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The role of cyclic AMP production, calcium channel activation and enzyme activities in the inhibition of testosterone secretion by amphetamine

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1 The aim of this study was to investigate the mechanism by which amphetamine exerts its inhibitory effect on testicular interstitial cells of male rats.

2 Administration of amphetamine (10^{-12}–10^{-6} M) *in vitro* resulted in a dose-dependent inhibition of both basal and human chorionic gonadotropin (hCG, 0.05 iu ml^{-1})-stimulated release of testosterone.

3 Amphetamine (10^{-9} M) enhanced the basal and hCG-increased levels of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) accumulation *in vitro* ($P<0.05$) in rat testicular interstitial cells.

4 Administration of SQ22536, an adenylyl cyclase inhibitor, decreased the basal release ($P<0.05$) of testosterone *in vitro* and abolished the inhibitory effect of amphetamine.

5 Nifedipine (10^{-6} M) alone decreased the secretion of testosterone ($P<0.01$) but it failed to modify the inhibitory action of amphetamine (10^{-10}–10^{-6} M).

6 Amphetamine (10^{-10}–10^{-6} M) significantly ($P<0.05$ or $P<0.01$) decreased the activities of 3β-hydroxysteroid dehydrogenase (3β-HSD), P450c17, and 17-ketosteroid reductase (17-KSR) as indicated by thin-layer chromatography (t.l.c.).

7 These results suggest that increased cyclic AMP production, decreased Ca^{2+} channel activity and decreased activities of 3β-HSD, P450c17, and 17-KSR are involved in the inhibition of testosterone production induced by the administration of amphetamine.

Keywords: Amphetamine; testosterone; cyclic AMP; calcium; steroidogenesis

Introduction

It is well documented that amphetamine is an indirectly acting dopamine agonist which stimulates dopamine release (Crowley & Zemlan, 1981; Dluzen & Ramirez, 1990a,b; Keiser, 1990). Our previous results have suggested that amphetamine inhibits testosterone release via a mechanism associated with increased testicular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Tsai *et al*., 1996a). Amphetamine has a biphasic action on an inward calcium current in neurones of snail *Lymnaea*, causing activation at 10^{-9}–10^{-7} M, and inhibition at higher concentrations (Vislobokov *et al*., 1993). Combined nimodipine, a L-type calcium channel antagonist, and haloperidol treatment have been shown to block the unconditioned and to attenuate the conditioned locomotor response to amphetamine in rats (DiLullo & Martin-Iverson, 1992). However, (±)-Bay K8644 and nicardipine have no effects on the amphetamine-induced hyperactivity (Moore *et al*., 1993).

The intracellular mechanism by which amphetamine mediates steroidogenesis has not yet been established, but a number of *in vitro* studies have shown that many compounds may directly or indirectly target the enzymes required for the biosynthesis of testosterone in the Leydig cell, including cholesterol side chain cleavage enzymes (cytochromes $P450_{SCC}$), 17α-hydroxylase/C_{17}-C_{20} lyase ($P450_{C}17$), 3β-hydroxysteroid dehydrogenase (3β-HSD), and 17-ketosteroid reductase (17-KSR) (Payne & O'Shaughnessy, 1996). As shown by immunohistochemistry, the lead-decreased production of testosterone and progesterone are correlated with the lower expression of the cytochromes $P450_{SCC}$, $P450_{C}17$, and 3β-HSD (Thoreux-Manlay *et al*., 1995). Glucocorticoids directly inhibit processes in the Leydig cell critical to the biosynthesis of testosterone, including 17α-hydroxylation and cholesterol side-chain cleavage (Welsh *et al*., 1982; Hales & Payne, 1989; Payne & Sha, 1991).

The biosynthesis of steroid hormones by Leydig cells requires the sequential actions that convert cholesterol into various steroid classes (Payne & O'Shaughnessy, 1996). Cytochrome $P450_{SCC}$ is a mitochondrial enzyme which catalyzes the first side chain cleavage of cholesterol to yield pregnenolone. The synthesis of testosterone requires the action of the microsomal enzyme cytochrome P450c17 which proceeds in two steps, 17α-hydroxylation and cleavage of the C17-20 bond to yield the C19 steroid dehydroepiandrosterone (DHEA) or androstenedione. 3β-HSD catalyzed 3β-hydroxy-5-ene steroids into 3-keto-4-ene steroids (i.e. pregnenolone→progesterone, 17α-hydroxypregnenolone→17α-hydroxyprogestrone, DHEA→androstenedione). The interconversion of androstenedione to testosterone is catalyzed by microsomal enzyme 17-KSR.

The present study was carried out to examine if the production of cyclic AMP, the activity of calcium channel and/or the enzyme required for steroidogenesis are involved in the action of amphetamine on the production of testosterone in rat testicular interstitial cells. Our data suggest that amphetamine inhibits testosterone production through the mechanisms involving increased cyclic AMP production, decreased calcium channel activity and decreased activities of 3β-HSD, P450c17, and 17-KSR in the testicular interstitial cells.

Methods

Animals

Male Sprague-Dawley rats weighing 300–350 g were housed in a temperature controlled room (22±1°C) with 14 h of ar-

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tificial illumination daily (06 h 00 min – 20 h 00 min) and given food and water *ad libitum*

Preparation of testicular interstitial cells

The method of collagenase dispersion of testicular interstitial cells was modified from the procedure described by Liao *et al.* (1991). Five decapsulated testes were added to a 50 ml polypropylene tube containing 5 ml preincubation medium and 700 μg collagenase (bovine, Type IA). Preincubation medium was made up of 1% bovine serum albumin (BSA, Fraction V) in Hank's balanced sodium solution (HBSS), with N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES, 25 mM), sodium bicarbonate 0.35 g l^{-1}, penicillin-G 100 iu ml^{-1}, streptomycin sulphate 50 μg ml^{-1}, heparin 2550 USP K units l^{-1}, pH 7.3, and aerated with 95% O_2 and 5% CO_2. The tube was laid horizontally in a 34°C water bath, parallel to the direction of the shaking. Fifteen min after shaking at 100 cycles min^{-1}, the digestion was stopped by adding 35 ml of cold preincubation medium and inverting the tube several times. The tube was allowed to stand for five min and was then filtered through a four-layer fine nylon mesh. Cells were collected by centrifugation at 4°C, $100 \times g$ for 10 min. The cell pellets were washed with deionized water to disrupt red blood cells (RBCs) and the osmolarity immediately restored with 10 fold HBSS. Hypotonic shock was repeated twice for disrupting RBCs and cell pellets resuspended in preincubation medium (substitution of HBSS in preincubation medium with Medium 199, and sodium bicarbonate 2.2 g l^{-1}). Cell concentration (1.0×10^6 cells ml^{-1}), viability (over 97%), and the sperm cells (less than 5%) were determined by use of a haemocytometer and the trypan blue method. Total cell proteins were determined by the method of Lowry *et al.* (1951).

To measure the abundance of Leydig cells in our preparation, the 3β-HSD staining method was used (Dirami *et al.*, 1991; Krummen *et al.*, 1994). The cells (1.0×10^6 cells ml^{-1}) were incubated with a solution containing 0.2 mg ml^{-1} nitro blue tetrozolium (Sigma, U.S.A.), 0.12 mg ml^{-1} 5-androstane-3β-ol-one (Sigma, U.S.A.), and 1 mg ml^{-1} NAD^+ (Sigma, U.S.A.) in 0.05 M PBS, pH 7.4 at 34°C for 90 – 120 min. Upon development of the blue formazan deposit sites of 3β-HSD activity, the abundance of Leydig cells was determined by use of a haemocytometer (Dirami *et al.*, 1991; Krummen *et al.*, 1994). Our preparation was found to contain approximately 20% Leydig cells.

Effect of amphetamine on testosterone and cyclic AMP production in testicular interstitial cells

Aliquots (1 ml) of cell suspensions (1.0×10^6 cells ml^{-1}) were preincubated with incubation medium in polyethylene tubes for one hour at 34°C under a controlled atmosphere (95% O_2 and 5% CO_2), shaken at 100 cycles min^{-1}. The supernatant fluid was decanted after centrifugation of the tubes at $100 \times g$ for 10 min. For studying the accumulation of cyclic AMP in response to amphetamine, aliquots (1 ml) of cell suspensions (1.0×10^6 cells ml^{-1}) were primed for 30 min with 1 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma, U.S.A.). Amphetamine (10^{-12} – 10^{-6} M), hCG (0.05 iu ml^{-1}), or hCG plus amphetamine in 200 μl fresh medium in the absence or presence of IBMX was then added to the tubes. After 2 h of incubation, 2 ml ice-cold PBSG buffer (0.1% gelatin in 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.5) was added to stop the incubation. The spent medium was centrifuged at $100 \times g$ and stored at −20°C until analysed for testosterone by radioimmunoassay (RIA). In the presence of IBMX, the cell pellets were mixed with 1 ml of 65% ice-cold ethanol, homogenized by polytron (PT3000, Kinematica Ag., Switzerland), and centrifuged at $1500 \times g$ for 15 min. The supernatant fluids were lyophilized in a vacuum concentrator (Speed Vac, Savant, U.S.A.) and stored at −20°C until analysed for cyclic AMP by RIA.

Effect of cyclic AMP on the ability of amphetamine to decrease testosterone release

Aliquots (1 ml) of cell suspensions were primed for 30 min with IBMX, or SQ22536 (9-(tetrahydro-2-furanyl)-9H-purine-6-amine), an adenylyl cyclase inhibitor (10 μM, Research Biochemicals International, U.S.A.) (Goldsmith & Abrams, 1992; Fiebich *et al.*, 1996; Mallat *et al.*, 1996), and then incubated (2 h) with amphetamine. At the end of the incubation, cells were mixed with 2 ml ice-cold PBSG buffer and immediately followed by centrifugation at $100 \times g$ for 10 min at 4°C. The supernatant fluids were stored at −20°C until analysed for testosterone by RIA.

Effect of Ca^{2+} on the ability of amphetamine to decrease testosterone release

Experiments were conducted following the same protocol as above with the exception that the L-type calcium channel blocker, nifedipine (100 nM or 1 μM, Research Biochemicals International, U.S.A.) was added into the medium. At the end of incubation (2 h), 2 ml ice-cold PBSG buffer were added and the supernatant fluids collected for measurement of testosterone by RIA.

Effect of amphetamine on steroidogenesis

Aliquots (1 ml) of cell suspensions, containing amphetamine (0, 10^{-8} or 10^{-9} M) were incubated for 2 h in the absence or presence of androstenedione at doses ranging from 10^{-13} to 10^{-7} M, respectively. In another experiment aliquots (1 ml) of cell suspensions, containing amphetamine (0, 10^{-12} to 10^{-6} M) were incubated with androstenedione (10^{-9} M). Two hours later, incubation was stopped by the addition of 2 ml ice-cold PBSG buffer. After centrifugation, the supernatant fluids were stored at −20°C until analysed for testosterone by RIA.

In addition, aliquots (1 ml) of cell suspension were incubated with [^{3}H]-pregnenolone (10000 c.p.m., 9 pmol, Amersham, U.K.) in the absence or presence of amphetamine (10^{-12} to 10^{-6} M) for 2 h. The reaction was then stopped by vigorous agitation in 1 ml diethyl ether, then quick frozen in a mixture of acetone and dry ice. The diethyl ether layer was collected, dried and reconstituted in 100 μl 100% ethanol containing 5 μg of each of the unlabelled carriers, including pregnenolone, progesterone, 17α-hydroxyprogesterone, DHEA, androstenedione and testosterone. Aliquots (50 μl) were applied to a thin layer chromatography plate (0.25 μg thick silica gel G sheets precoated with fluorescent indicator, Macherey-Nagel, Germany) and developed in a mixture of carbon tetrachloride and acetone (4:1; vol/vol). The sheets were dried and the location of steroid-containing spots were indicated under u.v. light. The R_f values were 0.55 for pregnenolone, 0.71 for progesterone, 0.50 for 17α-hydroxyprogesterone, 0.33 for DHEA, 0.65 for androstenedione and 0.44 for testosterone. The spots were cut off and transferred into vials containing 1 ml of liquid scintillation fluid (Ready Safe, Beckman, U.S.A.) before the radioactivity was counted in an automatic beta counter (Wallac 1409, Pharmacia, Finland).

RIA of testosterone

The concentration of testosterone in the medium was determined by RIA as described previously (Wang *et al.*, 1994; Tsai *et al.*, 1996a). With anti-testosterone serum No. W8, the sensitivity of testosterone RIA was 2 pg per assay tube. The intra- and interassay coefficients of variation (CV) were 4.1% ($n = 6$) and 4.7% ($n = 10$), respectively.

RIA of cyclic AMP

The concentration of cyclic AMP was determined by RIA as described elsewhere (Tsai 1996b; Lu *et al.*, 1996). With anti-cyclic AMP serum No. CV-27 pool, the sensitivity of cyclic

AMP was 2 fmol per assay tube. The intra- and interassay coefficients of viability were 6.9% ($n=5$) and 11.9% ($n=5$), respectively.

Materials

Bovine serum albumin (BSA), N-2-hydroxyethylpiperazine-N′-2-ethane-sulphonic acid (HEPES), Hank's balanced sodium salt (HBSS), medium 199, sodium bicarbonate, penicillin-G, streptomycin, heparin, collagenase, amphetamine, human chorionic gonadotropin (hCG), 3-isobutyl-1-methylxanthine (IBMX), nifedipine, pregnenolone, progesterone, 17-OH-progesterone, dehydroepiandrosterone (DHEA), androstenedione, testosterone were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [^{3}H]-pregnenolone, [^{3}H]-testosterone, and [^{125}I]-Na were obtained from Amersham International plc. (Bucks, U.K.). SQ22536 was purchased from Research Biochemical International (Natick, MA, U.S.A.). The doses of drugs are expressed in their final molar concentrations in the flask.

Statistical analysis

All values are given as 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 amphetamine on testosterone and cyclic AMP production

As compared with the control group, amphetamine (10^{-10}–10^{-6} M) produced a concentration-dependent inhibition of testosterone release from testicular interstitial cells (6.76 ± 0.81 to 5.32 ± 0.25 ng ml^{-1} protein 2 h^{-1}, $n=8$, versus basal level 10.19 ± 1.00 ng ml^{-1} protein 2 h^{-1}, $n=8$, $P<0.05$ or $P<0.01$) (Figure 1). Incubation of testicular interstitial cells with hCG (0.05 iu ml^{-1}) for 2 h increased the level of testosterone secretion (hCG treated group 45.80 ± 3.67 ng ml^{-1} protein 2 h^{-1}, $n=8$, versus basal group, $P<0.01$). A combination of hCG with amphetamine of 10^{-7}–10^{-6} M resulted in a significant inhibition of the hCG-stimulated release of testosterone (35.80 ± 3.61 to 31.87 ± 2.34 ng mg^{-1} protein 2 h^{-1}, $n=8$, versus hCG alone treated group, $P<0.05$).

Following priming with IBMX levels of testosterone in the medium were significantly ($P<0.01$) higher than those of the corresponding IBMX-free controls (Figure 2). Amphetamine (10^{-12} to 10^{-6} M) significantly inhibited testosterone release (18.66 ± 0.36 to 14.79 ± 0.87 ng mg^{-1} protein 2 h^{-1}, versus 27.93 ± 0.55 ng mg^{-1} protein 2 h^{-1} at amphetamine = 0 M, $n=7$, $P<0.01$) in the presence of IBMX. Administration of SQ22536, an adenylyl cyclase inhibitor, decreased the production of cyclic AMP, and decreased the basal release of testosterone *in vitro* (7.01 ± 0.53 ng mg^{-1} protein 2 h^{-1}, $n=7$, versus basal group, 10.16 ± 1.15 ng mg^{-1} protein 2 h^{-1}, $n=7$, $P<0.05$) (Figure 2). Amphetamine did not alter the release of testosterone in the presence of SQ22536 (6.95 ± 0.32 to 7.80 ± 0.18 ng mg^{-1} protein 2 h^{-1}, $n=7$–8, versus 7.01 ± 0.53 ng mg^{-1} protein 2 h^{-1} at amphetamine = 0 M, $n=8$).

Administration of hCG (0.05 iu ml^{-1}) significantly ($P<0.01$) increased the accumulation of cyclic AMP in testicular interstitial cells (hCG-treated group 62.08 ± 5.17 fmol mg^{-1} protein 2 h^{-1}, $n=8$, versus control group 38.21 ± 2.72 fmol mg^{-1} protein 2 h^{-1}, $n=8$) (Figure 3). Amphetamine (10^{-12} to 10^{-6} M) also increased the cyclic AMP content of the testicular interstitial cells (50.06 ± 1.66 to 64.30 ± 4.04 fmol mg^{-1} protein 2 h^{-1}, $n=8$, versus control group, $P<0.05$

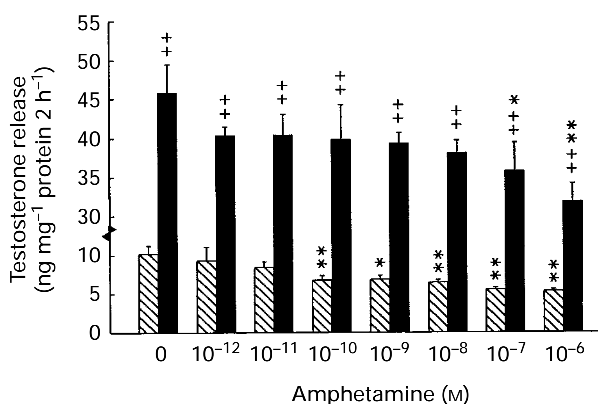

Figure 1 The release of testosterone in rat testicular interstitial cells at different doses of amphetamine in the presence (solid columns, $n=8$) or absence (hatched columns, $n=8$) of hCG (0.05 iu ml^{-1}). *$P<0.05$ and **$P<0.01$ compared with control (amphetamine 0 M). $^{++}P<0.01$ compared with non-hCG treated group. Each column represents mean ± s.e.mean.

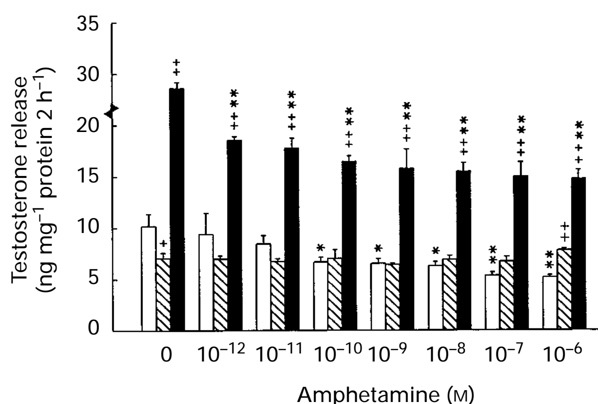

Figure 2 Effects of amphetamine (10^{-12}–10^{-6} M) on testosterone release in rat testicular interstitial cells pretreated with vehicle (open columns, $n=7$), SQ22536 (hatched columns, $n=7$) or IBMX (solid columns, $n=7$). *$P<0.05$ and **$P<0.01$ compared with control (amphetamine 0 M). $^{+}P<0.05$ and $^{++}P<0.01$ compared with vehicle-treated group. Each column represents mean ± s.e.mean.

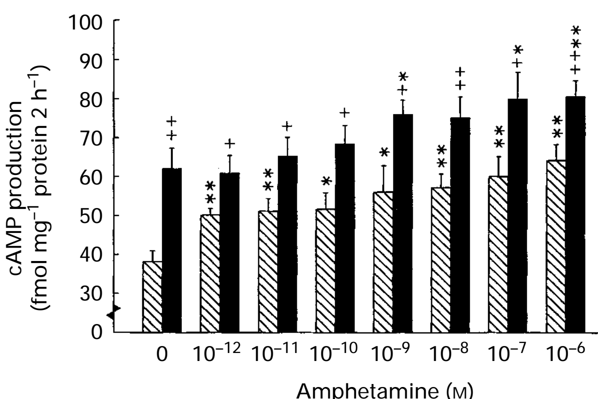

Figure 3 Dose-dependent effect of amphetamine on the accumulation of cyclic AMP in rat testicular interstitial cells after incubation in the absence (hatched columns, $n=8$) and presence (open columns, $n=8$) of hCG (0.05 iu ml^{-1}). *$P<0.05$ and **$P<0.01$ compared with control (amphetamine 0 M). $^{+}P<0.05$ and $^{++}P<0.01$ compared with control group. Each column represents mean ± s.e.mean.

or 0.01). In addition, amphetamine (10^{-9}, 10^{-7} and 10^{-6} M) enhanced the hCG-stimulated cyclic AMP accumulation (76.08 ± 3.63 to 80.7 ± 3.95 fmol mg^{-1} protein 2 h^{-1}, $n=8$, versus hCG-treated group, $P<0.05$ or 0.01).

Effects of calcium on amphetamine decreased testosterone secretion

Administration of nifedipine (a L-type calcium channel blocker) 10^{-6} M resulted in a significant decrease in the release of testosterone (5.17 ± 0.96 ng mg^{-1} protein 2 h^{-1}, $n=8$, versus nifedipine = 0 M 10.04 ± 1.33 fmol mg^{-1} protein 2 h^{-1}, $n=8$, $P<0.01$) (Figure 4). However, nifedipine (10^{-6} M) failed to enhance the inhibition of testosterone release induced by amphetamine ($10^{-10}-10^{-6}$ M).

Effect of amphetamine on the activities of 3β-HSD, P450c17 and 17-KSR

Administration of androstenedione (10^{-9} and 10^{-7} M) increased testosterone release (30.56 ± 1.63 to 46.47 ± 2.15 ng mg^{-1} protein 2 h^{-1}, $n=8$, versus control group 10.72 ± 0.80 ng mg^{-1} protein 2 h^{-1}, $n=8$, $P<0.01$, respectively, Figure 5). Amphetamine (10^{-8} or 10^{-6} M) not only decreased the basal release of testosterone (7.65 ± 0.94 and 6.59 ± 1.10 ng mg^{-1} protein 2 h^{-1}, $n=8$, $P<0.01$), but also attenuated the testosterone response to androstenedione (37.07 ± 1.42 and 32.65 ± 1.97 ng mg^{-1} protein 2 h^{-1}, versus 46.47 ± 2.15 ng mg^{-1} protein 2 h^{-1}, at androstenedione = 10^{-7} M, $n=8$, $P<0.01$. Figure 5).

In the presence of 10^{-9} M androstenedione, amphetamine produced a concentration-dependent decrease in the release of testosterone from the interstitial cells (31.18 ± 1.50 to 22.63 ± 1.44 ng mg^{-1} protein 2 h^{-1}, $n=8$, versus 37.64 ± 1.46 ng mg^{-1} protein 2 h^{-1}, $n=8$, $P<0.01$) (Figure 6).

Incubation of interstitial cells with [^{3}H]-pregnenolone for 2 h resulted in the formation of [^{3}H]-progesterone, [^{3}H]-17α-hydroxyprogestrone, [^{3}H]-DHEA, [^{3}H]-androstenedione, [^{3}H]-testosterone (Figure 7).

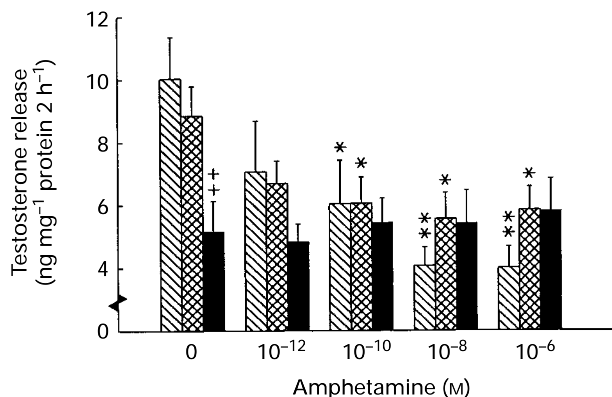

Figure 4 Effect of amphetamine on the release of testosterone following incubation of rat testicular interstitial cells with graded concentrations of nifedipine (hatched columns, 0 M; cross-hatched columns, 10^{-7} M; solid columns, 10^{-6} M). *$P<0.05$ and **$P<0.01$ compared with control (amphetamine 0 M). $^{++}P<0.01$ compared with control group. Each column represents mean ± s.e.mean.

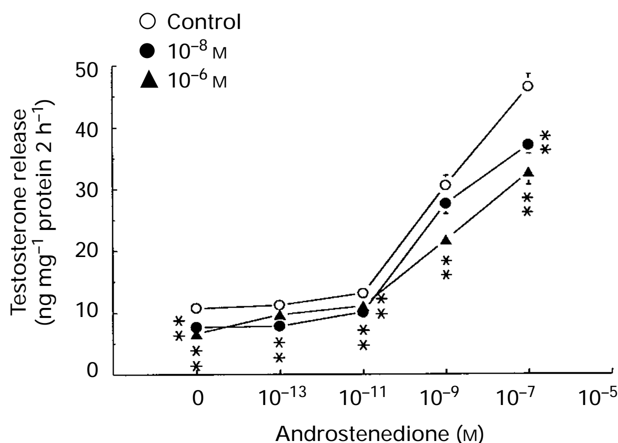

Figure 5 Effect of amphetamine (control, 0 M; 10^{-8} M; 10^{-6} M) on the release of testosterone following incubation of rat testicular interstitial cells with androstenedione at different doses. **$P<0.01$ compared with control. Each symbol represents the mean and vertical lines show s.e.mean.

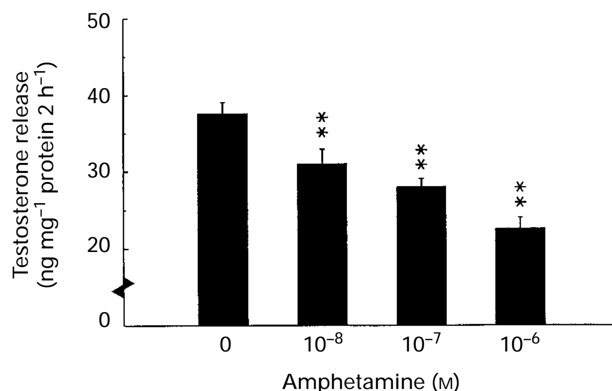

Figure 6 Dose-dependent effect of amphetamine on the release of testosterone after incubation of rat testicular interstitial cells with 10^{-9} M androstenedione. **$P<0.01$ compared with control (amphetamine 0 M). Each column represents mean ± s.e.mean.

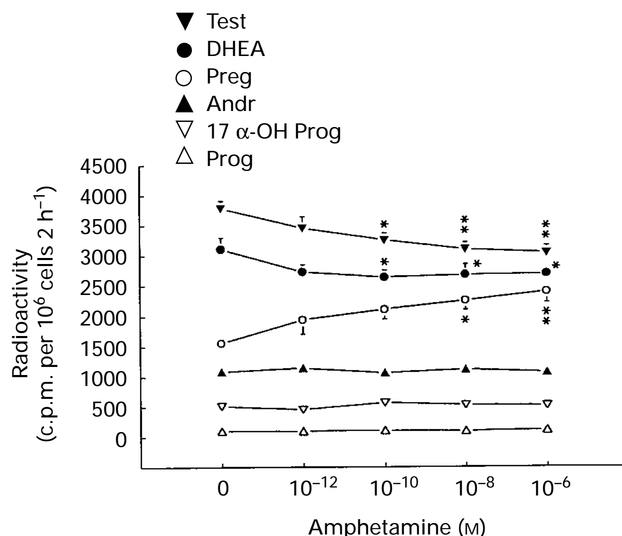

Figure 7 Effects of amphetamine ($10^{-12}-10^{-6}$ M) on the activities of P450c17, 3β-HSD and 17-ketosteroid reductase in testicular interstitial cells. Rat testicular interstitial cells were incubated with [^{3}H]-pregnenolone (10000 c.p.m.) and different doses of amphetamine at 37°C for 2 h. The medium was extracted by ether, dried and then reconstituted in ethanol before analysis by thin layer chromatography (t.l.c.). [^{3}H]-testosterone (Test) [^{3}H]-DHEA, [^{3}H]-pregnenolone (Preg), [^{3}H]-androstenedione (Andr), [^{3}H]-17α-hydroxyprogesterone (17α-OHProg), and [^{3}H]-progesterone (Prog) levels were measured. *$P<0.05$ and **$P<0.01$ compared with control (amphetamine 0 M). Each symbol represents mean ± s.e.mean.

Amphetamine dose-dependently increased the accumulation of [^{3}H]-pregnenolone, but decreased the production of [^{3}H]-DHEA and [^{3}H]-testosterone. The production of [^{3}H]-17α-hydroxyprogesterone and [^{3}H]-androstenedione was not altered by amphetamine. Only trace amounts of [^{3}H]-progesterone were detected and the 3β-HSD activity (conversion of [^{3}H]-pregnenolone to [^{3}H]-progesterone) appeared to be inhibited. Since the accumulation of [^{3}H]-pregnenolone was increased by 45–55%, the P450c17 activity (conversion of [^{3}H]-pregnenolone into [^{3}H]-DHEA) was inhibited with 10^{-8}–10^{-6} M amphetamine. Moreover, the 17-KSR activity (conversion of [^{3}H]-androstenedione to [^{3}H]-testosterone) was decreased by 14–19% with 10^{-10}–10^{-6} M amphetamine.

Discussion

In the present study we found that increased cyclic AMP accumulation, decreased Ca^{2+} channel activity and decreased activity of 3β-HSD, P450c17, and 17-KSR contributed to the inhibition of testosterone production induced by the administration of amphetamine in rat testicular interstitial cells.

We previously demonstrated that administration of amphetamine diminishes both basal and hCG-stimulated testosterone release *in vitro* and *in vivo*, but increases the accumulation of testicular cyclic AMP (Tsai *et al*., 1996a). In the present study, dispersed testicular interstitial cells, a cell population containing $\approx$20% Leydig cells, were employed to determine whether amphetamine had a direct effect on Leydig cells and to explore the mechanisms through which amphetamine could exert its action. The present experiments demonstrated that both the basal and the hCG-stimulated production of testosterone by testicular interstitial cells are markedly reduced, but that cyclic AMP accumulation is increased by the administration of amphetamine. It is established that hCG stimulates testosterone secretion both *in vivo* (Saez & Forest, 1979; Padron *et al*., 1980; Wang *et al*., 1994; Tsai *et al*., 1996a) and *in vitro* (Simpson *et al*., 1987; Nakhla *et al*., 1989; Liao *et al*., 1991; Wang *et al*., 1994; Tsai *et al*., 1996a), by mechanisms dependent on cyclic AMP generation (Avallet *et al*., 1987; Petersson *et al*., 1988; Sakai *et al*., 1989; Wang *et al*., 1994; Tsai *et al*., 1996a). However, the stimulant effect of hCG on cyclic AMP generation in rat testes is enhanced by amphetamine (Tsai *et al*., 1996a). The increase in testicular cyclic AMP induced by amphetamine reflects a correlation between activation of adenylyl cyclase and inhibition of testosterone production following administration of amphetamine. In the present study, IBMX, a non-selective phosphodiesterase inhibitor, significantly increased the basal release of testosterone and potentiated the inhibitory effect of amphetamine. Furthermore, SQ22536, an adenylyl cyclase inhibitor (10^{-6} M) effectively inhibited the basal release of testosterone, but failed to modify the depression of testosterone release induced by amphetamine. SQ22536 may prevent the effect of amphetamine by inhibiting the activity of adenylate cyclase. These results confirm our previous findings and suggest that an upregulation of cyclic AMP production is involved in the mechanisms of action of amphetamine.

It is now apparent that the Ras pathway, including the protein product of the Ras oncogene, provides an important path for transmitting hormones, cytokines, and growth factors (Marx, 1993) and a mechanism for biological 'crosstalk' between cyclic AMP and Ras pathway has been proposed. Thus cyclic AMP may act through protein kinase A (PKA) to block transmission of Ras to Raf-1 signalling in some cells, e.g. adipocytes, fibroblasts and cancer cells; it therefore prevents activation of mitogen activated protein (MAP) kinase, and thus may inhibit the cellular responses to hormones, cytokines or growth factors (Cook & McCormick, 1993; Sevetson *et al*., 1993; Wu *et al*., 1993; Häfner *et al*., 1994; Gallo *et al*., 1995). It is possible that as in other cells, both cyclic AMP and Ras pathways are linked in rat Leydig cells, and activated by amphetamine. The cyclic AMP produced in response to amphetamine may act through PKA to block the Ras pathway and subsequently inhibit the release of testosterone. This hypothesis requires further investigation.

Calcium ions play an important role in the control of Leydig cell steroidogenesis (Hall *et al*., 1981; Themmen *et al*., 1986; Foresta & Mioni, 1993; Foresta *et al*., 1995) and some studies have suggested that this ion contributes to the amphetamine signal transduction pathway (Dilullo & Martin-Iverson, 1992; Giambalvo, 1992; Vislobokov *et al*., 1993). We evaluated the role of transmembrane Ca^{2+} flux on amphetamine-decreased steroidogenesis by use of the Ca^{2+} channel blocker, nifedipine, and found that nifedipine reduced the basal release of testosterone but did not alter the effect induced by amphetamine. These results suggest that nifedipine and amphetamine share the same mechanism for regulation of testosterone production.

The final step in testosterone biosynthesis is the reduction of androstenedione to testosterone through the activity of the microsomal enzyme 17-KSR, also referred to as 17β-hydroxysteroid dehydrogenase. Administration of androstenedione produced a concentration-dependent increase in the release of testosterone, but this response was attenuated by amphetamine. The interconversion of androstenedione and testosterone is reversible with substrate and product concentrations regulating the direction of the action (Hall, 1988). Deficiency in testicular 17-KSR appears to account for most defects in testosterone synthesis in man.

In the present study, amphetamine increased the accumulation of [^{3}H]-pregnenolone, indicating that amphetamine inhibits the activity of P450c17. The amount of [^{3}H]-progesterone detected was very low and not altered by amphetamine, but the formation of the downstream metabolite-[^{3}H]-DHEA was decreased by amphetamine, indicating that the activities of P450c17 and 3β-HSD were decreased by administration of amphetamine. The decreased production of [^{3}H]-testosterone demonstrated the inhibitory effect of amphetamine on the activity of 17-KSR. The inhibition of 17-KSR in response to amphetamine reflects a strong correlation between the decreased steroidogenesis and the inhibition of testosterone production in testicular interstitial cells. Our data thus demonstrate that amphetamine, at a concentration greater than 10^{-10} M is effective in increasing cyclic AMP production but that it decreases the activity of 17-KSR and the release of testosterone. These phenomena suggest that amphetamine inhibits testosterone secretion via mechanisms involving an increased cyclic AMP production and decreased enzyme activities of steroidogenesis.

Anakwe & Payne (1987) demonstrated that cyclic AMP induced P450c17 synthesis and increased the rate of P450scc *de novo* synthesis. Keeney and Mason (1992) demonstrated increased 3β-HSD activity, protein synthesis and mRNA levels in cultures of Leydig cells from adult rats treated with luteinizing hormone (LH), cyclic AMP or forskolin. Purified Leydig cells isolated from adult rats treated with 5 iu hCG for 6 days exhibited approximately a two fold increase in 3β-HSD, P450c17 activities with no change or a slight decrease in 17-KSR activity. In contrast to the effect observed with daily low doses of hCG, administration of a single high dose of hCG or LH resulted in a decrease in LH receptors, a decrease in *in vitro* hCG-stimulated testosterone production as well as a marked decrease in the P450c17 activity (O'Shaughnessy & Payne, 1982). Payne and Sha (1991) found high basal expression of 3β-HSD mRNA in mouse cultured Leydig cells maintained in the absence of cyclic AMP. Increasing the concentration of cyclic AMP for 24 h resulted in a marked decrease in the 3β-HSD level.

The testicular cell preparation we employed included a population of macrophages. Since macrophage-conditioned medium is inhibitory to both basal (Lombard-Vignon *et al*., 1991) and LH/hCG-stimulated testosterone production (Sun *et al*., 1993; Sum & Risbridger, 1994) and cytokines, such as interleukin-1 and tumour necrosis factor-α, either added exogenously or generated *in vivo*, inhibit steroidogenesis in Leydig cells (Sun & Risbridger, 1994; Hales, 1996), it is also possible

that the effects of amphetamine on testosterone production described here are mediated in part by cytokines.

Our results suggest that amphetamine inhibits testosterone secretion in Leydig cells through a mechanism involving increased cyclic AMP production, decreased Ca^{2+} influx through L-type Ca^{2+} channel and decreased activity of 3β-HSD, P450c17 and 17-KSR.

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