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Evidence for the requirement of proteolysis in LH stimulated cyclic AMP production and steroidogenesis in Leydig cells

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We have investigated the effect of protease activity on cyclic AMP production and steroidogenesis in rat testis, mouse testis and mouse tumour Leydig (MA10) cells. LH-, dibutyryl cyclic AMP-, and forskolin-stimulated steroidogenesis, but not 22R(OH) cholesterol conversion to pregnenolone, was inhibited by protease inhibitors. In mouse Leydig cells, LH but not forskolin or cholera toxin stimulated cyclic AMP production was inhibited by protease inhibitors. These results suggest that steroidogenesis in Leydig cells requires proteolysis before the conversion of cholesterol to pregnenolone. In the mouse but not rat Leydig cells, LH-stimulated cyclic AMP production is also dependent on proteolysis.

Luteinizing hormone; Steroidogenesis; Cyclic AMP; Proteolysis

1. INTRODUCTION

Luteinizing hormone (LH) interacts with a specific cell surface receptor to stimulate steroidogenesis in Leydig cells. The hormonally regulated, rate limiting steps in steroid formation is thought to be the transport of cholesterol [1]. A possible protein candidate for this role was isolated from a Leydig cell tumor; the 'steroidogenesis activator polypeptide' (SAP) [2] was proposed to be cleaved from a glucose-regulated protein (GRP78) by a regulated process that is related to steroidogenesis [3], indicating the possible involvement of protease activity in steroidogenesis. Previously we have shown that inhibition of protease activity prevented the loss of receptors during down-regulation in MA10 cells and caused a 2–3-fold increase in LH receptor numbers in rat Leydig cells, with a similar increase in cyclic AMP production [4]. We have therefore investigated the effect of inhibiting protease activity in Leydig cells on the subsequent stimulated cyclic AMP production and steroidogenesis.

2. MATERIALS AND METHODS

Stock cultures of MA10 cells were maintained in Waymouth's MB752/1 medium according to the method of Ascoli (1981) [5]. Adult male Sprague–Dawley rat Leydig cells were prepared by collagenase dispersion, centrifugal elutriation and Percoll density gradient centrifugation and incubated as previously described [6] in DMEM (Gibco)-0.1% BSA (bovine serum albumin, Sigma). Adult male BALB/C mice Leydig cells were prepared by mechanical dispersion using a 50 ml syringe, followed by Percoll density gradient centrifugation [7] and incubated as for the rat Leydig cells. Leydig cell purity was established by 3β-hydroxysteroid dehydrogenase cytochemistry [8].

The addition of cyanoketone (5 μM) and SU-10603 (20 μM) (pregnenolone metabolism inhibitors) (Sterling Research Ltd.) and protease inhibitors was made 30 min prior to the addition of other reagents. The latter inhibitors (leupeptin (100 μM), PMSF (10 μM) and aprotinin (900 units/ml) (Sigma) were added as 100-fold concentrated aliquots to the incubation medium. LH (NIAMDD-oLH-22, 2.3 IU/mg, Bethesda, MD, USA), dibutyryl cyclic AMP, forskolin and cholera toxin (Sigma) were dissolved in incubation medium and 22R(OH) cholesterol and pregnenolone (Sigma) were dissolved in DMSO. Incubations were stopped with $HClO_4$ and stored frozen at −20°C. Samples were thawed and neutralized with K_3PO_4 prior to radioimmunoassay of testosterone [9], pregnenolone [10] and cyclic AMP [11,12]. Statistical analysis was by the Student's *t*-test.

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3. RESULTS

MA10 and rat Leydig cells were preincubated for 30 min with leupeptin (100 μM), PMSF (10 μM), and aprotinin (900 units/ml) in 1 ml of media at 34°C. LH (0.033 nM and 0.33 nM), 22R(OH)-cholesterol (5 μM), cholera toxin (1.19 nM), forskolin (8 μM) or dibutyryl cyclic AMP (1 mM) were then added for 2 h. The reactions were stopped and measured for pregnenolone, testosterone and cyclic AMP production. Fig. 1 shows the effect of the protease inhibitors on pregnenolone production in rat Leydig (Fig. 1A) and MA10 (Fig. 1B) cells. LH, forskolin and dibutyryl cyclic AMP stimulated pregnenolone were all inhibited in both cell types. There was no effect on the conversion of 22R(OH)-cholesterol to pregnenolone. Fig. 2 shows the effect of protease inhibitors on testosterone production

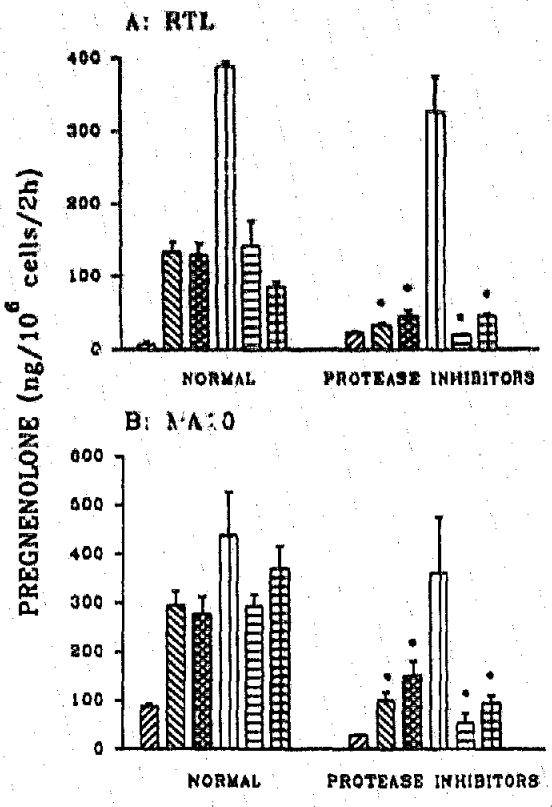

Fig. 1. The effect of protease inhibitors on pregnenolone production in Leydig cells. Rat Leydig (A) and MA10 (B) cells were plated at 10^5/well with 1 ml incubation medium. The cells were preincubated for 30 min with or without protease inhibitors before the addition of reagents. Protease inhibitors were present through the incubations. Cells were incubated for 2 h in the absence (▨) or presence of 0.033 nM (▧), 0.33 nM (▩) LH, 5 μM (▥) 22R(OH) cholesterol, 1 mM (▤) dibutyryl cyclic AMP or 8 μM (▦) forskolin. Data represent the mean ± SD of 2 experiments. Significant differences are shown for inhition only (*$P < 0.001$).

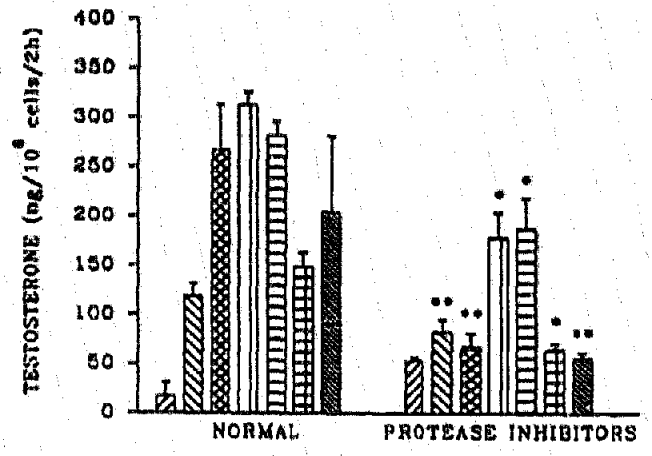

Fig. 2. The effect of protease inhibitors on testosterone production in rat testis Leydig cells. Cells were plated at 10^5/well with 1 ml incubation medium. The cells were preincubated for 30 min with or without protease inhibitors before the addition of reagents. Protease inhibitors were present through the incubations. Cells were incubated for 2 h in the absence (▨) or presence of 0.033 nM (▧), 0.33 nM (▩) LH, 5 μM (▥) pregnenolone, 5 μM (▤) 22R(OH) cholesterol, 8 μM (▦) forskolin or 1 mM (▧) dibutyryl cyclic AMP. Data represent the mean ± SD of 2 experiments. Significant differences are shown for inhibition only (*$P < 0.05$, **$P < 0.001$).

in rat Leydig cells. In these experiments LH, dibutyryl cyclic AMP, and forskolin stimulated testosterone were inhibited. Pregnenolone and 22R(OH)-cholesterol conversion to testosterone were inhibited but to a lesser extent.

Previously we have shown that LH-stimulated, but not cholera toxin- or forskolin-stimulated, cyclic AMP production in the rat Leydig cells was increased by two- to three-fold in the presence of protease inhibitors [4]. Fig. 3 shows cyclic AMP production in MA10 cells in the absence and presence of protease inhibitors. Both 0.33 nM and 3.3 nM LH-stimulated cyclic AMP production was inhibited by 70%, but forskolin- and cholera toxin-stimulated cyclic AMP production was unaffected.

As the difference in the effect of protease inhibitors on cyclic AMP production between the MA10 cell and the normal rat testis Leydig cell, may have been a result of the MA10 cell being a tumour cell, we repeated our studies on normal mouse testis Leydig cells. The following results which are the mean ± SD of 3 replicates were obtained. LH-stimulated cyclic AMP production was inhibited from 205.8 ± 58 to 21.2 ± 5.7 pmol/10^6 cells/2 h. Stimulation of cyclic AMP by forskolin was unaffected (untreated; 132.0 ± 1.1 pmol/10^6 cells/2 h :

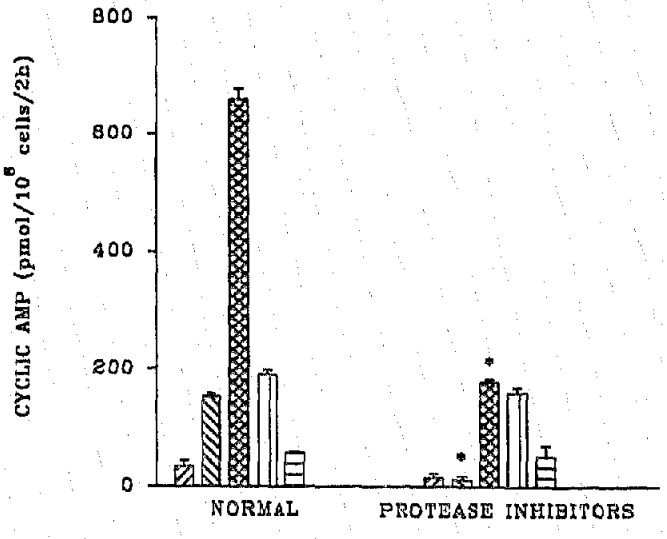

Fig. 3. The effect of protease inhibitors on cyclic AMP production in MA10 cells. Cells were plated at 10^5/well with 1 ml incubation medium. The cells were preincubated for 30 min with or without protease inhibitors before the addition of reagents. Protease inhibitors were present through the incubations. Cells were incubated for 2 h in the absence (▨) or presence of 0.033 nM (▧), 0.33 nM (▩) LH, 8 μM (▥) forskolin or 1.19 nM (▤) cholera toxin. Data represent the mean ± SD of 2 experiments. Significant differences are shown for inhibition only (*$P < 0.001$).

protease inhibitor-treated; 124 ± 9.3 pmol/10^6 cells/2 h). LH-stimulated (711 ± 32 ng/10^6 cells/2 h) and db-cAMP-stimulated (759 ± 88 ng/10^6 cells/2 h) testosterone production was also inhibited in the presence of the protease inhibitors (LH; 259 ± 146 ng/10^6 cells/2 h : db-cAMP; 279 ± 52 ng/10^6 cells/2 h). There was partial inhibition of 22R(OH)-cholesterol conversion to testosterone (1019 ± 10 to 748 ± 11 ng/10^6 cells/2 h). This is similar to the results for 22R(OH)-cholesterol conversion to testosterone in rat Leydig cells (Fig. 2).

4. DISCUSSION

The present experiments indicate that serine proteases are involved in the control of steroidogenesis in mouse and rat Leydig cells at a step after cyclic AMP production and before side-chain cleavage of cholesterol. In addition, in the mouse Leydig cells, proteases are required for the stimulation of cyclic AMP production by LH.

The evidence for the location of the action of the proteases on steroidogenesis is based on the found inhibition of both LH- and db-cAMP-stimulated testosterone production and the lack of effect on 22R(OH)-cholesterol conversion to pregnenolone. It is recognized that the rate-limiting step in steroidogenesis is the transport of cholesterol from the outer to the inner mitochondrial membrane and that a carrier protein is required for this transport [1]. 22R(OH)-Cholesterol bypasses this rate-limiting step and has direct access to the inner mitochondrial membrane where it undergoes side-chain cleavage. It has also been shown that cholesterol stored in the plasma membrane is utilized for mitochondrial steroidogenesis [12] and that cyclic AMP regulates its transport in MA10 cells. Our experiments indicate, therefore, that the transport of cholesterol from either the plasma membrane and/or from the outer to inner mitochondrial membrane may require the proteolysis of a cholesterol carrier precursor protein.

The inhibition of LH-stimulated cyclic AMP production by protease inhibitors in the MA10 and normal mouse Leydig cells is in contrast to the results obtained for the rat, where a 2–3-fold increase in cyclic AMP was observed [3] under the same conditions. As no effect of the protease inhibitors is seen on forskolin- or cholera toxin-stimulated cyclic AMP, the protease inhibitors most probably modulate the LH receptor. This may indicate that in mouse Leydig cells proteolysis of the LH receptor is required for activation or that the inhibitors are affecting the binding of LH. The latter is unlikely however, because protease inhibitors have no effect on the binding characteristics of [^{125}I]hCG [3]. We have previously demonstrated that proteases have a direct effect on the LH receptor by causing truncation of its extracellular domain, and that truncation is regulated by an inhibition of internalization of the receptor in the mouse but not the rat [3]. Further studies are required to determine if there is a relationship between truncation and activation of adenylate cyclase in the mouse Leydig cell.

To conclude, the present report and our previous studies indicate that proteases have an important role in the regulation of steroidogenesis via modulation of the LH receptor, causing both truncation and activation of adenylate cyclase, and by regulating cholesterol transport.

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