Characterization of atrial-natriuretic-factor-receptor-coupled membrane guanylate cyclase from rat and mouse testes

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Studies with isolated adrenal cells and mouse testicular cells have supported a mediatory role of cyclic GMP in ANF (atrial natriuretic factor)-dependent steroidogenic signal transduction. This concept has been strengthened by the purification and biochemical characterization of a 180 kDa protein, which appears to contain both ANF receptor and guanylate cyclase activities, from rat adrenocortical carcinoma cells. Utilizing the antibody to 180 kDa membrane guanylate cyclase as a probe, we now demonstrate the direct presence of ANF-dependent membrane guanylate cyclase in mouse and rat testes. The antibody blocks the ANF-dependent guanylate cyclase activity in isolated membranes, and Western-blot analysis of the partially purified enzyme reveals a single 180 kDa protein. The presence of this enzyme in mouse and rat testes, together with its previous demonstration in rat adrenocortical carcinoma, represent an important potential biochemical role for this enzyme in receptor-mediated steroidogenic signal transduction.

INTRODUCTION

Atrial natriuretic factor (ANF) is a peptide hormone that is released from atria and regulates sodium excretion, water balance and blood pressure (Cantin & Genest, 1985; deBold, 1985; Schwartz *et al.*, 1985; Atlas & Laragh, 1986). The putative 'second messenger' of certain ANF-signal transductions is cyclic GMP (Winquist *et al.*, 1984). In a number of steroid-secreting cells, ANF inhibits basal and hormone-stimulated steroidogenesis, which is accompanied by an elevation in cyclic GMP level and by a decline in cyclic AMP. For instance, aldosterone secretion in rat adrenoglomerulosa cells (Atarashi *et al.*, 1984; Chartier *et al.*, 1984; DeLean *et al.*, 1984; Kudo & Baird, 1984), both cortisol and aldosterone secretions in human adrenal tissue *in vitro* (Naruse *et al.*, 1987) and gonadotropin-stimulated progesterone production in murine tumour cells (Pandey *et al.*, 1985) are decreased. These results implicate cyclic GMP in the modulation of steroidogenesis, but the mechanism by which the cyclic nucleotide mediates ANF action is now known. It is noteworthy that, to date, the sole example of a link between any metabolic activity and the ANF-receptor-mediated signal is the process of steroidogenesis.

In direct contrast with the above results, infusion studies *in vivo* with rat adrenal gland (Nakamura *et al.*, 1985) and studies *in situ* with mouse interstitial cells (Bex & Corbin, 1985) show that ANF stimulates the production of corticosterone and testosterone in the respective systems. The process of steroidogenesis is accompanied both by an elevation of membrane guanylate cyclase activity and cyclic GMP levels in isolated fasciculata cells of rat adrenal cortex (Jaiswal *et al.*, 1986) and by an elevation of cyclic GMP levels in mouse Leydig cells (Mukhopadhyay *et al.*, 1986a; Pandey *et al.*, 1986), supporting the mediatory role of this nucleotide in both adrenocortical and testicular steroidogenesis. In further

support of this concept, we have demonstrated the presence of ANF-dependent membrane guanylate cyclase in rat adrenocortical carcinoma and have purified a 180 kDa protein from this tissue that contains guanylate cyclase activity and is coupled to an ANF receptor (Paul *et al.*, 1987). This provides a potential biochemical link between the ANF-receptor steroidogenic signal and cyclic GMP. In support of the close coupling of the ANF signal with testicular steroidogenesis (Bex & Corbin, 1985; Mukhopadhyay *et al.*, 1986b), in which cyclic GMP is a potential second messenger (Mukhopadhyay *et al.*, 1986a; Pandey *et al.*, 1986), we have extended these studies by directly demonstrating, in mouse and rat testis, an ANF-dependent membrane guanylate cyclase which is biochemically and immunologically indistinguishable from the rat adrenocortical carcinoma 180 kDa protein. Implications of these results for the plausible mechanism by which cyclic GMP mediates steroidogenic signal transduction have been explored.

EXPERIMENTAL

Materials and methods

Membranes isolated from rat and mouse testes were solubilized as described by Nambi *et al.* (1982a). The ANF-coupled membrane guanylate cyclase was purified to the GTP-affinity step as described in Paul *et al.* (1987).

Rabbit anti-(membrane guanylate cyclase) antibody was prepared by immunizing the rabbits with purified rat adrenocortical-carcinoma 180 kDa membrane guanylate cyclase (Paul *et al.*, 1987). IgG fractions were prepared from serum pools by a threefold precipitation with $(\text{NH}_4)_2\text{SO}_4$, followed by DEAE-cellulose chromatography (Garvey *et al.*, 1977). These IgG fractions from immune and normal serum pools were used for all experiments.

Abbreviation used: ANF, atrial natriuretic factor.

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In these studies, synthetic ANF consisting of the 26-amino-acid peptide *H*-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH was used.

RESULTS AND DISCUSSION

ANF stimulates crude membrane guanylate cyclase isolated from both rat and mouse testes, and the hormonal stimulation is blocked by the antibody to the 180 kDa rat adrenocortical-carcinoma membrane guanylate cyclase (Table 1), indicating the hormonal dependence of the 180 kDa enzyme. The hormonal dependence is lost upon solubilization and further purification of the enzyme (Table 2). These results are similar to those obtained for the rat adrenocortical carcinoma enzyme (Paul *et al.*, 1987). Since the loss in response to ANF stimulation and elevation of basal activity occurs during the detergent solubilization of the receptor, as speculated previously (Paul *et al.*, 1987), it is possible that a lipid component or an accessory protein necessary for hormonal stimulation is lost in this purification step. This loss would also account for the

release of otherwise-constrained enzyme activity in the non-solubilized membranes.

The basal guanylate cyclase activity in rat testis particulate fraction is ~ 7-fold less than that in mouse testis, but the extent of stimulation is the same in both species (Table 1). This may provide an explanation for the enigmatic behaviour of the rat and mouse cells in the production of steroids in response to ANF signal (Mukhopadhyay *et al.*, 1986b), although both cell types contain the hormonally dependent membrane guanylate cyclase. In the case of rat (in contrast with the mouse), the level of cyclic GMP produced in response to the hormone may be below the threshold to cause steroidogenesis. Our results support this previous interpretation, which was based on the observation that 8-bromo cyclic GMP stimulates testosterone production in rat as well as in mouse testicular cells (Mukhopadhyay *et al.*, 1986a).

There was no significant inhibition of rat or mouse basal guanylate cyclase activity by the 180-kD membrane guanylate cyclase antibody (Table 1). One possible explanation for this is that the basal activity of the crude membrane guanylate cyclase comprises the accumulative activity of as-yet-unknown multiple multiple isoenzyme

Table 1. Stimulation of rat and mouse testis membrane (non-solubilized) guanylate cyclases by ANF, and its inhibition by anti-(180 kDa membrane guanylate cyclase) polyclonal antibody

Membranes were preincubated with or without antibody, as appropriate, for 1 h on ice. The assay system contained 10 mM-theophylline, 15 mM-phosphocreatine, 20 μ g of creatine kinase, 1 mM-CaCl₂, 50 mM-Tris/HCl, pH 7.5. The total assay volume was 100 μ l. The assay mixture was preincubated with or without 1 μ M-ANF for 10 min on ice. The reaction was initiated by the addition of substrate solution containing 1 mM-MnCl₂ and 4 mM-GTP. Incubation (37 °C, 10 min) was terminated by addition of 0.9 ml of 50 mM-sodium acetate buffer, pH 6.2, followed by boiling in a water bath for 3 min. The amount of cyclic GMP was quantified by radioimmunoassay. Results are shown as means $\pm$ S.E.M. ($n = 3$).

Additions to membranes	Guanylate cyclase activity (pmol of cyclic GMP/min per mg of protein)	
	Rat testis	Mouse testis
Basal	40.36 $\pm$ 1.45	269.05 $\pm$ 6.10
Anti-(180 kDa guanylate cyclase) antibody	40.75 $\pm$ 1.45	270.32 $\pm$ 4.60
ANF (1 μ M)	72.20 $\pm$ 6.70	487.46 $\pm$ 21.70
ANF (1 μ M) + anti-(180 kDa guanylate cyclase) antibody	42.50 $\pm$ 3.10	349.88 $\pm$ 18.90

Table 2. Activities of solubilized and GTP-affinity-purified rat and mouse testicular membrane guanylate cyclases and the effect of ANF and anti-(180 kDa membrane guanylate cyclase) polyclonal antibody on these enzyme fractions

Experimental conditions were identical with those described in Table 1. Results are means $\pm$ S.E.M. ($n = 3$).

Additions to:	Guanylate cyclase activity (pmol of cyclic GMP/min per mg of protein)	
	Rat testis	Mouse testis
(a) Solubilized fraction		
Basal	301.88 $\pm$ 12.26	406.35 $\pm$ 9.41
ANF (1 μ M)	301.71 $\pm$ 10.40	405.68 $\pm$ 7.10
Anti-(180 kDa guanylate cyclase) antibody	302.16 $\pm$ 12.72	316.31 $\pm$ 2.60
(b) GTP-affinity-purified fraction		
Basal	516.99 $\pm$ 19.60	515.55 $\pm$ 5.13
ANF (1 μ M)	517.51 $\pm$ 19.30	514.81 $\pm$ 6.20
Anti-(180 kDa guanylate cyclase) antibody	245.99 $\pm$ 24.24	226.6 $\pm$ 14.20

forms, among which the 180 kDa enzyme represents a minor component. This interpretation is supported by the fact that antibody to the 180 kDa protein blocks almost all of the guanylate cyclase activity of the purified enzyme (Paul *et al.*, 1987).

Biochemical authenticity of the rat and mouse membrane guanylate cyclase with the 180 kDa membrane guanylate cyclase is shown by the following criteria: (i) the antibody to the 180 kDa membrane guanylate cyclase (Paul *et al.*, 1987) blocks up to 60% of the partially purified guanylate cyclase activity from both sources (Table 2). It is noteworthy that this antibody is highly specific for particulate guanylate cyclase, since it blocks neither soluble guanylate cyclase nor adenylate cyclase activities (Paul *et al.*, 1987); (ii) Western-blot analysis of the GTP-affinity-purified enzyme shows a single 180 kDa band, although the SDS/polyacrylamide-gel electrophoresis of the GTP-affinity proteins shows multiple Coomassie Blue-stained bands (Figs. 1a and 1b). These results demonstrate that the 180 kDa rat adrenocortical-carcinoma membrane guanylate cyclases from rat and mouse testis are biochemically and immunologically indistinguishable.

Subsequent to our original report (Paul & Sharma, 1985) on the complete purification of the 180 kDa adrenocortical-carcinoma membrane guanylate cyclase, we have demonstrated that this protein is tightly coupled to an ANF receptor (Paul *et al.*, 1987). Although the receptor and guanylate cyclase activities are inseparable, antibody to the 180 kDa protein blocks guanylate cyclase activity but does not inhibit the binding of ANF to the protein, which indicates that either the antibody reacts solely against the guanylate cyclase epitope of the protein or that there are two tightly coupled 180 kDa proteins which are inseparable by the present techniques (Paul *et al.*, 1987).

It is noteworthy that lung guanylate cyclase, which apparently is also tightly coupled to an ANF receptor (Kuno *et al.*, 1986), is strikingly different both biochemically and kinetically from the 180 kDa membrane guanylate cyclase (Paul *et al.*, 1987): the subunit molecular mass of the lung protein is 120 kDa; in contrast with the 180 kDa guanylate cyclase, the lung enzyme is stimulated by haemin (Waldman *et al.*, 1985); the pI of the lung enzyme is 6, whereas that of the 180 kDa protein is 4.7. Finally, in contrast with the near 1:1 stoichiometry of the binding of ANF to the tumour enzyme, the lung enzyme binds only 14.5% of ANF at the noted theoretical value.

Very recently Takayanagi *et al.* (1987) and Carrier *et al.* (1987) have also purified and characterized a guanylate cyclase-coupled ANF receptor from bovine adrenal cortex. The enzyme and the receptor activities occur in a single subunit of 135 kDa. Biochemical similarity of this protein either to lung or to the 180 kDa membrane guanylate cyclase is not known, but the molecular size is closer to that of the lung enzyme.

Previous studies have shown that 8-bromo cyclic GMP stimulates steroidogenesis in testicular (Mukhopadhyay *et al.*, 1986b) and isolated fasciculata cells of rat adrenal cortex (Ahrens *et al.*, 1982), and that, in the latter cells, (i) cyclic GMP stimulates cyclic GMP-dependent protein kinase and corticosterone production (Sharma *et al.*, 1976), (ii) cyclic GMP directly stimulates the transformation of cholesterol to corticosterone (Sharma & Sawhney, 1978) and (iii) the steroidogenic potential of

Fig. 1. Immunoblot analysis of the GTP-affinity-purified fractions of rat and mouse membrane guanylate cyclases

A 20 μ g portion of the crude enzymes was subjected to SDS/7.5%-(w/v)-polyacrylamide-gel electrophoresis. (a) Rat testes: lane 1, Coomassie Blue-stained M_r markers; lane 2, Coomassie Blue-stained GTP-affinity-purified fraction; lane 3, immunogenic band of the GTP-affinity-purified fraction as revealed by Western-blot analysis utilizing the 180 kDa rat adrenocortical-carcinoma membrane guanylate cyclase antibody. (b) Mouse testes: lane 1, Coomassie Blue-stained M_r markers; lane 2, Coomassie Blue-stained GTP-affinity-purified fraction; lane 3, immunogenic band of the GTP-affinity-purified fraction as revealed by Western-blot analysis utilizing the 180 kDa rat adrenocortical-carcinoma membrane guanylate cyclase antibody. Western-blot analysis was performed as described by Towbin *et al.* (1979), by subjecting GTP-affinity-purified fraction to SDS/7.5%-(w/v)-polyacrylamide-gel electrophoresis, followed by electrophoretically transferring the proteins to a nitrocellulose membrane. The protein-binding sites were blocked by nitrocellulose membrane with 3% bovine serum albumin. The membrane was incubated for 2 h at room temperature with 180 kDa rat adrenocortical-carcinoma membrane guanylate cyclase polyclonal antibody (dilution 1:200), followed by 2 h incubation at room temperature with conjugated anti-(rabbit IgG)-horse-radish peroxidase. The colour was developed by soaking the blot in horseradish peroxidase (600 μ g/ml)/0.015% H_2O_2 /10 mM-Tris/HCl, pH 7.4. The reaction was terminated by extensive washing with water. The standard M_r markers used were myosin (200000), β -galactosidase (116000), phosphorylase *b* (94000), bovine serum albumin (68000) and ovalbumin (43000). Abbreviation: DF, dye front.

cyclic GMP and its analogues correlates closely with their ability to stimulate cyclic GMP-dependent protein kinase (Ahrens *et al.*, 1982). In addition, cyclic GMP-dependent protein kinase has been purified to homogeneity and biochemically characterized in bovine adrenal cortex and isolated rat adrenal cells (Ahrens *et al.*, 1982). Although all these results provided strong evidence for the mediatory role of cyclic GMP in steroidogenic signal transduction, the crucial support for this concept came with the first demonstration of a hormonally dependent membrane guanylate cyclase which was different from the non-specific soluble guanylate cyclase (Nambi & Sharma, 1981a,b; Nambi *et*

al., 1982a,b). Our present results on the characterization of the 180 kDa membrane guanylate cyclase, which is tightly coupled to an ANF receptor in mouse and rat testis, as well as in the adrenal gland (Paul *et al.*, 1987), provide the identification of an important missing biochemical link which transduces the transmembrane ANF steroidogenic signal.

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