Steroidogenic effect of atrial natriuretic factor in isolated mouse Leydig cells is mediated by cyclic GMP

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The effects of different atrial natriuretic peptides on cyclic GMP formation and steroidogenesis have been studied in Percoll-purified mouse Leydig cells. Rat atrial peptides rANP (rat atrial natriuretic peptide), rAP-I (rat atriopeptin I) and rAP-II (rat atriopeptin II), in the presence of a phosphodiesterase inhibitor, stimulated cyclic GMP formation in a concentration-dependent manner. In the presence of saturating concentrations of the peptides, a 400–600 fold stimulation of cyclic GMP accumulation was observed. Among the peptides, rAP-II appeared to be the most potent. ED_{50} values (concentration causing half-maximal effect) for rAP-II, rANP and rAP-I were 1×10^{-9} M, 2×10^{-9} M and 2×10^{-8} M respectively. A parallel stimulation of cyclic GMP formation and testosterone production by the cells was observed after incubation of the cells with various concentrations of rAP-II. In the presence of a saturating concentration of rAP-II (2×10^{-8} M), maximum stimulation of intracellular cyclic GMP content was obtained within 5 min of incubation. Testosterone production by mouse Leydig cells could be stimulated by 8-bromo cyclic GMP in a concentration-related manner. At a 10 mM concentration of the cyclic nucleotide, steroidogenesis was stimulated to a similar extent as that obtained with a saturating concentration of human chorionic gonadotrophin (5 ng/ml). On the basis of these results we conclude that cyclic GMP acts as a second messenger in atrial-peptide-stimulated steroidogenesis in mouse Leydig cells. The steroidogenic effect of atrial peptides appears to be species-specific, since none of these peptides stimulated testosterone production by purified Leydig cells of rats, though in these cells a 40–60-fold stimulation of cyclic GMP formation in response to each of the three peptides was observed. However, 8-bromo cyclic GMP could stimulate testosterone production in rat Leydig cells. Therefore we conclude that the lack of steroidogenic response in rat Leydig cells to atrial-natriuretic-factor-stimulation results from an insufficient formation of cyclic GMP in these cells. This species difference would appear to result from a lower guanylate cyclase activity in rat Leydig cells.

INTRODUCTION

Mammalian atria secrete a group of polypeptide hormones responsible for cardiovascular homeostasis that have been collectively termed 'atrial natriuretic factors' (ANFs). These peptides have been identified and characterized [see review by Cantin & Genest (1985)]. ANF has recently been shown to modulate the function of a variety of steroidogenic cells, including, for example, inhibition of both basal and hormone-stimulated aldosterone secretion by rat adrenoglomerulosa cells (Atarashi *et al.*, 1984; Chartier *et al.*, 1984; De Lean *et al.*, 1984; Kudo & Baird, 1984). This effect of ANF appears to be mediated by activation of guanylate cyclase (Matsuoka *et al.*, 1985). Similarly, Pandey *et al.* (1985) have reported that ANF inhibits gonadotrophin-stimulated progesterone production by murine tumour Leydig cells. The inhibition of steroidogenesis in these cells was accompanied by an inhibition of cyclic AMP formation and a stimulation of cyclic GMP accumulation by ANF. On the other hand, in mouse Leydig cells, ANF has been shown to stimulate testosterone production (Bex & Corbin, 1985; Mukhopadhyay *et al.*, 1986). The latter authors observed that a phosphodiesterase inhibitor markedly enhanced the steroidogenic effect of ANF,

indicating the involvement of a cyclic nucleotide as a second messenger, but an increase in the level of cyclic AMP after the treatment of the cells with ANF was not observed. In the present study we have extended our investigations on the mechanism of action of ANF on testosterone production by mouse Leydig cells and report here that, in these cells, the stimulation of steroidogenesis by ANF is linked to its ability to stimulate cyclic GMP formation. The effects of ANF on Leydig cells from mouse and rat have been compared in order to determine its specificity of action.

MATERIALS AND METHODS

The following synthetic atrial peptides were obtained from Sigma, Munich, Germany: (1) rANP, (2) rAP-I and (3) rAP-II corresponding to ANF-(99–126)-peptide, ANF-(103–123)-peptide and ANF-(103–125)-peptide respectively (Cantin & Genest, 1985).

The sources of other chemicals were as follows: 8-bromo cyclic GMP and highly purified hCG (13 500 i.u./mg) from Boehringer, Mannheim, Germany; 8-bromo cyclic AMP and collagenase Type IA from Sigma; IBMX from Aldrich Europe, Beerse, Belgium;

Abbreviations used: ANF, atrial natriuretic factor; rANP, rat atrial natriuretic peptide; rAP-I, rat atriopeptin I; rAP-II, rat atriopeptin II; hCG, human chorionic gonadotrophin; IBMX, 3-isobutyl-1-methylxanthine; ED_{50} , concentration causing half-maximal effect.

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minimum essential medium with Earle's salts from Gibco Europe, Karlsruhe, Germany, and Hepes from Serva, Heidelberg, Germany.

Leydig cells from the testes of adult NMRI mice were isolated and purified as described by Schumacher *et al.* (1984). The procedure for the isolation of Leydig cells from the testes of adult Wistar rats was based upon the collagenase-dispersion method of Dufau & Catt (1975). In brief, a crude suspension of interstitial cells was prepared by digestion of decapsulated rat testes with collagenase (0.25 mg/ml) in minimum essential medium containing 25 mM-Hepes, pH 7.4, and 0.1% bovine serum albumin (henceforth referred to as 'medium'), for 20 min at 36 °C. The Leydig cells were further purified by centrifugation on a linear 0–90% (w/v) Percoll gradient as described for mouse Leydig cells (Schumacher *et al.*, 1984).

Purified Leydig cells (50 000–100 000 cells) were incubated in 500 μ l of medium for 3 h or as indicated at 36 °C with or without test substances. Radioimmunoassay procedures for testosterone and cyclic AMP have been described previously (Schumacher *et al.*, 1984). For measurement of total cyclic GMP, the incubation was terminated by addition of 2 ml of cold 100% ethanol, followed by vortex-mixing. The ethanol extract was evaporated to dryness, the residue was dissolved in 200–500 μ l of 0.1% NaN_3 and the content of cyclic GMP was determined by a specific radioimmunoassay (Schumacher *et al.*, 1977). For the determination of the intracellular content of cyclic GMP, the incubation was stopped by placing the tubes in an ice/water bath, followed immediately by centrifugation at 2500 g for 5 min at 4 °C. The supernatant was drained off as completely as possible, the cyclic GMP extracted from the cell pellet and assayed as described above.

RESULTS

Incubation of mouse Leydig cells with various concentrations of ANF peptides in the presence of a phosphodiesterase inhibitor, IBMX, resulted in a dose-related stimulation of cyclic GMP formation (Fig. 1). The concentrations of peptides required for maximal stimulation of cyclic GMP accumulation were 10^{-8} M-rAP-II, 5×10^{-8} M-rANP and 10^{-7} M-rAP-I. With concentrations of rAP-II and rANP exceeding 5×10^{-8} M a decline in the level of cyclic GMP accumulated was observed. The unstimulated cells produced 0.43 ± 0.07 pmol of cyclic GMP/3 h per 10^5 cells. The maximally stimulated cells produced between 400- and 600-fold more cyclic GMP than that produced by unstimulated cells as observed in five separate experiments using different batches of cell preparations. In contrast with the marked stimulation of cyclic GMP levels, there was no increase in the level of cyclic AMP in response to any of the peptides tested over the entire dose range (results not shown).

The results shown in Fig. 2 provide a comparison between the testosterone production and cyclic GMP accumulation in mouse Leydig cells stimulated with various concentrations of rAP-II, the most potent peptide. Both the parameters increased in a parallel and dose-dependent manner. However, the stimulation of cyclic GMP accumulation required twice the amount of rAP-II compared with that required for steroidogenesis

Fig. 1. Effect of atrial peptides on cyclic GMP accumulation by mouse Leydig cells

The cells were incubated with various concentrations of rAP-II (▲), rANP (□) or rAP-I (●) in the presence of 0.25 mM-IBMX for 3 h at 36 °C and cyclic GMP accumulated was measured. The results are means $\pm$ S.D. of triplicate determinations. The vertical bars represent S.D. and have been omitted where they are smaller than the symbols.

Fig. 2. Comparison of the testosterone production (▲) with cyclic GMP accumulation (●) in the mouse Leydig cells incubated with various concentrations of rAP-II

The incubation conditions and other details are same as those in Fig. 1. The maximally stimulated cells produced 175 ± 22.5 pmol of cyclic GMP/3 h per 10^5 cells and 102 ± 16.3 ng of testosterone/3 h per 10^5 cells.

(ED_{50} for cyclic GMP response, 2×10^{-9} M; for testosterone production, 1×10^{-9} M-rAP-II).

The time course of rAP-II-induced intracellular cyclic GMP accumulation by the cells incubated in the presence or absence of IBMX is shown in Fig. 3. It is evident that the rAP-II effects a rapid stimulation of cyclic GMP level in both the presence and absence of IBMX. The maximum response was attained within 5 min. In the absence of IBMX, the cellular cyclic GMP content declined

Fig. 3. Time course of intracellular cyclic GMP formation in mouse Leydig cells stimulated with rAP-II

The cells were incubated with 2×10^{-8} M-rAP-II in the absence (open symbols) or in the presence (closed symbols) of 0.25 mM-IBMX for various periods of time. The inset shows the kinetics of testosterone production (○, without IBMX; ●, with 0.25 mM-IBMX) by the cells incubated in the presence of rAP-II; ■, basal testosterone production. Other details are same as those in Fig. 1.

thereafter to a plateau after 20 min. In the presence of IBMX the levels of cyclic GMP at all time points were markedly higher than those obtained in the absence of IBMX. The rate of rAP-II-induced testosterone produc-

tion was increased by IBMX, although to a lesser extent (Fig. 3, inset).

Since the effects of the atrial peptides on steroidogenesis by mouse Leydig cells appear to be mediated by a rise in the level of intracellular cyclic GMP content, we have investigated the effects of incubating the cells with various concentrations of 8-bromo cyclic GMP. There was a dose-related stimulation of testosterone production by 8-bromo cyclic GMP (Fig. 4) and, with 10 mM-8-bromo cyclic GMP, nearly 20-fold stimulation of steroidogenesis was obtained, an effect comparable with that produced by a saturating concentration of hCG and even higher than that produced by a maximally effective concentration of rAP-II. In a separate experiment, where 8-bromo cyclic GMP was used at 10 mM and 20 mM concentrations, no further increase in steroidogenesis was observed (results not shown).

In order to determine the species specificity of the action of ANF, we have investigated whether ANF could produce similar effects on Leydig cells of rat. As shown in Fig. 5, all three peptides stimulated cyclic GMP formation in these cells as well, and rAP-II was evidently the most potent peptide. The other two peptides, rANP and rAP-I, were equipotent, in contrast with the observation made in case of mouse Leydig cells. However, none of the peptides had any effect on testosterone production by rat Leydig cells. The amounts of testosterone produced by rat Leydig cells in the absence of any addition and in the presence of 1×10^{-10} M-, 1×10^{-9} M-, 1×10^{-8} M- and 1×10^{-7} M-rAP-II were 1.71 ± 0.28 , 1.68 ± 0.29 , 1.72 ± 0.05 , 1.98 ± 0.08 and 1.81 ± 0.08 ng/

Fig. 4. Effect of 8-bromo cyclic GMP on testosterone production by mouse Leydig cells

The cells were incubated with various concentrations of 8-bromo cyclic GMP (●) or with 2×10^{-8} M-rAP-II (■) or hCG (2ng/ml) (▨) and the amount of testosterone produced was measured. For other details, see the legend to Fig. 1.

Fig. 5. Effects of atrial peptides on cyclic GMP accumulation in rat Leydig cells

Incubation conditions and other details are similar to those in Fig. 1.

3 h per 10^5 cells respectively. A 9-fold stimulation of steroidogenesis was obtained in these cells incubated with a saturating dose of hCG (5 ng/ml). The incubations were carried out in the presence of 0.25 mM-IBMX.

It may be noted from the data presented in Fig. 5 that the capacity of rat Leydig cells to accumulate cyclic GMP in response to atrial peptides was at least 10 times less than that of mouse Leydig cells (for comparison, see Fig. 1). In order to discern whether the lack of stimulation of steroidogenesis in rat Leydig cells in response to ANF could be explained on the basis of an inadequate formation of cyclic GMP in these cells, we have examined the effect of various concentrations of 8-bromo

Table 1. Effects of hCG, 8-bromo cyclic AMP and 8-bromo cyclic GMP on testosterone production by rat Leydig cells

Rat Leydig cells were incubated for 3 h in the presence of various concentrations of hCG, 8-bromo cyclic AMP and 8-bromo cyclic GMP and testosterone produced was measured as described in the Materials and methods section. Results are means $\pm$ S.D. of triplicate determinations.

Stimulator	Testosterone production (ng/3 h per 10^5 cells)
None	2.69 $\pm$ 0.40
hCG	
0.1 ng/ml	15.90 $\pm$ 0.79
1.0 ng/ml	21.70 $\pm$ 0.90
2.0 ng/ml	25.01 $\pm$ 1.60
8-Bromo cyclic AMP	
0.1 mM	13.30 $\pm$ 1.20
1.0 mM	22.80 $\pm$ 1.10
5.0 mM	25.80 $\pm$ 1.70
8-Bromo cyclic GMP	
1.0 mM	3.80 $\pm$ 0.60
5.0 mM	8.20 $\pm$ 0.30
10.0 mM	16.20 $\pm$ 1.20
20.0 mM	22.90 $\pm$ 0.40

cyclic GMP on testosterone production by rat Leydig cells. The results summarized in Table 1 show that 8-bromo cyclic GMP stimulates steroidogenesis by rat Leydig cells in a dose-related manner, and the amount of testosterone produced in response to 20 mM-8-bromo cyclic GMP was comparable with that produced in response to maximal steroidogenic concentrations of hCG or 8-bromo cyclic AMP.

DISCUSSION

The results obtained in the present study demonstrate for the first time the existence of a cyclic GMP-mediated pathway for stimulation of testosterone production in mouse Leydig cells, distinct from the well-established cyclic AMP-mediated pathway (as in the case of gonadotrophin-responsive steroidogenesis). It was observed that all three atrial peptides produced a stimulation of cyclic GMP accumulation by mouse Leydig cells in a concentration-dependent manner. The cyclic GMP-stimulating potencies of the peptides could be ranked in the following order: rAP-II > rANP > rAP-I. Interestingly, a similar order of steroidogenic potencies in these cells was reported for these three peptides (Mukhopadhyay *et al.*, 1986). Furthermore, there was a strong correlation between the stimulation of cyclic GMP formation and testosterone production by the most potent peptide, rAP-II. The testosterone production by mouse Leydig cells could be stimulated by 8-bromo cyclic GMP in a concentration-related manner. On the basis of these results and the fact that ANF has no effect on the cyclic AMP level, it is concluded that steroidogenic action of ANF on mouse Leydig cells is mediated by cyclic GMP acting as the second messenger. The role of cyclic GMP as a second messenger in the action of ANF has been previously reported in a variety of tissues (Hamet *et al.*, 1984; Waldman *et al.*, 1984; Winkvist *et al.*, 1984; Heisler *et al.*, 1986). In a number of steroidogenic tissues ANF has previously been reported to stimulate cyclic GMP accumulation, and in these tissues the ANF-mediated elevation of cyclic GMP levels has been shown to result in an inhibition of basal as well as hormone-stimulated steroid production (De Lean *et al.*, 1984; Matsuoka *et al.*, 1985; Pandey *et al.*, 1985). However, in mouse Leydig cells, the increase in the cyclic GMP level is associated with an increase in testosterone production.

The concentration of 8-bromo cyclic GMP required to elicit a steroidogenic response comparable with that obtained with a maximally effective concentration of hCG was high (for comparison 0.2 mM-8-bromo cyclic AMP was required for a maximal steroidogenic response). This could be due to the possibility that only a small fraction of 8-bromo cyclic GMP offered to the cells gained access to the relevant intracellular compartment necessary to initiate the steroidogenic cascade.

From a mechanistic point of view, the stimulation of testosterone production by ANF in mouse Leydig cells could be explained in two ways. Possibly, these cells possess a specific cyclic GMP-dependent protein kinase which, upon activation, phosphorylates a set of proteins. These proteins may be the common substrates for cyclic AMP-dependent protein kinase and their phosphorylation could then start the steroidogenic cascade. Alterna-

tively it can be postulated that the cyclic AMP-dependent protein kinase in these cells may be less specific for the requirement of the cyclic nucleotides in that this kinase is also stimulated by high concentrations of cyclic GMP in addition to cyclic AMP. Further studies are needed to test these possibilities.

The rat Leydig cells differ from the mouse Leydig cells by the absence of any steroidogenic response to atrial peptides, in spite of having a definite response in terms of cyclic GMP accumulation. However, the capacity of rat Leydig cells to accumulate cyclic GMP was markedly less than that of mouse Leydig cells. This may be due to a low guanylate cyclase activity in rat Leydig cells. This is not surprising, since it is known that the capacity of mouse Leydig cells in terms of both testosterone production and cyclic AMP accumulation in response to gonadotrophin is several times higher than that of rat Leydig cells (Dufau *et al.*, 1978; Schumacher *et al.*, 1979; Hunter *et al.*, 1982). The fact that 8-bromo cyclic GMP could stimulate testosterone production in rat Leydig cells as well indicates that a stimulation of the steroidogenic cascade in both species could be brought about by this cyclic nucleotide. The differences observed in rat and mouse Leydig cells with respect to the testosterone response to ANF stimulation may, therefore, be explained on the basis of the lower capacity of rat Leydig cells to accumulate cyclic GMP in response to atrial peptides.

Future research will be required to elucidate the exact mechanism of action of atrial peptides on mouse Leydig cells and to determine whether the ANF-responsive cyclic GMP-mediated pathway may be linked to the regulation of some other important, but as yet unknown, physiological functions of Leydig cells.

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