao *et al.*, 1991; Wang *et al.*, 1994), and increases testicular cyclic AMP content (Avallet *et al.*, 1987; Petersson *et al.*, 1988; Sakai *et al.*, 1989; Wang *et al.*, 1994). In the present study, we found that the stimulatory effect of hCG on plasma testosterone and testosterone production *in vitro* was diminished by amphetamine, whereas the stimulatory effect of hCG on cyclic AMP generation in rat testes was enhanced by amphetamine. These results suggest that amphetamine regulates testosterone production through a cyclic AMP-dependent mechanism which is independent of hCG regulation in post-cyclic AMP events. Although there is no direct evidence, it seems that the Ras pathway is not involved in the action of gonadotropin on Leydig cells. Based on the present *in vivo* and *in vitro* evidence, we cannot exclude the possibility that binding of gonadotropins to their receptors in the plasma membrane of rat Leydig cells (either receptor number or binding affinity) might be attenuated by amphetamine.

Numerous studies performed on rats have shown that dopamine is implicated in the control of male sexual behaviour with controversial results since both stimulatory and inhibitory effects of dopamine have been reported (Wilson, 1993). Similarly, amphetamine has been shown to both increase and de-



**Figure 2** The *in vitro* release of testosterone from rat testes at different doses of amphetamine in the presence (solid columns, hCG  $0.5$  iu ml<sup>-1</sup>,  $n=8$ ) or absence (hatched columns,  $n=7$ ). †  $P < 0.05$  and ‡  $P < 0.01$  compared with amphetamine at  $0$  M. Each column represents mean  $\pm$  s.e. mean.



**Figure 3** Dose-dependent effect of amphetamine on the accumulation of testicular cyclic AMP after *in vitro* incubation of rat testes with (●) or without (○) hCG ( $0.5$  iu ml<sup>-1</sup>);  $n=8$ . †  $P < 0.05$  and ‡  $P < 0.01$  compared with amphetamine at  $0$  M. Each value represents mean  $\pm$  s.e. mean.

crease sexual behaviour (Carter & Davis, 1976; Saito *et al.*, 1991). In human subjects most drugs of abuse disturb normal sexual function (Buffum, 1986). Perhaps in the case of amphetamine, inhibition of sexual function may be related to its ability to inhibit testosterone secretion in the testes through a mechanism involving cyclic AMP production.

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