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THE DYNAMICS OF THE TESTOSTERONE RESPONSE OF PERIFUSED MOUSE LEYDIG CELLS TO hCG AND ARGININE VASOPRESSIN

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The effect of hCG and Arginine-Vasopressin (AVP) on testosterone production by purified mouse Leydig cells was examined under dynamic conditions in a perifusion sytem. A rapid and dose-dependent increase in testosterone release was induced by a 5 min exposure of the cells to increasing concentrations of hCG (0.01 to 1 ng/ml). The testosterone response to hCG was Gaussian in distribution with a peak value by 100 min. A 12 h pretreatment of Leydig cells with 10^{-5} M AVP enhanced testosterone accumulation in the perifusate under basal conditions, but markedly reduced the hCG-stimulated testosterone production. The basal and hCG-stimulated testosterone secretion profiles by freshly isolated Leydig cells were, however, unaffected by the continuous presence of the same dose of AVP. These results support the finding that AVP acts directly on Leydig cells. They support the hypothesis of a possible role of neurohypophysial peptides on reproductive functions in the mouse by modulating steroidogenesis at the testicular level.

It has been previously reported that administration of the pineal peptide Arginine-Vasotocin (AVT) to the male, exerts inhibitory effects on the reproductive function (1). These effects appeared to result from the suppression of pituitary gonadotropins by AVT (2). Recently, the neurohypophysial hormones Arginine-Vasopressin (AVP) and oxytocin have been identified in adrenal glands (3,4), ovaries (5) and testes (4,6,7) from several species including the human. The large amount of vasopressin-like peptide found in the testes of Brattleboro rats which are deficient in pituitary and circulating AVP (8) indicated that this peptide could be synthesized locally within the testis itself. These observations, together with the characterization of specific AVP receptor in Leydig cells (8) suggested that these

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neurohypophysial peptides might exert some physiologic function in the normal animal. Recently we have established an in vitro perifusion system of pure mouse Leydig cells (10). In the present investigation, the direct effect of AVP on basal and hCG-stimulated testosterone production by purified mouse Leydig cells was examined under dynamic conditions as a function of time.

MATERIALS AND METHODS

MATERIALS. Human chorionic gonadotropin (hCG, 9000 IU/mg) and Arginine^8-vasopressin (AVP) were provided respectively by Boehringer, Mannheim France and Calbiochem (San Diego, CA, U.S.A.). Bovine serum albumin (BSA) and Collagenase type I (144 u/mg) were from ICN Pharmaceuticals Inc. (Cleveland, OH, U.S.A.), Glucose, Deoxyribonuclease I (DNA-ase I) from Sigma Chemical Co (St Louis, MO, U.S.A.). L-Glutamine, Sodium bicarbonate, N-$_2$ hydroxyethyl-piperazine-N'-$_2$ ethanesulfonic acid (Hepes) and medium 199 were obtained from Eurobio (Paris, France). Bio-Gel P$_2$ (200-400 mesh) and Percoll were purchased respectively from Bio-Rad Laboratories (Richmond, CA, U.S.A.) and Pharmacia (Uppsala, Sweden).

Leydig cells purification. Adult male Swiss mice (Janvier, France) were killed by cervical dislocation and testes were immediately excised and decapsulated. Cell suspension of whole testes was prepared as previously reported (11). Ten decapsulated testes were sustained to collagenase digestion in 10 ml medium 199 containing 0.1 % BSA, 3 mg collagenase and 40 µg DNAase I at 37°C in a shaking water bath for 30 min. After dispersion, the cell suspension was washed twice with 0.9 % NaCl, decanted, filtered through nylon gauze (86 µm) and centrifuged at 150 g for 15 min. The crude intertubular cell preparation was resuspended in 2 ml BSA-medium 199 and layered on the top of a continuous Percoll-gradient as previously described (12). Bands of highly purified Leydig cells were aspirated and 3 vol BSA-medium 199 were added. After centrifugation at 150 g for 15 min, the cell pellets were resuspended in BSA-medium 199 and the total number of purified Leydig cells was determined by counting aliquots in a haemocytometer. Cell viability assessed by trypan blue exclusion was greater than 95 %. The percentage of Leydig cells as determined by histochemical staining for 3β-hydroxysteroid dehydrogenase was 90-95 %.

Perifusion of purified Leydig cells. The perifusion method used was as previously described (13). Approximately 10^6 Leydig cells were mixed with 0,5 g Bio-Gel P$_2$. A 2 ml plastic syringe served as the perifusion column and a peristaltic pump (Bioblock - G 31098) was used to propel the medium through the perifusion chamber at approximately 0.1 ml/min. The perifusate used consisted of medium 199 containing 0.1 % BSA, 7 µmol/ml L-Glutamine, 350 µg/ml $NaHCO_3$, 1 mmol/ ml Hepes, 50 µg/ml gentamycin-sulfate and 4.5 mg/ml glucose. The columns were placed in a 37°C water bath and the out-flow line was connected to a fraction collector with which eluate samples were obtained at 5 min intervals. When indicated in the results section AVP or hCG were added to the medium. The eluates collected were stored at -20°C until testosterone assay. Since the rates of testosterone secretion varied somewhat from one experiment to another, each experiment (as represented by one panel in the figures) was performed with parallel columns using the same preparation of purified Leydig cells at the same time.

Steroid assay. Testosterone levels in the eluate from columns were assayed after appropriate dilutions by a specific radioimmunoassay as previously

described (11) and expressed as ng/10^6 3β-HSD-positive cells. The sensitivity of the assay was 7 pg/sample. The inter and intra-assay coefficients of variation for the assay were < 8 %. The cross-reaction with 5α-dihydrotestosterone was 36 % but was < 2 % with other steroid hormones.

RESULTS

Dose-response of Leydig cells to hCG. A stable basal release of testosterone was achieved when Leydig cells were perifused for about 60 min, at which time the level of testosterone was approximately 0.5 ng per fraction (Fig. 1A). After this equilibrium time, a 5 min infusion of hCG at concentrations ranging from 0.01 to 1 ng/ml, induced a dose-dependent increase in testosterone production. The testosterone profiles after acute hCG-stimulation, presented a rapid rise (lag period < 15 min) to a peak value by 100 min. As shown in Fig.1A a maximal response was obtained with 1 ng/ml hCG. At the end of the experiment lasting around 6 h, the testosterone levels remai-

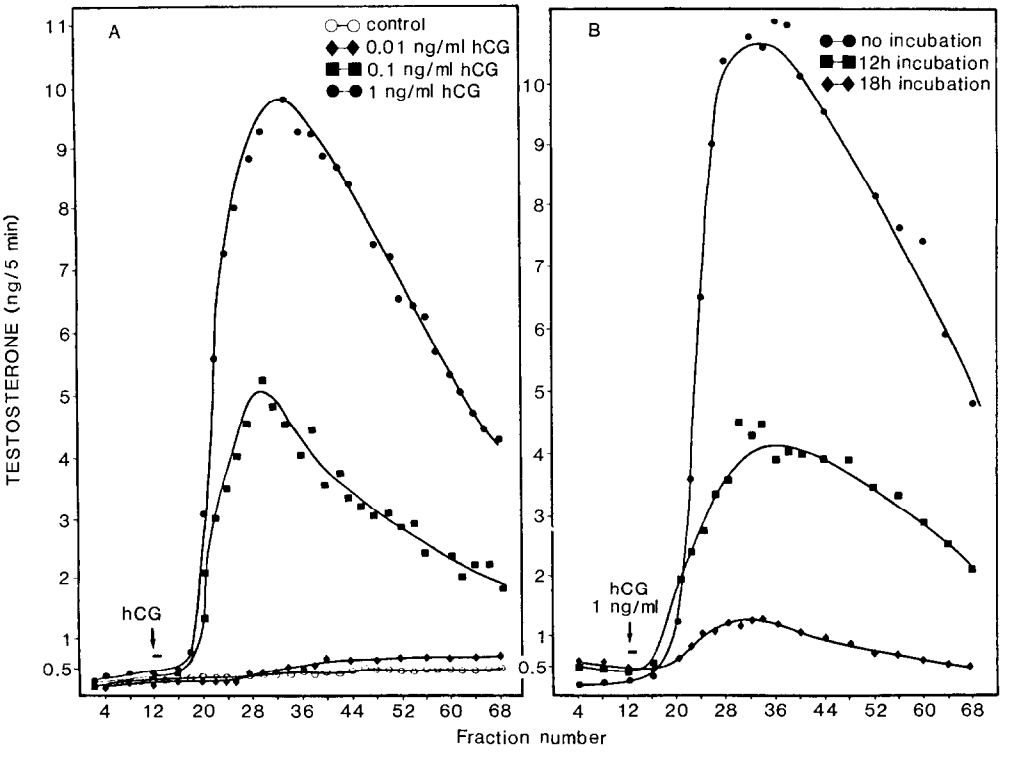

Figure 1. Effect of hCG and of preincubation period on the testosterone response of perifused mouse Leydig cells. Parallel columns containing freshly isolated (A) or preincubated mouse Leydig cells for 0, 12 and 18 h in hormone free-medium (B), were perifused for 6 h. Eluate samples were collected at 5 min intervals. Where indicated by the arrow, the columns were infused for 5 min with hCG. The data were typical of 3 separate experiments.

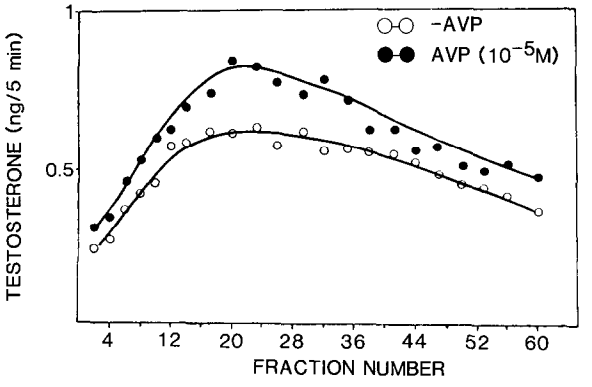

Figure 2. Effect of AVP pretreatment on basal testosterone secretion. Purified mouse Leydig cells were preincubated for 12 h in the presence or absence of 10^{-5}M AVP. After this incubation period, the columns were washed for 30 min and perifused in parallel for 6 h with hormone-free medium. The data were typical of 3 separate experiments.

ned, however, higher than the basal values. When concentrations higher than 1 ng/ml of hCG were used, the stimulation was maintained at a plateau during a longer time period and a slow time-dependent decrease was observed (data not shown).

Effect of the incubation period on testosterone response to hCG. Fig.1B shows that testosterone response to hCG after cell-incubation was markedly decreased compared to that of freshly isolated Leydig cells. The loss of the response to hCG at the peak values was about 60 and 86 % after cell-incubation for 12 h and 18 h respectively. Microscopic examination of the cells at the end of the experiment showed that cell-viability was unaffected by the incubation period.

Effect of AVP treatment on basal and hCG-stimulated testosterone production. To determine the effect of AVP on basal testosterone secretion, two columns containing Leydig cells preincubated with or without 10^{-5}M AVP for 12 h, were perifused simultaneously with hormone-free medium. The testosterone secretion profiles show that pretreatment of the cells by AVP enhanced testosterone secretion over controls throughout of the perifusion period (Fig. 2). Statistical analysis showed that testosterone secretion in the fraction corresponding to 60 min after the start of perifusion was significantly higher ($P < 0.02$, $n = 5$; paired t-test) in AVP-pretreated cells than in

Table 1

Purified mouse Leydig cells were preincubated for 12 h with or without 10^{-5}M AVP. After this time, the two separate columns were washed for 30 min and perifused in parallel with hormone-free medium. Each value represents testosterone secretion in the fraction corresponding to 60 min after the start of perifusion. Values given were from 5 different experiments.

	TESTOSTERONE (ng/5 min)	
	TREATMENT	
EXP.	CONTROLS	AVP (10^{-5}M)
1	0.581	0.873
2	0.463	0.582
3	0.593	0.720
4	0.696	0.989
5	0.532	0.618

AVP-treated significantly different from controls ($P < 0.02$; paired t-test).

controls (Table 1). The means $\pm$ s.e.m. (ng testosterone/5 min) were 0.573 ± 0.038 and 0.756 ± 0.077 in control and in AVP-pretreated Leydig cells respectively. In contrast, the pretreatment of Leydig cells by AVP

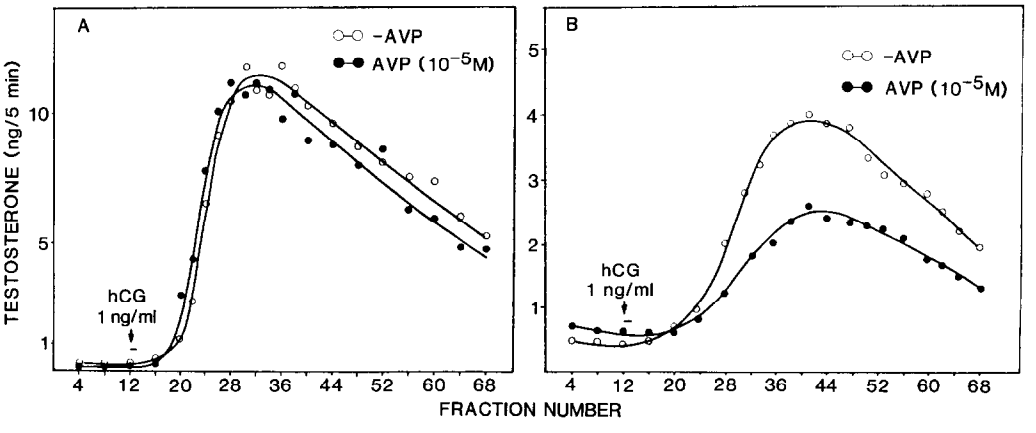

Figure 3. Effect of AVP treatment on the hCG-stimulated testosterone response. Freshly purified mouse Leydig cells were continuously perifused in parallel columns with medium containing or not 10^{-5}M AVP (A). Mouse Leydig cells were preincubated for 12 h in the presence or absence of 10^{-5}M AVP. After this incubation period, columns were washed and perifused simultaneously with hormone-free medium (B). Where indicated by the arrow, the columns were infused for 5 min with 1 ng/ml hCG. These data were typical of 3 separate experiments.

induced a marked decrease of the hCG-stimulated testosterone production compared to the untreated cells (Fig.3B). This reduction was approximately 40 % under control levels at the peak values. Similar results were obtained with a longer AVP-pretreatment period of the cells (data not shown). Such stimulatory and inhibitory effects of AVP were, however, not observed when freshly isolated Leydig cells were continuously perifused with AVP (Fig.3A).

DISCUSSION

The present study clearly indicates that AVP can directly influence testosterone production by the adult mouse testis. This is strongly supported by the fact that this neurohypophysial peptide affects the steroidogenic capacity of purified Leydig cells in a perifusion system. In addition our data show that this hormone exerts positive and negative effects respectively on basal and hCG-stimulated testosterone production. These results are partly consistent with recent data which show that both AVP and oxytocin inhibit gonadotropin-stimulated testosterone production in cultured rat testicular cells (14). They are also in agreement with the hypothesis that this inhibitory effect on testosterone synthesis could result from a decrease of the 17α-hydroxylase and 17,20_desmolase activities, since our own preliminary results (unpublished observations) showed an accumulation of progesterone in the perifusate of Leydig cells exposed to hCG. A more surprising observation is, however, that basal testosterone levels were increased after pretreatment of the Leydig cells by AVP alone. To our knowledge this is the first demonstration of a direct stimulatory effect of this peptide on testosterone production by a pure Leydig cell preparation. It is interesting to note that such an effect has been reported for human luteal cells exposed to low concentrations of oxytocin (15). Our kinetic observations that AVP stimulates basal testosterone production differ from those of other authors (16) who did not observe such a stimulatory effect in cultured testicular interstitial cells derived from the rat. Whether this discrepancy is due to methodological or species differences is presently unknown. The perifusion system has been largely used for the study of the

steroidogenic regulatory mechanisms (17). This model which presents some analogies with the in vivo situation could point out phenomena that are not revealed in static long-term incubations. On the other hand, a species specificity could explain this discrepancy. It is noteworthy that mouse Leydig cells are unresponsive to LHRH and its agonists which are known to locally affect the testicular androgenic capacity in the rat (18). The present study has failed to identify the precise cause for the increase of basal testosterone production in response to AVP exposure of the Leydig cells. A likely explanation could be that the peptide increases the cholesterol side-chain cleavage activity. Such a regulation by AVP has been suggested in the rat testis (16). As yet we have no information regarding the step of the steroidogenic pathway affected by AVP exposure, since in basal conditions, C_{21} steroid hormones were undetectable in the perifusate. The present results that both stimulatory and inhibitory effects of AVP occurred only after pretreatment for 12 h of Leydig cells with AVP are also puzzling. A similar phenomenon has been reported for other testicular regulatory agents such as catecholamines (19). Whether the appearance of the AVP response as a function of time is due to de-novo induction of new AVP-receptors, regulatory proteins or to other processes needs further investigations.

In conclusion, our results that AVP affects both positively and negatively the Leydig cell steroidogenic capacity support the finding that neurohypophysial peptides act as local modulators of testicular function. Additional studies are required to elucidate the exact physiological significance of these peptides in the overall function of the testis.

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