Angiotensin II Receptors and Inhibitory Actions in Leydig Cells*

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Rat Leydig cells possess functional high-affinity receptors for angiotensin II (AII). AII inhibits adenylate cyclase activity in Leydig cell membranes and reduces basal and human chorionic gonadotropin (hCG)-stimulated cAMP pools and testosterone production in intact cells. Treatment of cells with an inhibitory dose of forskolin (10⁻⁹ M) and a submaximal dose of AII caused additive inhibition of hCG-stimulated events. The inhibitory action of AII was largely prevented by pertussis toxin prior to the addition of AII alone or in the presence of hCG. This study and our recent report on inhibitory action of low doses of forskolin, 10⁻¹⁲-10⁻⁹ M (Khanum, A., and Dufau, M. L. (1986) J. Biol. Chem. 261, 11456–11459) are indicative of a pertussis toxin-sensitive subunit of adenylate cyclase available for acute regulation of Leydig cell function. 8-bromo-cAMP bypasses the inhibitory effect of forskolin as well as AII. We have, therefore, demonstrated functional AII high-affinity receptor and an acute inhibitory effect of AII on hCG action in Leydig cells. Our results have provided evidence for a pertussis toxin-sensitive guanine nucleotide inhibitory protein as mediator of the effect of AII. These findings further emphasized the importance of the cAMP pathway in the Leydig cells, and studies also suggest that tubular and locally produced AII could negatively modulate luteinizing hormone stimulation of Leydig cells.

A number of studies have provided evidence for the presence of the renin-angiotensin system in reproductive tissues. Immunoreactive renin has been detected in Leydig cells of rat and human testes and was found to be pituitary-dependent in the rat (1, 2). Similarly, more recent studies have shown the presence of renin, angiotensin I and II (AII) and AII in normal rat Leydig cells and a murine Leydig cell line (3, 4). Furthermore, angiotensin-converting enzyme activity was demonstrated in rat testis and shown to be localized predominantly in the seminal vesicles, whereas only minor activity was found in the purified adult rat Leydig and Sertoli cells (5). Also, [H]captopril bound specifically to cellular fractions enriched in seminal vesicles (6). Velletri et al. (5) have suggested that the pituitary gland is required for development and maintenance of the rat testicular angiotensin-converting enzyme through stimulation of steroidogenesis in the testes. The biochemical evidence (5) was consistent with immunofluorescence studies

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1 The abbreviations used are: AII and AII, angiotensin I and II; hCG, human chorionic gonadotropin; Nn, guanine nucleotide inhibitory unit; HPLC, high pressure liquid chromatography.

in swine testis showing the presence of converting enzyme in spermatids and other stages of germinal cells (6). On the other hand, there is strong evidence that Leydig cells possess a membrane system with the potential for negative modulation of gonadotrophic action. In this regard, we have demonstrated a novel, high-affinity inhibitory action of low doses of forskolin (range 10⁻¹²-10⁻⁹ M) upon adenylate cyclase activity and cAMP generation, an effect that appears to be mediated by the pertussis toxin-sensitive guanine nucleotide inhibitory unit (Nn) of adenylate cyclase (7). Since AII has been shown to exert an inhibitory influence on adenylate cyclase system of liver (8, 9), adrenal cortex (10, 11), renal cortex (12), and smooth muscle (13), this hormone could be a potential regulator of gonadal function. It is, therefore, conceivable that tubular and locally produced AII could modulate the action of gonadotrophin in Leydig cells.

EXPERIMENTAL PROCEDURES

Materials

Medium-199 and elutriation medium were obtained from Whittaker M. A. Bioproduct, Inc., Walkersville, MD and National Institutes of Health Media Supply Units, Bethesda, MD respectively. hCG (CR-121) was kindly provided by the Center of Population Research, National Institute of Child Health and Human Development, Bethesda, MD. AII was purchased from Sigma. [Sar¹,Ala⁸]AII was obtained from Vega Biotechnologies, Tucson, AZ, and forskolin from Behring Diagnostics. AII (5–8), LHRH, AII, and [des-Asp¹]AII were obtained through Peninsula Laboratories, San Carlos, CA. Pertussis toxin was purchased from List Biological Laboratories, Inc., Campbell, CA. [⁹⁴⁷]AII (2200 Ci/mmol) and succinyl cAMP [¹²⁵]tyrosine methyl ester (2000 Ci/µg) were prepared by Meloy Laboratories, Springfield, VA, and by Hazleton Biotechnologies, Vienna, VA, using a modification of the chloramine-T method (14). Followed by purification by HPLC (15). [¹¹⁴⁷]F]ATP (800 Ci/mmol) were obtained from Du Pont-New England Nuclear.

Methods

Preparation of Leydig Cells and Membranes—Adult male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, MA) were killed and testes were removed and placed in ice-cold PBS, pH 7.4. Intestinal cells were obtained by collagenase digestion of decapsulated testes, as described previously (16). Crude cell suspension was washed and then pelleted at 200 x g for 10 min. The cell pellet was resuspended with elutiation buffer consisting of regular medium-199 with Hanks' salts and L-glutamine containing 1.4 g/liter NaHCO₃, 0.5% bovine serum albumin-, 1 mM EDTA, 50 units/ml heparin, 12.5 μg/ml DNase and 50 μg/ml gentamycin, pH 7.4. The purified cells were obtained by centrifugal elutiation (17). Cells were centrifuged and resuspended in medium-199 containing 0.1% bovine serum albumin and were incubated at 34°C with shaking at 100 cycles/min under an atmosphere of O₂:CO₂ (95:5, v/v) in the presence and in the absence of various concentrations of hCG with or without AII, forskolin, and 8-bromo-cAMP. For some experiments, cells were pretreated for 60 min with pertussis toxin under the same incubation conditions prior to the addition of hCG and/or AII. The incubations were terminated by transferring the incubation tubes to an ice bath; all further steps were carried out at 0°C and processed for the analysis of cAMP (intracellular and receptor-bound) and testosterone; and
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FIG. 1. Time course specific binding of 125I-AII to Leydig cells during incubation at 4 °C, 22 °C and 37 °C for 120 min. Nonspecific binding for each time interval was determined by addition of unlabeled AII (10 μM). Each point represents the mean ± S.E. of triplicate determinations.

FIG. 2. Scatchard analysis and saturation curve (inset) of AII binding to Leydig cells. Cells were incubated at 22 °C for 20 min with increasing concentrations of 125I-AII. Each point represents the mean of triplicate determinations. The S.E. was less than 10%.

their measurements were performed by radioimmunoassay as described previously (18).

Purified Leydig cell plasma membranes were prepared in the presence of 5 mM EDTA (19) and stored in liquid nitrogen until use. Adenylate cyclase assay was carried out by methods previously described (9). For some experiments, membranes were also preincubated for 20 min with activated pertussis toxin.

Binding Studies—Binding assays with 125I-AII were performed with intact cells which were incubated in 500 μl of Dulbecco's PBS in the presence of 0.2% bovine serum albumin and 100 μM phenylmethylsulfonyl fluoride for various time periods: 0-120 min at different temperatures: 4 °C, 22 °C and 37 °C. For equilibrium studies, cells were incubated at 22 °C for 20 min with increasing concentrations of 125I-AII in the presence or in the absence of 10 μM of AII or with 125I-AII and increasing concentrations of unlabeled AII. In some displacement studies, AII-related and -unrelated peptides were used. The reaction was terminated by adding 2 ml of ice-cold PBS, pH 7.4, in the tube and immediately filtered under vacuum through GF/C filters (Whatman, Maidstone, Great Britain). Filters were washed twice with 3 ml of PBS and bound radioactivity was measured in a γ counter. For kinetic studies, Leydig cells were also incubated with 125I-AII at various times: 0-30 min. The specific binding of 125I-AII reached to equilibrium after 14 min of incubation. Therefore, the rate of dissociation of 125I-AII receptor complex was obtained by adding 10 μM of unlabeled AII after 14 min of incubation. The binding studies were analyzed by the method of Scatchard (20) using computer analysis of binding data by a nonlinear model curve fitting program (21).

RESULTS AND DISCUSSION

In this study, we have demonstrated the presence of functional AII receptors in rat Leydig cells and have provided the evidence of its acute inhibitory effects. Cells were incubated with 125I-AII for various time periods at 37 °C, 22 °C, and 4 °C. The highest specific binding was observed when the cells were incubated at 22 °C, and the binding reached to maximum within 30 min of incubation (Fig. 1). Thereafter, there was subsequent decrease in binding. At 37 °C, the specific binding increased rapidly after 5 min of incubation followed by a rapid decline. This is most likely due to tracer degradation by proteolytic enzymes as indicated by others (22) or may be due
to internalization of receptor complex and subsequent receptor degradation. In fact, this receptor-mediated internalization has been indicated in cases of various peptide hormones (22). However at 4 °C, the specific binding was increased gradually and reached equilibrium after 90 min of incubation. In this case the specific binding was only 10% of that observed at 22 °C during 20 min of incubation. Therefore, for binding studies, the incubation was carried out at 22 °C for 20 min. Scatchard plot derived from equilibrium binding data obtained at 22 °C showed the presence of high-affinity binding sites with \( K_0 \) of \( 1.7 \times 10^{-10} \text{ M}^{-1} \) ± 0.41 (n = 4) and number of 2018 ± 408 (n = 4) receptor sites per cell (Fig. 2). The binding was saturable and reversible as indicated by saturation analysis of binding data and association and dissociation studies, respectively. The rates of association (\( k_a \)) and dissociation (\( k_d \)) of \(^{125}\text{I}-\text{AII} \) binding to Leydig cells were 0.885 nM\(^{-1}\) min\(^{-1}\) and 0.04 min\(^{-1}\), respectively (Fig. 3). Furthermore, the calculated half-life was 17.5 min. From the kinetic data, the calculated equilibrium constant, \( K_0 \), was \( 2.2 \times 10^{10} \text{ M}^{-1} \). This value is very close to the value obtained by equilibrium analysis. Fig. 4 showed the specificity of AII binding to Leydig cell receptor sites. No inhibition in binding was observed with the neurohypophyseal peptide, arginine vasopressin and dipterene, forskolin. On the other hand, the binding-inhibition potency of various analogues and fragment was noted. [Sar\(^1\),Ala\(^8\)]AII, AII, and [des-Asp\(^\prime\)]AII were potent displacers of specific \(^{125}\text{I}-\text{AII} \) binding and their \( K_0 \) values were similar to the observed \( K_0 \) for AII (1.7 × 10\(^{-10}\) M\(^{-1}\)). AII was found to be 100 times less potent than AII in the binding assay, and the smaller fragment of AII (AII, Refs. 5–8) displayed weak inhibitory effect on \(^{125}\text{I}-\text{AII} \) binding. LHRH (hypothalamic gonadotropin-releasing hormone), an unrelated decapeptide, competed quite effectively with \(^{125}\text{I}-\text{AII} \) for binding to Leydig cell receptor sites when present in relatively high concentrations, although it was less potent (1000 times) than the AII. The potency order of these compounds for AII receptors is AII> [Sar\(^1\),Ala\(^8\)]AII> [des-Asp\(^\prime\)]AII> AII>LHRH> AII (5–8). LHRH (hypothalamic gonadotropin-releasing hormone) has shown its ability to inhibit \(^{125}\text{I}-\text{AII} \) binding in Leydig cells. This is consistent with earlier studies by Capponi and Catt (25) in the adrenal cortex and uterus suggesting that the binding inhibition of AII by LHRH is probably due to a common structural feature in the COOH-terminal sequence of LHRH and AII.

Although a number of studies have indicated the presence of the renin-angiotensin system in reproductive tissues on the basis of immunohistochemical, immunofluorescence, HPLC, and radioimmunoassay studies (1–4, 6, 26), its physiological significance and possible role in gonadal function are still unclear. Our studies have provided direct evidence for the
Fig. 7. Left, inhibitory effect of AII on testosterone production. Leydig cells (1 x 10^6 cells/ml) were incubated for 120 min with various concentrations of AII (10^{-10}-10^{-8} M) in the presence of hCG and hCG plus forskolin (F). Each point represents the mean ± S.E. of triplicate incubations. Right, effect of pertussis toxin (PT) on AII-induced inhibition on testosterone production. Leydig cells (1 x 10^6 cell/ml) were preincubated with pertussis toxin (30 ng/ml) for 60 min. Incubation was further carried out for 60 min in the presence or in absence of the indicated hCG doses with or without AII. Each point represents the means ± S.E. of triplicate incubations.

**TABLE II**

**Effect of pertussis toxin (PT) on AII-induced inhibition of adenylate cyclase activity**

Adenylate cyclase activity was determined in the membranes of Leydig cells, pretreated and untreated with pertussis toxin (µg/ml). The membranes were incubated with GTP (10^{-8} M) and LH (10^{-8} M) in the presence and in the absence of AII (10^{-8} M). Mean values between groups by student’s t test are as follows: b versus a and c (p < 0.05); e versus d and f (p < 0.01).

| Additions | Adenylate cyclase activity pmol/mg protein/15 min |
|-----------|--------------------------------------------------|
| Control   | 29 ± 2                                           |
| Control + PT | 39 ± 3                                      |
| GTP       | 71 ± 7 (a)                                      |
| GTP + PT  | 88 ± 7                                           |
| GTP + AII | 51 ± 4 (b)                                      |
| GTP + AII + PT | 113 ± 10 (c)                              |
| LH + GTP  | 121 ± 14 (d)                                    |
| LH + GTP + AII | 82 ± 8 (e)                              |
| LH + GTP + AII + PT | 154 ± 12 (f)                              |

presence of functional AII receptors, since AII acutely inhibits (as early as 15 min) gonadotrophin stimulation of cyclic AMP pools and testosterone production in Leydig cells. Fig. 5, left, shows AII-induced inhibition of intracellular CAMP production and the effect of pertussis toxin on this inhibition. Cells were stimulated with a submaximal dose of hCG in the presence and in the absence of increasing concentrations of AII, 10^{-11}-10^{-9} M. The inhibitory effect of AII was dose-dependent with an ID_{50} of 0.5 x 10^{-9} M. However, this effect was found to be largely prevented when cells were incubated with pertussis toxin prior to the addition of AII and/or hCG. The inhibitory effect of AII affected the hormonal stimulus over the entire dose-response range of hCG concentrations. As a result of addition of a submaximal dose of AII to the Leydig cells stimulated by various doses of hCG, the dose-response curve showed a significant increase in ED_{50} (2-fold), Fig. 5, right. Our studies have also provided evidence for an inhibitory effect of AII on receptor-bound cAMP (Fig. 6). Both basal and hCG-stimulated cAMP production were inhibited by the addition of AII to the cells. However, this inhibition was prevented when cells were incubated with pertussis toxin prior to the addition of AII and/or hCG. As a result of reduced production of cAMP, AII (10^{-10}-10^{-6} M), in a dose-dependent manner, inhibited testosterone production by Leydig cells stimulated by hCG (ID_{50} 1 x 10^{-10} M). This was commensurate with its binding affinity. Further addition

**Fig. 8. Effect of 8-bromo-cAMP on AII-induced inhibition of testosterone production.** Leydig cells (1 x 10^6 cells/ml) were incubated for 60 min with 8-bromo-cAMP in the presence and in the absence of hCG and/or AII. Each point represents the mean ± S.E. of triplicate incubations.
of forskolin ($10^{-8}$ M) to a submaximal inhibitory dose of AII caused an additive inhibition of testosterone production (Fig. 7, left), and this finding, therefore, indicated that both substances are exerting their inhibitory effects through a common pathway. This inhibitory action of AII was found to be prevented when cells were pretreated with pertussis toxin prior to the addition of hCG and/or AI1 (Fig. 7, right). This finding resembles our previous studies, demonstrating the involvement of a pertussis toxin-sensitive regulatory protein in the inhibitory action of low doses of forskolin (7). Moreover, our results are consistent with early studies on the adrenal (22) and more recent studies in liver (27) and kidney (28) which showed that AII receptors are functionally linked to the inhibitory unit of the adenylate cyclase system.

Furthermore, 8-bromo-cAMP ($10^{-3}$ M) bypassed the inhibitory effect of AII in hCG-stimulated cells (Fig. 8). This cAMP derivative was also shown to exert a similar effect on the inhibitory action of a low dose of forskolin (Table I). Also, AII significantly decreased GTP and luteinizing hormone plus GTP-stimulated adenylate cyclase activities in membranes, and this inhibition was prevented by pretreatment of membranes with pertussis toxin (Table II). Thus, the reduction in cAMP levels by AII is attributable to the inhibition of adenylate cyclase activity in the plasma membrane. It is, therefore, proposed on the basis of our studies that the N unit of adenylate cyclase is involved in the inhibitory action of AII on adenylate cyclase and consequently on cAMP pools and testosterone production. However, we cannot rule out the participation of other additional signal-transducing systems in mediating the inhibitory action of AII in the Leydig cells, since the role of calcium as a second messenger in the action of AII is well documented in liver (29), adrenals (30, 31), smooth muscle (32), and kidney (28). Also, the existence of a pertussis toxin-independent guanine nucleotide inhibition has been postulated for several systems (27, 33, 34). Furthermore, in liver (27) and in kidney (28), two different kinds of receptor-dependent mechanisms were delineated for the cellular responses of AII including phospholipase C/increase in intracellular calcium and adenylate cyclase system. However, in the present study, the finding that 8-bromo-cAMP bypassed the inhibitory effect of AII on hCG-stimulated testosterone production is suggestive that the pertussis-sensitive regulatory protein (N) mainly mediates the inhibitory effect of AII and modulates LH stimulation of Leydig cells. Because of the predominant localization of angiotensin-converting enzyme in the testicular tubular elements, it is likely that AII possesses a physiological paracrine regulatory function and the locally produced hormone would also effectively exert homologous negative modulatory influence on hormonal-stimulated events in the Leydig cells.

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