Testosterone production by mouse Leydig cells is stimulated in vitro by atrial natriuretic factor

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The synthetic atrial peptides, rat atrial natriuretic peptide, atriopeptin I and atriopeptin II, stimulated testosterone production by mouse Leydig cells in a time- and concentration-dependent manner. The maximum stimulation of the steroidogenesis in response to the peptides was 6–10-fold over the basal level, as compared with 20–24-fold stimulation obtained with saturating concentrations of hCG. The stimulation of steroidogenesis by the most potent peptide, atriopeptin II, was markedly enhanced in the presence of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, suggesting an involvement of cyclic nucleotides. However, neither basal nor hCG-stimulated levels of cAMP were altered by the peptide, though testosterone production in response to submaximal concentrations of hCG was increased in the presence of atriopeptin II. The nature of the second messenger involved and the mechanism of action of the atrial peptides may by elucidated by further research in progress.

(Mouse Leydig cell) Atrial natriuretic factor Testosterone production cyclic AMP

1. INTRODUCTION

In 1981, De Bold et al. [1] reported that the injection of rat atrial muscle extract into rats produced a marked diuresis, natriuresis and hypotension. Since then, several forms of the polypeptide, atrial natriuretic factor (ANF) have been isolated and identified [2–6]. The molecular mechanism of action of ANF remains unclear. However, on the basis of the reported data [7–10], it has been suggested that the effects of ANF are mediated through cyclic GMP (cGMP). Waldman et al. [9] showed that the guanylate cyclase activity in a membrane preparation from kidney was rapidly stimulated by ANF and that the phenomenon of guanylate cyclase activation by ANF was not restricted to kidney alone but was also observed in other tissues such as aorta, lung, intestine and testis. This observation implied that ANF may be involved in the regulation of cellular functions in diverse tissues. Indeed, ANF has recently been shown to inhibit steroidogenesis in adrenocortical cells [11–15] and murine tumour Leydig cells [16]. Atarashi et al. [11] reported that the crude extract of rat atria could inhibit both basal as well as ACTH- and angiotensin II-stimulated aldosterone production by rat zona glomerulosa cells. Similar observations have been reported by other authors [12–15] using synthetic ANF. The suppression of steroidogenesis has been attributed to the ability of ANF to stimulate cGMP formation [15]. In murine tumour Leydig cells synthetic ANF inhibited both basal and gonadotrophin-stimulated progesterone production, accompanied by a marked inhibition of cyclic AMP (cAMP) formation and stimulation of cGMP accumulation [16]. These observations on the effect of ANF on steroidogenesis prompted us to investigate whether testosterone production by purified mouse Leydig cells could be modulated by ANF. Here three different forms of ANF have been used, which are as follows: (a) rat atrial natriuretic peptide (rANP), (b) atriopeptin I (rAP-I) and (c) atriopeptin II (rAP-II).

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2. MATERIALS AND METHODS

The following synthetic peptides were obtained from Serva, Heidelberg: rANP(1–28) [2], rAP-I(5–25) [4] and rAP-II(Phe-22,Arg-23) [4]. Highly purified human chorionic gonadotrophin (hCG, 13500 IU/mg) was from Boehringer, Mannheim.

The sources of other chemicals used were as follows: bovine serum albumin from Sigma, Munich; 3-isobutyl-1-methylxanthine (IBMX) from Aldrich-Europe, Beerse, Belgium; minimum essential medium with Earle's salts from Gibco Europe, Karlsruhe; Hepes from Serva, Heidelberg.

Preparation and purification of Leydig cells from the testes of adult NMRI mice have been described [17,18]. In general, percoll-purified Leydig cells (50000–100000 cells) were incubated in 500 μl minimum essential medium containing 25 mM Hepes, pH 7.4, and 0.1% bovine serum albumin (medium) for 3 h or as indicated at 36°C, with or without various test substances. All peptides were dissolved in 10 mM sodium phosphate buffer, pH 7.0, containing 0.15 mM NaCl and 0.1% bovine serum albumin, at a concentration of 10^{-4} M. This stock solution was appropriately diluted with medium prior to the addition to the incubation medium.

Testosterone produced in the medium was assayed by a radioimmunoassay as described in [17,18]. For the determination of cAMP, the incubation was stopped by the addition of 2.0 ml cold absolute ethanol followed by vortex-mixing. The ethanol extract was evaporated to dryness, the residue was dissolved in 200–500 μl of 0.1% sodium azide and the content of cAMP was measured by a specific radioimmunoassay [17,18].

Each experiment was performed at least twice. The results presented are mean ± SD of triplicate or quadruplicate determinations, as indicated in the legends to the figures.

3. RESULTS

The results presented in fig.1 show that a dose-related stimulation of testosterone production (A) occurred following incubation of mouse Leydig cells with varying concentrations of rAP-II, rAP-I and rANP. The amount of testosterone produced by these cells in the presence of maximal steroidogenic concentration of hCG (2 ng/ml) was

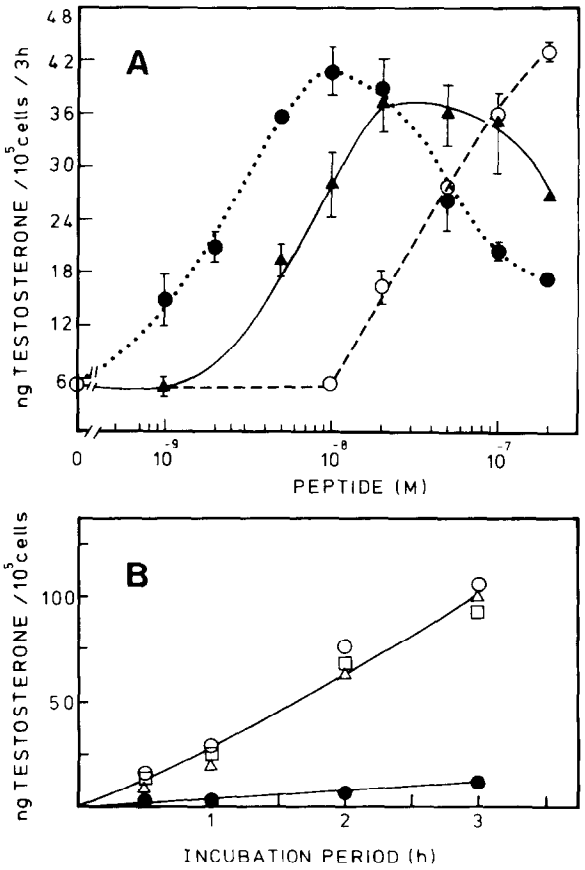

Fig.1. (A) Dose response of testosterone production by ANF in mouse Leydig cells. Percoll-purified mouse Leydig cells were incubated at 36°C for 3 h in the presence of varying concentrations of either rANP (▲), rAP-I (○) or rAP-II (●) and the amount of testosterone produced was measured by radioimmunoassay as mentioned in section 2. The data represent means ± SD of triplicate determinations. The vertical bars show the SD and have been omitted where they were smaller than the symbols. (B) Time course of stimulation of steroidogenesis in response to ANF. The cells were incubated in the absence of any addition (●) or in the presence of 1×10^{-7} M rANP (□), 1×10^{-7} M rAP-I (△) or 1×10^{-8} M rAP-II (○) for varying periods of time and testosterone produced was measured. The results are the means of three determinations. The SD in each case was less than 10% of the mean value and is not shown for the sake of clarity.

91.4 ± 9.0 ng/10^5 cells per 3 h. Thus, the maximum stimulation of steroidogenesis achieved with the peptides was about 40% of that obtained with 2 ng/ml hCG and was comparable to the level of steroidogenesis obtained with 0.1 ng/ml hCG. The

cells incubated with 0.1 ng/ml hCG produced 43.0 ± 4.6 ng testosterone/10^5 cells per 3 h. The maximal effective concentrations of rAP-II, rANP and rAP-I were 10^{-8}, 2×10^{-8} and 2×10^{-7} M, respectively. At higher concentrations of both rAP-II and rANP, a decline in steroidogenesis was noted. In an experiment where higher concentrations of rAP-I were used, a decline in the extent of stimulation of testosterone production was observed at concentrations of rAP-I beyond 5×10^{-7} M (not shown).

Half-maximum stimulation of steroidogenesis was achieved with 2.6×10^{-9} M rAP-II, 8.0×10^{-9} M rANP and 3.8×10^{-8} M rAP-I. The extent of maximum stimulation of steroidogenesis by each of the peptides varied somewhat between experiments using different batches of the cell preparations. In 6 separate experiments, the amounts of testosterone produced in the presence of maximal steroidogenic concentrations of the peptides varied between 6- and 10-times that produced in the absence of the peptides.

Fig.1B shows the time course of stimulation of testosterone production by Leydig cells in the presence of rAP-II (1×10^{-8} M), rAP-I (1×10^{-7} M) and rANP (1×10^{-7} M). It is evident that each of the three peptides caused a time-related increase in the amount of testosterone produced, and a significant difference between the stimulated and basal levels of steroidogenesis was observed as early as 1 h from the start of the incubation. Having shown that all three peptides can stimulate steroidogenesis, further experiments were carried out with the most potent one, rAP-II.

In the presence of a phosphodiesterase inhibitor, IBMX (0.25 mM), rAP-II-induced testosterone production by Leydig cells was markedly enhanced (fig.2). The concentration of rAP-II required to elicit a half-maximal steroidogenic response was reduced from 3.0×10^{-9} M in the absence of IBMX to 6.0×10^{-10} M in the presence of IBMX. There was little effect of IBMX on basal steroidogenesis. Low concentrations of rAP-II (2×10^{-10} and 5×10^{-10} M), which did not stimulate testosterone production at all in the absence of IBMX, effected an enhanced steroidogenic response when IBMX was present.

The effect of rAP-II on hCG-induced testosterone production by mouse Leydig cells is shown in fig.3. The basal level of testosterone pro-

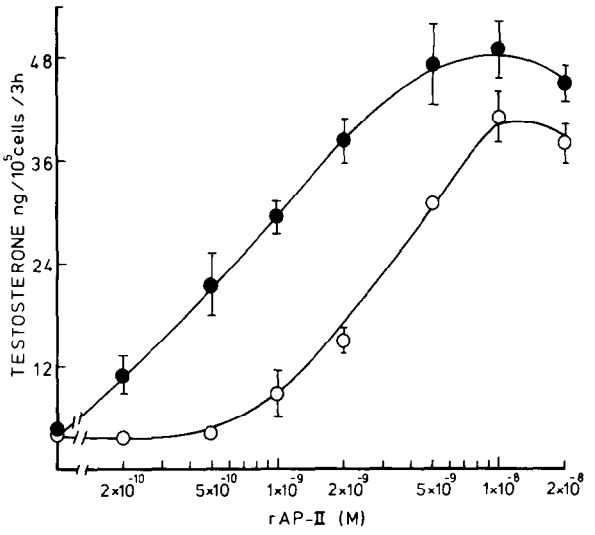

Fig.2. Effect of the phosphodiesterase inhibitor, IBMX, on the testosterone production by mouse Leydig cells in response to varying concentrations of rAP-II. The Leydig cells were incubated with varying concentrations of rAP-II in the absence (○) or presence (●) of 0.25 mM IBMX and the amount of testosterone produced was measured. Other details are the same as in the legend to fig.1.

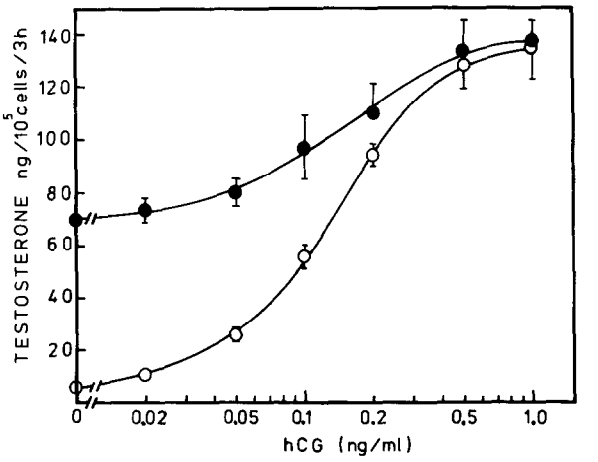

Fig.3. Effect of rAP-II on the dose-response curve of hCG. Mouse Leydig cells were incubated with varying concentrations of hCG in the absence (○) or presence (●) of 1×10^{-8} M rAP-II and testosterone produced was measured. Other details are the same as in the legend to fig.1.

duction was increased 10-fold by rAP-II. In the presence of rAP-II, low concentrations of hCG (0.02–0.1 ng/ml) produced increased amounts of testosterone. However, the differences in the amounts of the steroid produced in the presence or absence of rAP-II decreased gradually with increasing doses of hCG. At saturating concentrations of hCG, no effect of rAP-II was seen. Since it has been reported that only low concentrations of ANF (1×10^{-9} M) produced a strong inhibition of hCG and LH-stimulated progesterone production by mouse tumour Leydig cells and that higher concentrations of ANF (1×10^{-7}–1×10^{-6} M) were ineffective [16], we have examined the effect of addition of varying concentrations of rAP-II (10^{-10}–10^{-6} M) on testosterone production by mouse Leydig cells stimulated with a submaximal concentration of hCG (0.2 ng/ml). No inhibitory effect was observed at any concentration of rAP-II (not shown).

Fig.4 shows the effect of rAP-II on hCG-induced cAMP responses in mouse Leydig cells. In the presence of increasing concentrations of hCG, the amount of cAMP accumulated increased in a dose-related manner. The addition of increasing concentrations of rAP-II in the presence of 2 ng/ml hCG had no effect on the amount of cAMP accumulated. In a separate experiment, the effect of varying concentrations of rAP-II (10^{-9}–10^{-6} M) on the cAMP accumulation by the cells in the absence of hCG was examined and no increase in the basal level of cAMP was effected by rAP-II (Mukhopadhyay, unpublished).

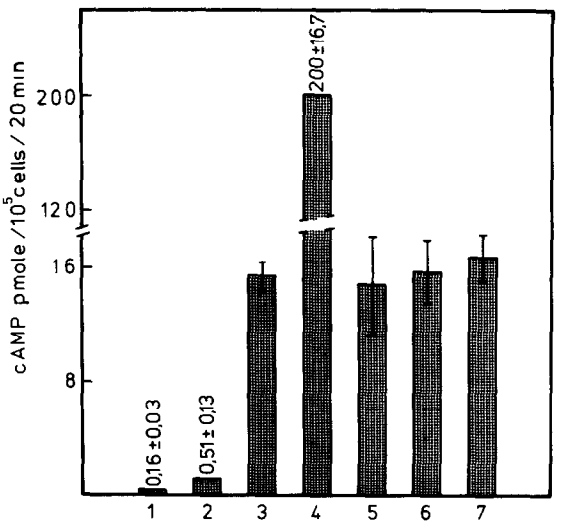

Fig.4. Effect of rAP-II on the hCG-stimulated cAMP response in mouse Leydig cells. The Leydig cells were incubated for 20 min in the presence of 0.25 mM IBMX with the following additions: (1) none; (2) 0.2 ng/ml hCG; (3) 2 ng/ml hCG; (4) 20 ng/ml hCG; (5) 2 ng/ml hCG plus 5×10^{-9} M rAP-II; (6) 2 ng/ml hCG plus 1×10^{-8} M rAP-II and (7) 2 ng/ml hCG plus 2×10^{-8} M rAP-II. cAMP produced was assayed as described in section 2. The data are means ± SD of quadruplicate determinations.

4. DISCUSSION

The results obtained demonstrate that ANF can stimulate steroidogenesis by percoll-purified mouse Leydig cells in a time- and dose-related manner. Among the peptides used, rAP-II was the most potent one and the least active peptide was rAP-I. In terms of natriuretic potency, rAP-I has been reported to be 4-times less potent than rAP-II [4]. In mouse Leydig cells, the steroidogenic potency of rAP-II was 15-times greater than rAP-I. The amino acid composition of rAP-I is identical to that of rAP-II except for the presence of Phe-Arg extension at the carboxy-terminal of rAP-II. The carboxy-terminal amino acids, therefore, appear to be important determinants for steroidogenic activity. The longer peptide, rANP, differs from rAP-II in having an extended chain of Ser-Leu-Arg-Arg residues at the amino-terminal end and an additional Tyr residue at the carboxy-terminal end. Whether the lower steroidogenic potency of rANP compared to rAP-II results from the addition of extra amino acid residues at the amino-terminal or at the carboxy-terminal, cannot at present be clarified. Further studies with other synthetic ANF peptides having various chain lengths are necessary to identify the activity-determinant region.

The stimulation of basal steroidogenesis by rAP-II was strongly potentiated by a phosphodiesterase inhibitor, IBMX, indicating therefore, that a cyclic nucleotide is involved in mediating the effect of rAP-II in mouse Leydig cells. However, neither basal nor hCG-stimulated levels of cAMP were increased by rAP-II, though in addition to stimulating the steroidogenesis in the absence of hCG, rAP-II also increased the amount of testosterone produced in response to submaximal hCG concentrations. Thus a role for cAMP ap-

pears to be unlikely. Of course, the possibility that rAP-II might have caused a small but undetectable increase in cAMP level that was sufficient to effect a stimulation of steroidogenesis, cannot be excluded yet. Alternatively, a rAP-II-induced shift from free to bound levels of cAMP might also be responsible for the stimulation of steroidogenesis. A role for cGMP has been indicated in mediating the effects of ANF in a variety of tissues [8–10,15,16]. The possibility remains that in mouse Leydig cells also, cGMP may be the second messenger involved. Available data do not offer as yet a choice between these possibilities and further studies are in progress to determine the nature of the second messenger.

We do not yet understand the reason for the discrepancy between the present result and that reported recently in murine tumour Leydig cells [16], where ANF markedly inhibited both steroidogenesis as well as cAMP accumulation. Possibly the action of ANF is specific to the type of target tissue under investigation. Indeed, the neurohypophysial peptide, GnRH, has been shown to stimulate steroidogenesis in rat Leydig cells [19] while in rat luteal cells progesterone production was inhibited by GnRH [20]. In an analogous manner, it may not be surprising if ANF produces opposite effects in different tissues.

The evidence on the effect of ANF on cAMP accumulation is rather equivocal. No effect of ANF on cAMP level was observed in single nephron segments and glomeruli [21], or in arterial plasma following infusion of ANF into canine renal artery [22] or in isolated rabbit aortic segments [8]. On the other hand, in murine tumour Leydig cells [16] and in the rat pituitary [23], a marked inhibitory effect of ANF on cAMP formation has been reported. The latter report was contradicted by Heisler et al. [24] who did not observe any effect of ANF on adenylate cyclase activity in the rat pituitary homogenate. The results in the present study also exclude an inhibitory effect of ANF on cAMP levels in mouse Leydig cells.

The physiological relevance of the observed effects of ANF on mouse Leydig cells still remains a matter of speculation. Further studies are necessary to define the role of ANF in the regulation of steroidogenesis.

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