Atrial natriuretic peptide-induced stimulation of cyclic GMP formation by isolated bovine luteal cells*

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Summary. Incubation of bovine luteal cells with an atrial natriuretic peptide (rat atriopeptin II, rAP-II) did not affect hCG-stimulated or basal cyclic AMP accumulation and progesterone production, but cyclic GMP formation was stimulated by rAP-II in a dose-dependent manner, being maximally stimulatory in the nanomolar range. This stimulatory influence of rAP-II on cyclic GMP formation results from a specific stimulation of particulate guanylate cyclase. We suggest that, although rAP-II mediated cyclic GMP formation can be demonstrated in bovine luteal cells, there appear to be no acute effects of the atrial peptide on the regulation of progesterone production by these cells.

Keywords: atrial natriuretic peptide; luteal cells; steroidogenesis; guanylate cyclase; cow

Introduction

Mammalian atrial cardiocytes are known to produce and secrete a family of peptides called atrial natriuretic peptide (ANP), having potent natriuretic, diuretic and vasorelaxant properties (Cantin & Genest, 1985; Anderson & Bloom, 1986). Apart from their classical role in the regulation of cardiovascular homeostasis, these peptides have also been shown to modulate the function of a variety of steroidogenic tissues. ANP inhibits basal as well as hormone-stimulated aldosterone production by rat and bovine adrenoglomerulosa cells (Kudo & Baird, 1984; Racz *et al.*, 1985) and progesterone production in mouse Leydig tumour cells (Pandey *et al.*, 1985). However, a stimulation of steroidogenesis in response to ANP was observed in mouse Leydig cells (Bex & Corbin, 1985; Pandey *et al.*, 1986; Mukhopadhyay *et al.*, 1986a, 1988) and in human granulosa cells (Pandey *et al.*, 1987). No influence of ANP on either testosterone or cyclic AMP production was detected in rat Leydig cells, although a stimulation of cyclic GMP formation in response to ANP was observed (Mukhopadhyay *et al.*, 1986b). Similarly, in rat luteal cells no effect of ANP could be demonstrated on cyclic AMP accumulation or progesterone production although an activation of guanylate cyclase was reported (Budnik *et al.*, 1987). Although the general effects of ANP in steroidogenic tissues remain conflicting, stimulation of cyclic GMP by ANP seems to be common in all tissues so far examined. This stimulation always appears to result from a specific activation of a particulate guanylate cyclase activity (Waldman *et al.*, 1984; Tremblay *et al.*, 1985). Vollmar *et al.* (1988) have reported that a significant quantity of ANP could be measured in an acidic extract of bovine corpus luteum tissue and that a single class of high-affinity receptors for ANP was present in this tissue; the authors suggested that ANP may play a role in the regulation of luteal steroidogenesis, but this possibility has not been examined. We have therefore investigated the effect of ANP on cyclic nucleotide formation and progesterone production in luteal cells from cows.

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Materials and Methods

The synthetic rat atriopeptin II (rAP-II, corresponding to ANF 103–123 according to Cantin & Genest, 1985) was obtained from Sigma (Deisenhofen, FRG) and human chorionic gonadotrophin (hCG, 2nd International Standard Preparation) was generously provided by National Institute for Biological Standards and Control (Holly Hill, London, UK).

Preparation of luteal cells. Suspensions of bovine luteal cells were prepared by the method described by Brunswig *et al.* (1986). In brief, bovine ovaries from the local slaughterhouse were transported to the laboratory and the mid-luteal phase corpora lutea (stage II), as judged by the criteria of Ireland *et al.* (1980), were dissected out. About 5 g tissue were minced with scissors and enzymically dispersed by incubation with collagenase (3 mg/ml) and DNase (20 µg/ml) for 90 min at 37°C in 25 ml minimum essential medium (Gibco Europe, Eggenstein, FRG) containing 25 mM-Hepes pH 7.4 and 0.1% BSA (MEM). Dispersed cells were collected by centrifugation, washed twice with MEM and finally enriched by centrifugation on 63% Percoll. The number of cells was counted with a haemocytometer and cell viability, determined by the trypan blue exclusion test (Tennant, 1964), was > 80%.

Incubation of luteal cells. A suspension of luteal cells (200 000/500 µl MEM) was incubated at 37°C for 2 h in a shaking water bath with or without test substances. Incubation was terminated by the addition of 2 ml 100% ethanol. The ethanol was evaporated and the residue was taken up in 500 µl 0.1% NaN₃. Cyclic AMP, cyclic GMP and progesterone were measured by radioimmunoassay as described previously (Budnik *et al.*, 1987).

Guanylate cyclase assay. Guanylate cyclase was measured in a particulate fraction of luteal cells as described previously (Budnik *et al.*, 1987). In brief the purified cell suspension was centrifuged at 90 g for 15 min, homogenized in 10 mM-Tris/HCl pH 7.5 and 0.25 mM-sucrose, sonicated (Branson Sonifier B 12) and centrifuged at 105 000 g for 60 min. The high-speed pellet was resuspended in 1.5 ml 10 mM-Tris-HCl pH 7.5. Protein concentrations were determined by the method of Bradford (1976) using the reagents provided by BioRad Laboratories. The assay was performed in a total volume of 0.1 ml. The reaction mixture contained 10 mM-Tris-HCl pH 7.5, creatine phosphate (5 mM), creatine phosphokinase (66 U/ml), GTP (2 mM), divalent cations as indicated and 5 µg protein equivalent of particulate fraction. The incubation was carried out at 30°C for 20 min and was terminated by the addition of 1 ml cold 100% ethanol. The cyclic GMP produced was extracted and measured by radioimmunoassay as described above (Budnik *et al.*, 1987).

Radioimmunoassays. Progesterone was measured with a commercial kit supplied by RSL (Carson, CA, USA). According to the information provided with the kit the antibody had the following cross-reactions: 20 α -dihydroprogesterone 5.41%, desoxycorticosterone 3.80%, corticosterone 0.70%, 17-hydroxyprogesterone 0.67%, pregnenolone 0.41%, androstendione 0.23%, testosterone 0.16%, and oestrogens 0.01%. The assay sensitivity, expressed as the minimum detectable amount, was 0.02 ng progesterone/ml. The intra- and interassay coefficients were 4.9% and 7.6%, respectively.

The cyclic nucleotides were measured with commercial assay kits provided by IBL (Hamburg, FRG). The cyclic AMP antibody cross-reacted 0.00028% with cyclic GMP, 0.00020% with adenosine, 0.00013% with 2',3'-cAMP and 0.00001% with ATP and GTP. The antibody to cyclic GMP cross-reacted 0.001% with cyclic AMP, 0.0001% with GTP and 0.0001% with ATP. The detection limits of the assays were 5 fmol cyclic AMP/tube and 7 fmol cyclic GMP tube, respectively. The intra and interassay coefficients were, respectively, < 5% and < 10% for cyclic AMP and 5.2% and 7.5% for cyclic GMP.

Statistics. Every incubation and enzyme assay was performed in triplicate if not otherwise stated and each experiment was repeated at least twice. Results are expressed as mean + s.e. Student's *t* test was used throughout for statistical analysis.

Results

Formation of cyclic GMP in luteal cells stimulated with rAP-II

The results summarized in Fig. 1 show that rAP-II was able to stimulate significantly cyclic GMP formation by bovine luteal cells *in vitro*. This stimulatory effect was dose-dependent and the maximally stimulating dose of rAP-II was 10 nM. For comparison, the amount of cyclic GMP measured in the cells incubated with 20 mi.u. hCG/ml is also presented in Fig. 1. In previous experiments, hCG at this concentration stimulated significantly the production of progesterone and accumulation of cyclic AMP by bovine luteal cells. However, there was no increase in the amount of cyclic GMP formed in response to stimulation with hCG.

To examine whether this rAP-II-dependent stimulation was due to a stimulation of particulate guanylate cyclase activity, the enzyme activity in the membrane fraction prepared from bovine luteal cells in the presence or absence of 10 nM-rAP-II was measured, with various concentrations

Fig. 1. Stimulation of cyclic GMP in a suspension of luteal cells (200 000 cells/500 μ l medium) incubated without any addition (C), with 20 mi.u. hCG/ml (hCG) or with various concentrations of rAP-II ($\circ$) for 2 h at 37°C. Values are mean $\pm$ s.e. for triplicate determinations. * P < 0.025, ** P < 0.01 compared with values for control incubations (C).

Fig. 2. Activation of particulate guanylate cyclase enzyme in bovine luteal cells by rAP-II in the presence of various concentrations of (a) MgCl₂ or (b) MnCl₂, without (open symbols) or with 10 nM-rAP-II (closed symbols). Values are mean $\pm$ s.e. of triplicate determinations. * P < 0.05, ** P < 0.01, *** P < 0.001 compared with values without rAP-II.

Table 1. Progesterone production by hCG-stimulated and unstimulated bovine luteal cells incubated in the presence of various concentrations of rAP-II

rAP-II (nM)	Progesterone (ng/200 000 cells/2 h)		
	Basal	hCG	
		2 mi.u./ml	20 mi.u./ml
0	1.20 ± 0.12	9.20 ± 0.61	24.04 ± 1.50
0.1	1.05 ± 0.03	8.93 ± 0.83	27.84 ± 1.43
1	1.22 ± 0.08	10.01 ± 1.21	25.65 ± 0.86
10	1.08 ± 0.07	8.89 ± 0.27	23.03 ± 0.60
100	1.34 ± 0.12	9.33 ± 0.07	22.73 ± 1.83

Values are mean ± s.e. for quadruplicate determinations.

of Mg^{2+} (Fig. 2a) or Mn^{2+} (Fig. 2b) added. In the presence of rAP-II, a significant stimulation of guanylate cyclase activity occurred when each cation was present. In the absence of any cation the enzyme activity was close to the limit of detection of the assay. The enzyme activity measured in the presence of Mg^{2+} was only one-third of that measured in the presence of Mn^{2+} .

The effect of rAP-II on basal and hCG induced steroidogenesis

Luteal cells were incubated without or with hCG (2 mi.u./ml or 20 mi.u./ml) in the presence of various concentrations of rAP-II. The amounts of progesterone produced by the cells were measured. As shown in Table 1, hCG at both dose levels stimulated progesterone production significantly. However, there was no effect of rAP-II on basal or on hCG-stimulated progesterone production, regardless of whether a submaximal or maximal steroidogenic dose of hCG was used.

The amounts (mean ± s.e.m.) of cyclic AMP accumulated (fmol/2 × 10⁵ cells/2 h) in luteal cells left untreated or stimulated with 2 or 20 mi.u. hCG/ml did not differ in the absence (59 ± 11, 110 ± 8 and 539 ± 17 respectively) or presence (10 nM) of rAP-II (93 ± 19, 90 ± 17 and 551 ± 16 respectively), although the effect of hCG was significant. When the phosphodiesterase inhibitor, 1-isobutyl-3-methyl xanthine (IBMX) was included in the incubation medium, rAP-II did not affect formation of progesterone or cyclic AMP in the absence or presence of hCG (data not shown).

Discussion

Specific receptor-mediated stimulation of progesterone secretion and cGMP accumulation by ANP in human granulosa cells has been reported (Pandey *et al.*, 1987). In these cells ANP did not affect cAMP formation. On the other hand, we have reported previously that ANP markedly stimulates cyclic GMP formation by rat luteal cells through an activation of a particulate guanylate cyclase, without affecting progesterone or cyclic AMP formation (Budnik *et al.*, 1987). In the present study, no effect of ANP on basal or hCG-stimulated cyclic AMP accumulation by bovine luteal cells was observed in the presence or absence of IBMX, although in mouse Leydig tumour cells (Pandey *et al.*, 1985) and pituitary cells (Anand-Srivastava *et al.*, 1985) an inhibition of cyclic AMP accumulation was reported. The atrial peptide also had no acute effect on steroidogenesis by bovine luteal cells. An effect of ANP on steroidogenesis and cAMP accumulation therefore appears to be specific to certain types of steroidogenic cells only. We have shown here that nanomolar concentrations of rAP-II can elicit a significant stimulation of cyclic GMP formation in bovine luteal cells. The maximally effective concentration was 10 nM, which is comparable to that reported for other tissues (Mukhopadhyay *et al.*, 1986b; Budnik *et al.*, 1987). The presence of ANP receptors in bovine

corpus luteum has been demonstrated (Vollmar *et al.*, 1988). Our results, showing that the particulate guanylate cyclase prepared from purified bovine luteal cells could be stimulated with rAP-II measured in the presence of Mg^{2+} or Mn^{2+} , corroborate the previous findings using total luteal tissue (Vollmar *et al.*, 1988).

However, although bovine luteal cells can be included in among the target organs for atrial peptides, the physiological significance of this observation remains obscure. The fact that steroidogenesis by luteal cells was not affected by rAP-II would indicate that, if ANP has a function in the bovine corpus luteum, it does not appear to include the regulation of progesterone production.

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