Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 399-404

ATRIAL NATRIURETIC FACTOR REGULATES STEROIDOGENIC RESPONSIVENESS AND CYCLIC NUCLEOTIDE LEVELS IN MOUSE LEYDIG CELLS IN VITRO

Kailash N. Pandey[1], Spyros N. Pavlou[2], William J. Kovacs[2], and Tadashi Inagami[1]

Departments of Biochemistry[1] and Medicine[2]
Vanderbilt University, School of Medicine, Nashville, TN 37232

Received May 16, 1986

SUMMARY: The effects of synthetic atrial natriuretic factor (ANF) on the regulation of mouse Leydig cell steroidogenesis have been studied in vitro. ANF in nanomolar concentration increased testosterone production by more than 30-fold over basal levels. Concomitantly, cyclic guanosine monophosphate levels were increased 35-fold; cyclic adenosine monophosphate levels fell minimally (15-20%). ANF at low concentration ($1x10^{-11}$ M) inhibited testosterone production by luteinizing hormone-stimulated cells, while at higher concentration (> $2x10^{-9}$ M) ANF stimulated steroidogenesis beyond the level attained by luteinizing hormone alone. These results indicate that ANF can exert stimulatory effects on testosterone steroidogenesis in vitro, and that the mechanism may involve an intracellular messenger other than cyclic adenosine monophosphate.

Atrial natriuretic factors (ANF) are a group of peptides synthesized in specific secretory granules of mamalian atrial cardiocytes. These peptides exert natriuretic as well as diuretic actions on the kidney (1,2) and vasorelaxant activity in vascular smooth muscle cells (3,4). Several atrial natriuretic peptides have recently been purified and their amino acid sequences determined (5-9). The amino acid sequences deduced from the nucleotide sequences of cDNA clones (10-12) have suggested that these peptides are derived from a common precursor molecule. While the mechanism of ANF action has not been clarified, ANF has been found to stimulate cGMP accumulation (13-15) and to inhibit cAMP generation (16) in different cell types. It has also been shown that ANF inhibits aldosterone synthesis and secretion from the adrenal gland (17-19). Recently we found that ANF also

ABBREVIATIONS: ANF: atrial natriuretic factor; cGMP: guanosine-3', 5'-monophosphate; cAMP: adenosine-3',5'-monophosphate; LH: luteinizing hormone; MIX: 3-isobutyl-1-methyl xanthine.

0006-291X/86 $1.50

affects progesterone synthesis in clonally derived mouse Leydig tumor cell line (20). Thus, it was of immediate interest to investigate the possible role of ANF on testosterone synthesis in normal Leydig cells. In these experiments we show that ANF exerts profound effects in vitro on testosterone synthesis by normal mouse Leydig cells, both in basal and gonadotropin-stimulated state.

MATERIALS AND METHODS

Hormones and Supplies: Rat ANF IV (6) with residues 107-131 was synthesized by a solid phase method as described previously (21). LH was obtained from National Pituitary Agency. Cell culture supplies were from Gibco Laboratories (Grand Island, N.Y.). Neutral alumina resine (AG-7, 100-200 mesh), Dowex resin (AG50W-x8, 100-200 mesh, hydrogen form) and Dowex columns were from Bio-Rad (Richmond, CA). All other chemicals were reagent grade.

Preparation and Treatment of Cells: Interstitial cells from testes of adult Swiss mice (30 g body weight) were prepared as previously described (22). Enriched Leydig cells were suspended in medium-199 containing 0.2 mM 3-isobutyl-1-methylxanthine (MIX), 0.1% BSA and 20 mM HEPES, pH 7.3. Cells were treated with indicated concentrations of LH and/or ANF and were incubated under 95% O_2/5% CO_2 for varying time periods. For cyclic nucleotide assays the reaction was stopped by the addition of perchloric acid at a final concentration of 1N.

Assay of testosterone: Testosterone was measured in cell culture medium by a solid phase radioimmunoassay (22) using ^{125}I-labeled testosterone.

Cyclic nucleotides assay: cAMP and cGMP were determined using radioimmunoassay after purification on Al_2O_3 and Dowex column by the method of Steiner et al. (23) with modifications as described by Harper and Brooker (24). The recovery of cyclic nucleotides from column chromatography was between 70-75%.

Statistical analyses: Data were analyzed using one-way analysis of variance and Duncan's multiple range test.

RESULTS

ANF stimulated the testosterone production by more than 30-fold in normal mouse Leydig cells (Fig. 1a). The concentration of ANF required for half-maximal response (ED_{50}) was $4x10^{-9}$M. LH stimulated testosterone production with an ED_{50} of $2x10^{-10}$M. When Leydig cells were treated with varying concentrations of ANF ($1x10^{-12}$-$1x10^{-6}$M) in combination with LH ($5x10^{-10}$M) a biphasic response in testosterone production was observed. As shown in Fig. 1b, ANF at a concentration of $1x10^{-11}$M exerted a slight

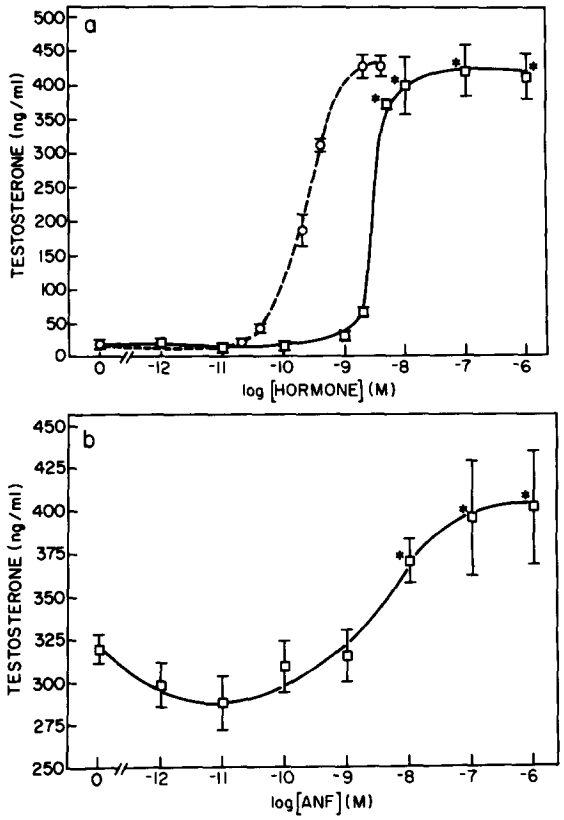

Fig. 1 a) Dose-response curve for testosterone production in isolated normal mouse Leydig cells treated with ANF (□) or LH (○). The enriched Leydig cell suspensions were treated with indicated concentrations of hormones for 3h at 37° C and testosterone measured in cultured supernatant by solid phase radioimmunoassay. b) Cells were treated in combination of LH (1ng/ml) and varying concentrations of ANF as above. Testosterone was assayed as described in Materials and Methods. Results are the mean ± SE of 3 independent experiments.

inhibitory effect on LH-stimulated testosterone production (not statistically significant). In contrast, at higher concentrations of ANF, testosterone production was stimulated by more than 75% ($P < 0.005$) over the LH-stimulated levels in the absence of ANF.

We also examined the effect of ANF on the accumulation of cyclic nucleotides in freshly isolated Leydig cells. As shown in Fig. 2a and b, ANF stimulated cGMP accumulation in both time- and dose-dependent manner. ANF (1×10^{-8}M and above) increased cGMP accumulation by more than 35-fold in comparison to the untreated cells (Fig. 2b). In the presence of LH, the stimulatory effect of ANF on cGMP accumulation was slightly diminished

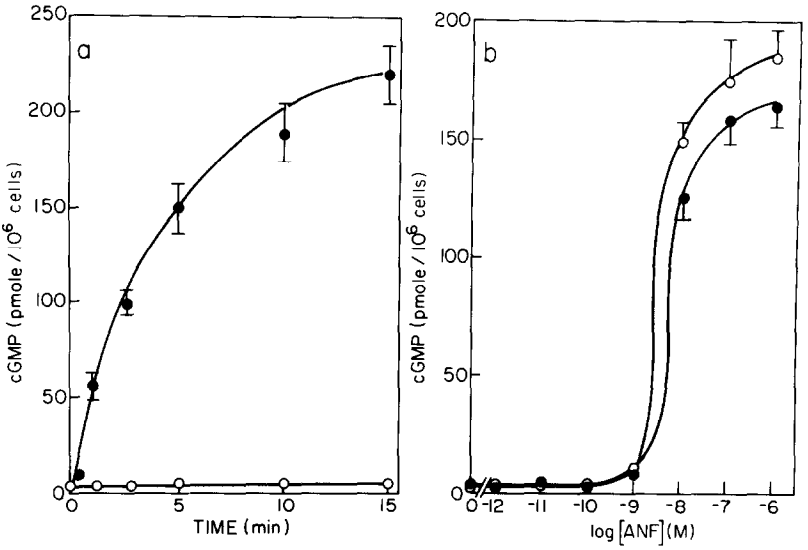

Fig. 2 Time- and dose-dependent response of ANF on accumulation of cGMP in isolated normal Leydig cells. a) Cells were treated for indicated time with ANF ($1x10^{-7}$M). b) Cells were treated with indicated concentrations of ANF for 10 min. All treatment received 0.2 mM MIX and were incubated at 37° C. cGMP was assayed as described in Materials and Methods. Data represents the mean of 2-3 determinations from duplicate experiments. Bars represent mean ±SE.

(Fig. 2b). The effect of ANF on cAMP accumulation was minimal, and showed only 15-20% inhibition in the cAMP levels as compared to the controls (data not shown).

DISCUSSION

These experiments show that ANF can affect steroidogenesis in normal mouse Leydig cells in two ways. First, ANF alone increases testosterone production in these cells by more than thirty-fold; the maximum level attained equals the maximal stimulatory effect seen with authentic gonadotropins. ANF achieves this stimulation of steroidogenesis with an ED_{50} approximately 20-fold higher than that of luteinizing hormone. Second, ANF exerts an apparent biphasic effect on steroidogenesis in mouse Leydig cells under stimulation by LH. At low concentration of ANF (10^{-11}M), testosterone production is slightly inhibited; as ANF concentration is increased the inhibition is reversed and a stimulatory effect (75% increase) in testosterone production is observed. However, the overall effect of ANF on testosterone production is stimulatory.

The mechanisms by which ANF exerts these effects on testicular steroidogenesis, and the physiologic relevance of the concentrations of ANF at which the effects are observed remain to be established. We have observed increases in cGMP levels in these isolated Leydig cells over the range of ANF concentrations in which stimulatory effects on steroidogenesis are seen (10^{-9}-10^{-6}M). Cyclic AMP levels were unchanged or fell. These data suggest that the observed effects of ANF on Leydig cell steroidogenesis do not occur via classic mechanisms of cAMP mediated regulation of steroidogenic activity. However, these effects seem to involve specific testicular ANF receptors. In preliminary studies we found that intact normal mouse interstitial cells bound radiolabelled ANF with high affinity (K_a=8.2x$10^9 M^{-1}$) and low capacity (0.31 fmoles/10^6 cells).

Previous studies have examined effects of ANF on testicular tissue. Guanylate cyclase activity is known to be increased in rat testis treated with ANF (14). In addition, we previously found that ANF markedly stimulated cGMP accumulation in cultured Leydig tumor cells, and profoundly suppressed cAMP generation. The Leydig tumor cells exhibited a biphasic effect of ANF on progesterone synthesis, the inhibitory effects at lower ANF concentrations being most pronounced (20). While the present work was in progress a preliminary report also showed that a very high concentration (10^{-6}-10^{-5}M) of ANF stimulated testosterone production in mouse interstitial cells (25). The results of this study clearly reveal that ANF affects the testicular steroidogenesis both in basal and gonadotropin-stimulated state. The stimulatory effect of ANF on the normal Leydig cell steroidogenesis is in marked contrast with its inhibitory action on aldosterone synthesis and release from adrenal gland (17-19) and progesterone synthesis in cultured Leydig tumor cells (20).

ACKNOWLEDGEMENTS

We acknowledge the excellent technical assistant of Sam Morley Jr. This work was supported by NIH Research Grants HL-14192 and HD-05797. K.P. is Research Fellow of the American Heart Association Middle Tennessee Chapter.

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