REGULATION OF THE LUTEINIZING HORMONE RECEPTOR IN LEYDIG CELLS

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Abstract

The structure-function relationships and regulation of luteinizing hormone (LH)/chorionic gonadotropin (CG) receptors in Leydig cells was investigated. Four Leydig cell types were used: two mouse tumour Leydig cells (MA10 and MLTC-1), a rat tumour Leydig cell (R2C) and rat testis Leydig cells. Desensitization, up- and down-regulation of receptors were studied including the role of proteolysis in the mechanism of down-regulation. A new method was developed to study these processes, which generates truncated, but functional receptors using antisense oligodeoxynucleotides.

The up-regulation of functional LH/CG receptors was found to occur only in MA10 cells, which continually synthesize their LH/CG receptor, after a 24h incubation. This is a cyclic AMP-dependent mechanism and occurs at low doses of agonists. The increase was inhibited by cycloheximide and actinomycin D.

The mechanism for the loss of binding sites in Leydig cells over 2h was found to be different in the rat and mouse Leydig cells. No loss of receptors was observed in rat Leydig cells, but continual proteolysis of LH/CG receptors occurred at the plasma membrane. In mouse Leydig cells LH-induced loss of binding sites was due to a cyclic AMP-dependent mechanism - which occurred at high doses of agonists and resulted in an inhibition of internalization allowing for loss of binding sites at the plasma membrane by proteolytic cleavage.

Using antisense oligodeoxynucleotides, truncated but functional receptors in the MA10 cell were generated. Six different antisense oligodeoxynucleotides were used to show that the phosphorylation sites on the intracellular C-terminal tail are required for desensitization. Furthermore the conformation of the 7th
transmembrane domain and intracellular C-terminal tail were shown to be important for the mechanism of desensitization. Also, the final 9 amino acids on the intracellular C-terminal tail are essential for LH-induced loss of receptors. MA10 cells secrete high affinity LH/CG binding proteins, which were shown to be coded for by LH/CG receptor mRNA.
## Contents:

<table>
<thead>
<tr>
<th>Section Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Contents</td>
<td>4</td>
</tr>
<tr>
<td>Figures</td>
<td>9</td>
</tr>
<tr>
<td>Tables</td>
<td>17</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>18</td>
</tr>
<tr>
<td><strong>Chapter 1: General introduction</strong></td>
<td>19</td>
</tr>
<tr>
<td>1.1 The Testis</td>
<td>20</td>
</tr>
<tr>
<td>1.2 Spermatogenesis</td>
<td>23</td>
</tr>
<tr>
<td>1.3 Interactions of cells involved in the control of spermatogenesis</td>
<td>23</td>
</tr>
<tr>
<td>1.4 Steroidogenesis</td>
<td>28</td>
</tr>
<tr>
<td>1.5 Interactions between cells involved in the control of steroidogenesis</td>
<td>33</td>
</tr>
<tr>
<td>1.6 Luteinizing hormone</td>
<td>35</td>
</tr>
<tr>
<td>1.6.1 LH/CG structure</td>
<td>38</td>
</tr>
<tr>
<td>1.6.2 LH/CG interaction with its receptor</td>
<td>38</td>
</tr>
<tr>
<td>1.6.3 Control of LH release by gonadotropin hormone (GnRH)</td>
<td>39</td>
</tr>
<tr>
<td>1.6.4 LH/CG action in the Leydig cell</td>
<td>41</td>
</tr>
<tr>
<td>1.6.5 Desensitization of LH/CG responses</td>
<td>43</td>
</tr>
<tr>
<td>1.7 The LH/CG receptor</td>
<td>44</td>
</tr>
<tr>
<td>1.7.1 The structure of the LH/CG receptor: biochemical approaches</td>
<td>44</td>
</tr>
<tr>
<td>1.7.2 The structure of the LH/CG receptor: cloning of cDNA</td>
<td>46</td>
</tr>
<tr>
<td>1.7.3 The gene for the LH/CG receptor</td>
<td>58</td>
</tr>
<tr>
<td>1.7.4 Messenger RNA for the LH/CG receptor</td>
<td>59</td>
</tr>
<tr>
<td>1.7.5 Hormone binding region of the LH/CG receptor</td>
<td>61</td>
</tr>
<tr>
<td>1.7.6 LH/CG binding proteins</td>
<td>62</td>
</tr>
</tbody>
</table>
1.7.7 LH/CG receptor interaction with G-proteins
1.7.8 Regulation of LH/CG receptors
1.7.9 Regulation of LH/CG receptor mRNA
1.7.10 The role of phosphorylation of the LH/CG receptor

1.8 Aims

Chapter 2: Materials and Methods

2.1 Materials
2.2 Methods
2.2.1 Isolation of rat testis Leydig cells
2.2.2 Percoll density gradients
2.2.3 Isolation of mouse testis Leydig cells
2.2.4 Culture of tumour Leydig cells
2.2.5 Cell incubations
2.2.6 Diaphorase histochemistry
2.2.7 3β-Hydroxysteroid dehydrogenase histochemistry
2.2.8 Iodination of hCG
2.2.9 Iodination of cyclic AMP
2.2.10 Cell surface [125I]-hCG binding assay
2.2.11 Membrane and solubilized cell [125I]-hCG binding assay
2.2.12 Internalization studies
2.2.13 Membrane preparation
2.2.14 Bio-Rad protein assay
2.2.15 SDS-PAGE
2.2.16 Amino acid incorporation
2.2.17 Cyclic AMP RIA
2.2.18 Pregnenolone and testosterone RIA
2.2.19 Inhibitors of pregnenolone metabolism

Chapter 3: Characterization of cultured tumour Leydig cells

3.1 Summary
3.2 **Introduction**

3.2.1 MA10 cells

3.2.2 MLTC-1 cells

3.2.3 R2C cells

3.3 **Results**

3.3.1 Growth rates and cell density

3.3.2 Receptor content of culture tumour Leydig cells

3.3.3 Stimulation of cyclic AMP and pregnenolone production

3.3.4 Desensitization in rat and mouse Leydig cells

3.4 **Discussion**

Chapter 4: The up-regulation of the LH/CG receptor

4.1 **Summary**

4.2 **Introduction**

4.3 **Results**

4.3.1 Initial characterization of a cyclic AMP-dependent pathway for the up-regulation of LH/CG receptors in MA10 cells

4.3.2 Comparative binding studies on the up-regulation of the LH/CG receptors in MA10 cells

4.3.3 The effect of transcription and translation inhibitors on the up-regulation of LH/CG receptors

4.3.4 Cyclic AMP and pregnenolone production in LH/CG receptor up-regulated cells
| 4.3.5 | Comparison of the effects of LH and db-cAMP on the up-regulation of LH/CG receptors in MLTC-1 and R2C cultured tumour cells | 171 |
| 4.4 | Discussion | 171 |

**Chapter 5: Mechanism involved in LH/CG receptor down-regulation**

| 5.1 | Summary | 175 |
| 5.2 | Introduction | 176 |
| 5.3 | Results | 177 |
| 5.3.1 | The effect of inhibition of receptor internalization on the levels of LH/CG receptors | 177 |
| 5.3.2 | The role of protein kinase A and C in the regulation of LH/CG receptors | 199 |
| 5.4 | Discussion | 210 |

**Chapter 6: The role of proteases in LH/CG action**

| 6.1 | Summary | 213 |
| 6.2 | Introduction | 214 |
| 6.3 | Results | 215 |
| 6.3.1 | The effect of protease inhibitors on the level of LH/CG receptors | 215 |
| 6.3.2 | Detection of LH/CG receptor proteolytic fragment | 234 |
| 6.3.3 | The effect of protease inhibitors on cyclic AMP production and steroidogenesis | 240 |
| 6.3.4 | The effect of soybean trypsin inhibitor on cyclic AMP and testosterone production | 241 |
| 6.3.5 | The effect of protease inhibitors on LH/CG action in mouse testis Leydig cells | 245 |
| 6.4 | Discussion | 249 |
Chapter 7: Determination of structure-function-relationships of the LH/CG receptor using antisense oligodeoxynucleotides

7.1 Summary

7.2 Introduction

7.3 Results

7.3.1 Characterization of a protocol for the addition of LH/CG receptor antisense oligodeoxynucleotides

7.3.2 The effect of antisense oligodeoxynucleotides on the level of LH/CG receptors and LH-stimulated cyclic AMP and pregnenolone production

7.3.3 The effect of antisense oligodeoxynucleotides on LH/CG receptor desensitization and down-regulation

7.3.4 Demonstration of secreted high affinity LH/CG binding sites using antisense oligodeoxynucleotides

7.4 Discussion

Chapter 8: General discussion

8.1 Discussion

8.2 Future work

References

List of publications
<table>
<thead>
<tr>
<th>Figures</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1.1 Diagram of the parts of the testis</td>
<td>21</td>
</tr>
<tr>
<td>1.1.2 Cell biology of the testis</td>
<td>22</td>
</tr>
<tr>
<td>1.2.1 The seminiferous epithelium</td>
<td>24</td>
</tr>
<tr>
<td>1.2.2 Schematic representation of the spermatogenic cycle in the adult rat</td>
<td>25</td>
</tr>
<tr>
<td>1.4.1 Factors involved in the intermitochondrial transport of cholesterol</td>
<td>30</td>
</tr>
<tr>
<td>1.4.2 Pathways of androgen biosynthesis in rat testis</td>
<td>32</td>
</tr>
<tr>
<td>1.5.1 Schematic diagram to summarize some of the paracrine interactions that may modulate the production and action of testosterone (T) in the adult rat testis</td>
<td>34</td>
</tr>
<tr>
<td>1.6.1 The interpolated tertiary structure of hCG during receptor interaction</td>
<td>36</td>
</tr>
<tr>
<td>1.6.2 Amino acid sequence of loop peptides from LH and hCGß</td>
<td>37</td>
</tr>
<tr>
<td>1.6.3 Structure of human GnRH</td>
<td>39</td>
</tr>
<tr>
<td>1.6.4 Overview of gonadotrope action in the pituitary</td>
<td>40</td>
</tr>
<tr>
<td>1.7.1 The cDNA sequence and deduced amino acid sequence of the rat luteal LH/CG receptor</td>
<td>47</td>
</tr>
<tr>
<td>1.7.2 Hydropathy plot of the LH/CG receptor</td>
<td>50</td>
</tr>
<tr>
<td>1.7.3 The amino acid sequence of the LH/CG receptor</td>
<td>51</td>
</tr>
<tr>
<td>1.7.4 Depiction of zonal characteristics of the glycoprotein hormone receptor chain</td>
<td>53</td>
</tr>
<tr>
<td>1.7.5 Differentially spliced forms of the rat ovarian LH/CG receptor cDNA</td>
<td>55</td>
</tr>
<tr>
<td>1.7.6 Model of glycoprotein hormone-receptor interaction</td>
<td>60</td>
</tr>
<tr>
<td>1.7.7 Schematic representation of the possible routes of receptor and hormone during RME</td>
<td>66</td>
</tr>
</tbody>
</table>
2.2.1 The elutriator system
2.2.2 Profile obtained for the iodination of hCG
2.2.3 Profile obtained for the iodination of cyclic AMP
3.3.1 Growth rates for MA10, MLTC-1 and R2C cells
3.3.2 Time course of the recovery of LH/CG receptors after trypsin treatment
3.3.3 The effect of cycloheximide on the level of hCG binding sites in MA10 and R2C cells
3.3.4 Scatchard analysis of $[^{125}\text{I}]$-hCG in MA10, MLTC-1, rat testis Leydig and R2C cells
3.3.5 The effect of LH, hCG, cholera toxin and forskolin on cyclic AMP production in MA10 cells
3.3.6 The effect of LH, hCG, cholera toxin and forskolin on cyclic AMP production in MLTC-1 cells
3.3.7 The effect of LH, hCG, cholera toxin and forskolin on cyclic AMP production in R2C cells
3.3.8 The effect of LH, hCG, cholera toxin and forskolin on cyclic AMP production in rat testis Leydig cells
3.3.9 The effect of LH, hCG, db-cAMP, cholera toxin and forskolin in MA10 cells
3.3.10 The effect of LH, hCG, db-cAMP, cholera toxin and forskolin in MLTC-1 cells
3.3.11 The effect of LH, hCG, db-cAMP, cholera toxin and forskolin in R2C cells
3.3.12 The effect of LH, hCG, db-cAMP, cholera toxin and forskolin in rat testis Leydig cells
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.13</td>
<td>The effect of LH, db-cAMP and PMA pretreatment on subsequent pregnenolone and cyclic AMP production in MA10 cells</td>
<td>138</td>
</tr>
<tr>
<td>3.3.14</td>
<td>The effect of LH, db-cAMP and PMA pretreatment on subsequent pregnenolone and cyclic AMP production in mouse testis Leydig cells</td>
<td>140</td>
</tr>
<tr>
<td>3.3.15</td>
<td>The effect of LH, db-cAMP and PMA pretreatment on subsequent pregnenolone and cyclic AMP production in rat testis Leydig cells</td>
<td>142</td>
</tr>
<tr>
<td>4.3.1</td>
<td>The effect of LH, db-cAMP, cholera toxin and forskolin on the level of ( ^{125}\text{I})-hCG binding sites in MA10 cells</td>
<td>150</td>
</tr>
<tr>
<td>4.3.2</td>
<td>The effect of LH, db-cAMP, cholera toxin and forskolin on the growth of MA10 cells</td>
<td>151</td>
</tr>
<tr>
<td>4.3.3</td>
<td>Time course of the recovery of LH/CG receptors after trypsin treatment</td>
<td>153</td>
</tr>
<tr>
<td>4.3.4</td>
<td>The level of ( ^{125}\text{I})-hCG binding sites in MA10 cells over time</td>
<td>154</td>
</tr>
<tr>
<td>4.3.5</td>
<td>Time course of the effect of LH, cholera toxin, forskolin and db-cAMP on the levels of binding sites in MA10 cells</td>
<td>156</td>
</tr>
<tr>
<td>4.3.6</td>
<td>Time course of the effect of ( 1)mM db-cAMP with LH, cholera toxin or forskolin on the levels of binding sites in MA10 cells</td>
<td>157</td>
</tr>
<tr>
<td>4.3.7</td>
<td>Scatchard analysis of LH and db-cAMP treatment on the MA10 cells</td>
<td>158</td>
</tr>
<tr>
<td>4.3.8</td>
<td>The effect of horse serum on the level of LH/CG receptors in MA10 cells</td>
<td>161</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.3.9</td>
<td>The effect of translation and transcription inhibitors on the level of protein synthesis in MA10 cells</td>
<td>163</td>
</tr>
<tr>
<td>4.3.10</td>
<td>The effect of translation and transcription inhibitors on the level of $[^{125}]$-hCG binding sites in MA10 cells</td>
<td>164</td>
</tr>
<tr>
<td>4.3.11</td>
<td>Pregnenolone production by cells pretreated for 24h to induce LH/CG receptors</td>
<td>165</td>
</tr>
<tr>
<td>4.3.12</td>
<td>Scatchard analysis of the acid washing treatment on MA10 cells</td>
<td>168</td>
</tr>
<tr>
<td>4.3.13</td>
<td>The effect of LH and db-cAMP on the level of LH/CG receptors in MA10, MLTC-1 and R2C cells</td>
<td>170</td>
</tr>
<tr>
<td>5.3.1</td>
<td>The time course of the effect of LH and hCG on the levels of LH/CG receptors in MA10 cells</td>
<td>178</td>
</tr>
<tr>
<td>5.3.2</td>
<td>The effect of sodium azide on the internalization of $[^{125}]$-hCG in MA10 cells</td>
<td>183</td>
</tr>
<tr>
<td>5.3.3</td>
<td>Regulation of $[^{125}]$-hCG binding sites: The effect of inhibiting receptor internalization</td>
<td>185</td>
</tr>
<tr>
<td>5.3.4</td>
<td>Scatchard analysis of the effect of LH and sodium azide on MA10 cells</td>
<td>187</td>
</tr>
<tr>
<td>5.3.5</td>
<td>Scatchard analysis of the effect of LH and sodium azide on rat testis Leydig cells</td>
<td>189</td>
</tr>
<tr>
<td>5.3.6</td>
<td>The effect of db-cAMP on the levels of $[^{125}]$-hCG binding sites in Leydig cells</td>
<td>191</td>
</tr>
<tr>
<td>5.3.7</td>
<td>Scatchard analysis of the effect of db-cAMP and PMA on MA10 cells</td>
<td>195</td>
</tr>
</tbody>
</table>
5.3.8 Scatchard analysis of the effect of db-cAMP and PMA on R2C cells 197
5.3.9 The effect of cycloheximide on LH and db-cAMP induced loss of LH/CG receptors in MA10 cells 200
5.3.10 The effect of db-cAMP on the internalization of [125I]-hCG in MA10 cells 202
5.3.11 The effect of db-cAMP on the internalization of [125I]-hCG in MLTC-1 cells 204
5.3.12 The effect of PMA on the internalization of [125I]-hCG in MA10 cells 206
5.3.10 The effect of db-cAMP and PMA on the internalization of [125I]-hCG in rat testis Leydig cells 208
6.3.1 The effect of trypsin treatment of MLTC-1 cells on pregnenolone production 216
6.3.2 Scatchard analysis of trypsin treatment of MLTC-1 cells 217
6.3.3 The effect of protease inhibitors on the regulation of [125I]-hCG binding sites in mouse tumour Leydig cells 220
6.3.4 The effect of protease inhibitors on the regulation of [125I]-hCG binding sites in rat testis Leydig and rat tumour Leydig cells 221
6.3.5 The effect of protease inhibitors on the regulation of [125I]-hCG binding sites in rat testis Leydig cells with time 224
6.3.6 Scatchard analysis of the effect of protease inhibitors on MA10 cells 226
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3.7</td>
<td>The effect of protease inhibitors on the regulation of $^{125}$I-hCG binding sites in MA10 Leydig cells</td>
</tr>
<tr>
<td>6.3.8</td>
<td>Autoradiograph and densitometric trace of proteins derived from culture medium of rat testis Leydig cells prelabelled with $^{125}$I-hCG</td>
</tr>
<tr>
<td>6.3.9</td>
<td>Autoradiograph and densitometric trace of proteins derived from culture medium of MA10 cells prelabelled with $^{125}$I-hCG</td>
</tr>
<tr>
<td>6.3.10</td>
<td>Autoradiographs of Leydig cell culture medium incubated with $^{125}$I-hCG</td>
</tr>
<tr>
<td>6.3.11</td>
<td>The effect of protease inhibitors on cyclic AMP production in rat testis and MA10 Leydig cells</td>
</tr>
<tr>
<td>6.3.12</td>
<td>The effect of protease inhibitors on pregnenolone production in Leydig cells</td>
</tr>
<tr>
<td>6.3.13</td>
<td>The effect of protease inhibitors on testosterone production in rat testis Leydig cells</td>
</tr>
<tr>
<td>6.3.14</td>
<td>The effect of soybean trypsin inhibitor on LH-stimulated cyclic AMP production in rat testis Leydig cells</td>
</tr>
<tr>
<td>6.3.15</td>
<td>The effect of soybean trypsin inhibitor on LH-stimulated testosterone production in rat testis Leydig cells</td>
</tr>
<tr>
<td>6.3.16</td>
<td>The effect of soybean trypsin inhibitor on db-cAMP-stimulated testosterone production in rat testis Leydig cells</td>
</tr>
<tr>
<td>6.3.17</td>
<td>The effect of protease inhibitors on the regulation of $^{125}$I-hCG binding sites in mouse testis Leydig cells</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>6.3.18</td>
<td>The effect of protease inhibitors on cyclic AMP production in mouse testis Leydig cells</td>
</tr>
<tr>
<td>6.3.19</td>
<td>The effect of protease inhibitors on testosterone production in mouse testis Leydig cells</td>
</tr>
<tr>
<td>7.3.1</td>
<td>The amino acid sequence of the LH/CG receptor</td>
</tr>
<tr>
<td>7.3.2</td>
<td>The effect of one addition of Oligo 1 at time zero on the level of [125I]-hCG binding sites</td>
</tr>
<tr>
<td>7.3.3</td>
<td>The effect of two additions of Oligo 1 added at time zero and 24h on the level of [125I]-hCG binding sites</td>
</tr>
<tr>
<td>7.3.4</td>
<td>The effect of two additions of Oligo 1 added at time zero and 24h on LH-stimulated cyclic AMP</td>
</tr>
<tr>
<td>7.3.5</td>
<td>The effect of two additions of Oligo 1 added at time zero and 24h on LH-stimulated pregnenolone</td>
</tr>
<tr>
<td>7.3.6</td>
<td>Theoretical deletions caused by the antisense oligodeoxynucleotides encoding regions of the C-terminus of the LH/CG receptor</td>
</tr>
<tr>
<td>7.3.7</td>
<td>The effect of antisense oligodeoxy-nucleotides 2, 3 and 4 on cyclic AMP desensitization</td>
</tr>
<tr>
<td>7.3.8</td>
<td>The effect of antisense oligodeoxy-nucleotides 2, 3 and 4 on desensitization of pregnenolone production</td>
</tr>
<tr>
<td>7.3.9</td>
<td>The effect of antisense oligodeoxy-nucleotide 5 on desensitization of pregnenolone production</td>
</tr>
</tbody>
</table>
7.3.10 The effect of antisense oligodeoxy-nucleotides 2, 3, 4 and 5 on receptor regulation by LH and db-cAMP 285
7.3.11 Scatchard analysis of [125I]-hCG binding in the culture medium and on the surface of MA10 cells 288
7.3.12 The amino acid sequence of the LH/CG receptor 290
7.3.13 An autoradiograph of [125I]-hCG cross-linked to proteins derived from the culture medium of MA10 cells 295
7.3.14 Scatchard analysis of [125I]-hCG binding in solubilized MA10 cells 297
7.3.15 An autoradiograph of [125I]-hCG cross-linked to proteins of solubilized cells and culture medium derived from cells treated with Oligo 6 299
8.1.1 Proteolytic cleavage 1 308
8.1.2 Proteolytic cleavage 2 and 3 309
8.1.3 Release of the LH receptor extracellular domain 312
8.1.4 Binding of LH to its receptor 315
8.1.5 Activation of LH receptors 316
8.1.6 Desensitization of LH receptors 317
8.1.7 Acute loss of LH receptors 318
<table>
<thead>
<tr>
<th>Table</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.3.1</td>
<td>Density of cultured tumour Leydig cells</td>
<td>110</td>
</tr>
<tr>
<td>4.3.1</td>
<td>Cyclic AMP production by cells pretreated for 24h to induce LH/CG receptors</td>
<td>167</td>
</tr>
<tr>
<td>5.3.1</td>
<td>The effect of 0.28nM and 2.8nM $[^{125}I]$-hCG on the steady state levels of LH/CG receptors in MA10 cells</td>
<td>180</td>
</tr>
<tr>
<td>5.3.2</td>
<td>The effect of PMA on the levels of $[^{125}I]$-hCG binding sites in Leydig cells</td>
<td>193</td>
</tr>
<tr>
<td>6.3.1</td>
<td>The effect of protease inhibitors on the regulation of $[^{125}I]$-hCG binding sites in MA10 Leydig cells in the presence and absence of horse serum</td>
<td>223</td>
</tr>
<tr>
<td>7.3.1</td>
<td>The effect of one addition of Oligo 1 at time zero on LH-stimulated cyclic AMP production</td>
<td>263</td>
</tr>
<tr>
<td>7.3.2</td>
<td>The effect of one addition of Oligo 1 at time zero on LH-stimulated cyclic AMP production</td>
<td>264</td>
</tr>
<tr>
<td>7.3.3</td>
<td>The effect of adding the LH/CG receptor antisense oligodeoxynucleotides on the concentration of $[^{125}I]$-hCG binding sites</td>
<td>273</td>
</tr>
<tr>
<td>7.3.4</td>
<td>The effect of adding the LH/CG receptor antisense oligodeoxynucleotides on the LH-stimulated cyclic AMP production</td>
<td>274</td>
</tr>
<tr>
<td>7.3.5</td>
<td>The effect of adding the LH/CG receptor antisense oligodeoxynucleotides on the LH-stimulated pregnenolone production</td>
<td>275</td>
</tr>
<tr>
<td>7.3.6</td>
<td>The effect of antisense oligodeoxynucleotides on the surface expression of LH/CG receptors</td>
<td>293</td>
</tr>
</tbody>
</table>
7.3.7 Scatchard analysis of $[^{125}]$-hCG binding in culture medium of antisense oligodeoxynucleotide treated MA10 cells

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CHAPTER 1

GENERAL INTRODUCTION
Deciphering the structure-function relationships of the luteinizing hormone (LH)/chorionic gonadotropin (CG) receptor, and the regulation of the receptor in testis Leydig cells, is of fundamental importance in understanding the ultimate regulation of steroidogenesis and thus spermatogenesis.

1.1 The Testis

The testes are bilateral organs, consisting of coiled seminiferous tubules opening into the rete testis; from the rete testis arise ductuli efferentes, which drain into the epididymis as shown in figure 1.1.1. The epididymis is attached to the back of the testis and consists of a coiled tube, which continues into the vas (ductus) deferens. The seminiferous tubules and the convoluted ductus epididymis contain ciliated epithelium and smooth muscle. The seminiferous tubules contain Sertoli cells that provide structural and nutritional support (Fawcett, 1975) for the developing germinal cells. Peritubular myoid cells surround the tubule and are in contact with the basal surface of the Sertoli cells (figure 1.1.2). The seminiferous tubules are surrounded by the interstitium (figure 1.1.2). The interstitium contains Leydig cells, macrophages, together with collagen fibres, a rich blood supply, and a sympathetic nerve supply.

The testis has two distinct but related functions, both of which are under adenohypophyseal and hypothalamic control:

1. Production and storage of viable spermatozoa.
2. Synthesis and secretion of the androgenic hormone testosterone.
FIG. 1.1.2  *Cell biology of the testis* (Skinner, 1991)
1.2 Spermatogenesis

Spermatogenesis is the process by which spermatozoa are formed. Sertoli cells extend from the basement membrane to the centre of the lumen of seminiferous tubule. The developing germ cells lie along the Sertoli cells and are nourished by them as they grow from spermatogonia, on the basement membrane, to become in turn primary spermatocytes, secondary spermatocytes, spermatids, and finally spermatozoa (figure 1.2.1). Spermatogonia divide by mitosis but the division of primary spermatocytes is by meiosis in which the number of chromosomes is reduced from 46 to 23 in secondary spermatocytes, spermatids, and spermatozoa (Russell et al., 1987).

The presence of various generations of spermatocytes and spermatids derived from individual spermatogonia (Clermont, 1972) initiates waves of spermatogenesis that are not random but instead occur in a specific cyclic manner referred to as stages (Leblond and Clermont, 1952). In the rat, multiplication and differentiation of a germ cell from a diploid stem cell (spermatogonium A₁) into a clone of potentially 256 haploid spermatozoa (step 19 spermatids) takes approximately 8 weeks and involves the cell(s) passing 4.5 times through a spermatogenic cycle of 12.8 days, this cycle being divided itself into 14 stages (figure 1.2.2) (Sharpe, 1990).

1.3 Interactions of cells involved in the control of spermatogenesis.

Sertoli cells near the base of the epithelium have tight and gap junctions
FIG. 1.2.1 The seminiferous epithelium, showing the relation of the Sertoli cells to germ cells.
FIG. 1.2.2 *Schematic representation of the spermatogenic cycle in the adult rat.* The figure illustrates how each of the 14 stages (I-XIV) is characterized by a unique and fixed association of germ cell types. Each stage lasts for a fixed period of time and this varies from stage to stage. This is illustrated in the tubule segment in the diagram in which the length of each stage has been drawn in proportion to its duration. Stages follow one another in sequence along the length of a tubule and, with the passage of time, germ cells in stage I will move into stage II, stage VI into stage VII, stage XIV into stage I, and so on. Testosterone is believed to act selectively at stage VII. A<sub>1-4</sub>, A-type spermatogonia; In, intermediate; B, B-type spermatogonia; Pl, preleptotene; L, leptotene; Z, zygotene; P, pachytene and Di, diplotene spermatocytes; II, secondary spermatocytes; I-19, step 1 to 19 spermatids. (Sharpe, 1990).
(Dym and Fawcett, 1970). These tight junctions exclude the passage of macromolecules from the interstitial space to the lumen of the seminiferous tubules (Dym, 1972). The interaction between Sertoli cells is mediated by this tight junction and is essential in the creation of the blood-testis barrier for germinal cell development (Setchell, 1980). Sertoli cells can be in contact with 5 adjacent Sertoli cells at the basal surface of the cell and 47 adjacent germinal cells at various stages of development (Skinner, 1991). The junctional interactions between peritubular cells and the extracellular matrix provide a permeability barrier or prefilter while the tight junctional interactions between Sertoli cells create a functional blood-testis barrier. The extracellular matrix between the peritubular cells and Sertoli cells is produced co-operatively by the two cell types (Skinner et al., 1985; Pollanen et al., 1985). Sertoli cells produce laminin and collagen I and IV (Skinner et al., 1985), unique proteoglycans (Skinner, and Fritz, 1985a) and plasminogen activator (Lacroix et al., 1977). Peritubular cells produce collagen type I (Skinner et al., 1985), unique proteoglycans (Skinner and Fritz, 1985a), plasminogen activator inhibitor (Hettle et al., 1988), and fibronectin (Skinner et al., 1985; Tung et al., 1984).

General factors released by Sertoli, peritubular and Leydig cells that may regulate spermatogenesis are insulin growth factor 1 and 2, transforming growth factor α and β and interleukin-1 (for a review see Skinner, 1991). Specific factors isolated are the seminiferous growth factor (SGF) (Feig et al., 1983) and Sertoli cell-secreted growth factor (SCSGF) (Buch et al., 1988). These compounds are secreted by Sertoli cells, but their function is unknown. PModS (Skinner and Fritz, 1985b), which is secreted by peritubular cells, has dramatic effects on the
functions of Sertoli cells, and may be the factor that regulates spermatogenesis. However, the secretion of testosterone from Leydig cells, is still regarded as the main paracrine factor that regulates spermatogenesis. The effects of testosterone on spermatogenesis are highly specific and are thought to occur at discrete stages of germ cell development. Testosterone is thought to act only at stage VII (Sharpe, 1990). The production of the testicular paracrine factor PModS by peritubular cells also appears to be under the control of testosterone (Skinner and Fritz, 1985b). This therefore means that PModS may stimulate Sertoli cells to secrete factors that drive germ cells through the stages 1-6 and 8-14, whereas testosterone may act only at stage 7 and other factors may inhibit PModS action at this stage.

1.4 Steroidogenesis

Leydig cells are responsible for the production of testosterone in the testis. The first stage of testosterone synthesis is the transport of cholesterol to the mitochondria (for a review see Gower, 1988). Cholesterol is derived either from de novo synthesis, lipoproteins, internal stores of cholesterol esters or the plasma membrane. The cytoskeleton, including the array of microfilaments, may play a role in the intracellular transport of cholesterol. There is also evidence for the involvement of cholesterol carrier proteins, which may transport cholesterol from the cytosol or plasma membrane. Candidates for this role include the sterol carrier protein-2 (SCP2), the steroidogenesis activator polypeptide (SAP), and the 8.2 K protein (for a review see Strott, 1990). SCP2 (Noland et al., 1980) consists of a single polypeptide chain of 122 amino acids, and was shown to promote the
transfer of cholesterol from lipid droplets to mitochondria. SAP (Pedersen and Brownie, 1987) was shown to directly stimulate cholesterol side chain cleavage activity by isolated mitochondria in a dose-dependent fashion. SAP has homology with the carboxyl-terminal region of the Mr 78,000 heat shock protein or glucose-regulated protein (GRP78). This led to the proposal that SAP is cleaved from GRP78 by a regulated process that is related to steroidogenesis. The 8.2 K protein (Yanagibashi et al., 1990) was shown to directly stimulate cholesterol side-chain cleavage activity in isolated mitochondria in a dose dependent fashion. The 8.2 K protein was found to have homology with the brain protein called endozapine, which inhibits the binding of diazepam to benzodiazepine/GABA<sub>A</sub> receptors. The transport of cholesterol from the plasma membrane was also shown to be cyclic AMP dependent (Freeman, 1987b).

The second stage of androgen synthesis is the intramitochondrial transport of cholesterol. The transport of cholesterol from the outer to the inner mitochondrial membrane is dependent on protein synthesis (Freeman, 1987a). The proteins SCP<sub>2</sub>, SAP and 8.2 K protein can also be implicated in the transfer of cholesterol from the outer to the inner mitochondrial membrane. Phospholipids are also thought to be involved in cholesterol transference. The mechanism of transference involves several factors, including phospholipids and Ca<sup>2+</sup> ions, which are involved in the hexagonal phase-mediated transport of cholesterol into the inner mitochondrial membrane - hexagonal phases are more favourable structures for lipid bilayers allowing effective solute permeability, without the assistance of translocating proteins (Gower, 1988). The non-polar groups of unsaturated fatty acyl chains will be orientated towards the outside of the membrane. As cholesterol
FIG. 1.4.1 Factors involved in the intermitochondrial transport of cholesterol.

The diagram shows a schematic representation of membrane fusion stimulating reversed hexagonal phase formation (Gower, 1988).
approaches this non-polar domain, free from the membrane surface, the rotatory action of the hexagonal phase clusters may transfer cholesterol from outside to inside. If fusion of outer and inner membranes occurs in the hexagonal phase (figure 1.4.1), this would result in transference of cholesterol in the outer membrane to the matrix side.

Stage three of androgen synthesis is the side-chain cleavage (SCC) of cholesterol. Once cholesterol is transferred to the inner mitochondrial membrane it encounters the enzyme system known as the cholesterol SCC system. This system comprises 20- and 22-hydroxylases and a C-20,22-lyase, all tightly bound to the inner face of the membrane and associated with a specific cytochrome P-450\textsubscript{SCC}. In addition, molecular O\textsubscript{2} is necessary together with NADPH reductase and non-haem iron sulphur protein. Cholesterol is converted first by 22-hydroxylation to 22R-hydroxycholesterol, then to 20R,22R-dihydroxycholesterol by 20-hydroxylation and, finally, to pregnenolone by means of the C-20,22-lyase reaction (Gower, 1988).

The final stages involve the conversion of pregnenolone to testosterone. Two pathways for testosterone synthesis exist (figure 1.4.2). After the formation of pregnenolone from cholesterol in the mitochondria, testosterone synthesis occurs in the endoplasmic reticulum from pregnenolone or progesterone. Pregnenolone is converted to progesterone by the 3\textbeta-hydroxysteroid dehydrogenase/4,5-isomerase. The C-21 precursors are 17-hydroxylated allowing a C-17,20-lyase to generate dehydroepiandrosterone (DHA) via 17-hydroxypregnenolone or 4-androstenedione from 17-hydroxyprogesterone. The 17-hydroxylase and C-17,20-lyase are associated with the smooth endoplasmic reticulum. In addition to the P-
FIG. 1.4.2 Pathways of androgen biosynthesis in rat testis. A→B→C and a→b→c are the delta 5 and delta 4 pathways, respectively, for testosterone biosynthesis. Enzymes A,a, 17-dehydroxylase; B,b, C-17,20-lyase; C,c, 17β-HSD. Reaction c is reversible (Gower, 1988).
which catalyses both enzymic functions, NADPH and O₂ are needed. The action of 17β-hydroxysteroid dehydrogenase (17β-HSD) on DHA provides 5-androstene-3β,17β-diol, which is converted to testosterone through the action of 5-ene-3β-HSD/4,5-isomerase activity. The action of 17β-HSD on 4-androstenedione also provides testosterone. The 3β-HSD requires NAD⁺ as a cofactor, whereas the 4,5-isomerase does not require a cofactor. Both enzymes are associated with the smooth endoplasmic reticulum (Gower, 1988).

Testosterone can also be converted to 5α-dehydrotestosterone (5α-DHT) by the action of the enzyme 4-ene-5α-reductase and requires NADPH as a cofactor. This enzyme is associated with the nucleus and microsomes. 4-Androstenedione can be further metabolized to oestrogen or oestradiol-17β.

The final stage is the secretion of testosterone. The release of steroid with time could be resolved into two components, one rapid and the second, much slower. The two phase release may reflect the presence of two pools of steroid, the initial phase representing passive diffusion and the slower phase being caused by pregnenolone binding to intracellular proteins (Gower, 1988).

1.5 Interactions between cells involved in the control of steroidogenesis.

The possible interactions between cells in the regulation of steroidogenesis are shown in figure 1.5.1. Leydig cell morphology changes with the seminiferous cycle being most dramatic at stage VII and VIII (Bergh, 1985a). Oestrogen, which is secreted from both the Sertoli cells and Leydig cells, generally has inhibitory
FIG. 1.5.1 Schematic diagram to summarize some of the paracrine interactions that may modulate the production and action of testosterone (T) in the adult rat testis. (Sharpe, 1990)
effects on androgen production. Sertoli cells also produce a gonadotropin releasing hormone (GnRH)-like substance (Sharpe and Fraser, 1980; Sharpe et al., 1981; Sharpe, 1984; Kerr and Sharpe, 1985; Sharpe and Cooper, 1987). Leydig cells have receptors for GnRH (Clayton et al., 1980; Sharpe and Fraser, 1980; Hunter et al., 1982), and GnRH generally has long term inhibitory effects on Leydig cell steroidogenesis. Inhibin (Rivier et al., 1990) is a peptide hormone produced by Sertoli cells under the control of follicle stimulating hormone (FSH) or agents that alter cyclic AMP levels. Although a major function for inhibin is to act on the pituitary to regulate FSH production, inhibin and its related protein activin both can influence Leydig cell steroidogenesis (Rivier et al., 1990). Leydig cells also produce inhibin and activin. Testicular macrophages physically associate with Leydig cells, and there may be interaction between the macrophage and Leydig cell (Bergh, 1985b).

1.6 Luteinizing hormone

Luteinizing hormone (LH) and its analogue chorionic gonadotropin (CG) are members of a glycoprotein family that includes thyroid stimulating hormone (TSH) and follicle stimulating hormone (FSH). These hormones have two subunits that are joined by noncovalent forces. The α subunit (92 amino acids) is common to all of the hormones. The β subunit (LH - 112 amino acids; CG - 144 amino acids; FSH - 124 amino acids; TSH - 112 amino acids) is specific for each hormone, although they show some degree of sequence homology, because they bind to a common α-subunit.
The interpolated tertiary structure of hCG during receptor interaction. The carbohydrate (CHO) collar of the hormone is distal to the receptor binding region. The β-subunit (stippled) provides the majority of the receptor-determinant loops, around the disulphide bonds at β34-57 and β38-72, with the α-subunit (unshaded) contributing its C-terminal residues. The αSer-38, αHis-83 and βAsp-99 surround the putative agonist surface of the hormone are depicted as S, H and D respectively.
FIG. 1.6.2  *Amino acid sequence of loop peptides from LH and hCGβ.* A) The 93-100 disulphide loop of LH and hCG with a glycine at position 101 and tyrosine at 102. B) The 38–57 loop of hCGβ (inner sequence) with the substitutions occurring in the hLHβ shown on the outside (Ryan et al., 1988).
LH and CG share common biological properties and their β-subunit possesses a high degree of sequence homology. CG has an additional proline and serine-rich carboxyl-terminal extension on the β-subunit. The subunits are coded for on separate genes and each has a propeptide sequence (Pierce and Parsons, 1981).

LH and CG have a high degree of disulphide bridging in each subunit (Pierce and Parsons, 1981) and contain carbohydrate side chains (Sairam, 1983) of asparagine (N)-linked chains on the α- and β-subunits. CG also has serine (O)-linked chains on the carboxyl-terminal extension of the β-subunit. The N-linked carbohydrates are complex biantennary structures with a mannose core and branches that terminate with galactose sialic acid or galactose sulphate.

1.6.2 LH/CG interaction with its receptor.

The gross morphology (Willey and Leidenberger, 1989) of the α and β subunits of CG and their interaction with the cells surface receptor is depicted in figure 1.6.1. Both subunits cooperate in the production of two relatively distinct reactive areas; the β subunit contributing most to receptor binding and the α-subunit mainly responsible for agonist action, with neither indispensable to either activity. The major region of the α-subunit involvement in receptor binding is the C-terminus. The putative agonist surface of the hormone includes the majority of the α-subunit and the loops βCys-93 to 100 and βCys-38 to 57 of the β-subunit.
Control of LH release by gonadotropin releasing hormone (GnRH).

Gonadotropin releasing hormone (GnRH) is a decapeptide with the primary structure shown in figure 1.6.3. It is secreted into the portal circulation from the medial basal hypothalamus with intrinsic pulse frequency and amplitude that are critical determinants not only of acute LH and FSH release, but also their synthesis (Redding et al., 1972). The pulsatile secretion of GnRH is chiefly influenced by gonadal sex steroids, neurotransmitters such as catecholamines (norepinephrine, epinephrine and dopamine), and the endogenous opioid peptides (Marshall and Kelch, 1986). Testosterone controls the secretion of GnRH in the male through an extensive series of negative feedback loops.
FIG. 1.6.4 Overview of gonadotrope action in the pituitary. The GnRH is functionally coupled to a calcium ion channel and to a GTP binding protein which can activate protein kinase C.
GnRH actions are initiated by binding to specific receptors on the gonadotrope membrane, activation involves the first three amino acids (figure 1.6.3) (Conn, 1986). The number of GnRH receptors on the gonadotrope is highest under conditions where endogenous GnRH secretion is greatest. After binding to pituitary GnRH receptors, signal transduction leading to gonadotropin release is accomplished through calcium-dependent processes (Conn et al., 1987). The current model for GnRH action is presented in figure 1.6.4 (Jennes and Conn, 1988). The receptor is also intimately coupled to a GTP-binding protein which can activate phospholipase C leading to the production of diacylglycerols, which in turn can activate protein kinase C (PKC). GnRH is then degraded by membrane-associated proteases, internalization of receptor-bound GnRH with subsequent lysosomal proteolysis, and recycling to the exterior with old GnRH receptors (Handelsman and Swerdloff, 1986).

1.6.4 \textit{LH/CG action in the Leydig cell}

LH/CG binds to its receptor on the surface of Leydig cells to stimulate steroidogenesis, growth/differentiation and the secretion of paracrine factors (Rommerts and Cooke, 1988). Several second messengers have been implicated in the action of LH/CG. Cyclic AMP levels increase within minutes of the addition of LH/CG to Leydig cells in a dose-dependent manner. The activation of adenylate cyclase is initiated by GTP binding protein (G-protein) $G_s$. The inhibitory G-protein $G_i$ is also present in Leydig cells (Platts et al., 1988) and is involved in the negative modulation of cyclic AMP and steroidogenesis.
The formation of inositol phosphates, including inositol 1,4,5-triphosphate (IP$_3$) has been demonstrated in ovarian cells, but not Leydig cells (Rommerts and Cooke, 1988). The levels of these compounds were raised for periods of seconds to minutes after the addition of LH/CG to isolated cells. The activated breakdown of phospholipid by phospholipase C, results in the liberation of diacylglycerol. This compound can directly stimulate protein kinase C (PKC) and can be further metabolized to release arachidonic acid. The release of arachidonic acid (Dix et al., 1984) can also involve phospholipase A$_2$, which is activated by calcium and protein kinase C. Arachidonic acid is metabolized to prostaglandins, prostacyclins and thromboxanes via the cyclooxygenase pathway and to the leukotrienes via the lipoxygenase pathway. Both prostaglandins and leukotrienes are synthesized in the testis. The lipoxygenase, but not the cyclooxygenase, pathway has been shown to be involved in the regulation of steroidogenesis (Cooke et al., 1989). Extracellular calcium is also required to give maximal LH/CG stimulated steroidogenesis. LH/CG and cyclic AMP analogues also increase intracellular calcium levels (Rommerts and Cooke, 1988).

Cyclic AMP is thought to be directly involved in the regulation of steroid production, however, there are several observations that do not fit this general concept.

1. At low concentrations of cholera toxin, cyclic AMP production is stimulated in purified Leydig cells without any detectable change in steroidogenesis.

2. Isoproterenol can stimulate cyclic AMP production more than 10-fold in freshly isolated Leydig cells without any change in steroid
production whereas LH/CG can stimulate steroid production more than 10-fold without any detectable change in cyclic AMP (for a review see Rommerts and Cooke, 1988).

These discrepancies may be explained by compartmentalization of cyclic AMP, which has been shown to occur in Leydig cells with LH stimulation but not with cyclic AMP analogue stimulation (Moger, 1991).

1.6.5 Desensitization of LH/CG responses.

In addition to the stimulatory actions of LH/CG on steroidogenesis in Leydig cells, LH/CG also causes a refractoriness or desensitization of that same steroidogenic response. This may involve a loss of LH/CG receptors (down-regulation), an uncoupling of the LH/CG receptor from the adenylate cyclase, an increase in the metabolism of cyclic AMP due to an increased phosphodiesterase activity and a decrease in the activities in some of the enzymes in the pathways of steroidogenesis (for a review see Rommerts and Cooke, 1988).

Desensitization of adenylate cyclase has been shown to involve a lesion between the receptor and the adenylate cyclase enzyme. This can be caused by incubating cells with LH, hCG, forskolin, cyclic AMP analogues and the phorbol ester phorbol 12-myristate-13-acetate (PMA) (Freeman and Ascoli, 1982; Dix et al., 1982; Lefèvre et al., 1985; Inoue and Rebois, 1989). However, it has been reported that PMA induced desensitization is via a different mechanism to LH/CG (Platts et al., 1988; Inoue and Rebois, 1989). The lesion is thought to occur between the LH/CG receptor and $G_s$ (for a review see Cooke et al., 1990). This
lesion may be necessary before internalization of the receptor can occur.

Desensitization of steroidogenesis in the rat also involves a decrease in the steroid enzymes 17,20-desmolase and 17α-hydroxylase activity. Oestrogen has also been shown to inhibit the activity of 17α-hydroxylase and 17,20-desmolase. In contrast in the mouse there is an earlier lesion, which results in a decrease in pregnenolone synthesis. Desensitization of steroidogenesis in the mouse is associated with a depletion of cellular cholesterol (for a review see Rommerts and Cooke, 1988).

1.7  

The LH/CG receptor.

The LH/CG receptor plays a pivotal role in reproductive physiology. On binding LH/CG, the LH/CG receptor activates a $G_s$ protein which stimulates adenylate cyclase (1.6.4). The responsiveness of the Leydig cell can be modulated by one of two mechanisms. One of these is the alteration in the number of cell surface LH/CG receptors. The other is independent of, or in addition to, changes in receptor numbers. This latter mechanism is the phenomenon of desensitization (1.6.5).

1.7.1  

The structure of the LH/CG receptor: biochemical approaches.

Direct labelling, immunoprecipitation of purified LH/CG receptors and chemical cross-linking of the LH/CG receptor to radiolabelled hCG indicated that
the LH/CG receptor has a molecular weight ($M_r$) of 93,000 on denaturing polyacrylamide gels (for a review see Ascoli, and Segaloff, 1989). However when the LH/CG receptor was characterized on non-denaturing gels the apparent $M_r$ was reported to be 190,000-220,000 (for a review see Segaloff et al., 1990). These data may reflect a different tertiary structure of the receptor under denaturing conditions and the receptor may also exist in the plasma membrane as a noncovalently associated oligomer of the 93,000 $M_r$ polypeptide.

The LH/CG receptor, like other proteins, is susceptible to protease activity. Treatment of Leydig cells that have been labelled with iodinated hCG, with a crude preparation of collagenase that contains numerous contaminating proteases, results in the generation of smaller fragments of the LH/CG receptor. The intact hCG-receptor complex has a $M_r$ of 132,000 whereas after collagenase treatment bands at 95,000, 75,000 and 63,000 $M_r$ were more apparent (Ascoli and Segaloff, 1986). Running collagenase treated LH/CG receptors on a non-denaturing gel, did not result in the appearance of the smaller bands. This indicated that proteases "nick" the LH/CG receptor and that these fragments are held together by disulphide bonds. It has also been shown that endogenous proteases in gonadal tissue nick the LH/CG receptor (Kellokumpu and Rajaniemi, 1985a). This activity was modulated by hCG binding (Kellokumpu and Rajaniemi, 1985b). The resultant labelled cross-linked fragments were 96,000 and 74,000 $M_r$. These two components were shown to contain receptor components of 64,000 and 38,000 $M_r$ as shown by direct labelling.

The finding that the LH/CG receptor can be absorbed to wheat germ agglutinin and eluted with acetylglucosamine shows that this receptor is a
glycoprotein (for a review see Ascoli and Segaloff, 1989). Affinity purified LH/CG receptors were treated with glycosidases that cleave N-linked carbohydrate chains (neuraminidase and peptide N-glycosidase F) and glycosidases that cleave O-linked carbohydrate chains (endo-α-N-acetylgalactosaminidase). Treatment with neuraminidase decreased the LH/CG receptor to 79,000 Mr and peptide N-glycosidase F resulted in a decrease to 67,000 Mr then to 62,000 Mr (Keinanen, 1988; Petaja-Repo et al., 1991). Endo-α-N-acetylgalactosaminidase had no effect. Treatment of hCG-receptor complex with the above glycosidases also decreased the Mr of the LH/CG receptor by the same degree, indicating that the carbohydrate chains are not involved in binding. The carbohydrate chains on both subunits of hCG were also removed (Petaja-Repo et al., 1991).

1.7.2 The structure of the LH/CG receptor: cloning of cDNA.

Two approaches were used to isolate the cDNA to the LH/CG receptor. The first involved isolating LH/CG receptors from rat ovaries. Female rats were made pseudopregnant, which gives a rich source of LH/CG receptors. The LH/CG receptors in ovarian membranes, solubilized in buffer that contained 20% glycerol (Ascoli, 1983), were then purified by affinity chromatography – first by using wheat germ and hCG linked to affi-gel 10 (Rosemblit et al., 1988). The 93,000 Mr receptor protein was electroblotted from the gel and used directly for determining the amino-terminal sequence. In other experiments the receptor protein was subjected to cleavage with lysyl C endopeptidase or with CNBr (McFarland et al., 1989). The resulting fragments were purified and then subjected to amino-terminal
FIG. 1.7.1 The cDNA sequence and deduced amino acid sequence of the rat luteal LH/CG receptor. Amino acids are numbered beginning with the amino-terminal sequence for the mature intact receptor, with negative numbers encoding the signal sequence. Solid bars above the sequence denote amino acid sequences determined from the purified receptor and receptor peptides, with residues differing from those predicted by the open bars. Potential sites for N-linked glycosylation are noted by inverted solid triangles, and the proposed membrane-spanning regions are enclosed in boxes. Lines above residues (e.g. amino acids 254-255) denote amino acids that are identical to a region of the soybean lectin (McFarland et al., 1989).
amino acid sequence analysis. Oligonucleotides based on the amino-terminal amino acid sequence of the intact receptor and on one of the internal amino acid sequences were used to prime a preparation of rat luteal cDNA. The polymerase chain reaction (PCR) was then used, generating a 622-nucleotide cDNA. The PCR fragment was used to screen a rat luteal lambda gt10 cDNA library, ultimately yielding a cDNA that has 43 nucleotides of a 5'-flanking region, 2100 nucleotides that represent the entire coding region, and 759 nucleotides of a 3'-flanking region (figure 1.7.1).

The second approach used cDNA libraries derived from porcine testis polyadenylated RNA (Loosfelt et al., 1989). Screening of a lambda gt11 library with monoclonal antibodies to the LH/CG receptor led to the isolation of three immunoreactive clones whose inserts were thereafter used to isolate 80 cross-hybridizing clones from a lambda gt10 library. Six of the clones were sequenced to derive the primary structure of the complete protein. The human LH/CG receptor sequence has also been isolated using probes derived from the rat luteal cDNA sequence (Minegish et al., 1990). There was 84% homology between the cloned cDNA for the rat, porcine and human LH/CG receptor.

The rat luteal LH/CG receptor will be used to discuss the characteristics of the isolated cDNA. The nucleotide sequence is shown in figure 1.7.1. The open reading frame is 2,100 nucleotides, encoding for a protein of 700 amino acids. The first 26 amino acids are the signal sequence. The mature receptor would therefore be 674 amino acids, with a predicted molecular weight of 75,000. The difference between this size and that observed for the purified receptor (i.e. 93,000 Mr) is presumably due to the glycoprotein nature of the receptor (McFarland et al., 1989).
FIG 1.7.2 Hydropathy plot of the LH/CG receptor. Shown are Kyte and Doolittle hydropathy plots (window of 20 residues) for (A) the substance K receptor (SKR) and (B) the LH/CG receptor (LH/CG-R). The seven putative transmembrane regions of the LH/CG receptor are denoted by the solid bars atop them (McFarland et al., 1989).
FIG. 1.7.3 THE AMINO ACID SEQUENCE OF THE LH RECEPTOR. The figure shows the amino acid sequence derived from the rat ovary LH/CG receptor (McFarland et al., 1989). Indicated are potential phosphorylation sites, PKC consensus phosphorylation sites, glycosylation sites and the point of divergence between the full length LH/CG receptor mRNA and shorter forms. The alternative amino acids shown in the phosphorylation areas are the differences between the rat (McFarland et al., 1989) and pig (Loosfelt et al., 1989) LH/CG receptors sequences.
FIG. 1.7.4 Depiction of zonal characteristics of the glycoprotein hormone receptor chain. Cysteine residues are #1 to #14 with #5 (boxed) unique to the LH/CG receptor, leucine-rich repeats are 1 to 14 with 12A and 12B unique to the TSH receptor, also depicted are biantennary glycosylation sites and arrows identifying dibasic residues.
The primary sequence of the receptor reveals six potential N-linked glycosylation sites.

The LH/CG receptor interacts with G-proteins, therefore a comparison was made between the hydropathy plot for the rat luteal LH/CG receptor and those of the rhodopsin-like G protein-coupled receptors. Figure 1.7.2 shows that the carboxyl-terminal half of the LH/CG receptor displays a strikingly similar hydropathy plot to that of substance K receptor, suggesting that this half of the LH/CG receptor possesses seven membrane-spanning regions (McFarland et al., 1989). The amino acid homology with the other rhodopsin-like G-protein coupled receptors is approximately 18-26% within the transmembrane region.

From the amino acid sequence of the rat luteal LH/CG receptor, and by analogy with the topography of other G-protein-coupled receptors, it was postulated that the LH/CG receptor existed in the plasma membrane, with the amino-terminal hydrophilic half being extracellular and the carboxyl-terminal half spanning the plasma membrane seven times, ending with a relatively short cytoplasmic tail (figure 1.7.4) (Segaloff et al., 1990). This hypothesis was confirmed by using antibodies directed to sequences in the amino-terminal (amino acids 194-206) and the carboxyl-terminal sequence (amino acids 661-674). Immunofluorescence was detected with the amino-terminal antibody on the surface of intact luteal cells. Immunofluorescence was only detected with the carboxyl-terminal antibody when cells were permeabilized (Rodriguez and Segaloff, 1990).

In contrast to the rhodopsin-like G-protein receptors the LH/CG receptor contains a large 341 amino acid amino-terminal hydrophilic domain. Prediction of
FIG. 1.7.5  *Differentially spliced forms of the rat ovarian LH/CG receptor cDNA.* The top scheme depicts the complete LH/CG receptor cDNA (A1) containing a region encoding the signal sequence (solid bar), a section encoding the extracellular receptor domain (open bar), and the sequence encoding transmembrane regions I-VII. Untranslated regions are represented by thin lines. A2 represents an alternatively spliced version of A1, deleting amino acid residues 206-268. B1-B4 are differentially spliced cDNAs, leading to the translation of a different reading frame (cross-hatched bar) with early termination. C1 and C2 contain 95 and 66 nucleotides, respectively, of presumptive intronic sequences. (*Segaloff et al.,* 1990)
the Mr for the extracellular domain from the amino acid sequence would be approx.
38,000 and with glycosylation 56,000. This extracellular domain contains a 14-fold
imperfectly repeated sequence of approximately 20 residues. The amino acids
which are aligned and the spacing between them are similar to a repeat motif,
termed a "leucine-rich repeat" (figure 1.7.3). The functional significance of this
repeat motif is not known, although it has been suggested that this structure
might, by forming amphipathic helices, be able to interact with both hydrophobic
and hydrophilic surfaces. The extracellular domain also contains several dibasic
residues that could act as potential cleavage sites. Another feature contained
within the extracellular domain is a site where 10 of 12 amino acids (244-255;
figure 1.7.1) are identical to a region of the soybean lectin. This is of interest
because it is well known that although deglycosylated forms of hCG or LH bind to
the LH/CG receptor with high affinity, they elicit little, if any, stimulation of
cyclic AMP production (Sairam, 1990).

Cytoplasmically orientated LH/CG receptor regions contain numerous
serines, threonines, and tyrosines. Consensus PKC sites have been reported within
this region (amino acids 668-670 and 675-677) (Loosfelt et al., 1989), there are also
other possible phosphorylation sites at 638-640, 659-661, 674-675 and 679-680,
where PKA could act. There is also a consensus PKC site on the third intracellular
loop at 560-561 (Loosfelt et al., 1989). This raises the possibility that the function
and/or levels of the LH/CG receptor are modulated by phosphorylation.

Interestingly, the sequence of the cloned LH/CG receptor cDNAs revealed
an unexpectedly high number of cDNAs encoding truncated forms of the LH/CG
receptor (Segaloff et al., 1990) (figure 1.7.5). One cDNA (A2) is missing 186
nucleotides encoding a variant receptor which lacks 62 residues of the extracellular domain. The deleted region includes residues homologous to the soybean lectin. Other cDNA variants displayed similar deletions and some carried insertions presumably of intronic origin. Many of these variants lead to frameshifts which introduce premature translation termination. Some of these variants are predicted to generate the extracellular domain of the LH/CG receptor lacking the transmembrane regions. These variants are likely to be created by alternative, incomplete, or incorrect splicing events (Segaloff et al., 1990).

1.7.3 The gene for the LH/CG receptor.

The LH/CG receptor gene was isolated from rat genomic libraries (Koo et al., 1991; Tsai-Morris et al., 1991) and spanned at least 75 kilobase pairs from nucleotide -2057 of the 5′-flanking region to the 3′-noncoding end. The structural configuration of the coding region of the LH/CG receptor gene consists of 11 exons separated by 10 introns that are all located within the putative extracellular domain prior to the first transmembrane region. The 5′-noncoding region contains several potential TATA boxes and SP1 promoter binding sites, as well as six palindromic elements, potential intron/exon splice junctions, and two extended open reading frames, which are in frame with the initiation codon. Primer extension studies indicate the presence of multiple transcriptional initiation sites. Truncated forms of the LH/CG receptor conform to alternative splicing patterns that are consistent either with the deletion of complete exons or alternate acceptor sites within exons. All splice site junctions corresponded to known donor
and acceptor consensus sequences. Exons 2-8 approximate the regions of the 14 consecutive 20 amino acid repeated motif. Exons 1, 9, 10 and 11, do not conform to this repetitive intronic motif. These exons, however, contain important structural elements including the conserved cysteines (exons 1, 9, 11), the soybean lectin motif (exon 9), the seven-transmembrane domain with cytoplasmic G protein coupling elements (exon 11), and three putative N-linked glycosylation sites (exon 10) (Tsai-Morris et al., 1991).

1.7.4 Messenger RNA for the LH/CG receptor.

Northern blot analysis of gonadal cells which express LH/CG receptors indicated that there are multiple LH/CG receptor mRNA transcripts (Segaloff et al., 1990). The number and sizes of these transcripts, however, are different between species. For example in the rat, four transcripts were detected (6.7, 4.3, 2.6 and 1.2 kb (Segaloff et al., 1990)) and in a mouse tumour Leydig (MA10) cells, six transcripts were identified (7.7, 4.3, 2.6, 1.9, 1.6, and 1.2 kb (Wang et al., 1991c). There have also been discrepancies in the size of the LH/CG receptor transcripts within a species because of the use of RNA markers rather than DNA markers (Segaloff et al., 1990). The multiple LH/CG receptor transcripts may arise from a number of different phenomenae including: 1) different sites of polyadenylation, 2) different lengths of polyadenylation, and/or 3) alternate or incorrect splicing of the LH/CG receptor gene. To eliminate contributions of the latter possibility, the LH/CG receptor cDNA was transfected into 293 kidney cells. Examination of the subsequent mRNA species generated a major transcript of 3.1
FIG. 1.7.6 Model of glycoprotein hormone-receptor interaction. Single receptor in its interaction with 2 hormone molecules and two adjacent receptor molecules (which probably have interchain disulphide links) and their interaction with 2 hormone molecules.
kb and less abundant transcripts both larger and smaller than the 3.1 kb species. These data suggest that even if alternate or incorrect gene splicing was contributing to different sizes of LH/CG receptor mRNA transcripts in gonadal cells, other factors such as differences in polyadenylation were also likely to be involved (Wang et al., 1991c). The open reading frame of the complete LH/CG receptor is 2.1 kb. Since one of the different LH/CG receptor mRNA species is only 1.2 kb, it might represent a mRNA that encodes for the truncated form of the receptor. It is important to note, however, that it is not known which of the larger mRNAs is used for the translation of the LH/CG receptor protein.

1.7.5 **Hormone binding region of the LH/CG receptor.**

Many studies have been carried out to determine the number of LH/CG binding sites per cell and binding affinity of the LH/CG receptor. Conflicting results have been obtained especially with regard to the number of receptors per cell. Using mature rat and mouse testis Leydig cells and rat and mouse tumour Leydig cells, approximately 13-30,000 LH/CG receptors per cell have been found and 23,000 per cell were reported using rat Leydig cell homogenates. In porcine Leydig cells 60,000 receptors per cell have been found (for a review see Cooke and Rommerts, 1988). Various values have also been reported for the binding affinity of LH or hCG for its receptor although this is consistently high and in the range of $10^9$ to $10^{11}$ M$^{-1}$ (Cooke and Rommerts, 1988).

Two mutant LH/CG receptors were constructed, one containing exons 1-10 (LH/CG receptor$_{exon\ 1-10}$) and the other containing exon 1 and 11 (LH/CG
receptor(exon 1&11). These mutants were functionally expressed in Cos 7A cells (Ji and Ji, 1991). The LH/CG receptor(exon 1-10, which lacks the transmembrane domains, showed high affinity binding. The LH/CG receptor(exon 1&11 also bound hCG, but with low affinity, and stimulated cyclic AMP production. These results demonstrated that exons 1-10 encode a high affinity LH/CG binding site and exon 11 codes for the site of G-protein activation. The results also indicated that there may be multisteps to hormone binding (Ji and Ji, 1991).

A series of LH/CG-TSH receptor chimars were constructed by homologous substitution of corresponding regions of the extracellular domain, to locate the site of high affinity hormone binding (Nagayama et al., 1990). Amino acids 1-83 and 316-367 (figure 1.7.1) were shown not to be involved, whereas amino acids 83-316 were essential for hormone binding and activation (Nagayama et al., 1990).

Though it is known that LH/CG binds to the receptor with high affinity, it is not known whether two molecules of hormone interact with one molecule of receptor, or whether the receptor is in a dimerized state before binding of hormone (figure 1.7.6).

1.7.6 LH/CG binding proteins.

In addition to detergent-extractable LH/CG receptor, there have also been reports that small proportions of receptors are "water-soluble" and can be extracted with buffers of low ionic strength (for a review see Roche and Ryan, 1985).
Identified cDNA for potential soluble LH/hCG binding proteins are derived from exons 1-9 of the LH/CG receptor gene (Tsai-Morris et al., 1991) and contain 307-316 amino acids. The potential soluble LH/CG binding proteins therefore have a Mr of 43,000-44,000. This estimation of the molecular weight assumes the same degree of glycosylation (Mr = 9,000) as the native LH/CG receptor, with respect to loss of exon 10 which contains three N-linked glycosylation sites.

Two studies have investigated whether a LH/CG binding protein can be synthesized and released from eukaryotic expression systems. Expression of a mutated construct of the rat luteal LH/CG receptor (Xie et al., 1990), demonstrated that high affinity binding sites for LH could only be detected in the solubilized cells of a eukaryotic cell expression system. However, expression of a LH/CG receptor cDNA containing a 266 bp deletion resulting in truncation of the open reading frame, and omission of the 1st transmembrane domain, demonstrated secretion of binding sites into the medium of a eukaryotic cell expression system (Tsai-Morris et al., 1990). These studies support the concept of a secreted soluble binding protein being synthesized in gonadal tissues.

1.7.7 LH/CG receptor interaction with G-proteins.

Although there are no studies on the interaction of LH/CG receptors with G-proteins, results obtained for the β-adrenergic receptor (βAR) can be
extrapolated, due to structural similarities. The regions at the N- and C-termini of the third intracellular loop of the βAR which were implicated in the coupling of the receptor to $G_S$ would be predicted by secondary structure prediction algorithms to form amphipathic α-helices. This structural feature was of interest in light of the observation that the amphipathic peptide mastoparan is able to activate the G-protein, $G_o$. Although this hydrophilic loop represents one of the most divergent regions of the primary sequence of G-protein coupled receptors, all of these receptors would be predicted to have amphipathic helices at this position. These observations suggest that the mechanism of coupling between receptors and G-proteins may involve interactions with these amphipathic domains. According to the structural model proposed for the βAR, these amphipathic regions would be predicted to form cytoplasmic α-helical extensions of the fifth and sixth transmembrane helices of the receptor. The interaction of the ligand with the 5th transmembrane helix could cause a conformational change in this helix, which could be transmitted to the amphipathic region at the bottom of helix 5, allowing the receptor to interact with $G_s$ (for a review see Strader et al., 1989). However, though the LH/CG receptor is thought to interact with $G_s$, it does not appear to share a greater percentage of amino acid identity with the βAR in these regions.

There is also a highly conserved region within the 7th transmembrane domain between all the G-protein linked receptors, which may be involved in the regulation of G-proteins. Structure predictions and homology comparisons suggest that three or four residues in transmembrane helix 7 could constitute a small polar loop or distortion in the helix (Findlay and Eliopoulos, 1990). This distortion could play a role in the regulation of G-protein activity.
Studies on the regulation and processing of LH/CG receptors are based on the fate of radiolabelled LH/hCG. Labelled LH/hCG injected in vivo or incubated with cultured cells has been localized to the plasma membranes, submembranous smooth and coated vesicles, lysosomes, and Golgi membranes (for a review see Roche and Ryan, 1985). This indicated that LH/CG receptor underwent receptor mediated endocytosis (REM).

Figure 1.7.7 shows a schematic overview of RME. The cell surface receptors are either located in areas of the plasma membrane referred to as coated pits or they are randomly distributed throughout the cell surface and migrate to coated pits upon binding of the hormone. Coated pits are indented areas of the plasma membrane where there is an intracellular "lining" of the membrane with the protein clathrin. Coated pits containing receptor bound hormone become invaginated and pinch off intracellularly to form what are called coated vesicles. There is no free hormone at this stage. With time the clathrin coat is shed and the vesicles fuse. The resulting vesicles are called endosomes or endocytic vesicles. Within the endosome, which has a pH of 5.5, the hormone dissociates from its receptor. When this occurs there is a subsequent sequestering of the free hormone from the receptor in a related vesicle and tubule compartment called the CURL - compartment for the uncoupling of receptor from ligand - free hormone is sequestered into the vesicular structure while the receptor accumulates in the membrane of the tubule structure. The free hormone is ultimately delivered to the lysosome where it is degraded. The free receptor may be recycled to the
FIG. 1.7.7  Schematic representation of the possible routes of receptor and hormone during RME.
cell surface, where it can rebind hormone or be sequestered intracellularly. Alternatively the hormone and receptor may be delivered directly to the lysosome (for a review see Segaloff and Ascoli, 1988).

The whole process of receptor mediated endocytosis for the LH/CG receptor is temperature dependent. At 4°C movement of the hormone receptor-complex inside the cells does not occur, and at 21°C hormone accumulated within the cytoplasm, but was not degraded or released from the cell. At 34°C, internalization, degradation and loss of degraded hormone fragments from the cell occurred (Habberfield et al., 1987).

Recycling of the LH/CG receptor has been demonstrated in the rat (Habberfield et al., 1986) and porcine (Genty et al., 1987) Leydig cells. In the mouse tumour Leydig (MA10) cells LH/CG receptors were indicated to be continually synthesized and not recycled (Lloyd and Ascoli, 1983). LH and hCG are internalized by the same pathway (Salesse et al., 1989), however, there is controversy over the rates of internalization. In porcine Leydig cells it was shown that LH was internalized much more rapidly than hCG (Salesse et al., 1983). In contrast in the mouse tumour Leydig (MA10) cell, LH and hCG were internalized at similar rates (Ascoli and Segaloff, 1987).

Like other receptors for polypeptide hormones, the LH/CG receptor exhibits ligand-induced loss or down-regulation of cell surface LH/CG binding capacity (Freeman and Ascoli, 1982). This negative modulation of receptor levels in vitro is time and hormone concentration dependent (Freeman and Ascoli, 1982). Decreased binding capacity is the result of a lowered LH/CG receptor level rather than an altered affinity of the receptor for the ligand (Freeman and Ascoli, 1982).
However, the time course for the loss of LH/CG receptors is different in in vivo and isolated cells, where incubations of 24-72h are required (Sharpe, 1976; Hsueh et al., 1977; Cigorraga et al., 1978), to cultured mouse tumour Leydig (MA10) cells, where only incubations of 90-180 min are required (Freeman and Ascoli, 1982; Lloyd and Ascoli, 1983).

The mechanism for homologous up-regulation of LH/CG receptors in Leydig cells is not clearly understood, but reports indicate that it might be only a transient phenomenon that precedes homologous down-regulation (Tsuruhara et al., 1977; Huhtaniemi et al., 1978; Huhtaniemi et al., 1981). The increases observed were due to changes in the number of receptors without change in affinity (Tsuruhara et al., 1977; Huhtaniemi et al., 1981). Under optimal conditions, the LH/CG receptors can be up-regulated 1.5- to 2.5-fold (Tsuruhara et al., 1977; Huhtaniemi et al., 1981). The general consensus of opinion at present, however, is that the increases in LH/CG receptor in Leydig cells is due to either an externalization of preformed receptors or to general changes in membrane conformation (Huhtaniemi et al., 1981; Barañao and Dufau, 1983).

1.7.9 Regulation of LH/CG receptor mRNA.

The regulation of testis LH/CG receptor mRNAs was examined in vivo in adult rats that received 10 or 200 IU hCG (LaPolt et al., 1991). 6h and 12h of treatment with 200 or 10 IU hCG, respectively, decreased LH/CG receptor mRNA transcripts 7, 4.2 and 2.5 kb by more than 60%. This preceded a loss in labelled
hCG binding. These transcripts were further inhibited (>93%) between 24-72 h after hCG treatment and returned to 40% and 100% of control levels by days 6 and 9, respectively (LaPolt et al., 1991). In contrast, the truncated 1.8 kb LH/CG receptor transcript was not affected by hCG treatment, indicating a differential suppressive effect of the ligand on its receptor mRNA levels (LaPolt et al., 1991).

Using MA10 Leydig tumour cells as a model system it was shown that LH and hCG caused a rapid loss of labelled hCG binding. This was followed by a slower loss of mRNA for LH/CG receptors (Wang et al., 1991a). There was only a 20% loss of LH/CG receptor mRNA with LH treatment by 16h, compared to a 50-60% loss with hCG. Treatment with deglycosylated hCG also caused a loss of LH/CG receptors, however, there was a 40% increase in mRNA. Treatment with the cyclic AMP analogue (8-bromo-cAMP) caused a loss of LH/CG receptor mRNA (50% loss by 6h) before there was a loss of labelled hCG binding sites (Wang et al., 1991a). This profile was mimicked with epidermal growth factor and PMA treatment, but the time courses were longer (50% loss by 20h) (Wang et al., 1991b). These results indicated that the down-regulation of LH/CG receptors involves two stages. The first stage is the loss of surface receptors by RME, and the second is a cyclic AMP dependent loss of LH/CG receptor mRNA. PMA and EGF are able to modulate the second stage of LH/CG receptor down-regulation.
Phosphorylation of the β-adrenergic receptor (βAR) has been shown to be involved in the mechanism of desensitization (Lefkowitz et al., 1990). At low concentrations of agonist, the cyclic AMP-dependent protein kinase (PKA), is activated. This phosphorylates one or both of two sites adjacent to the region of the receptor involved in coupling to the α-subunit of Gs (αs), and disrupts coupling. At high concentrations of agonist, when most receptors are occupied, the β adrenergic receptor specific kinase (βARK) is also activated. This enzyme phosphorylates sites primarily on the distal portion of the carboxyl terminus of the βAR. Phosphorylation by βARK in itself may further disrupt coupling to αs, but it has been postulated that an arrestin-like protein (48,000 Mr) may also be needed for the full extent of desensitization to occur (Lefkowitz et al., 1990).

Purified LH/CG receptors have been shown to be phosphorylated by the catalytic subunit of PKA, in a hCG concentration dependent manner (Minegishi et al., 1989) on serine and threonine residues. A novel rhodopsin kinase subtype has been isolated from rat ovary that phosphorylates the LH/CG receptor in a ligand dependent manner (Funatsu and Dufau, 1991). Low concentration of hCG facilitated phosphorylation of LH/CG receptor by this kinase, while higher concentrations markedly inhibited receptor phosphorylation. This contrasts with the phosphorylation of βAR by βARK, which was only induced by high ligand concentrations. This novel kinase, underwent autophosphorylation and was not under the control of cyclic AMP, cyclic GMP or Ca^{2+}/calmodulin. The kinase activity was inhibited by heparin, Ca^{2+}, sodium chloride and phosphatidylserine.
(Funatsu and Dufau, 1991). This would indicate that phosphorylation of LH/CG receptors may play a role in the regulation of LH/CG receptors.

1.8 Aims

This work was carried out to elucidate the structure-function relationships of the LH/CG receptor and mechanisms of LH/CG receptor regulation in the Leydig cell. Studies were concentrated on four Leydig cell types: two mouse tumour Leydig cells (MA10 and MLTC-1), a rat tumour Leydig cell (R2C) and rat testis Leydig cells. Mouse testis Leydig cells were also used to confirm data obtained in the cultured mouse tumour Leydig cells. Desensitization, up- and down-regulation of receptors were studied including the role of proteolysis in the mechanism of down-regulation and LH/CG action on Leydig cells. To study the structure-function relationships of the LH/CG receptor with respect to the above mechanisms, investigations to develop a new method, that did not require site directed mutagenesis and expression in non-host cell systems were carried out.

Though the cDNA sequence for the LH/CG receptor has been isolated and expression of functional receptors has been obtained, very little is known about the regulation of LH/CG receptors in Leydig cells. Numerous studies on the β-adrenergic receptor elucidated the interactions of this receptor with its ligand, G-protein and the mechanisms of desensitization. The LH/CG receptor is a member of the G-protein coupled receptor family, but has very distinct differences, e.g. large extracellular domain and short third intracellular loop, suggesting that the regulatory mechanisms are different from those of the β-adrenergic receptor.
CHAPTER 2

MATERIALS AND METHODS
MA10 cells were a gift from Dr. M. Ascoli of the University of Iowa, Iowa, USA. The MLTC-1 and R2C cells were a gift from Dr I. Mason, University of Texas, Dallas, Texas, USA. Testosterone and pregnenolone antisera were a gift from the Dept. Biochemistry, Medical Faculty, Erasmus University, Rotterdam. Cyclic AMP antisera was a gift from Dr. Matthias Schumacher, Hamburg. Antisense oligodeoxynucleotides were purchased from British Biotechnology Ltd. (Oxford, England). Ovine luteinizing hormone (batch oLH-26, potency: 2.3 U/mg) and hCG (batch CR-127, potency: 14,900 IU/mg) were obtained from the National Institute of Child Health and Human Development (Bethesda, USA). Powdered media and sera were obtained from Gibco Ltd. (Middlesex, England). Collagenase (197 u/mg, Batch 49C948) was obtained from Worthing Biochemical Corporation (New Jersey, USA). Disuccinimidy suberate was purchased from Pierce Chemicals (Cheshire, England). Carrier free Na$^{125}$I was obtained from the Radiochemical Centre, Amersham (Amersham, England). Kodak X-Omat S autoradiographic film was obtained from Kodak-Pathe (France). Centriprep 30 concentrators were purchased from Amicon Division, W.R. Grace and Co. (Danvers, USA). Phorbol 12-myristate-13-acetate (PMA), sodium azide, trypsin inhibitor, cyclic AMP standard, pregnenolone and testosterone standards, bovine serum albumin (BSA) fraction V, cycloheximide, actinomycin D, percoll, forskolin, cholera toxin, 3-isobutyl-1-methylxanthine (IBMX), dibutyryl cyclic 3',5'-adenosinemonophosphate (db-cAMP), leupeptin, phenylmethylsulphonyfluoride (PMSF), and aprotinin were purchased from
the Sigma Chemical Company Ltd (Dorset, England). Dextran T 500 and PD-10 columns were from Pharmacia (Milton Keynes, England). Glass microfibre filters (GF/C) were purchased from Whatman Ltd (Maidstone, England). [\(^{3}\text{H}\)]-Methionine, [\(^{3}\text{H}\)]-pregnenolone and [\(^{3}\text{H}\)]-testosterone were purchased from NEN, Dupont (UK) Ltd (Hertfordshire, England). Cyanoketone and SU10603 (inhibitors of pregnenolone metabolism) were obtained from Sterling Research Group-Europe (Surrey, England). Chloramine T, polyethylene glycol 6000 were obtained from BDH (Dorset, England). All other reagents were purchased from Sigma or BDH (Dorset, England). All sterile plastic ware was from Bibbey (Staffordshire, England).
2.2 Methods

2.2.1 Isolation of rat testis Leydig cells.

Rat testis Leydig cells were isolated essentially as described by Platts et al. (1988) and modified by Choi and Cooke (1991).

2.2.1.1 Buffers:

A) Dissection Medium: (pH 7.4)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dulbecco's Modified Eagles Medium (DMEM)</td>
<td>13.4g</td>
</tr>
<tr>
<td>HEPES 10mM</td>
<td>2.381g</td>
</tr>
<tr>
<td>BSA 0.1%</td>
<td>1g</td>
</tr>
<tr>
<td>Streptomycin 500μg/ml</td>
<td>0.5g</td>
</tr>
<tr>
<td>Penicillin 250 IU/ml</td>
<td>250 x10³ IU</td>
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<tr>
<td>Double distilled Water</td>
<td>to 11</td>
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</table>

B) Elutriation medium: (pH 7.4)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>DMEM</td>
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<td>HEPES 10mM</td>
<td>2.381g</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>0.5g</td>
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<td>Penicillin</td>
<td>250 x10³ IU</td>
</tr>
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<td>EDTA 1mM</td>
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<td>Heparin 25,000 U/l</td>
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<td>BSA 0.5%</td>
<td>5g</td>
</tr>
<tr>
<td>Double distilled Water</td>
<td>to 11</td>
</tr>
</tbody>
</table>

Buffers A and B were sterilised by filtering through a 0.22μm filter and stored at 4°C.
C) Collagenase dispersion medium: (pH 7.4)

<table>
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<tr>
<th>Component</th>
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</tr>
</thead>
<tbody>
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<td>Dissection medium</td>
<td>7ml/rat</td>
</tr>
<tr>
<td>Collagenase</td>
<td>0.5mg/ml</td>
</tr>
<tr>
<td>Trypsin inhibitor (1% solution)</td>
<td>750μl/35ml</td>
</tr>
</tbody>
</table>

2.2.1.2 Method:

200 - 250g Sprague Dawley rats were anaesthetised with a mixture of oxygen and halothane (1500:5) and killed by cervical dislocation. The rat's abdomen was washed with ethanol and the testes dissected out into a small volume of dissection medium. The testes were decapsulated and placed in 7ml of collagenase medium, and incubated at 37°C in a shaking water bath (60-65 strokes/min) until the Leydig cell clumps were dispersed (approx. 45 min.) - the time of digestion depended on the age of the rats. The medium appeared cloudy.

Dissection medium was added to make the volume up to 25ml and the universals were inverted 40 times. The suspension was left to settle and the medium removed with a 50ml syringe and filtered through a fine mesh gauze (60μm) into a 50ml universal. The settled suspension was washed again with dissection medium by inverting 20 times, and then left to settle again. The supernatant was removed and filtered as above. The filtered supernatant was then centrifuged for 10min at 300xg. The resultant cell pellet was resuspended in a small volume of elutriation medium.

The cell suspension was placed in a syringe and injected into the elutriation system (figure 2.2.1) and pumped (25ml/min) to the chamber located in the rotor, which is spinning at 1,500xg with the pump speed of 25ml/min, 100ml was collected.
FIG. 2.2.1 The elutriator system. Essential elements of the system are shown. The buffer reservoir, pump, sample injection syringe, and collection flask, inlet/bubble trap/sample reservoir.
The pump speed was then increased to 55ml/min and 150ml collected.

The cells collected at 55ml/min were centrifuged for 10min at 300xg and resuspended in a minimum volume of elutriation medium. Resuspended cells were added to the top of a previously prepared percoll density gradient (see 2.2.2) and centrifuged at 1,500xg for 25min.

At this point 3 layers of cells should be present - 2 tight bands (1.051 and 1.064 g/ml) and a third slightly dispersed band (Leydig cells - 1.076 g/ml). The percoll was removed from above the first layer of cells and then bands 1 & 2 were removed. The 3rd band was then placed in a sterile universal. The percoll was diluted at least 1:4 with dissection medium and centrifuged at 300xg for 10 min. The supernatant was discarded and the cell pellet resuspended in a small volume of dissection medium. The cells were counted in a haemocytometer and diluted to the required concentration.

2.2.2 Percoll density gradients

Percoll gradients were prepared by mixing a solution of 90% percoll (90% Percoll: 10% Earles balanced salt solute (x10) (v/v)) with an equal volume of dissection medium.

25ml of 90% percoll: 10% Earles balanced salt solute (x10) (v/v) solution is adjusted to a pH of 7.4 by the addition of several drops of 1M HEPES (pH 7.4) - a red colour should appear.

A 0-90% percoll density gradient is prepared by pumping dissection medium
into 90% percoll solution. As the first drops of dissection medium enter the 90% percoll solution the percoll solution was also pumped into a 2x25ml sterile universals.

2.2.3 Isolation of mouse testis Leydig cells.

Mouse testis Leydig cells were isolated essentially as described by Cooke et al. (1981).

2.2.3.1 Buffer:

A) Dissection Medium: (pH 7.4)

<table>
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<tr>
<th>Component</th>
<th>Quantity</th>
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<tbody>
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</tr>
<tr>
<td>Penicillin 250 IU/ml</td>
<td>250 x10^3 IU</td>
</tr>
<tr>
<td>Double distilled Water</td>
<td>to 1l</td>
</tr>
</tbody>
</table>

2.2.3.2 Method:

30 - 50g Balb/c mice were anaesthetised with a mixture of oxygen and halothane (1500:5) and killed by cervical dislocation. The mouse's abdomen was washed with ethanol and the testes dissected out into a small volume of dissection medium. The testes were decapsulated and 6 testes were placed in 20ml dissection medium. The testes were then drawn up into a 50ml syringe through a plastic cannula.
(2mm diameter). This process was repeated 15 times. The seminiferous tubules were allowed to settle and the supernatant was removed. The supernatant was filtered through a 60μm nylon gauze. The supernatant was centrifuged at 300xg for 10 min and the pellet was resuspended in 10ml of dissection medium and the suspension recentrifuged. The pellet was resuspended again in 2ml of dissection medium and added to the top of a previously prepared percoll density gradient (see 2.2.2) and centrifuged at 1,500xg for 25min.

At this point 3 layers of cells should be present - 2 tight bands (1.051 and 1.064 g/ml) and a third slightly dispersed band (Leydig cells - 1.076 g/ml). The percoll was removed from above the first layer of cells and then bands 1 & 2 were removed. The 3rd band was then placed in a sterile universal. The percoll was diluted at least 1:4 with dissection medium and centrifuged at 300xg for 10 min. The supernatant was discarded and the cell pellet resuspended in a small volume of dissection medium. The cells were counted in a haemocytometer and diluted to the required concentration.

2.2.4 Culture of tumour Leydig cells.

Tumour Leydig cells were cultured according to the method of Ascoli (1981).

2.2.4.1 Buffer:

Growth medium:

<table>
<thead>
<tr>
<th>g</th>
<th>Substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>138.4</td>
<td>Waymouth MB752/1 powder</td>
</tr>
<tr>
<td>47.7</td>
<td>HEPES (20mM)</td>
</tr>
<tr>
<td>22.4</td>
<td>NaHCO₃ (2.24g/l)</td>
</tr>
<tr>
<td>10l</td>
<td>double distilled water</td>
</tr>
</tbody>
</table>

80
Solids were dissolved and then 1.5l of medium was removed and replaced with 1.5l of horse serum (15%) and mixed well. 10ml of Gentamycin (50mg/ml) was then added. pH was adjusted to 7.7 - 7.8 with saturated NaOH. The solution was sterilized by filtration through a sterile 0.22μm filter.

2.2.4.2 Method:

Tumour Leydig cells were cultured in growth medium at 37°C in air/CO₂ (95%/5%). The medium was changed every two days. The cells were subcultured by washing the cells twice with 5ml of Ca²⁺-Mg²⁺ free phosphate buffered saline before the addition of 2ml of trypsin (0.05% w/v)/EDTA (0.53mM). After 1 min the trypsin was removed and the cells were incubated at 37°C for 3 min. The cells were then removed from the cultures with fresh growth medium and plated either in 6, 12, 24, or 96 well plates for assay cultures or T25 flasks for stock cultures. Assay cultures were used two days later and stock culture were split every 4 days.

2.2.5 Cell incubations.

Rat and mouse testis Leydig cells were preincubated for 2h, to allow the cells to stick to the culture surface, before being treated with reagents. Cultured tumour cells, after being treated with trypsin 0.05% (w/v)/EDTA 0.53mM, were plated in culture dishes. These cells were used 2 days later.
2.2.6 *Diaphorase histochemistry.*

This method is essentially as described by Aldred and Cooke (1983).

2.2.6.1 **Buffer:**

A) Reaction buffer

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate buffer</td>
<td>0.1M</td>
</tr>
<tr>
<td>Nitroblue Tetrazolium</td>
<td>1mg/ml</td>
</tr>
<tr>
<td>NADH</td>
<td>3mg/ml</td>
</tr>
</tbody>
</table>

B) Fixative

<table>
<thead>
<tr>
<th>Fixative</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Formaldehyde</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>Ethanol</td>
<td>50% (v/v)</td>
</tr>
</tbody>
</table>

2.2.6.2 **Method:**

Plated cells (1x10^5) were washed with 0.01M PBS and then incubated with the reaction buffer for 30 min at 37°C. After this incubation the reaction buffer was removed and replaced with the fixative. The cells were then incubated at room temperature for 30 min.

A minimum of 5 fields were counted. Cells which stained for diaphorase are those which have membrane damage, i.e. they are unable to exclude the large molecules of NADH.
3β-Hydroxysteroid dehydrogenase histochemistry:

This method was derived from Cooke et al. (1983).

2.2.7.1 Buffers:

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Conc.</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-Androstan-3β-ol-17-one</td>
<td>2mg/ml DMF</td>
<td>0.1</td>
</tr>
<tr>
<td>Nitroblue tetrazolium</td>
<td>1mg/ml H₂O</td>
<td>1.0</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>1.6mg/ml H₂O</td>
<td>0.7</td>
</tr>
<tr>
<td>NAD⁺ free salt</td>
<td>3mg/ml H₂O</td>
<td>0.8</td>
</tr>
<tr>
<td>Phosphate buffer pH 7.3</td>
<td>0.1M</td>
<td>4.0</td>
</tr>
</tbody>
</table>

(DMF = Dimethylformamide)

Solution was mixed in a dark bottle.

Gelatine jelly (15g/100ml H₂O): Gelatine was dissolved by warming and 100g of glycerol added. Warm at 60°C for 5 min, and filter through glass wool to 100ml and add 1 drop of phenol.

2.2.7.2 Method:

50,000 cells per 100μl were centrifuged onto a slide using a cytopsin centrifuge, at 1,000xg for 5 min. One drop of the reaction mixture was added and incubated at 37°C for 20 min. The cells were fixed using a solution of 50% ethanol/10% formaldehyde. One drop of gelatine jelly was added and a cover-slip placed on top. A minimum of 5 fields of approximately 50-150 cells were counted. The colourless cells were negative and dark blue/purple cells were positive. Results were expressed as a percentage stained of total cell number.
2.2.8  

*iodination of hCG.*

Human chorionic gonadotropin was iodinated by a lactoperoxidase method reported by Thorell and Johansson (1971).

2.2.8.1  

*Buffers:*

- **F1:** 10µg gonadotropin in 50µl 0.2M PBS (pH 7.4)
- **F2:** Lactoperoxidase - 0.5mg/ml in 0.05M PBS
- **S1:** 0.01M PBS (0.9% NaCl, 0.1% BSA) pH 7.4
- **S2:** 5% BSA in 0.01M PBS (0.9% NaCl)
- **S3:** 0.1% Azide/10mM KI
- **S4:** 0.2M Phosphate buffer (pH 7.5)
- **S5:** 0.007% H$_2$O$_2$ (10µl in 150ml H$_2$O)

2.2.8.2  

*Method:*

A G25 sephadex chromatography column (PD-10) was washed with 30ml of S2 to block non-specific binding.

To F1, which was in a plastic tube containing a magnetic microflea, situated on top of a magnetic stirrer - the following was added:

1) 15µl S4.
2) 10µl Na$^{125}$I (Amersham IMS 30) 1mCi.
3) 10µl F2.
4) 10µl S5.

Upon addition of S5 the reaction was started and allowed to react for 1min. The reaction was stopped by the addition of 1ml S3. The reaction mixture was then added to the column and allowed to enter the PD-10 column before continuing. hCG
was eluted with S1 collecting fractions of 200μl. The fractions were then measured for radioactivity in a gamma-counted. The fractions that form the radioactive peak (figure 2.2.2) were pooled. The activity is determined in a bioactive assay and then diluted so that the concentration is 1ng $^{125}$I-hCG/μl. The bioassay involves the generation of a displacement curve using $1\times10^6$ MA10 cells in 1ml of growth medium with 10μl of radioactive solution and increasing concentration of pure hCG (0-1.4nM).
Non-specific binding was determined using 1μg pure hCG. The results were expressed as a percentage of control binding and the concentration of pure hCG that displaces 50% of the radioactivity gives the concentration of the radioactive hCG.

2.2.9 Iodination of cyclic AMP.

2'-Monosuccinyladenosine-3',5'-cyclic monophosphate Tyrosyl methyl ester (ScAMP-TME) was radioiodinated as follows and as described by Brooker et al. (1979).

2.2.9.1 Buffers:

A) ScAMP-TME was prepared as a 40μg/ml solution in 5mM acetate buffer, pH 4.75.

B) 0.5M Potassium phosphate, pH 7.0

C) Chloramine T was dissolved at a final concentration of 1mg/ml in 0.5M potassium phosphate, pH 7.0.

D) Sodium metabisulphite was prepared as a 5mg/ml solution in distilled water.

E) Solvent mixture:

Butan-1-ol 12 vol.
Glacial acetic acid 3 vol.
Distilled water 5 vol.

F) 50mM Sodium acetate buffer, pH 4.75
2.2.9.2 **Method:**

800ng of ScAMP-TME (20μl) was mixed with 20μl of 0.5M potassium phosphate, pH 7.0 and 1mCi of Na$^{125}$I. 5μg of chloramine T (5μl) was added and the reaction stopped 1 min later by the addition of 50μl of sodium metabisulphate.

The reaction mixture was then streaked onto a strip (2 x 40 cm) of Whatman 31 ET chromatography paper. This was developed by descending paper
chromatography over a 20 cm migrating distance in the solvent mixture. 100ml of solvent mixture was used for each separation. After chromatography the strip was dried, cut into 1 cm segments and the radioactive material eluted over 30 min, by the addition of 1 ml of 50 mM sodium acetate buffer. 5 μl of each fraction was counted in a gamma counter and a profile derived (figure 2.2.3). The largest peak contained the eluted [\(^{125}\)I]-ScAMP-TME. The peak fractions from this were pooled, diluted 1:1 with propan-1-ol.

2.2.10 Cell surface [\(^{125}\)I]-hCG binding assay.

2.2.10.1 Buffers:

A) Washing buffer: (11)

0.01 M PBS (pH 7.4) + 0.1% BSA

B) Binding buffer (pH 7.4): (11)

DMEM 13.4g
HEPES 10 mM 2.381g
BSA 0.1% 1 g
Streptomycin 500 μg/ml 0.5 g
Penicillin 250 IU/ml 250 x 10\(^3\) IU

Double distilled water to 11

Filter (0.22 μ filter) sterilised.

C) Pure hCG (CR127) - 10 μg/50 μl (0.2 M PBS pH 7.4)

D) [\(^{125}\)I]-hCG stock - 1 ng/μl
The number of cells required for the comparison of binding levels is $1.5 \times 10^5$ cells/well and for Scatchard analysis $1 \times 10^6$ cells/well. The final incubation volume was 1ml.

The cells were washed with washing buffer and then 500μl of binding buffer was added. Pure hCG was added at the concentration 0, 0.14 and 0.28 nM for comparison of binding levels whereas concentrations in the range of 0-1.4 nM were added for Scatchard analysis. Non-specific binding was determined by the addition of 1μg/ml pure hCG.

$[^{125}\text{I}]-\text{hCG}$ was added to all wells at a concentration of 0.56 nM and the total reaction volume made up to 1ml with binding buffer. The reaction was then incubated overnight at 4°C.

The binding buffer was aspirated and washed twice with washing buffer. The cells were then dissolved with 500μl of 0.5M NaOH and transferred to culture tubes to be counted in a gamma-counter. 5μl of $[^{125}\text{I}]-\text{hCG}$ was counted to determine the total counts added.

Scatchard analysis was interpreted using the EBDA/Ligand programme by G.A. McPherson (Elsevier - BIOSOFT).

Surface binding levels can be separated from internalized by washing the cells with 50mM glycine-buffered-saline (pH 3.0) (Ascoli, 1982) before dissolving the cells with 0.5M NaOH.
2.2.11 Membrane and solubilized cell $[^{125}\text{I}]-\text{hCG}$ binding assay.

Binding of $[^{125}\text{I}]-\text{hCG}$ to solubilized membranes was performed according to the method of Minegishi et al. (1987).

2.2.11.1 Buffer:

- 25mM HEPES pH 7.5
- 150mM NaCl
- 5mM N-ethylmaleimide
- 2mM PMSF
- 5mM EDTA
- 10mM Benamidine
- 1mg/ml Bacitracin
- 0.1% Triton X-100
- 20% Glycerol
- 0.1% BSA

2.2.11.2 Method:

50μg of protein from solubilized cells or membranes was added to 500μl of binding buffer. Pure hCG was added in the range 0-1.4nM. Non-specific binding was determined by the addition of 1μg/ml pure hCG.

$[^{125}\text{I}]-\text{hCG}$ was added to all tubes at a concentration of 0.56nM and the total reaction volume made up to 1ml with binding buffer. The reaction was then incubated overnight at 4°C.

Bovine gamma globulin was added to a final concentration of 1mg/ml. To this mixture 0.5ml of 30% (w/v) polyethylene glycol (PEG) in PBS was added. The
suspension was centrifuged at 1,500 xg for 15 min at 4°C. The supernatant was aspirated and the pellet resuspended with 0.1 ml of binding buffer and left to stand for 10 min at 4°C. Another 0.5 ml of 30% PEG was added and the suspension centrifuged at 1,500 xg for 15 min at 4°C. The supernatant was aspirated and the pellet counted in a gamma counter.

Scatchard analysis was interpreted using the EBDA/Ligand program by G.A. McPherson (Elsevier - BIOSOFT).

2.2.12 Internalization studies.

2.2.12.1 Method:

Cells were plated at 2 x 10^6/well in 6 well plates. Cells were incubated at 10°C, to inhibit internalization (Habberfield et al., 1987), in 1 ml fresh medium (see 2.2.1 or 2.2.4) with 1.4 nM [125I]-hCG for 1 h. The cells were then placed on ice and washed with 0.01 M PBS-BSA to remove unbound hormone. The cells were then recultured in 1 ml fresh medium at 37°C. To stop the reaction, cells were placed on ice, medium was removed and washed with 0.01 M PBS-BSA. The surface bound [125I]-hCG was determined by washing the cells twice with 50 mM glycine-buffered saline (pH 3.0) (Ascoli, 1982) and counting the combined washings. To measure the amount of internalized hormone, cells were dissolved in 1 ml of 0.5 M NaOH, and counted in a gamma-counter.
2.2.13 Membrane preparation.

2.2.13.1 Buffers:

A) Homogenising buffer: pH 7.5

50mM HEPES
150mM NaCl
250mM Sucrose
5mM N-ethylmaleimide
2mM PMSF
5mM EDTA
10mM Benzamidine
1mg/ml Bacitracin

B) Solubilizing buffer: pH 7.5

Buffer A - sucrose
20% Glycerol
1% Triton X-100

C) Storing buffer: pH 7.5

As for buffer B except 0.1% Triton X-100.

2.2.13.2 Method:

5x10⁷ - 1x10⁸ cells in 4ml of buffer A were homogenised with a "Polytron" tissue homogeniser (or equivalent). Homogenisation was performed for 30 sec on ice.

The homogenate was centrifuged at 600xg for 15min at 4°C. The supernatant was decanted and centrifuged at 30,000xg for 30min at 4°C.

The pellet was solubilized in buffer B for 60min at 4°C. The solubilized membranes were centrifuged for a further 2h at 100,000xg at 4°C. The pellet was resuspended in buffer C. Protein was determined by the Bio-Rad method (2.2.14).
2.2.14  Bio-Rad protein assay.

The stock solution of concentrated dye was diluted 1:5 in distilled water and filtered. Aliquots of solubilized membrane were diluted 1:10 with distilled water. A standard BSA curve was constructed in the range 0-1µg/ml in distilled water.

20µl of standard or sample were added to 480µl dye, mixed and incubated for 10-15min, then read at 595nm.

2.2.15  SDS-PAGE

Determination of molecular weights for proteins was carried out according to the method of Laemmli (1970).

2.2.13.1  Buffers:

A) Separating gel (10% polyacrylamide):

7.5 ml  2M Tris/HCl pH 8.3 (MW 157.6)
12.6ml  30% (w/v) acrylamide
        0.8% (w/v) N,N'-methylenebisacrylamide (bis)
12.4ml  Water
2.5ml   1.6% (w/v) SDS

Mix then add:-

2.8ml   1.2% ammonium persulphate
17.5µl  N,N,N',N'-tetramethylethylenediamine (TEMED).

This solution was then poured into a vertical slab gel and overlayed with water and left to set for 2h at room temp.
B) Stacking gel:

5ml  0.25M Tris/HCl pH 6.8
2.5ml 14% (w/v) acrylamide
      0.2% (w/v) bis
1.25ml 1.6% (w/v) SDS

Mix then add:

1.25ml 1.2% (w/v) ammonium persulphate
5µl TEMED

This solution was poured onto the separating gel and a spacer comb placed between the plates. This solution was allowed to set for 1h.

C) Running buffer (2l):

0.052M Tris/HCl (MW 157.6) pH 8.3
0.192M Glycine (MW 75.07)
0.2% SDS (w/v)

D) Laemmli's sample buffer:

0.0625M Tris/HCl pH 6.8
2% SDS (w/v)
10% Glycerol (v/v)
5% β-mercaptoethanol (v/v)
0.05% Bromophenol Blue (w/v)

E) Coomassie blue staining:

0.25% Coomassie blue (or brilliant blue) (w/v)
25% Isopropanol (v/v)
10% Glacial acetic acid (v/v)
F) Destain:

10% Isopropanol (v/v)
10% Glacial acetic acid (v/v)

2.2.15.2 Method:

After preparation of the gel the comb was removed and the gel placed in a tank containing the running buffer. Solubilized membrane or cells was added to an equal volume of Laemmli's buffer and boiled for 5 min. The resulting solution was then pipetted into the chambers left by the comb. Proteins were stacked within the gel using a power setting of 150 V, which was decreased to 100 V to run the proteins through the separating gel. The gel was run until the dye front was 1cm from base of the gel. The gel was removed and stained for 60min and then destained overnight. The gel was then dried on a gel slab dryer for 40-60 min.

If radioactively labelled proteins were used, the gel was placed in a lead autoradiographic film holder with autoradiographic film and an intensifying screen. The film was exposed at 70°C from 2 days to 3 weeks. The film was then developed in an automated developing machine.
2.2.16 Amino acid incorporation.

2.2.16.1 Buffers:

a) DMEM-F12 (1l) pH 7.7:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM-F12</td>
<td>13.4g</td>
</tr>
<tr>
<td>L-glutamine</td>
<td>350mg</td>
</tr>
<tr>
<td>L-lysine</td>
<td>240mg</td>
</tr>
<tr>
<td>L-leucine</td>
<td>50mg</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>90.61mg</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>97.67mg</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>112.6mg</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.1g</td>
</tr>
</tbody>
</table>

b) DMEM-F12 + Methionine (1l):

Buffer (a)
L-Methionine  1.49g (10mM)

c) DMEM-F12 + 15% Horse serum (270ml):

Buffer (a)  230ml
Horse serum  40ml

d) PBS-BSA-Methionine (500ml) pH 7.4:

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂HPO₄·7H₂O</td>
<td>7.06g</td>
</tr>
<tr>
<td>NaCl</td>
<td>4.5g</td>
</tr>
<tr>
<td>BSA</td>
<td>0.5g</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>746mg (10mM)</td>
</tr>
</tbody>
</table>
e) 10% TCA-Methionine (500ml):

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA</td>
<td>50g</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>746mg (10mM)</td>
</tr>
</tbody>
</table>

All buffers were filtered (0.22μm) sterilized.

2.2.16.2  Method:

Tumour Leydig cells were grown to a concentration of $2 \times 10^6$ cells/well. The cells were then incubated for 16h with 5ml DMEM-F12 + 15% horse serum. After the first incubation the cells were washed twice with methionine free medium (1ml) and reculture in 5ml DMEM-F12/15% horse serum + $^3$H-methionine (5μCi) for 6h.

To stop the reaction cells were washed twice with DMEM-F12 + methionine (5ml). Cells were then scraped from the culture dishes in 1ml PBS-BSA-methionine. The scraped cells were homogenised in a 1ml glass hand homogeniser and 100μl placed on a prewashed (PBS-BSA-methionine) GF/C filter. The proteins were precipitated on the GF/C filter under vacuum with TCA-methionine.

The filter was placed in a maxi scintillation vial and 10ml Ready Protein scintillant (Beckman) added. The vials were then counted for 5min in a β-counter.
2.2.17  *Cyclic AMP RIA.*

This method is derived from the method of Steiner *et al.* (1972) with the acetylation modification described by Harper and Brooker (1975).

### 2.2.17.1  Buffers:

A) 0.1M Phosphate buffer saline (PBS): pH 7.0

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaH₂PO₄·2H₂O</td>
<td>0.2M</td>
<td>215ml</td>
</tr>
<tr>
<td>Na₂HPO₄·12H₂O</td>
<td>0.2M</td>
<td>305ml</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.15M</td>
<td>8.78g</td>
</tr>
<tr>
<td>NaN₃</td>
<td>0.3mM</td>
<td>20mg</td>
</tr>
</tbody>
</table>

Made up to 1l with H₂O.

B) 0.3% Bovine gamma Globulin:

0.3g  Bovine gamma globulin in 100ml PBS

C) Acetylating Reagent:

- Acetic Anhydride: 1ml
- Triethylamine: 2ml

D) [¹²⁵I]-cyclic AMP:

Each assay tube required 20,000 cpm/100μl PBS/tube.

5μl of the stock was counted in a gamma-counter to calculate the amount required.

E) 16% Polyethylene Glycol

PEG (in 11 tap water): 160g

F) Antibody (MSI) diluted to required concentration in 0.3% bovine gamma globulin.
G) Standard cyclic AMP are prepared at the concentrations of 1, 10, 100 nM in ethanol.

H) Sample medium:
Medium as treated in experiment.

2.2.15.2 Method:

A standard curve (10-5000 fmol - final conc.) in ethanol was aliquoted and dried under nitrogen and redissolved in 100μl sample medium. 100μl of treated medium was aliquoted for totals, non-specific binding (NSB), and zero concentration of cyclic AMP (Bo). Samples were aliquoted up to 100μl or treated medium was added to make up the volume to 100μl.

3μl of the acetylating mixture was added, the mixture vortex mixed, and left for 3h at room temp. 100μl of 0.3% bovine gamma globulin was added to the totals and NSB.

Antibody was made up in 0.3% bovine gamma globulin and 100μl of antibody solution was added to all tubes except totals and NSB. Iodinated cyclic AMP in PBS, containing 20,000 cpn in 100μl was then added and the mixture vortexed and incubated overnight at 4°C.

1.6ml of 16% PEG was added and vortex mixed. The incubation mixtures were then centrifuged for 30 min at 3,000xg at 4°C. The supernatant was decanted and the pellets measured in a gamma counter.
2.2.18 *Pregnenolone and testosterone RIA.*

These assays were performed as described by van der Molen et al. (1975) and Verjans et al. (1973), respectively.

2.2.18.1 *Buffers:*

A) 0.01M Phosphate buffer: pH 7.0

\[\begin{align*} 
0.2\text{M } \text{NaH}_2\text{PO}_4.2\text{H}_2\text{O} & \quad 19.5\text{ml} \\
0.2\text{M } \text{Na}_2\text{HPO}_4.12\text{H}_2\text{O} & \quad 30.5\text{ml} \\
\text{Make up to 1l.} & \\
\end{align*}\]

B) Pas - Gel:

\[\begin{align*} 
0.01\text{M Phosphate buffer} & \quad 1l \\
0.14\text{M } \text{NaCl} & \quad 8.19\text{g} \\
0.3\text{mM } \text{NaN}_3 & \quad 20\text{mg} \\
0.1\% \text{ Gelatin} & \quad 1\text{g} \\
\text{Dissolved by warming.} & \\
\end{align*}\]

C) \(^{3}\text{H}\)-Pregnenolone and \(^{3}\text{H}\)-Testosterone:

Each assay tube required 10,000 cpm/100 \(\mu\)l Pas - Gel/ tube.

D) Pregnenolone and Testosterone antibodies were diluted in Pas - gel.

E) Standard Pregnenolone and Testosterone were at a concentration of 5ng/ml in ethanol.

F) Charcoal:

\[\begin{align*} 
5\text{mg Dextran T500/20ml phosphate buffer} & \\
50\text{mg Charcoal/ 20ml } & \\
\end{align*}\]
The dextran was dissolved and then charcoal added. The suspension was mixed with a magnetic stirrer at 4°C for 30min.

G) Sample medium:

Medium as treated in experiment.

2.2.16.2 Method:

A standard curve for pregnenolone (50-400 pg - final conc.) or testosterone (25-250 pg - final conc.) in ethanol was aliquoted and dried under nitrogen and redissolved in 100μl sample medium. 100μl of treated medium was aliquoted for totals, non-specific binding (NSB), and zero concentration of cyclic AMP (Bo). Samples were aliquoted up to 100μl or treated medium was added to make up the volume to 100μl.

100μl of Pas-Gel was added to totals and NSB. Antibody in Pas-gel (100μl) was added to all tubes except totals and NSB. Label containing 20,000 cpm (100μl) in 0.01M phosphate buffer was added to all tubes. The mixture was vortex mixed and incubated overnight at 4°C.

The charcoal solution (500μl) was added to all tubes except the totals and vortex mixed. The suspension was centrifuged at 3,000xg for 10min. 4°C. The supernatant was decanted into mini scintillation vial and 2ml of scintillation fluid was added. The vials were then counted for 5 min in a β-scintillation counter.
Inhibitors of pregnenolone metabolism.

The assay for pregnenolone production required the inhibition of pregnenolone metabolism. Cells were preincubated for 30 min with the inhibitors cyanoketone (inhibits 3β-hydroxysteroid dehydrogenase) and SU 10603 (inhibits 17α-hydroxylase) before stimulation. The inhibitors were made up in DMSO. Final concentration of inhibitors added was 20μM SU 10603 and 5μM cyanoketone.
CHAPTER 3

CHARACTERIZATION OF CULTURED TUMOUR LEYDIG CELLS
MA10, MLTC-1 and R2C cells were found to have similar growth rates. The density of the cells, as determined on a continuous 0-90% percoll gradient, varied but fell within the range of densities obtained for isolated rat testis Leydig cells. All the cell types contained high affinity LH/CG receptors (K_D = 2 - 5 nM). There was continual LH/CG receptor synthesis in the MA10 cells. Cyclic AMP and pregnenolone production in MA10 and MLTC-1 cells could be stimulated in a similar manner to isolated rat testis Leydig cells. Pregnenolone production in R2C cells could not be stimulated, however, basal production was equivalent to maximally stimulated rat testis Leydig cells. Cyclic AMP production in R2C cells could only be stimulated by LH, hCG, cholera toxin or forskolin in the presence of IBMX. Cyclic AMP and pregnenolone production in MA10 cells was desensitized with LH (3.3nM), db-cAMP (1mM) and PMA (10^{-7}M) in a similar manner to isolated mouse testis Leydig cells. LH (3.3nM) and PMA (10^{-7}M) caused desensitization of cyclic AMP production in rat testis Leydig cells, but testosterone production was unaffected.
3.2 Introduction

In this chapter the cultured tumour Leydig (MA10, MLTC-1, R2C) cells have been characterized with respect to cell density, growth rate, levels of LH/CG receptors, stimulation of cyclic AMP and pregnenolone, and finally desensitization. These characteristics were compared with those of isolated testis Leydig cells.

3.2.1 MA10 cells

MA10 cells are a clonal strain of mouse Leydig tumour cell adapted to culture in the laboratory of Dr. M. Ascoli (Ascoli, 1981). These cells were derived from a transplantable tumour (designated M5480P) that originated spontaneously in C57B1/6 mouse.

Like mouse testis Leydig cells, MA10 cells have the capacity to convert cholesterol into steroid hormones and respond to LH/CG with increased steroid biosynthesis. The major difference between MA10 cells and isolated testis Leydig cells is a decrease in the activity of 17α-hydroxylase enzyme, which is involved in the conversion of progesterone to testosterone. The major steroid produced by MA10 cells is progesterone rather than testosterone (Ascoli, 1981). In addition to this change the MA10 cells gained the ability to metabolize progesterone to 20α-hydroxyprogesterone. Under basal conditions, MA10 cells synthesize equivalent amounts of progesterone and 20α-hydroxyprogesterone. When exposed to hCG, however, the major steroid produced is progesterone (Ascoli, 1981). Steroidogenic cholesterol in MA10 cells is derived from the plasma membrane (Freeman, 1989)
and is under the control of cyclic AMP (Freeman, 1987b). Cyclic AMP can also increase the levels of the cholesterol side-chain cleavage cytochrome P450 (P450scc).

Desensitization of steroid production was shown to be mediated by both hCG and cyclic AMP analogues.

MA10 cells contain 10,000 - 20,000 LH/CG receptors per cell with high affinity ($K_D = 10^{-9} - 10^{-10} \text{ M}$). The receptors are under the negative modulation of hCG (>1.4nM) with 70% of receptors lost by 4h and 100% by 24h (Wang et al. 1991a). This loss involves two stages: Stage 1 is the acute loss of surface receptors (4h) and involves receptor-mediated-endocytosis (Wang et al., 1991a); Stage 2 involves the loss LH/CG receptor mRNA (Wang et al., 1991a). Stage 2 has been shown to be under the control of cyclic AMP, phorbol esters and epidermal growth factor (EGF) (Wang et al., 1991a;1991b).

It also appears that the population of cells known as MA10 include subclones. One subclone isolated (designated MA10-LP) only secretes 10% of the steroid synthesized by the parent MA10 cells (Kilgore and Stocco, 1989). These cells, however accumulate comparable amounts of cyclic AMP and have equivalent cholesterol side-chain cleavage activity as parent MA10 cells (Kilgore et al., 1990).

3.2.2 MLTC-1

MLTC-1 cells are a clonal strain of mouse Leydig tumour cell adapted to culture in the laboratory of Dr. R. Rebois (Rebois, 1982). These cells also originated from the M5480P transplantable tumour.

MLTC-1 cells were characterized in respect to LH/CG receptor content and
stimulation of cyclic AMP. Specific binding of hCG to isolated MLTC-1 membranes indicated a dissociation constant for hCG of $1.0 \pm 0.2 \times 10^{-10}$ M (Rebois, 1982). The receptors appeared identical to those from normal murine Leydig cells when analyzed by SDS PAGE and sucrose density gradient centrifugation. The molecular weight and sedimentation coefficient were 95,000 and 8.5 S, respectively (Rebois, 1982). MLTC-1 cells responded to hCG by producing cyclic AMP and progesterone. Desensitization of cyclic AMP production was shown to be mediated by hCG and phobol esters (Rebois and Patel, 1985).

### R2C

R2C cells are a clonal strain of rat Leydig tumour cell adapted to culture in the laboratory of Dr. S-L. Shin (Shin et al., 1968). These cells were derived from a rat transplantable tumour that originated spontaneously in Wistar-Furth rats. The original culture contained 2 distinct cell types. An epithelial-type and fibroblast-type. The epithelial-type clone isolated from this culture secreted a high level of steroid hormones, was nearly diploid and was functionally stable through extended serial subcultures (Shin et al., 1968). The major steroids synthesized by R2C are 20α-hydroxypreg-4-nen-3-one and progesterone (Shin et al., 1968). Cyclic AMP analogues could not stimulate any increase in steroid production (Shin et al., 1968). Cholera toxin increased cyclic AMP accumulation in the expected manner (Freeman, 1987a) and cycloheximide is able to inhibit steroidogenesis in a dose dependent manner (Freeman, 1987a).
3.3 Results

3.3.1 Growth rates and cell density

MA10, MLTC-1 and R2C cells were treated with 0.05% (w/v) trypsin/0.53mM EDTA for 1 min, then subcultured into 6 well culture dishes at a density of $2 \times 10^5$ cells per well in 2ml culture medium. Cells were cultured for 48h at 37°C. The cells in one 6 well culture dish were then treated with trypsin/EDTA, 6 wells combined, and cells counted with a haemocytometer. Cells were cultured over 120h and cell number determined every 24h. The results are shown in figure 3.3.1. All the cells exhibited a similar growth rate. In the exponential phase of cell growth the doubling time was approximately every 24h.

Determination of the density of the cultured Leydig cells was achieved using a 0-90% continuous percoll gradient. $50 \times 10^6$ cells were loaded onto the top of the percoll gradient. Standard density beads (1.034 - 1.096 g/ml) were also loaded to determine the density cell bands. Table 3.3.1 shows the densities obtained for MA10, MLTC-1 and R2C compared to the densities of elutriated isolated rat testis Leydig cells. Isolated rat testis Leydig cells were separated into three bands on percoll gradients. These bands were found at densities of 1.051 g/ml (40% pure), 1.064 g/ml (70% pure) and 1.076 g/ml (98% pure). MA10 cells separated into two bands 1.064 g/ml (70% total cell number) and 1.076 g/ml. MLTC-1 and R2C cells each separated as one band at 1.033 g/ml and 1.051 g/ml respectively.
FIG. 3.3.1 Growth rates for MA10, MLTC-1 and R2C cells. Cells were plated at 2x10^5/well in 6 well plates, incubated at 37°C. At the times indicated the cells were scraped from the wells in growth medium. 6 wells were combined, and the cells counted with a haemocytometer. Data is representative of 6 experiments.
<table>
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<th>Cell</th>
<th>Band</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>MLTC-1</td>
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</tr>
<tr>
<td>R2C</td>
<td>1</td>
<td>1.051</td>
</tr>
</tbody>
</table>

**TABLE 3.3.1 Density of cultured tumour Leydig cells.** MA10, MLTC-1 and R2C cells were scraped from culture flasks in 2ml of growth medium. A total of $50 \times 10^6$ cells were loaded onto the top of 0-90% continuous percoll gradients. The gradients were centrifuged at 1,500xg for 25min at room temperature. The percoll gradients also contained standard density beads (1.034 - 1.096 g/ml). The resulting cell bands were removed and the cell number counted.
LH/CG receptors are susceptible to proteolytic cleavage (for a review see Segaloff et al., 1990). To subculture tumour Leydig cells, the cells were treated for 1 min with 0.05% (w/v) trypsin/0.53mM EDTA. This procedure results in a loss of detectable $[^{125}\text{I}]-\text{hCG}$ binding sites. Figure 3.3.2 shows the recovery of LH/CG receptors. Both MA10 and R2C cells were treated for 1 min with the trypsin solution and subcultured in 24 well plates at a density of $2 \times 10^5$ cells per well. $[^{125}\text{I}]-\text{hCG}$ binding sites were then measured at the times indicated. Recovery of LH/CG receptors in R2C cells, to pretrypsinization levels, was reached by 6-12h. In MA10 cells there appeared to be a two phase recovery of pretrypsinization levels of LH/CG receptors. A 10% recovery was observed by 24h. This was followed by a rapid appearance of receptors to pretrypsinized levels between 24 and 48h. The involvement of protein synthesis in the maintenance of LH/CG receptors was also investigated (figure 3.3.3). Cycloheximide (35mM) was incubated with MA10 or R2C cells for 6h at 37°C. The cells were then placed on ice and the level of $[^{125}\text{I}]-\text{hCG}$ binding sites determined. No effect of cycloheximide was observed on R2C cells. In MA10 cells a 60% loss of receptors was observed. This indicated that there was continual synthesis of LH/CG receptors in MA10 cells.

Figure 3.3.4 shows Scatchard analysis for LH/CG receptors in MA10 (A), MLTC-1 (B), rat testis Leydig (C) and R2C (D) cells. $1 \times 10^6$ cells per well were incubated with 1.4nM $[^{125}\text{I}]-\text{hCG}$ with increasing concentrations of pure hCG (0-14nM) at 4°C overnight. Cells were then prepared for gamma-counting (2.2.10) and
FIG. 3.3.2  *Time course of the recovery of LH/CG receptors after trypsin treatment.* MA10 and R2C cells were subcultured after treatment with trypsin 0.05% (w/v) for 1 min, at a density of 2 x 10^5/well (24 well plates). The trypsinated cells were recultured in 1 ml growth medium at 37°C. At the times indicated the degree of [125I]-hCG binding was determined as described in the Materials and Methods (2.2.10). Results are the mean ± SD, n = 2, replicates = 12.
FIG. 3.3.3 The effect of cycloheximide on the level of hCG binding sites in MA10 and R2C cells. Cells were plated at a density of 2x10^5/well in 24 well plates in a volume of 1ml at 37°C and used 2 days later. Cells were incubated for 6h with 35mM cycloheximide. At the end of the total incubation time cells were placed on ice, washed, and [125I]-hCG binding determined. Results are the mean ± S.D. Statistical analysis was by the student paired t-test where n = 3, replicates = 6 (*P<0.05).
FIG. 3.3.4  Scatchard analysis of $[^{125}\text{I}]-\text{hCG}$ in MA10, MLTC-1, rat testis Leydig and R2C cells. Cells were plated at $1 \times 10^6$ cells per well in 6 well plates. Cells were incubated overnight at $4^\circ\text{C}$ with 1.4nM $[^{125}\text{I}]-\text{hCG}$ and increasing concentrations of unlabelled hCG (0-2.8nM). Non-specific binding was assessed with 14nM hCG. After the overnight incubation, cells were washed with 0.01M PBS-BSA to remove excess label and were solubilized with 0.5M NaOH. Results were interpreted using the McPherson EBDAB-ligand radioligand binding analysis program. Values are the mean ± S.D., n = 2.
A: MA10 cells

$K_D = 3.25 \pm 0.6 \text{ nM}$

$B_{\text{max}} = 19,571 \pm 2543$

Binding sites/cell

B: MLTC-1 cells

$K_D = 4.66 \pm 2.1 \text{ nM}$

$B_{\text{max}} = 16,861 \pm 3,547$

Binding sites/cell
C: Rat testis Leydig cells

\[ K_D = 2.5 \pm 0.6 \text{ nM} \]
\[ B_{\text{max}} = 30,110 \pm 4,154 \text{ Binding sites/cell} \]

D: R2C cells

\[ K_D = 2.5 \pm 1.2 \text{ nM} \]
\[ B_{\text{max}} = 12,044 \pm 3,854 \text{ Binding sites/cell} \]
the results analyzed by the EBDA/ligand computer programme. High affinity binding sites ($K_D = 2-5$ nM) were detected in all cell types. The $B_{max}$ for $[^{125}I]$-hCG binding sites varied between 12,000 - 30,000 sites/cell. Rat testis Leydig cells contained the most LH/CG receptors (30,000), whereas R2C cells had the least (12,000). Both MA10 and MLTC-1 cells had equivalent numbers of LH/CG receptors (16,000 - 19,000). Hill plot determinations (0.98-0.99) demonstrated that only one type of high affinity binding site was detected in all the cell types.

### 3.3.3  
**Stimulation of cyclic AMP and pregnenolone production.**

To investigate the regulation of cyclic AMP production, Leydig cells were stimulated for 2h with LH (0.0033 - 3.3nM), hCG (0.0028 - 2.8nM), cholera toxin (1.19 - 11.9nM) or forskolin (0.8 - 80 μM). Cyclic AMP was determined as described in Materials and Methods (2.2.15). Figure 3.3.5 to figure 3.3.8 show the results for stimulation of cyclic AMP in the absence (A) or presence (B) of 0.5mM isobutylmethyoxanthine (IBMX) in MA10 (figure 3.3.5), MLTC-1 (figure 3.3.6), R2C (figure 3.3.7) and rat testis Leydig (figure 3.3.8) cells. In MA10 (figure 3.3.5) cells all the reagents resulted in a dose dependent accumulation of cyclic AMP. LH at all the doses used was more potent than hCG. Maximal stimulation with LH (3.3nM) reached 250 pmol/10^6 cells/2h whereas hCG (2.8nM) reached 100 pmol/10^6 cells/2h. Maximal stimulation with hCG was achieved with 0.028nM. 1.19nM cholera toxin gave equivalent stimulation as LH, whereas cholera toxin (11.9nM) stimulation resulted in a one fold increase in the accumulation of cyclic AMP.
FIG. 3.3.5  The effect of LH, hCG, cholera toxin and forskolin on cyclic AMP production in MA10 cells. Cells were plated at 10⁵/well with 1ml incubation medium. Cells were incubated for 2h ± 0.5mM IBMX in the absence or presence of LH (0.0033, 0.033, 0.33, 3.3nM), hCG (0.0028, 0.028, 0.28, 2.8nM), forskolin (0.8, 8, 80μM) or cholera toxin (1.19, 11.9nM). Data represents the mean ± SD of 2 experiments.
A: Control
- cholera toxin

B: IBMX
- cholera toxin

Cyclic AMP (pmol/10^6 cells/2h)

- Forskolin
- LH
- hCG
FIG. 3.3.6  The effect of LH, hCG, cholera toxin and forskolin on cyclic AMP production in MLTC-1 cells. Cells were plated at 10^5/well with 1ml incubation medium. Cells were incubated for 2h ± 0.5mM IBMX in the absence or presence of LH (0.0033, 0.033, 0.33, 3.3nM), hCG (0.0028, 0.028, 0.28, 2.8nM), forskolin (0.8, 8, 80μM) or cholera toxin (1.19, 11.9nM). Data represents the mean ± SD of 2 experiments.

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<th>FORSKOLIN (μM)</th>
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<tr>
<td>3.3</td>
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</tr>
</tbody>
</table>
**FIG. 3.3.7** The effect of LH, hCG, cholera toxin and forskolin on cyclic AMP production in R2C cells. Cells were plated at $10^5$/well with 1ml incubation medium. Cells were incubated for 2h ± 0.5mM IBMX in the absence or presence of LH (0.0033, 0.033, 0.33, 3.3nM), hCG (0.0028, 0.028, 0.28, 2.8nM), forskolin (0.8, 8, 80μM) or cholera toxin (1.19, 11.9nM). Data represents the mean ± SD of 2 experiments.

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<table>
<thead>
<tr>
<th>LH (nM)</th>
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<td>0.28</td>
</tr>
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<td>3.3</td>
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</tr>
</tbody>
</table>
A: Control

B: IBMX

Cyclic AMP (pmol/10^6 cells/2h)
FIG. 3.3.8 The effect of LH, hCG, cholera toxin and forskolin on cyclic AMP production in rat testis Leydig cells. Cells were plated at $10^5$/well with 1ml incubation medium. Cells were incubated for 2h ± 0.5mM IBMX in the absence or presence of LH (0.0033, 0.033, 0.33, 3.3nM), hCG (0.0028, 0.028, 0.28, 2.8nM), forskolin (0.8, 8, 80μM) or cholera toxin (1.19, 11.9nM). Data represents the mean ± SD of 2 experiments.
above LH stimulated. Forskolin (0.8 - 80 μM) stimulation of cyclic AMP was similar to LH. Incubations in the presence of IBMX resulted in a one fold increase in cyclic AMP production above cells stimulated in the absence of IBMX.

In MLTC-1 (figure 3.3.6) cells all the reagents resulted in a dose dependent accumulation of cyclic AMP. Stimulation of cyclic AMP in MLTC-1 cells was 50% lower than stimulation in MA10 cells. LH and hCG at all doses were equipotent. Maximal stimulation with LH (3.3nM) and hCG (2.8nM) reached 50 pmol/10^6 cells/2h. 1.19nM and 11.9nM cholera toxin gave equivalent stimulation as LH or hCG. Forskolin (0.8 - 80 μM) stimulation of cyclic AMP was greater than LH or hCG resulting in a maximal stimulation of 150 pmol/10^6 cells/2h. Incubations in the presence of IBMX had no effect on cyclic AMP production.

In R2C (figure 3.3.7) cells all the reagents used were unable to stimulate cyclic AMP. In the presence of IBMX, however, a dose dependent stimulation of cyclic AMP could be observed. Stimulation of cyclic AMP in R2C cells, in the presence of IBMX was equivalent to those observed for MA10 cells. LH and hCG at all doses were equipotent. Maximal stimulation with LH (3.3nM) and hCG (2.8nM) reached 300 - 400 pmol/10^6 cells/2h. 1.19nM cholera toxin gave equivalent stimulation as LH or hCG, whereas cholera toxin (11.9nM) stimulation resulted in a 2 fold increase in the accumulation of cyclic AMP above LH stimulated cyclic AMP. Forskolin (0.8 - 80 μM) stimulation of cyclic AMP was greater than LH or hCG resulting in a maximal stimulation of 3000 pmol/10^6 cells/2h.

In rat testis Leydig (figure 3.3.8) cells all the reagents resulted in a dose dependent accumulation of cyclic AMP. hCG at all the doses used was more potent than LH. Maximal stimulation with hCG (2.8nM) reached 800 pmol/10^6
cells/2h whereas LH (3.3nM) reached 400 pmol/10^6 cells/2h. 1.19nM and 11.9nM cholera toxin gave equivalent stimulation as hCG. Forskolin (0.8 - 80 µM) stimulation of cyclic AMP was similar to hCG. Incubations in the presence of IBMX resulted in a one fold increase in cholera toxin stimulated cyclic AMP production and a four fold increase in forskolin stimulated cyclic AMP production. A one fold increase in LH stimulated cyclic AMP was also observed, however, there was no effect on hCG stimulated cyclic AMP.

To investigate the regulation of pregnenolone production, Leydig cells were stimulated for 2h with LH (0.0033 - 3.3nM), hCG (0.0028 - 2.8nM), db-cAMP (0.0 and 1mM), cholera toxin (1.19 - 11.9nM) and forskolin (0.8 - 80 µM). Pregnenolone was determined as described in Materials and Methods (2.2.16). Figure 3.3.9 to figure 3.3.12 show the results for stimulation of pregnenolone in the absence (A) or presence (B) of 0.5mM isobutylmethyxanthine (IBMX) in MA10 (figure 3.3.9), MLTC-1 (figure 3.3.10), R2C (figure 3.3.11) and rat testis Leydig (figure 3.3.12) cells.

In MA10 (figure 3.3.9) cells maximal stimulation of pregnenolone production was obtained with the lowest doses of cholera toxin (1.19nM), db-cAMP (0.1mM) and forskolin (0.8µM). Maximal stimulation was approximately 200ng/10^6 cells/2h. LH and hCG stimulation of pregnenolone production was in a dose dependent manner. Maximal stimulation (200 - 250 ng/10^6 cells/2h) was achieved with LH (3.3nM) and hCG (2.8nM). Incubations in the presence of IBMX resulted in a one fold increase in LH-, hCG-, and forskolin stimulated pregnenolone production. Cholera toxin stimulated pregnenolone production was inhibited.

In MLTC-1 (figure 3.3.10) cells, maximal stimulation of pregnenolone
FIG. 3.3.9  *The effect of LH, hCG, db-cAMP, cholera toxin and forskolin in MA10 cells.* MA10 cells were plated at $10^5$/well with 1ml incubation medium. The cells were preincubated for 30min with inhibitors of pregnenolone metabolism. Cells were incubated for 2h ± 0.5mM IBMX in the absence or presence of LH (0.0033, 0.033, 0.33, 3.3nM), hCG (0.0028, 0.028, 0.28, 2.8nM), db-cAMP (0.1, 1mM), forskolin (0.8, 8, 80μM) or cholera toxin (1.19, 11.9nM). Data represents the mean ± SD of 2 experiments.
FIG. 3.3.10  The effect of LH, hCG, db-cAMP, cholera toxin and forskolin in MLTC-1 cells. MLTC-1 cells were plated at $10^5$/well with 1ml incubation medium. The cells were preincubated for 30min with inhibitors of pregnenolone metabolism. Cells were incubated for 2h ± 0.5mM IBMX in the absence or presence of LH (0.0033, 0.033, 0.33, 3.3nM), hCG (0.0028, 0.028, 0.28, 2.8nM), db-cAMP (0.1, 1mM), forskolin (0.8, 8, 80μM) or cholera toxin (1.19, 11.9nM). Data represents the mean ± SD of 2 experiments.
A: Control

B: IBMX
FIG. 3.3.11  The effect of LH, hCG, db-cAMP, cholera toxin and forskolin in R2C cells. R2C cells were plated at 10^5/well with 1ml incubation media. The cells were preincubated for 30min with inhibitors of pregnenolone metabolism. Cells were incubated for 2h ± 0.5mM IBMX in the absence or presence of LH (0.0033, 0.033, 0.33, 3.3nM), hCG (0.0028, 0.028, 0.28, 2.8nM), db-cAMP (0.1, 1mM), forskolin (0.8, 8, 80μM) or cholera toxin (1.19, 11.9nM). Data represents the mean ± SD of 2 experiments.
A: Control

B: IBMX
FIG. 3.3.12 The effect of LH, hCG, db-cAMP, cholera toxin and forskolin in rat testis Leydig cells. Rat testis Leydig cells were plated at $10^5$/well with 1ml incubation media. The cells were preincubated for 30min with inhibitors of pregnenolone metabolism. Cells were incubated for 2h ± 0.5mM IBMX in the absence or presence of LH (0.0033, 0.033, 0.33, 3.3nM), hCG (0.0028, 0.028, 0.28, 2.8nM), db-cAMP (0.1, 1mM), forskolin (0.8, 8, 80μM) or cholera toxin (1.19, 11.9nM). Data represents the mean ± SD of 2 experiments.
production (200 ng/10^6 cells/2h) was obtained with the lowest doses of LH (0.0033nM), hCG (0.0028nM), cholera toxin (1.19nM), and db-cAMP (0.1mM). Forskolin demonstrated a dose dependent stimulation of pregnenolone production. Maximal db-cAMP- and forskolin-stimulation of pregnenolone production (600 ng/10^6 cells.2h) was 2 fold greater than LH or hCG. Incubations in the presence of IBMX had no effect on LH-, hCG-, or forskolin- stimulated pregnenolone production. Cholera toxin stimulated pregnenolone production was increased by 2 fold whereas db-cAMP stimulation was inhibited.

In R2C (figure 3.3.11) cells all the reagents used were unable to stimulate pregnenolone. In the presence of IBMX, however, a dose dependent stimulation of pregnenolone was observed for forskolin. There was a one fold increase in maximal forskolin stimulated pregnenolone production.

In rat testis Leydig (figure 3.3.12) cells all the reagents resulted in a dose dependent accumulation of pregnenolone. hCG and LH were equipotent in maximally stimulating pregnenolone production. Maximal stimulation with hCG (2.8nM) or LH (3.3nM) reached 150 ng/10^6 cells/2h. However, LH was more potent at lower doses, i.e. maximal stimulation was achieved with 0.033nM. Cholera toxin, db-cAMP and forskolin produced similar stimulatory profiles. Incubations in the presence of IBMX resulted in a 2 fold increase in hCG stimulated pregnenolone production and a one fold increase in cholera toxin stimulated pregnenolone production. No effect was observed on db-cAMP, forskolin or LH stimulated pregnenolone.
3.3.4  Desensitization in rat and mouse Leydig cells

Figure 3.3.13 and 3.3.14 show the effect of preincubating MA10 cells (figure 3.3.13) or mouse testis Leydig cells (figure 3.3.14) for 2h with either LH (3.3nM), db-cAMP (1mM) or PMA (10⁻⁷M) on subsequent LH-stimulated cyclic AMP (A) and pregnenolone (B) production. After preincubation, the cells were stimulated for 2h with or without LH (3.3nM). Pretreatment with LH or PMA markedly decreased the ability of LH to stimulate cyclic AMP production above basal levels (figure 3.3.13 A and 3.3.14 A). Basal pregnenolone production in cells pretreated with LH or db-cAMP, but not PMA was significantly higher than in control cells (figure 3.3.13 B and figure 3.3.14 B). Further stimulation of pregnenolone production above basal, however, was inhibited by pretreatment with LH, db-cAMP or PMA (figure 3.3.13 B and figure 3.3.14 B).

Dix et al. (1982,1987) reported that PMA, but not cyclic AMP can cause desensitization in rat Leydig cells. In figure 3.3.15 the effect of preincubating rat testis Leydig cells for 2h with either LH (3.3nM) or PMA (10⁻⁷M) on subsequent LH-stimulated cyclic AMP (A) and testosterone production (B). After preincubation, the cells were stimulated for 2h with or without LH (3.3nM). Pretreatment with LH or PMA markedly decreased the ability of LH to stimulate cyclic AMP production. Basal levels in LH (3.3nM), but not PMA (10⁻⁷M), pretreated cells were elevated. Basal testosterone (figure 3.3.15 B) production in cells pretreated with LH, but not PMA, was also significantly higher than in control cells. However there was no apparent loss of LH-stimulated testosterone production (figure 3.3.15 B).
The effect of LH, db-cAMP and PMA pretreatment on subsequent pregnenolone and cyclic AMP production in MA10 cells. MA10 cells were plated at 1.5 x10^5 cells/well in 1ml medium. The cells were preincubated for 2h in the absence or presence of LH (3.3nM), db-cAMP (1mM) or PMA (10^{-7}). Medium was removed and cells were washed with glycine-buffered-saline (pH 3.0) to remove excess hormone. Cells were then incubated for 2h in the absence or presence of 3.3nM LH. Incubations were stopped with HClO₄ and stored frozen at -20°C. Samples were thawed and neutralized with K₃PO₄ prior to radioimmunoassay of cyclic AMP (A) and pregnenolone (B). Values given are the mean, ± SD, n=2, replicates=3,*P<0.01, paired student t-test.
PREGNENOLONE (ng/10 cells/2h) CYCLIC AMP (pmol/10 cells/2h)

A: Cyclic AMP Production

CONTROL LH PMA (3.3nM) (10^{-7}M)

B: Pregnenolone Production

CONTROL LH db-cAMP PMA (3.3nM)(1mM)(10^{-7}M)
FIG. 3.3.14  The effect of LH, db-cAMP and PMA pretreatment on subsequent pregnenolone and cyclic AMP production in mouse testis Leydig cells. Mouse testis Leydig cells were plated at 1.5 x 10^5 cells/well in 1ml medium. The cells were preincubated for 2h in the absence or presence of LH (3.3nM), db-cAMP (1mM) or PMA (10^-7). Medium was removed and cells were washed with glycine-buffered-saline (pH 3.0) to remove excess hormone. Cells were then incubated for 2h in the absence or presence of 3.3nM LH. 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 cyclic AMP (A) and pregnenolone (B). Values given are the mean, ± SD, n=2, replicates=3,*P<0.01, paired student t-test.
FIG. 3.3.15  The effect of LH, db-cAMP and PMA pretreatment on subsequent testosterone and cyclic AMP production in rat testis Leydig cells. Rat testis Leydig cells were plated at 1.5 x10^5 cells/well in 1ml medium. The cells were preincubated for 2h in the absence or presence of LH (3.3nM), db-cAMP (1mM) or PMA (10^{-7}). Medium was removed and cells were washed with glycine-buffered-saline (pH 3.0) to remove excess hormone. Cells were then incubated for 2h in the absence or presence of 3.3nM LH. Incubations were stopped with HClO₄ and stored frozen at -20°C. Samples were thawed and neutralized with K₃PO₄ prior to radioimmunoassay of cyclic AMP (A) and pregnenolone (B). Values given are the mean, ± SD, n=2, replicates=3,*P<0.01, paired student t-test.
A: Cyclic AMP Production

B: Testosterone Production

CONTROL  LH  PMA
(3.3nM) (10^{-7}M)
We have shown that the MA10, MLTC-1 and R2C cells have similar growth rates. They contain high affinity LH/CG receptors. R2C cells contain a lower density of LH/CG receptors as compared to the mouse tumour Leydig cells and rat testis Leydig cells. Only high affinity binding sites were detected in all the cell types. Cycloheximide had no effect on the level of LH/CG receptors in R2C cells that had not been treated with trypsin to remove surface binding. The synthesis of LH/CG receptors in R2C cells, therefore, appears to occur after down-regulation. In these experiments down-regulation was simulated with trypsin treatment. However, in the MA10 cell continual synthesis of LH/CG receptors occurs.

The effect of IBMX on the stimulation of cyclic AMP and pregnenolone production in these Leydig cells was variable, and interpretation of the results was difficult. The results for IBMX treatment do indicate, however, that there is more phosphodiesterase activity in the rat Leydig cells. The degree of LH and hCG stimulation of cyclic AMP or pregnenolone production is different depending on the cell type used. This may be due to the differences in the affinity of LH and hCG for their receptor (Huhtaniemi and Catt, 1981; Buettner, and Ascoli, 1984). It may also indicate that other mechanisms are involved in the regulation of cyclic AMP or pregnenolone production (Rommerts and Cooke, 1988). Db-cAMP, cholera toxin and forskolin have similar effects in all the cell types. Though the accumulation of cyclic AMP was greater in MA10 cells than MLTC-1 cells, pregnenolone production was the same. The differences in the dose curves for LH or hCG
stimulated cyclic AMP and pregnenolone production for rat testis Leydig cells was not observed (Rommerts and Cooke, 1988). As reported before (Freeman, 1987a) R2C pregnenolone production could not be stimulated. Cyclic AMP production in R2C cells could only be stimulated in the presence of IBMX. This would indicate that there is a high cyclic AMP phosphodiesterase activity in these cells.

It has been reported that in mouse Leydig cells LH-induced desensitization and down-regulation are concurrent (Freeman and Ascoli, 1982). LH-induced desensitization was mimicked by forskolin and PMA (Rebois and Patel, 1985; Inoue and Rebois, 1989; Lefèvre et al., 1985). In our studies LH, PMA and db-cAMP caused desensitization in MA10 and mouse testis Leydig cells. LH-induced desensitization in rat Leydig cells is not concurrent with down-regulation. In rat Leydig cells, PMA can cause desensitization (Dix et al., 1987), but cyclic AMP does not (Dix et al., 1982). Our studies show that both LH and PMA caused desensitization of cyclic AMP production, but not testosterone production. These results indicate that there may be differences in the sites and mechanisms of phosphorylation of the LH/CG receptor in the mouse and rat Leydig cells. There were also differences in the regulation of steroidogenic enzymes.
CHAPTER 4

THE UP-REGULATION OF THE LH/CG RECEPTOR
The role of cyclic AMP in the up-regulation of LH/CG receptors was investigated in cultured tumour (MA10, MLTC-1, R2C) Leydig cells. The LH/CG receptors were quantified by measuring the binding of $^{[125]}$I-human chorionic gonadotropin (hCG). LH (0.033nM) in the presence of 1mM db-cAMP, caused a 3-8 fold increase in subsequent $^{[125]}$I-hCG binding. Db-cAMP (1mM), cholera toxin (11.9nM) and forskolin (1μM) each caused a 2-4 fold increase in binding. In the presence of translation (cycloheximide) and transcription (actinomycin-D) inhibitors there was a loss in detectable binding sites. Db-cAMP increased the rate of recovery of binding sites after trypsinization of MA10 cells with a concomitant one fold increase in the level of binding sites. Under conditions where receptor levels were increased by 3-8 fold there was also a significant increase in pregnenolone production. It was concluded that LH and cyclic AMP have positive regulatory effects on LH/CG receptors in MA10 cells by inducing the synthesis of new functional receptors. No effect was observed in MLTC-1 or R2C cells.
In this chapter the involvement of a cyclic AMP-dependent mechanism, similar to that found in granulosa cells, for the up-regulation of LH/CG receptors was investigated. The methodology for inducing receptor synthesis was developed in the MA10 cells which was then applied to the other cell lines (MLTC-1 and R2C).

The direct addition of cyclic AMP analogues or the addition of other compounds that increase cyclic AMP by activating adenylate cyclase, e.g. cholera toxin or forskolin, to granulosa cells initiated LH/CG receptor induction (Knecht and Catt, 1982). The increase in LH/CG receptors was due to an increase in the number of receptors and not to an increase in the affinity of the receptor for its ligand (May et al., 1980). The increase in number was also derived from de novo protein synthesis and not from a preformed pool of receptors (Segaloff and Limbird, 1983b).

The mechanism for homologous up-regulation of LH/CG receptors in Leydig cells is not clearly understood, but reports indicate that it might be only a transient phenomenon that precedes homologous down-regulation (Tsuruhara et al., 1977; Huhtaniemi et al., 1978; Huhtaniemi et al., 1981). The increases previously observed were due to changes in the number of receptors without changes in affinity (Tsuruhara et al., 1977; Huhtaniemi et al., 1981). Under optimal conditions, the LH/CG receptors could be up-regulated 1.5- to 2.5-fold (Tsuruhara et al., 1977; Huhtaniemi et al., 1981). The general consensus of opinion was that the found increases in LH/CG receptors in Leydig cells was due to either an
The following studies have investigated the effects of the agonists LH, hCG, cholera toxin and forskolin (all of which will increase cyclic AMP production) and the cyclic AMP analogue, dibutyryl cyclic AMP, on the levels of LH/CG receptors in cultured Leydig cells. Incubations were carried out over time periods greater than 24h. Receptor numbers were assessed using $[^{125}]$-hCG binding at 4°C.

4.3 Results

4.3.1 Initial characterization of a cyclic AMP-dependent pathway for the up-regulation of LH/CG receptors in MA10 cells.

The effect of different doses of the agonists LH, db-cAMP, cholera toxin and forskolin on the level of LH/CG receptors in MA10 cells over 24h is shown in figure 4.3.1. Incubations for 24h with 0.0033nM, 0.33nM or 3.3nM LH had no effect on LH/CG receptors. In contrast, 0.033nM LH caused a 50% decrease by 24h. 2.5mM and 5mM db-cAMP had no effect on receptor levels whereas 0.5mM and 1mM db-cAMP significantly increased the levels of binding sites. Both concentrations of cholera toxin (1.19 and 11.9nM) used had a significant effect on LH receptor levels. Only 1μM forskolin had any effect on binding sites. From these studies the concentrations for LH (0.033nM), db-cAMP (1mM), cholera toxin (11.9nM) and forskolin (1μM) were chosen.
FIG. 4.3.1 The effect of LH, db-cAMP, cholera toxin and forskolin on the level of $[^{125}\text{I}]-\text{hCG}$ binding sites in MA10 cells. Cells were plated at $2\times 10^5$/well in 12 well plates, incubated at 37°C and used 48h later. At 48h LH, db-cAMP, cholera toxin or forskolin was added at the concentrations indicated and incubated for 24h. $[^{125}\text{I}]-\text{hCG}$ binding sites were assessed as described in Materials and Methods (2.2.10). The results are the mean ± S.E.M. Statistical analysis was by the student paired t-test, $n = 3$, replicates = 6.
FIG. 4.3.2 The effect of LH, db-cAMP, cholera toxin and forskolin on the growth of MA10 cells. Cells were plated at 2×10^5/well in 12 well plates, incubated at 37°C and used 48h later. At 48h 0.033nM LH, 1mM db-cAMP, 11.9nM cholera toxin or 1µM forskolin was added and cell number determined at the times indicated. The results are the mean ± S.E.M., n = 6, replicates = 6.
To assess whether any increases in receptor levels were due to an increase in receptor protein and not due to an effect on the growth of MA10 cells, the latter were incubated with LH (0.033nM), db-cAMP (1mM), cholera toxin (11.9nM) and forskolin (1μM) for 72h (figure 4.3.2). LH had no effect on the growth of MA10 cells, whereas all the other agonists inhibited growth with db-cAMP having the greatest effect.

The process of "splitting" cells involves the use of trypsin to detach the cells from their surface support. The cells are non-responsive after this process. It is also known that LH receptors are sensitive to proteolytic cleavage. Figure 4.3.3 shows the effect of a 4 minute trypsin (0.05% w/v) treatment of MA10 cells and the time course for subsequent recovery of LH/CG receptors. Figure 4.3.3 also shows the effect of db-cAMP (1mM) treatment on the rate of recovery of LH receptors after trypsin treatment. Trypsin treatment resulted in a >98% loss of binding sites. In control cells, binding returned to pre-trypsin-treated levels by 48h. In the presence of db-cAMP, receptor levels reached a maximum by 24h, which was maintained for 72h and was one fold higher than control levels. Incubations with reagents were over long time periods, therefore it was important to investigate the basal level of binding sites in MA10 cells. The MA10 cells were therefore incubated in 1ml of growth medium over the time periods shown in figure 4.3.4 and at the times indicated the medium was removed and \(^{[125]}\)-hCG binding sites assessed. The results in figure 4.3.4 show that the levels of binding sites varied in a cycling fashion every 12h, between 15 and 100 fmol/10^6 cells.
FIG. 4.3.3  Time course of the recovery of LH/CG receptors after trypsin treatment. Cells were grown up to a density of $2 \times 10^5$/well (24 well plates) and trypsinized with 0.05% (w/v) trypsin for 4min. The trypsinized cells were recultured in 1ml growth medium at 37°C in the absence or presence of 1mM db-cAMP. At the times indicated the degree of $[^{125}\text{I}]$-hCG binding was determined as described in the Materials and Methods (2.2.10). Results are the mean ± S.D. Statistical analysis was by the student paired t-test where $n = 2$, replicates = 12 (*P<0.05).
FIG. 4.3.4  The level of $^{125}$I-hCG binding sites in MA10 cells over time. Cells were plated at a density of 1.5x10^5/well in 24 well plates and used two days later. At indicated times the cells were placed on ice, washed and $^{125}$I-hCG binding determined as described in Materials and Methods (2.2.10). Results are the mean ± S.E.M., n = 6, replicates = 6.
Comparison of the effects of LH, cholera toxin, forskolin and db-cAMP on the up-regulation of LH/CG receptors in MA10 cells.

Figures 4.3.5 and 4.3.6 show the effect on LH/CG receptor levels when MA10 cells were incubated in the presence of LH, db-cAMP, cholera toxin or forskolin. LH at all concentrations (0.033-3.3 nM) caused a significant loss of receptors (figure 4.3.5A). The degree of loss was both concentration - and time - dependent. There was also a significant recovery of binding sites, back to control levels, in the incubations containing 0.033 and 0.33 nM LH. Cholera toxin (11.9nM) caused a 4 fold increase above basal levels of binding sites by 24h. Forskolin (1μM) and db-cAMP (1mM) produced a similar profile, inducing a 3 fold increase above basal levels by 48h (figure 4.3.5B).

Figure 4.3.6 shows the effect of adding 1mM db-cAMP with LH, cholera toxin or forskolin. Db-cAMP in the presence of LH (0.033nM) induced an 8 fold increase in receptor number. The average increase in binding sites, from different experiments, of cells treated with LH (0.033nM) and db-cAMP (1mM) was 5 ± 2 fold (mean ± SD, n = 5). Db-cAMP had no effect on the loss of binding sites induced by 3.3nM LH, but increased the rate of loss of receptors with 0.33nM (figure 4.3.6A) i.e. by 48h there was a total loss in binding sites compared with only 30% loss with 0.33nM LH (figure 4.3.6A) on its own at the same time point. Incubating cells with 1mM db-cAMP and cholera toxin (figure 4.3.6B) enhanced the induced levels of binding sites from 4 to 6 fold. Db-cAMP (1mM) had no effect on forskolin-(1μM) stimulated induction of binding sites (figure 4.3.6B).
FIG. 4.3.5  Time course of the effect of LH, cholera toxin, forskolin and db-cAMP on the levels of binding sites in MA10 cells. Cells were plated at a density of $1.5 \times 10^5$/well in 24 well plates and used two days later. A) Cells were incubated at 37°C, with LH 0.033nM, 0.33nM, and 3.3nM. At indicated times the cells were place on ice, washed and $[^{125}]$-hCG binding determined. B) Cells were treated as above with 1mM db-cAMP, 11.9nM cholera toxin, and 1μM forskolin. Results are the mean ± S.E.M. Statistical analysis was by the student paired t-test where $n = 3$, replicates = 6 (*P<0.05).
FIG. 4.3.6  *Time course of the effect of 1mM db-cAMP with LH, cholera toxin or forskolin on the levels of binding sites in MA10 cells.* Cells treated as in Fig. 4.3.5 in the presence of 1mM db-cAMP with A) varying LH concentration 0.033nM, 0.33nM, 3.3nM, and B) 11.9nM cholera toxin and 1μM forskolin. Results are the mean ± S.E.M. Statistical analysis was by the student paired t-test where n = 3, replicates = 6 (*P<0.05).
FIG. 4.3.7  *Scatchard analysis of LH and db-cAMP treatment on MA10 cells.*

MA10 cells were plated at 1×10^6 cells per well in 6 well plates and used 2 days latter. Cells were incubated in the absence (A) or presence (B) of LH (0.033nM) plus db-cAMP (1mM) for 24h at 37°C. Cells were then placed on ice and washed with 50mM glycine buffered saline (pH 3.0) for 2min. The cells were then incubated in 1ml of medium in the presence of 1.4nM[^125I]-hCG and increasing concentration of unlabelled hCG (0-2.8nM) overnight at 4°C. Non-specific binding was assessed with 14nM hCG. After the overnight incubation, cells were washed with 0.01M PBS-BSA to remove excess label and were solubilized with 0.5M NaOH. Results were interpreted using the McPherson EBDA-ligand radioligand binding analysis program. Values are the mean ± S.D., n = 2.
A: Control cell binding

K_D = 3.25 ± 0.6 nM

B_max = 19,571 ± 2543

Binding sites/cell

B: LH (0.033nM) + db-cAMP (1mM) for 24h

K_D = 6.6 ± 2.3 nM

B_max = 210,770 ± 30,436

Binding sites/cell
Scatchard analysis of the up-regulation of LH/CG receptors in MA10 cells is shown in figure 4.3.7. Cells were incubated for 24h in the absence or presence of LH (0.033nM) and db-cAMP (1mM). The cells were then washed with 50mM glycine-buffered-saline (pH 3.0) and incubated overnight at 4°C with 1.4nM [125I]-hCG and unlabelled hCG (0-14 nM). Control cell binding gave a dissociation constant of 3.25 ± 0.6 nM and a Bmax of 19,571 ± 2,543 binding sites per cell. Cells treated with LH and db-cAMP had a similar dissociation constant (6.6 ± 2.3 nM) but the Bmax had increased to 210,770 ± 30,436 binding sites per cell.

The role of serum was also investigated in the up-regulation of LH receptors (figure 4.3.8). MA10 cells were incubated for 24h in the presence or absence of 15% serum. Horse serum was replaced with 0.1% (w/v) BSA, 4mg/l insulin and 10mg/l transferrin. Under these conditions the up-regulation of receptors by db-cAMP, hCG and LH was also observed. In control cells the level of binding sites detected was decreased by 70% in horse serum free medium. Incubation with 1mM db-cAMP showed no increase in binding sites in horse serum medium, but prevented the loss of binding sites in horse serum free conditions. Both LH (0.033nM) and hCG (0.028nM) caused a loss in binding sites. The increases caused by LH and db-cAMP or hCG and db-cAMP in both horse serum and horse serum free conditions were equivalent compared to control binding levels.
FIG. 4.3.8 The effect of horse serum on the level of LH/CG receptors in MA10 cells. MA10 cells were plated at 2x10^5/well in 24 well plates, incubated at 37°C in horse serum medium and used 48h later. The cells were then recultured in the presence of either horse serum medium or horse serum free medium. The cells were also incubated in the presence or absence of db-cAMP (1mM), LH (0.033nM), hCG (0.028nM), LH (0.033nM) + db-cAMP (1mM) or hCG (0.028nM) + db-cAMP (1mM). At the end of 24h the cells were placed on ice, washed with 50mM glycine buffered saline (pH 3.0) and [125I]-hCG assessed as described in Materials and Methods (2.2.10). The results are the mean ± S.E.M. Statistical analysis was by the student paired t-test, n = 3, replicates = 6 (*P<0.05, **P<0.01).
The effect of transcription and translation inhibitors on the up-regulation of LH/CG receptors.

In order to determine the mechanism involved in the observed increase in receptors, the effects of transcription and translation inhibitors on binding sites and protein synthesis were investigated. Binding studies and protein synthesis studies were carried out in parallel on the same population of cells. Cells were preincubated for 14h in the absence or presence of LH (0.033nM) and db-cAMP (1mM) and then further incubated for 6h after the addition of cycloheximide (3.5 and 35 mM) or actinomycin D (3.98 mM). The incorporation of labelled amino acid into protein is shown in figure 4.3.9. Cycloheximide inhibited labelled amino acid incorporation up to 80% and actinomycin D inhibited by 40%. Labelled amino acid incorporation into protein was increased during the final 6h of the incubation when LH and db-cAMP were present together. When LH was removed for the last 6h, i.e. cells incubated in the presence of db-cAMP only, this was not detectable. The effects of cycloheximide and actinomycin D on receptor levels in MA10 cells during the 6h incubation period are shown in figure 4.3.10. Both inhibitors caused a loss of binding sites in control cells. In cells that had been pretreated for 14h with LH and db-cAMP, and then for 6h in the presence of inhibitors, a 50% decrease in the levels of induced binding sites was observed. At the concentrations of inhibitors used there was only a 5% decrease in the viability of the cells, which would not account for the observed losses of binding sites.
FIG. 4.3.9  The effect of translation and transcription inhibitors on the level of protein synthesis in MA10 cells. MA10 cells were plated at 2x10^6 cells per well in 6 well plates and used 2 days latter. Cells were incubated for 14h in methionine free medium (DMEM-F12) containing 15% (v/v) horse serum in the absence or presence of LH (0.033nM) + db-cAMP (1mM). The cells were then washed twice with methionine free medium and recultured in methionine free medium/15% horse serum either in the absense or presence of LH (0.033nM) + db-cAMP (1mM) or db-cAMP (1mM). The incubations also included ^3H-metionine (5μCi) for 6 hours. The inhibitors actinomycin-D (3.98mM) and cycloheximide (3.5mM and 35mM) were also added during the final 6h of the incubation. At the end of the incubations cells were washed twice with medium containg 10mM methionine. Determination of the level of incorporation of radiolabelled methionine was as described in Materials and Methods (2.2.16). Results are the mean ± S.D. Statistical analysis was by the student paired t-test where n = 4, replicates = 6 (*P<0.05).
FIG. 4.3.10 The effect of translation and transcription inhibitors on the level of \([^{125}\text{I}]\text{-hCG}\) binding sites in MA10 cells. Cells were plated at a density of 1.5x10^5/well in 24 well plates in a volume of 1ml at 37°C and used 2 days later. Cells were incubated for 14h in absence or presence of LH (0.033nM) + db-cAMP (1mM). At the end of 14h the inhibitors actinomycin-D (3.98mM) and cycloheximide (3.5mM and 35mM) were added, and incubated for a further 6h. At the end of the total incubation time cells were placed on ice, washed, and \([^{125}\text{I}]\text{-hCG}\) binding was determined. Results are the mean ± S.D. Statistical analysis was by the student paired t-test where n = 2, replicates = 6 (*P<0.05, **P<0.01).
FIG. 4.3.11 Pregnenolone production by cells pretreated for 24h to induce LH/CG receptors. Cells were plated at a density of 1.5x10^5/well and used 2 days later. The cells were preincubated with LH (0.033nM), db-cAMP (1mM) or LH + db-cAMP for 24h. At the end of this incubation period the cells were washed 2 times with 50mM glycine buffered saline (pH 3.0) and 4 times with medium and recultured with 1ml of fresh growth medium. Non-pretreated cells were also given an acid wash or not treated to act as controls. Cells were then further incubated for 30min with inhibitors of pregnenolone metabolism before being incubated for 2h ± LH (3.3nM). The reaction was stopped using perchloric acid. Pregnenolone was determined as described in Materials and Methods (2.2.18). Results are the mean ± S.E.M. Statistical analysis was by the paired student t-test, n = 3, replicates = 6 (*P<0.01).
4.3.4  
*Cyclic AMP and pregnenolone production in LH/CG receptor up-regulated cells.*

The effect of preincubating MA10 cells for 24h with LH (0.033nM) and/or db-cAMP (1mM) on the subsequent LH-stimulated pregnenolone production of these cells when incubated with 3.3nM LH is shown in figure 4.3.11. The results show that in cells treated with db-cAMP alone or with LH there was a marked increase in pregnenolone production both in the presence and the absence of LH in the second incubation (figure 4.3.11). These increases correlated with the increases in receptor numbers (figures 4.3.5B and 4.3.6A). In order to remove residual bound LH the cells were acid-washed before the second incubation; this was found to decrease pregnenolone production in control cells by 50% (figure 4.11).

Table 4.3.1 shows the production of cyclic AMP in pretreated cells on stimulation with LH (3.3nM). The washing procedure used appears to have inhibited the production of cyclic AMP, though there is still a 50% maximal stimulated production of pregnenolone observed in these cells. Scatchard analysis of the binding sites in cells that have been acid-washed is shown in figure 4.3.12. No change in the number of binding sites or dissociation constant was observed in these cells.
TABLE 4.3.1  Cyclic AMP production by cells pretreated for 24h to induce LH/CG receptors. Cells were plated at a density of 1.5x10^5/well and used 2 days later. The cells were preincubated in the absence or presence of LH (0.033nM) for 24h. At the end of this incubation period the cells were washed 2 times with 50mM glycine buffered saline (pH 3.0) and 4 times with media and recultured with 1ml of fresh growth media. Non-pretreated cells were also given an acid wash or not treated to act as controls. Cells were then further incubated for 2h ± LH (3.3nM). The reaction was stopped using perchloric acid. Cyclic AMP was determined as described in the Materials and Methods (2.2.17). Results are the mean ± S.E.M., n = 3, replicates = 6.
FIG. 4.3.12  *Scatchard analysis of the acid washing treatment on MA10 cells.*

MA10 cells were plated at 1x10⁶ cells per well in 6 well plates and used 2 days latter. Cells were then placed on ice and either washed with 50mM glycine buffered saline (pH 3.0) for 2 min or not treated. The cells were then incubated in 1ml of medium in the presence of 1.4nM [¹²⁵I]-hCG and increasing concentration of unlabelled hCG (0-2.8nM) overnight at 4°C. Non-specific binding was assessed with 14nM hCG. After the overnight incubation, cells were washed with 0.01M PBS containing 0.1% (w/v) BSA to remove excess label and were solubilized with 0.5M NaOH. Results were interpreted using the McPherson EBDA-ligand radioligand binding analysis program. Values are the mean ± S.D., n = 2.
A: Control cell binding

$K_D = 1.8 \pm 0.06 \text{ nM}\\
B_{\text{max}} = 22,258 \pm 165 \text{ Binding sites/cell}$

B: Acid wash treated cells

$K_D = 1.1 \pm 0.1 \text{ nM}\\
B_{\text{max}} = 21,185 \pm 4,938 \text{ Binding sites/cell}$
FIG. 4.3.13  The effect of LH and db-cAMP on the level of LH/CG receptors in MA10, MLTC-1 and R2C cells. Cells were plated at 2x10^2/well in 24 well plates, incubated at 37°C and used 48h later. The cells were then incubated in the presence or absence of db-cAMP (1mM), LH (0.033nM), LH (0.033nM) + db-cAMP (1mM). At the end of 24h the cells were placed on ice, washed with 50mM glycine buffered saline (pH 3.0) and [^{125}I]-hCG assessed as described in Materials and Methods (2.2.10). The results are the mean ± S.E.M., n = 3, replicates = 6.
Comparison of the effects of LH and db-cAMP on the up-regulation of LH/CX receptors in MLTC-1 and R2C cultured tumour cells.

MA10, MLTC-1 and R2C cells were incubated for 24h in the presence of either LH (0.033nM), db-cAMP (1mM) or LH and db-cAMP. Binding sites were assessed after this time period. It was found, as previous results have shown, that LH and db-cAMP caused an increase in the level of binding sites in MA10 cells. No effect was seen on the level of binding sites in MLTC-1 or R2C cells.

Discussion

Data is presented for the positive modulation of the synthesis of LH/CX receptors by LH and cyclic AMP in cultured mouse tumour (MA10) Leydig cells. The up-regulation of LH/CX receptors in rat testis Leydig cells has previously been shown to occur over a short period of time and to be due to the presence of cryptic receptors on the plasma membrane (Huhtaniemi et al., 1981; Huhtaniemi and Catt, 1981). LH in our studies did not exhibit this short term effect, and with all concentrations of LH used, it eventually resulted in down - regulation. This was similar to the rat testis Leydig cells where it was established that LH - induced desensitization and eventually down regulation, regulates the LH/CX receptor (Rommerts and Cooke, 1988). These events are not thought to be mediated by cyclic AMP but probably involve LH-regulated protein kinase C (PKC) (Dix et al., 1987).
Cyclic AMP in the present experiments prevented the loss of LH/CG receptors in serum free conditions and in the presence of low doses of LH increased the levels of binding sites. This may explain the recovery of binding sites observed after down-regulation with 0.033nM LH (48h) and 0.33nM LH (24h). Generation of cyclic AMP by activation of the GTP binding protein (G_s) by cholera toxin and direct stimulation of adenylate cyclase by forskolin also resulted in an increase in binding sites.

LH and cyclic AMP have previously been shown to induce the synthesis of mitochondrial proteins involved in steroid synthesis in MA10 cells, but it was found that the overall protein synthesis capacity was not affected during a 6h incubation period (Stocco and Kilgore, 1988). In our study, preincubation with LH and cyclic AMP for 14h resulted in a detectable increase in total protein synthesis, during a further 6h incubation. The constant presence of LH was required for this increase to occur. The effect of the translation and transcription inhibitors on the levels of LH/CG receptors indicate that for the maintenance of receptors, continual protein synthesis is required over a 6-12h period. The initiation of this process may be derived at the gene level, as was demonstrated in porcine granulosa cells (Segaloff and Limbird, 1983a; Segaloff and Limbird, 1983b), and not from a pool of mRNA. The stimulatory effect of cyclic AMP on the induction of binding sites was also shown in cells which had been trypsinized; cyclic AMP increased the rate of recovery of binding sites in these cells, as well as raising the number of detectable binding sites.

The results for the inhibition of MA10 cell growth rates by cyclic AMP are consistent with observations found in other cell systems, where cyclic AMP either
stimulates (Dumont et al., 1989) or inhibits (Pastan et al., 1975) growth.

The results obtained by pretreating the cells for 24h with LH and db-cAMP and then stimulating with LH (3.3nM) show that there is an increase in functionally coupled LH/CG receptors. The results also indicated that there was no detectable production of cyclic AMP in cells that have been acid washed. The maximal pregnenolone production in control cells could therefore be the result of two pathways acting together; one of these pathways may not be cyclic AMP - dependent. Previously it was shown that intracellular levels of cyclic AMP could not be restimulated after exposure to 1.12nM hCG for 15, 30 or 120 min. using an acid wash to remove the hCG (Segaloff and Ascoli, 1981).

The lack of effect in the MLTC-1 and R2C cells may be due to differences in the rate of synthesis of the LH receptor, as was shown in chapter 3 for the R2C cells.

To conclude, the results of the present study support the concept of a role for cyclic AMP in the maintenance and regulation of LH/CG receptors in MA10 cells. These cells were similar to granulosa cells in that factors acting via cyclic AMP maintain the LH/CG receptor. When cyclic AMP is added in the presence of low doses of LH (<0.033nM), up-regulation occurs which is probably due to an increased rate of synthesis of the LH/CG receptor.
CHAPTER 5

MECHANISMS INVOLVED IN LH/CG RECEPTOR DOWN-REGULATION
5.1 Summary

Down-regulation of LH/CG receptors was investigated in two types of mouse tumour Leydig cells (MA10 and MLTC-1), rat testis Leydig cells and a rat tumour Leydig cell (R2C). Receptor numbers were measured by binding $[^{125}\text{I}]$-hCG to the cells cultured in monolayers. Addition of 3.3nM LH or 2.8nM hCG for 2h at 37°C had no detectable effect on binding sites in rat testis Leydig cells or R2C cells, but in MA10 and MLTC-1 cells it caused a 50% loss in binding sites. The effect on MA10 and MLTC-1 cells could be mimicked by inhibiting receptor internalization with 5mM NaN$_3$. Dibutyryl-cyclic AMP (0.01, 0.1 and 1mM) caused a 30-50% loss of $[^{125}\text{I}]$-hCG binding sites and an inhibition of receptor-$[^{125}\text{I}]$-hCG complex internalization in mouse tumour Leydig (MA10, MLTC-1) cells during 2 hours. The effect of db-cAMP on the level of LH/CG receptors was inhibited by cycloheximide. In contrast, db-cAMP had no effect on the level of binding sites or internalization of the hormone receptor complex in rat testis Leydig cells or R2C Leydig cell. Phorbol 12-myristate-13-acetate at concentrations from $10^{-9}$ to $10^{-5}$M had no effect on hormone binding or hormone-receptor complex internalization in any of the Leydig cells. These results indicated that LH/CG receptor down-regulation was mediated by cyclic AMP dependent kinases, but not protein kinase C, in mouse Leydig cells. No down-regulation of rat Leydig cell LH/CG receptor occurred with either of these kinases.
LH and hCG interact with a specific cell surface receptor and like other receptors for polypeptide hormones, exhibits ligand-induced loss or down-regulation of cell surface LH/Cg binding capacity (Freeman and Ascoli, 1982). This negative modulation of receptor levels *in vitro* is time and hormone concentration dependent (Freeman and Ascoli, 1982). Decreased binding capacity is the result of a lowered LH/Cg receptor level rather than an altered affinity of the receptor for the ligand (Freeman and Ascoli, 1982).

It is well established that binding of several protein ligands to specific cell-surface receptors is followed by the internalization and/or degradation of the bound protein (Segaloff and Ascoli, 1988). LH/Cg is taken up by receptor-mediated endocytosis, and the hormone-receptor complex is transported to the lysosomes (Ascoli, 1984), and after dissociation of this complex (Ascoli, 1984) the hormone is degraded (Ascoli, and Segaloff, 1987). In some cases, recycling of the intact LH/Cg receptors has been demonstrated (Habberfield *et al.*, 1986; Genty *et al.*, 1987). In other cases of polypeptide hormones, at least part of the hormone-receptor complexes may be recycled to the plasma membrane.

In this chapter the role of internalization in the mechanism of regulating LH/Cg receptors was investigated. Also studied were the possible effects of protein kinase A and C on the loss of LH/Cg receptors and internalization of these receptors.
5.3 Results

5.3.1 The effect of inhibition of receptor internalization on the levels of LH/CG receptors.

Figure 5.3.1 is a demonstration of the effect of LH (0.33 and 3.3nM) or hCG (0.28 and 2.8nM), over a 4h incubation period, on the levels of $^{125}$I-hCG binding sites in MA10 cells. At the times indicated the cells were placed on ice and washed with 50mM glycine buffered saline (pH 3.0) (Ascoli, 1982) - to remove residual hormone bound - and twice with 0.1M PBS. LH (0.33nM) and hCG (0.28nM) had no effect on the levels of binding sites. LH (3.3nM) and hCG (2.8nM) caused a 50% loss of binding sites by 60 min. No further loss occurred with LH treatment, however, there was further loss with hCG (2.8nM) treatment. hCG (2.8nM) caused an 80% loss by 120 min.

The loss of LH/CG receptors in MA10 cells was previously reported to be due to internalization and degradation of the receptor (Lloyd and Ascoli, 1983). Table 5.3.1 shows the radioactivity associated with the surface membrane of MA10 cells and internalized levels of radioactivity, in a steady state reaction over 4h, using $^{125}$I-hCG at the concentrations of 0.28nM and 2.8nM. At the times indicated the cells were placed on ice and washed twice with 0.1M PBS. Surface levels of radioactivity were assessed by washing the cells twice with 50mM glycine buffered saline (pH 3.0) for 2min and combining the two washes. Internalized levels were assessed by dissolving the cells with 0.5M NaOH. Addition of 2.8nM $^{125}$I-hCG saturated all the available binding sites by 30 min (approx. 15,000
FIG. 5.31  The time course of the effect of LH and hCG on the levels of LH/CG receptors in MA10 cells. MA10 cells were plated at a concentration of 2x10^5/well in 24 well plates. Cells were incubated in 1ml growth medium at 37°C for the times indicated in the presence of LH (0.33nM and 3.3nM) (A) or hCG (0.28nM and 2.8nM) (B). Cells were placed on ice washed with 50mM glycine buffered saline (pH 3.0) for 2 min, then twice with 0.1M PBS. The level of [125I]-hCG binding site was assessed as described in Materials and Methods (2.2.10). Values are the mean ± SD, n = 2, replicates = 3.
TABLE 5.31  The effect of 0.28nM and 2.8nM $^{125}$I-hCG on the steady state levels of LH/CG receptors in MA10 cells. Cells were plated at 2x10^6/well in 6 well plates. Two days later the cells were incubated at 37°C in 1ml fresh growth medium with 0.28nM or 2.8nM $^{125}$I-hCG. The cells were incubated for the times indicated and then placed on ice, medium removed and washed with 0.01M PBS-BSA. The surface bound $^{125}$I-hCG was determined by washing the cells twice with 50mM glycine-buffered saline (pH 3) (Ascoli, 1982) and counting the combined washings. To measure the amount of internalized hormone, cells were dissolved in 1 ml of 0.5M NaOH, and counted in a gamma-counter. Values represent the mean ± SD, n=2, replicates=2.
<table>
<thead>
<tr>
<th>[(^{125}\text{I})]-hCG (nM)</th>
<th>Radioactivity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min.</td>
</tr>
<tr>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>Surface</td>
<td>288 ± 27</td>
</tr>
<tr>
<td>Internalized</td>
<td>252 ± 59</td>
</tr>
<tr>
<td>Total</td>
<td>480 ± 86</td>
</tr>
<tr>
<td>2.8</td>
<td></td>
</tr>
<tr>
<td>Surface</td>
<td>2687 ± 1081</td>
</tr>
<tr>
<td>Internalized</td>
<td>445 ± 120</td>
</tr>
<tr>
<td>Total</td>
<td>3132 ± 1201</td>
</tr>
</tbody>
</table>
sites/cell), whereas 0.28nM $^{125}$I-hCG had only occupied 15% of the binding sites. Distribution between internalized levels and surface bound levels of radioactivity for 0.28nM $^{125}$I-hCG remained constant over 4h, with approximately 50% of the radioactivity detected in both. Total radioactivity detected was constantly at 15% over the 4h. Internalized levels of radioactivity for 2.8nM $^{125}$I-hCG were constant over the 4h. However, the level of surface associated radioactivity decreased by 65% over the 4h and total detected radioactivity decreased by 50%. Internalized levels of radioactivity for 0.28nM $^{125}$I-hCG were 65% of the levels detected for 2.8nM $^{125}$I-hCG.

The effect of inhibiting internalization of the LH/CG receptor with energy depleting agents was also investigated. Figure 5.3.2 shows the internalization profile obtained for $^{125}$I-hCG in the presence of 5mM NaN$_3$ in MA10 cells. In control cells there was a loss of surface bound radioactivity, which was paralleled by an equivalent increase in internalized levels. There was also a continual loss of surface bound radioactivity in the presence of NaN$_3$, however, internalized levels were decreased. This indicated that internalization of hormone was inhibited, however there was still a loss in surface bound levels. The effect of 5mM NaN$_3$ on surface levels of $^{125}$I-hCG binding sites was therefore observed in different Leydig cell types (figure 5.3.3). In order to desensitize the MA10, MLTC-1, R2C and rat testis Leydig cells with respect to steroid production (i.e. uncouple the receptor from its transducing system), the cells were incubated for 2h with LH (3.3nM) (Freeman and Ascoli, 1982; Dix et al., 1982). In addition, cells were incubated with NaN$_3$ (5mM) (with and without LH). Incubating the cells with NaN$_3$ (5mM) caused a small decrease in the viability of the cells from 99% to 86% and
FIG. 5.3.2  *The effect of sodium azide on the internalization of $[^{125}\text{I}]-\text{hCG}$ in MA10 cells.* Cells were plated at 2x10^6/well in 6 well plates. Two days later the cells were incubated at 10°C, to inhibit internalization, in 1ml fresh growth medium with 1.4nM $[^{125}\text{I}]-\text{hCG}$ alone or with 5mM NaN$_3$ for 1h. The cells were then placed on ice and washed with 0.01M PBS-BSA to remove unbound hormone. The cells were then recultured in 1ml fresh growth medium at 37°C with the same additions as before, but excluding the $[^{125}\text{I}]-\text{hCG}$. At the times indicated the plates were placed on ice, medium removed and washed with 0.01M PBS-BSA. The surface bound $[^{125}\text{I}]-\text{hCG}$ (A) was determined by washing the cells twice with 50mM glycine-buffered saline (pH 3.0) and counting the combined washings. To measure the amount of internalized hormone (B), cells were dissolved in 1 ml of 0.5M NaOH, and counted in a gamma-counter. Values represent the mean ± SD, n=2, replicates=2, *P<0.05 -student t-test.
O Control
● 5mM NaN₃

A: Surface

% of Initial CPM Bound

Time (h)

B: Internalized

% of Initial CPM Bound

Time (h)
FIG 5.3.3  Regulation of [125I]-hCG binding sites: The effect of inhibiting receptor internalization. MA10 and MLTC-1 cells (figure 5.3.3 A), R2C and rat testis Leydig (figure 5.3.3 B) cells were plated at 1.5x10^5 in 1ml of medium. The cells were incubated either without hormone; with 5mM NaN_3; with 3.3nM LH; or with 3.3nM LH + 5mM NaN_3. After 2h the cells were placed on ice and washed twice with 50mM glycine-buffered saline (pH 3.0) before [125I]-hCG binding was determined. Values given are the mean, ± S.E.M., replicates = 6, n = 2, *P<0.05 - student t-test. Due to the variance in control binding of cultured Leydig cells (Chapter 4) only experiments where control binding was approximately the same have been represented. Experiments were repeated four times with similar results for the MA10, MLTC-1 and rat testis Leydig cells and twice for the R2C cells.
would, therefore, have no deleterious effect on the detection of $[^{125}\text{I}]-\text{hCG}$ binding sites. These cells were discounted from the total number of cells, before determining the number of binding sites. Cell surface bound hormone was removed with 50mM glycine-buffered saline (pH 3.0) and the cells washed before $[^{125}\text{I}]-\text{hCG}$ was added and incubations carried out at 4°C, to determine the residual numbers of binding sites and dissociation constants ($K_D$).

A 2h incubation of MA10 and MLTC-1 cells (figure 5.3.3 A) in the presence of LH (3.3nM) caused a 50% loss in binding of $[^{125}\text{I}]-\text{hCG}$. The loss in binding caused by LH was mimicked when NaN$_3$ (5mM) was present either with or without LH. The loss of binding that occurred with NaN$_3$ treatment alone in the MA10 and MLTC-1 cells indicated that in the mouse tumour cells loss of receptors had occurred at the cell surface rather than by internalization, since NaN$_3$ inhibits internalization.

Similar experiments were also carried out on the rat Leydig cells. A 2h incubation of rat testis Leydig and R2C cells (figure 5.3.3 B) with either NaN$_3$ or LH did not cause a loss in binding sites.

To investigate whether the effects on binding were due to a change in the affinity of the LH/CG receptor, Scatchard analysis was carried out on cells treated as above. The effect of NaN$_3$ (5mM) and LH (3.3nM) treatment on the $K_D$ for $[^{125}\text{I}]-\text{hCG}$ in the MA10 and rat testis Leydig cells are shown in figure 5.3.4 and 5.3.5. In MA10 cells (figure 5.3.4) LH (3.3nM) and NaN$_3$ (5mM) caused equivalent losses in the Bmax obtained for $[^{125}\text{I}]-\text{hCG}$ binding sites, however, there was no effect on the $K_D$. In rat Leydig cells (figure 5.3.5) LH (3.3nM) and NaN$_3$ (5mM) had no effect on the Bmax obtained for $[^{125}\text{I}]-\text{hCG}$ binding sites, however, there was
FIG. 5.3.4  Scatchard analysis of the effect of LH and sodium azide on MA10 cells. MA10 cells were plated at 1x10^6 cells per well in 6 well plates and used 2 days later. Cells were incubated in the absence (A) or presence of 3.3nM LH (B) 5mM NaN₃ (C) for 2h at 37°C. The cells were then washed with 50mM glycine buffered saline (pH 3.0) and twice with 0.1M PBS before incubating overnight at 4°C with 1.4nM [¹²⁵I]-hCG and increasing concentrations of unlabelled hCG (0-2.8nM). Non-specific binding was assessed with 14nM hCG. After the overnight incubation, cells were washed with 0.01M PBS-BSA to remove excess label and were solubilized with 0.5M NaOH. Results were interpreted using the McPherson EBDA-ligand radioligand binding analysis program. Values are the mean ± S.D., n = 2.

A: MA10 control binding

\[ K_D = 4.53 \pm 2.2 \text{ nM} \]
\[ B_{\text{max}} = 21,068 \pm 770 \text{ Binding sites/cell} \]
B: 3.3nM LH treatment on MA10 cells

\[ K_D = 4.84 \pm 1.21 \text{ nM} \]
\[ B_{\text{max}} = 5,694 \pm 1,607 \text{ Binding sites/cell} \]

C: 5mM NaN₃ treatment on MA10 cells

\[ K_D = 3.07 \pm 0.56 \text{nM} \]
\[ B_{\text{max}} = 4,510 \pm 1,547 \text{ Binding sites/cell} \]
FIG. 5.3.5 *Scatchard analysis of the effect of LH and sodium azide on rat testis Leydig cells.* Rat testis Leydig cells were plated at $1 \times 10^6$ cells per well in 6 well. After a 2h preincubation the cells were incubated in the absence (A) or presence of 3.3nM LH (B) 5mM NaN$_3$ (C) for 2h at 37°C. The cells were then washed with 50mM glycine buffered saline (pH 3.0) and twice with 0.1M PBS before incubating overnight at 4°C with 1.4nM $[^{125}]$-hCG and increasing concentrations of unlabelled hCG (0-2.8nM). Non-specific binding was assessed with 14nM hCG. After the overnight incubation, cells were washed with 0.01M PBS-BSA to remove excess label and were solubilized with 0.5M NaOH. Results were interpreted using the McPherson EBDA-ligand radioligand binding analysis program. Values are the mean ± S.D., n = 2.

\[ K_D = 2.33 \pm 1.9 \text{ nM} \]
\[ B_{\text{max}} = 30,531 \pm 2,604 \text{ Binding sites/cell} \]
B: 3.3nM LH treatment on rat testis Leydig cells

\[ K_D = 0.37 \pm 0.42 \text{ nM} \]
\[ B_{\text{max}} = 27,701 \pm 3,869 \text{ Binding sites/cell} \]

C: 5mM NaN₃ treatment on rat testis Leydig cells

\[ K_D = 0.63 \pm 0.33 \text{ nM} \]
\[ B_{\text{max}} = 30,110 \pm 4,253 \text{ Binding sites/cell} \]
FIG. 5.3.6  *The effect of db-cAMP on the levels of $[^{125}]$-hCG binding sites in* Leydig cells. Leydig cells were plated at 1.5 x 10$^5$ cells/well in 1ml medium. The cells were incubated for 2h in the absence or presence of 0.01mM, 0.1mM, 1mM db-cAMP or 3.3nM LH. At the times indicated cells were placed on ice and washed twice with 0.01M PBS-BSA before $[^{125}]$-hCG binding was determined as described in Materials and Methods (2.2.10). Values given are the mean, ± SD, n=2, replicates=6, *P<0.05.
TABLE 5.3.2  The effect of PMA on the levels of \([^{125}\text{I]}-\text{hCG}\) binding sites in Leydig cells. Leydig cells were plated at 1.5x10^5 cells/well in 1ml growth medium. The cells were incubated for 2h with PMA at concentrations from 10^{-9} to 10^{-5} M (initially dissolved in DMSO at 10^{-2} M and diluted to the concentrations required with medium). After 2h the cells were placed on ice and washed twice with 0.01M PBS-BSA before \([^{125}\text{I]}-\text{hCG}\) binding was determined as described in Materials and Methods (2.2.10). Values given are the mean, ± SD, n = 2, replicates = 6.
<table>
<thead>
<tr>
<th>PMA (M)</th>
<th>R2C</th>
<th>MLTC-1</th>
<th>Rat testis</th>
<th>MA10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.7 ± 2.11</td>
<td>19.5 ± 1.2</td>
<td>26.7 ± 1.97</td>
<td>21.2 ± 4.9</td>
</tr>
<tr>
<td>10-9</td>
<td>7.9 ± 2.1</td>
<td>20.8 ± 4.2</td>
<td>26.0 ± 2.8</td>
<td>17.8 ± 4.2</td>
</tr>
<tr>
<td>10-8</td>
<td>6.3 ± 1.3</td>
<td>16.2 ± 3.2</td>
<td>28.0 ± 2.4</td>
<td>17.0 ± 7.2</td>
</tr>
<tr>
<td>10-7</td>
<td>6.6 ± 2.2</td>
<td>18.6 ± 3.3</td>
<td>27.1 ± 3.3</td>
<td>28.4 ± 9.1</td>
</tr>
<tr>
<td>10-6</td>
<td>12.6 ± 0.6</td>
<td>20.8 ± 4.2</td>
<td>20.3 ± 3.6</td>
<td>17.0 ± 5.6</td>
</tr>
<tr>
<td>10-5</td>
<td>9.2 ± 1.3</td>
<td>16.7 ± 6.1</td>
<td>23.9 ± 2.55</td>
<td>23.9 ± 7.6</td>
</tr>
</tbody>
</table>
FIG. 5.3.7  Scatchard analysis of the effect of db-cAMP and PMA on MA10 cells. MA10 cells were plated at 1x10^6 cells per well in 6 well plates and used 2 days latter. Cells were incubated in the absence (A) or presence of 1mM db-cAMP (B) 10^-7M PMA (C) for 2h at 37°C. The cells were then washed with 50mM glycine buffered saline (pH 3.0) and twice with 0.1M PBS before incubating overnight at 4°C with 1.4nM [125I]-hCG and increasing concentrations of unlabelled hCG (0-2.8nM). Non-specific binding was assessed with 14nM hCG. After the overnight incubation, cells were washed with 0.01M PBS-BSA to remove excess label and were solubilized with 0.5M NaOH. Results were interpreted using the McPherson EBDA-ligand radioligand binding analysis program. Values are the mean ± S.D., n = 2.

A: MA10 control binding

\[ K_D = 4.53 \pm 2.2 \text{ nM} \]

\[ B_{\text{max}} = 21,068 \pm 770 \text{ Binding sites/cell} \]
B: Cyclic AMP (1mM) treated MA10 cells

\begin{align*}
K_D &= 2.13 \pm 0.55 \text{ nM} \\
B_{\text{max}} &= 13,850 \pm 3,842 \\
\text{Binding sites/cell}
\end{align*}

C: PMA (10^{-7} \text{M}) treated MA10 cells

\begin{align*}
K_D &= 2.7 \pm 1.2 \text{ nM} \\
B_{\text{max}} &= 24,088 \pm 12253 \\
\text{Binding sites/cell}
\end{align*}
FIG. 5.3.8 Scatchard analysis of the effect of db-cAMP and PMA on R2C cells. R2C cells were plated at 1x10^6 cells per well in 6 well plates and used 2 days latter. Cells were incubated in the absence (A) or presence of 1mM db-cAMP (B) 10^{-7}M PMA (C) for 2h at 37°C. The cells were then washed with 50mM glycine buffered saline (pH 3.0) and twice with 0.1M PBS before incubating overnight at 4°C with 1.4nM [^{125}I]-hCG and increasing concentrations of unlabelled hCG (0-2.8nM). Non-specific binding was assessed with 14nM hCG. After the overnight incubation, cells were washed with 0.01M PBS-BSA to remove excess label and were solubilized with 0.5M NaOH. Results were interpreted using the McPherson EBDA-ligand radioligand binding analysis program. Values are the mean ± S.D., n = 2.

A: R2C control binding

\[ K_D = 0.93 \pm 0.014 \text{ nM} \]
\[ B_{\text{max}} = 13,549 \pm 1,740 \text{ Binding sites/cell} \]
B: Cyclic AMP (1mM) treated R2C cells

\[ K_D = 1.88 \pm 0.3 \text{ nM} \]
\[ B_{\text{max}} = 19,679 \pm 375 \text{ Binding sites/cell} \]

C: PMA (10^{-7} M) treated R2C cells

\[ K_D = 2.8 \pm 0.5 \text{ nM} \]
\[ B_{\text{max}} = 27,701 \pm 6443 \text{ Binding sites/cell} \]
a significant increase in the affinity of the receptor. These results indicated that in mouse tumour Leydig cells there was a loss in binding sites when internalization of the LH/CG receptor was inhibited at 37°C.

5.3.2 The role of protein kinase A and C in the regulation of LH/CG receptors.

Dix et al. (1982, 1987) reported that PMA, but not cyclic AMP can cause desensitization in rat Leydig cells. We have shown that preincubating cultured mouse tumour Leydig (MA10 and MLTC-1) cells for 2h with either LH (3.3nM), db-cAMP (1mM) or PMA (10⁻⁷M) markedly decreased the ability of LH to stimulate cyclic AMP or pregnenolone production (Chapter 3).

To study the levels of LH/CG receptors during desensitization, mouse and rat Leydig cells were incubated for 2h at 37°C, with db-cAMP (0.01, 0.1 and 1mM) or PMA (10⁻⁹ - 10⁻⁵M). Db-cAMP caused a 30-50% loss in detectable [¹²⁵I]-hCG binding sites by 120 min in MA10 and MLTC-1 cells (figure 5.3.6 A), with no effect on binding sites in rat Leydig and R2C cells (figure 5.3.6 B). PMA had no significant effect on binding sites, by 120 min, in any of the Leydig cells (table 5.3.2).

To investigate whether the effects on binding were due to a change in the affinity of the LH receptor, Scatchard analysis was carried out on cells treated as above. The effect of db-cAMP (1mM) and PMA (10⁻⁷M) treatment on the $K_D$ for [¹²⁵I]-hCG in the MA10 and R2C cells are shown in figure 5.3.7 and 5.3.8. In MA10
FIG. 5.3.9 The effect of cycloheximide on LH and db-cAMP induced loss of LH/CG receptors in MA10 cells. MA10 cells were plated at a concentration of 2x10^5/well in 24 well plates. Cells were preincubated for 30 min in 1ml growth medium at 37°C with 35mM cycloheximide. The cells were further incubated in the presence or absence of cycloheximide with 3.3nM LH or 1mM db-cAMP for 2h. Cells were then placed on ice, washed with 50mM glycine buffered saline (pH 3.0) for 2 min, then twice with 0.1M PBS. The levels of [125I]-hCG binding sites were assessed as described in Materials and Methods (2.2.10). Values are the mean ± SD, n = 2, replicates = 6, *P<0.05 - student t-test.
cells (figure 5.3.7) db-cAMP (1mM) caused a 50% decrease in the Bmax obtained for \([^{125}\text{I}]\)-hCG binding sites with no effect on the \(K_D\). PMA \((10^{-7}\text{M})\) had no effect on the Bmax of \([^{125}\text{I}]\)-hCG binding sites or \(K_D\) in MA10 cells. In the R2C cells (figure 5.3.8) db-cAMP (1mM) and PMA \((10^{-7}\text{M})\) had no effect on the Bmax obtained for \([^{125}\text{I}]\)-hCG binding sites, however, there was a significant decrease in the affinity of the receptor. These results indicated that in mouse tumour Leydig cells a loss in binding sites occurs with db-cAMP treatment.

The effect of cycloheximide, an inhibitor of mRNA translation, on the loss of LH/CG receptors caused by LH or db-cAMP is shown in figure 5.3.9. MA10 cells were preincubated for 30 min with 35mM cycloheximide before the addition of LH \((3.3\text{nM})\) or db-cAMP \((1\text{mM})\). The cells were further incubated for 2h. Both LH and db-cAMP caused a 50-60% loss of \([^{125}\text{I}]\)-hCG binding sites, however, in the presence of cycloheximide the loss of receptors was not apparent. This indicated that acute loss of LH receptors involved protein synthesis.

We also studied the profile of receptor internalization in the presence of db-cAMP or PMA. Cells were preincubated for 1h at 10°C with 1.4nM \([^{125}\text{I}]\)-hCG and either db-cAMP \((0.1\text{ and }1\text{mM})\) or PMA \((10^{-8}\text{ or }10^{-6}\text{ M})\). Excess hormone was removed after 1h and the cells recultured in the presence of either db-cAMP or PMA at 37°C. Figures 5.3.10 and 5.3.11 shows the effect of db-cAMP on the internalization of \([^{125}\text{I}]\)-hCG in MA10 cells (figure 5.3.10) and MLTC-1 cells (figure 5.3.11). Surface associated radioactivity (figure 5.3.10 A and 5.3.11 A) in the presence of db-cAMP demonstrated a more rapid decline than in control cells, though there was no increase in internalized levels (figure 5.3.10 B and 5.3.11 B). A more pronounced effect on the lack of internalization of hormone was observed
FIG. 5.3.10  The effect of db-cAMP on the internalization of $[^{125}\text{I}]-hCG$ in MA10 cells. Cells were plated at $2 \times 10^6$/well in 6 well plates. Two days later the cells were incubated at $10^\circ C$, to inhibit internalization, in 1ml fresh growth medium with 1.4nM $[^{125}\text{I}]-hCG$ alone or with either 0.1 or 1mM db-cAMP for 1h. The cells were then placed on ice and washed with 0.01M PBS-BSA to remove unbound hormone. The cells were then recultured in 1ml fresh growth medium at $37^\circ C$ with the same additions as before, but excluding the $[^{125}\text{I}]-hCG$. At the times indicated the plates were placed on ice, medium removed and washed with 0.01M PBS-BSA. The surface bound $[^{125}\text{I}]-hCG$ (A) was determined by washing the cells twice with 50mM glycine-buffered saline (pH 3.0) (Ascoli, 1982) and counting the combined washings. To measure the amount of internalized hormone (B), cells were dissolved in 1 ml of 0.5M NaOH, and counted in a gamma-counter. Values represent the mean ± SD, n=2, replicates=2.
**A: Surface**

- Control
- db-cAMP (0.1mM)
- db-cAMP (1mM)

**B: Internalized**

- Control
- db-cAMP (0.1mM)
- db-cAMP (1mM)
FIG. 5.3.11  The effect of db-cAMP on the internalization of $[^{125}\text{I}]$-hCG in MLTC-1 cells. Cells were plated at $2 \times 10^6$/well in 6 well plates. Two days later the cells were incubated at $10^\circ\text{C}$, to inhibit internalization, in 1ml fresh growth medium with 1.4nM $[^{125}\text{I}]$-hCG alone or with either 0.1 or 1mM db-cAMP for 1h. The cells were then placed on ice and washed with 0.01M PBS-BSA to remove unbound hormone. The cells were then recultured in 1ml fresh growth medium at $37^\circ\text{C}$ with the same additions as before, but excluding the $[^{125}\text{I}]$-hCG. At the times indicated the plates were placed on ice, medium removed and washed with 0.01M PBS-BSA. The surface bound $[^{125}\text{I}]$-hCG (A) was determined by washing the cells twice with 50mM glycine-buffered saline (pH 3.0) (Ascoli, 1982) and counting the combined washings. To measure the amount of internalized hormone (B), cells were dissolved in 1 ml of 0.5M NaOH, and counted in a gamma-counter. Values represent the mean ± SD, n=2, replicates=2.
A: Surface

B: Internalized

% of Initial CPM Bound

Time (h)

○ Control
● db-cAMP (0.1 mM)
△ db-cAMP (1 mM)
The effect of PMA on the internalization of $[^{125}\text{I}]-\text{hCG}$ in MA10 cells. Cells were plated at $2\times10^6$/well in 6 well plates. Two days later the cells were incubated at $10^\circ\text{C}$, to inhibit internalization, in 1ml fresh growth medium with $1.4\text{nM}$ $[^{125}\text{I}]-\text{hCG}$ alone or with either $10^{-8}\text{M}$ or $10^{-6}\text{M}$ PMA for 1h. The cells were then placed on ice and washed with 0.01M PBS-BSA to remove unbound hormone. The cells were then recultured in 1ml fresh growth medium at $37^\circ\text{C}$ with the same additions as before, but excluding the $[^{125}\text{I}]-\text{hCG}$. At the times indicated the plates were placed on ice, medium removed and washed with 0.01M PBS-BSA. The surface bound $[^{125}\text{I}]-\text{hCG}$ (A) was determined by washing the cells twice with 50mM glycine-buffered saline (pH 3.0) (Ascoli, 1982) and counting the combined washings. To measure the amount of internalized hormone (B), cells were dissolved in 1 ml of 0.5M NaOH, and counted in a gamma-counter. Values represent the mean ± SD, n=2, replicates=2.
- Control
- PMA (10^{-8} M)
- PMA (10^{-6} M)

A: Surface

B: Internalized

% of Initial CPM Bound

Time (h)
FIG. 5.3.13  The effect of db-cAMP and PMA on the internalization of $[^{125}]$-hCG in rat testis Leydig cells. Cells were plated at $2 \times 10^6$/well in 6 well plates. After a 2h preincubation the cells were incubated at $10^\circ$C, to inhibit internalization, in 1ml fresh medium with 1.4nM $[^{125}]$-hCG alone or with either $10^{-7}$M PMA or 1mM db-cAMP for 1h. The cells were then placed on ice and washed with 0.01M PBS-BSA to remove unbound hormone. The cells were then recultured in 1ml fresh medium at $37^\circ$C with the same additions as before, but excluding the $[^{125}]$-hCG. At the times indicated the plates were placed on ice, medium removed and washed with 0.01M PBS-BSA. The surface bound $[^{125}]$-hCG (A) was determined by washing the cells twice with 50mM glycine-buffered saline (pH 3.0) (Ascoli, 1982) and counting the combined washings. To measure the amount of internalized hormone (B), cells were dissolved in 1 ml of 0.5M NaOH, and counted in a gamma-counter. Values represent the mean ± SD, n=2, replicates=2.
A: Surface

B: Internalized
with MLTC-1 cells (figure 5.3.11). Incubating MA10 cells with PMA \(10^{-8} \text{ or } 10^{-6} \text{M}\) had no significant effect on the internalization of \(^{125}\text{I}\)-hCG (figure 5.3.12). The effect of db-cAMP (1mM) and PMA \(10^{-7}\text{M}\) on internalization of \(^{125}\text{I}\)-hCG in rat Leydig cells is shown in figure 5.3.13. No significant effect on the internalization profile of \(^{125}\text{I}\)-hCG was observed, however the degree of internalization of \(^{125}\text{I}\)-hCG occurred at a far slower rate in rat testis Leydig cells compared to the cultured mouse tumour Leydig (MA10 and MLTC-1) cells. These results supported the observation that in mouse Leydig cells an inhibition of internalization was involved in the acute loss of LH/CG receptors. This process appears to mediated by cyclic AMP.

5.4 Discussion

The results of this study establish that the regulation of LH/CG receptors during desensitization is different in the mouse and rat Leydig cells. The results obtained for the mouse tumour Leydig cells indicate that LH at desensitizing concentrations may inhibit internalization of LH/CG receptors by a cyclic AMP dependent mechanism. Possible mechanisms involved in the loss of receptors are: i) a conformational change in the receptor caused by binding of hormone which results in degradation at the plasma membrane; ii) phosphorylation of the receptor inhibiting internalization allowing degradation at the plasma membrane; or iii) initiation of the synthesis of a protein or phosphorylation of a protein that causes degradation of the LH/CG receptor. The synthesis of a protein that is involved in
LH- and db-cAMP-induced loss of binding sites was supported by the inhibitory effect of cycloheximide on the down-regulation of LH/CG receptors. In the rat testis Leydig cells rapid recycling of the receptor has been shown to occur (Habberfield et al., 1986; Genty et al., 1987). The internalization studies presented here would suggest that if rapid recycling occurs then the bound $[^{125}\text{I}]$-hCG is not released from the receptor. Alternatively the internalization of LH/CG receptors is a slow process in the rat testis Leydig cell. This demonstrates a further species difference in the regulation of LH/CG receptors between rat and mouse Leydig cells.

It has been reported that in mouse Leydig cells LH-induced desensitization and down-regulation were concurrent (Freeman & Ascoli, 1982). LH-induced desensitization was mimicked by forskolin and PMA (Rebois et al., 1985, Lefèvre et al., 1985). Previously though, no report of a loss in binding sites was reported with either PMA or forskolin. In our studies db-cAMP caused a loss in binding sites under the same conditions that caused desensitization (Chapter 3). LH-induced desensitization in rat Leydig cells was not concurrent with down-regulation. In rat Leydig cells, PMA caused desensitization (Dix et al., 1987), but cyclic AMP does not (Dix et al., 1982). Our studies showed that no loss of binding sites occurred in rat Leydig cells with db-cAMP or with LH under conditions that cause desensitization, though there was a significant decrease in the affinity of $[^{125}\text{I}]$-hCG binding with db-cAMP and PMA treatment, but an increase with LH and NaN$_3$ treatment. These results indicated that there may be differences in the sites and mechanisms of phosphorylation of the LH/CG receptor in the mouse and rat Leydig cells.
CHAPTER 6

THE ROLE OF PROTEASES IN LH/CG ACTION
The role of proteases in LH/CG regulation of its receptor, cyclic AMP production and steroidogenesis was investigated in Leydig cells. It was found that the loss of binding sites caused by LH (3.3nM), NaN₃ (5mM) and db-cAMP (1mM) in MA10 and MLTC-1 cells could be prevented by the addition of protease inhibitors. Incubating rat testis Leydig and R2C cells with protease inhibitors caused a 2-3 fold increase in binding sites. The increase in binding sites observed in rat testis was only observed over the first 4h of the incubation. When rat testis Leydig and MA10 cells were incubated in the presence of [¹²⁵I]-hCG, a radioactive-protein complex of approximate Mr of 80,000-90,000 was released into the incubation medium. In mouse Leydig cells, LH but not forskolin or cholera toxin stimulated cyclic AMP production was inhibited by protease inhibitors, whereas in rat testis Leydig cells a 2-3 fold increase in LH-stimulated cyclic AMP production was observed. LH-, dibutyryl cyclic AMP-, and forskolin-stimulated steroidogenesis, but not 22R(OH) cholesterol conversion to pregnenolone, was inhibited by protease inhibitors. It is concluded that LH/CG receptors are regulated by proteolysis at the plasma membrane in both mouse and rat Leydig cells. Furthermore, truncation of the LH/CG receptor in the mouse Leydig cells is involved in down-regulation whereas, in the rat it is a continuous process over the first 4h of the incubation. 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.
Introduction

In this chapter the role of protease activity in the action of LH/CG in relation to receptor regulation, cyclic AMP production and steroidogenesis has been investigated.

Several reports have shown that proteolytic cleavage of hormone receptors at the plasma membrane are involved in down-regulation, e.g. epidermal growth factor (EGF) receptors (Gross et al., 1983; Decker, 1989), acetylcholine receptors (Hatzfeld et al., 1982), and β-adrenergic receptors (Strulovici and Lefkowitz, 1984). There are also reports indicating that the binding of polypeptide hormones to their receptors causes a conformational change which exposes on the outer surface of the plasma membrane a trypsin-sensitive site on the receptor, e.g. insulin (Donner and Yonkers, 1983) and glucagon (Iyengar and Herberg, 1984).

The possible involvement of a membrane protease(s), as modulators of LH/CG receptor function has also been suggested. Protease inhibitors have been shown to inhibit the activation of adenylate cyclase, in rat ovarian and hepatic membrane preparations, at concentrations which inhibit proteolytic activity (McIlroy et al., 1980) and aprotinin (a serine protease inhibitor) markedly reduced the rate of association of hCG to its receptor in ovarian membrane preparations but not in solubilized preparations of the LH/CG receptor (Wilks and Hui, 1987). Kellokumpu and Rajaniemi (1985a, 1985b) demonstrated that when rat ovarian membranes were isolated and incubated with [125I]-hCG, proteolytic cleavage of the LH receptor occurred. This proteolytic cleavage resulted in the release of two distinct receptor-[125I]-hCG fragment complexes of Mr of 96,000 and 74,000.
Kellokumpu and Rajaniemi, 1985a). Susceptibility to this endogenous plasma membrane protease activity was increased with hCG binding (Kellokumpu and Rajaniemi, 1985b). Kellokumpu (1987), also investigated the existence of this process in a cultured mouse tumour Leydig cell line (MLTC-1). He concluded that the faster rate of internalization in the MLTC-1 cells (cf. cultured rat luteal cells) meant that this process of proteolytic cleavage was not evident in the MLTC-1 cells.

Protease activity has also been implicated in the control of the hormonally regulated, rate limiting step in steroid formation, i.e the transport of cholesterol (Gower, 1988). One of the protein candidates for this role is the "steroidogenesis activator polypeptide" (SAP) (Pedersen and Brownie, 1987) which was reported to be cleaved from a glucose-regulated protein (GRP78) by a regulated process that was related to steroidogenesis (Mertz and Pedersen, 1989).

6.3 Results

6.3.1 The effect of protease inhibitors on the level of LH/CG receptors.

The effect of treating MA10 cells with trypsin on surface binding was shown in chapter 4. Pregnenolone production in MLTC-1 cells that were treated with trypsin for 4 min to remove surface LH/CG receptors is shown in figure 6.3.1. No subsequent stimulation of pregnenolone was observed. This supports the data in chapter 4 showing that functional LH/CG receptors were susceptible to proteolytic
FIG. 6.3.1  The effect of trypsin treatment of MLTC-1 cells on pregnenolone production. MLTC-1 cells were plated at 1x10^5/well with 1ml incubation medium. Two days later the cells were either untreated or incubated for 4 min. at 37°C with 0.05% (w/v) trypsin. Cells were then preincubated for 30min with inhibitors of pregnenolone metabolism before being incubated for 2h ± LH (3.3nM). The reaction was stopped using perchloric acid. Pregnenolone was determined as described in Materials and Methods (2.2.18). Results are the mean ± S.E.M. Statistical analysis was by the paired student t-test, n = 3, replicates = 6.
FIG. 6.3.2 Scatchard analysis of trypsin treatment of MLTC-1 cells. MLTC-1 cells were grown to sub-confluent concentrations (10x10^6 cells) in T25 flasks. The cells were then incubated in the absence (A) or presence (B) of 0.05% (w/v) trypsin for 4 min. at 37°C. Cells were washed with 0.01M PBS on ice and then scraped from the flasks in 1ml homogenizing buffer. Homogenates were then prepared as described in Materials and Methods (2.2.13). 50µg of protein/tube/ml binding buffer was then incubated in the presence of 1.4nM [125I]-hCG and increasing concentration of unlabelled hCG (0-2.8nM) overnight at 4°C. Non-specific binding was assessed with 14nM hCG. After the overnight incubation 200µl of 5mg/ml Bovine gamma globulin and 500µl of 30% solution of polyethylene glycol (PEG) were added. Tubes were incubated for 10 min at 4°C and then spun for 15 min at 3000xg. The supernatant was aspirated and the pellets were resuspended with 1ml of binding buffer and incubated at 4°C for a further 10 min. 500µl of PEG was added and the tubes were spun at 3000 xg for 15 min and supernatant aspirated. The pellets were then counted in a gamma-counter. Results were interpreted using the McPherson EBDA-ligand radioligand binding analysis program. Values are the mean ± S.D., n = 2.
A: MLTC-1 homogenate

\[ B/F = \frac{1}{K_D} \]

\[ K_D = 5.3 \pm 0.28 \text{ nM} \]

\[ B_{\text{max}} = 1.97 \pm 0.28 \text{ pmol/mg protein} \]

B: Trypsinized MLTC-1 homogenate

\[ B/F = \frac{1}{K_D} \]

\[ K_D = 7.63 \pm 0.19 \text{ nM} \]

\[ B_{\text{max}} = 0.7 \pm 0.23 \text{ pmol/mg protein} \]
cleavage. Figure 6.3.2 shows the binding in homogenate preparations of MLTC-1 cells that were treated with trypsin for 4 min compared to $[^{125}\text{I}]-\text{hCG}$ binding in homogenates of control cells. Scatchard analysis indicated that binding in control cell homogenates had a dissociation constant $5.3 \pm 0.28 \text{ nM}$ and a Bmax of $1.97 \text{ pmol/mg protein}$. However, in homogenates of cell treated with trypsin that have no detectable surface binding, $[^{125}\text{I}]-\text{hCG}$ binding could be detected with a dissociation constant of $7.63 \pm 0.19 \text{ nM}$ with a Bmax of $0.7 \text{ pmol/mg protein}$. This indicates that binding sites are either located in folds of the plasma membrane and protected from trypsin treatment or that an intracellular pool of receptors exists.

In order to desensitize the MA10, MLTC-1, R2C and rat testis Leydig cells with respect to steroid production (i.e. uncouple the receptor from its transducing system), the cells were incubated for 2h with LH (3.3nM) (Freeman, and Ascoli, 1982; Dix et al., 1982). In addition, cells were incubated with NaN$_3$ (5mM) (with and without LH) to inhibit internalization as shown in chapter 5. Cell surface bound hormone was removed with glycine-buffered saline (pH 3.0) (Ascoli, 1982) and the cells washed before $[^{125}\text{I}]-\text{hCG}$ was added and incubations carried out at 4°C, to determine the residual numbers of binding sites and dissociation constants ($K_D$).

A 2h incubation of MA10 and MLTC-1 cells (fig. 6.3.3) in the presence of LH (3.3nM) or NaN$_3$ (5mM) caused a 50% loss in binding of $[^{125}\text{I}]-\text{hCG}$ as previously reported (chapter 5). Preincubating the MA10 and MLTC-1 cells with a cocktail of protease inhibitors (leupeptin 100µM, PMSF 10µM, aprotinin 900 units/ml) for 30 min before the addition of LH or NaN$_3$, prevented the observed loss of receptors.
**FIG. 6.3.3** The effect of protease inhibitors on the regulation of $[^{125}\text{I}]-\text{hCG}$ binding sites in mouse tumour Leydig cells. MA10 and MLTC-1 cells were plated at $1.5\times10^5$ in 1ml of medium and preincubated ± protease inhibitors (leupeptin 100μM, PMSF 10μM, aprotinin 900 units/ml) in 1ml fresh medium, for 30min. at 37°C. The cells were further incubated in the absence of protease inhibitors for 2h (either without hormone; with 5mM NaN$_3$; with 3.3nM LH; or with 3.3nM LH + 5mM NaN$_3$) or in the presence of protease inhibitors for 2h; (either without hormone; with 5mM NaN$_3$; with 3.3nM LH; or with 3.3nM LH + 5mM NaN$_3$). After 2h the cells were placed on ice and washed twice with 50mM glycine-buffered saline (pH 3.0) before $[^{125}\text{I}]-\text{hCG}$ binding was determined as described in Materials and Methods (2.2.10). Values given are the mean, ± S.E.M., replicates = 6, n = 3, *$P<0.05$ - student t-test. Due to the variance in control binding of cultured Leydig cells (chapter 4) only experiments where control binding was approximately the same have been represented. Experiments were repeated four times with similar results for the MA10 and MLTC-1.
FIG. 6.3.4 The effect of protease inhibitors on the regulation of $[^{125}I]$-hCG binding sites in rat testis Leydig and rat tumour Leydig cells. R2C and rat testis Leydig cells were plated at 1.5x10^5 in 1ml of medium and preincubated ± protease inhibitors (leupeptin 100μM, PMSF 10μM, aprotinin 900 units/ml) in 1ml fresh medium, for 30min. at 37°C. The cells were further incubated in the absence of protease inhibitors for 2h (either without hormone; with 5mM NaN_3; with 3.3nM LH; or with 3.3nM LH + 5mM NaN_3) or in the presence of protease inhibitors for 2h; (either without hormone; with 5mM NaN_3; with 3.3nM LH; or with 3.3nM LH + 5mM NaN_3). After 2h the cells were placed on ice and washed twice with 50mM glycine-buffered saline (pH 3.0) before $[^{125}I]$-hCG binding was determined. Values given are the mean, ± S.E.M., replicates = 6, n = 3, *P<0.05 - student t-test. Due to the variance in control binding of cultured Leydig cells (chapter 4) only experiments where control binding was approximately the same have been represented. Experiments were repeated four times with similar results for the rat testis Leydig cells and twice for the R2C cells.
over a 2h incubation period at 37°C - as determined by subsequent $^{125}$I-hCG binding at 4°C (in the absence of protease inhibitors). Similar results were obtained for the MA10 cells, when horse serum was replaced by 0.1% (w/v) BSA (Table 6.3.1), thus demonstrating that the protease activity was not derived from the serum.

Similar experiments were also carried out on the rat Leydig cells. A 2h incubation of rat testis Leydig and R2C cells (fig. 6.3.4) with either NaN$_3$ or LH did not cause a loss in binding sites. However, preincubating these cells with the protease inhibitors resulted in a one fold increase (R2C) and a two fold increase (rat testis Leydig) in binding sites (determined by $^{125}$I-hCG binding at 4°C in the absence of protease inhibitors) under all the conditions used, thus suggesting that there is a continual process of proteolysis of the LH/CG receptor in the rat testis Leydig and R2C cells. Figure 6.3.5 shows the effect of adding protease inhibitors to rat testis Leydig cells at the time of plating the cells or at 2, 4 or 6 h after plating. The cells were incubated for 2h in the presence of the protease inhibitors. The level of $^{125}$I-hCG binding sites were assessed at the end of this period. Addition at time zero resulted in a 3 fold increase in binding sites. Addition of protease inhibitors 2h after plating resulted in a one fold increase in binding sites whereas addition at 4 or 6 hours after plating the cells resulted in no change in the level of binding sites as compared to control cells. The protease inhibitors had no effect on the viability of the cells.

To investigate whether the effects of the protease inhibitors on binding were due to a change in the affinity of the LH/CG receptor, Scatchard analysis was carried out. The effect of the protease inhibitor treatment on the $K_D$ and
### TABLE 6.3.1

The effect of protease inhibitors on the regulation of $[^{125}\text{I}]-\text{hCG}$ binding sites in MA10 Leydig cells in the presence and absence of horse serum. MA10 cells were plated at $2 \times 10^5$/well in 24 well plates, incubated at 37°C in horse serum medium and used 48h later. The cells were then recultured in the presence of either horse serum medium or horse serum free medium ± protease inhibitors (leupeptin 100μM, PMSF 10μM, aprotinin 900 units/ml) for 30min. at 37°C. The cells were further incubated in serum or serum free conditions with or without protease inhibitors for 2h. The cells were also incubated in the presence or absence of NaN$_3$ (5mM) or LH (3.3nM). At the end of 2h the cells were placed on ice, washed with 50mM glycine buffered saline (pH 3.0) and $[^{125}\text{I}]-\text{hCG}$ assessed as described in Materials and Methods (2.2.10). The results are the mean ± S.E.M. Statistical analysis was by the student paired t-test, n = 3, replicates = 6 (*P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Serum</th>
<th>Serum Free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>20.5±0.13</td>
<td>18.1±0.9</td>
</tr>
<tr>
<td>NaN$_3$ (5mM)</td>
<td>11.1±0.3*</td>
<td>19.4±0.3</td>
</tr>
<tr>
<td>LH (3.3nM)</td>
<td>8.3±0.3*</td>
<td>16.3±0.2</td>
</tr>
</tbody>
</table>
FIG. 6.3.5 The effect of protease inhibitors on the regulation of [125I]-hCG binding sites in rat testis Leydig cells with time. Rat testis Leydig cells were plated at 1.5x10^5 in 1ml of medium and incubated ± protease inhibitors (leupeptin 100μM, PMSF 10μM, aprotinin 900 units/ml) added at time 0, 2h, 4h and 6h at 34°C. After the addition of protease inhibitors the cells were incubated for 2h before [125I]-hCG binding sites were assessed. Values given are the mean, ± SD, replicates = 6, n = 2, *P<0.05 - student t-test.
number of binding sites for $^{125}\text{I}}$-hCG in the MA10 is shown in figure 6.3.6. The protease inhibitors had no effect on the $K_D$ or the number of binding sites.

The above results indicated that in mouse tumour Leydig cells there was a loss in binding sites when internalization of the LH/CG receptor was inhibited at 37°C. Experiments were therefore also undertaken to see if proteolysis of the receptor occurred at 4°C which has been shown to inhibit internalization of the hormone-receptor complex (Habberfield et al., 1987). MA10 cells were incubated overnight at 4°C, with 0.56nM $^{125}\text{I}}$-hCG ± the protease inhibitors. Binding in untreated cells was 17.7 ± 0.56 fmol/10^6 cells, whereas binding in treated cells, i.e. incubated with the protease inhibitors, increased to 26.7 ± 0.56 fmol/10^6 cells ($n = 3$, replicates = 12). There was no effect on the $K_D$ of $^{125}\text{I}}$-hCG when MA10 cells were incubated under these conditions (figure 6.3.6). These results demonstrated that at 4°C (i.e. conditions that prevent internalization of receptors (Habberfield et al., 1987)) and in the presence of protease inhibitors the level of detectable binding was increased. The effect of the protease inhibitors is not as great as at 37°C, but this can be explained by a decreased proteolytic enzyme activity due to the lower temperature.

We reported in chapter 5 that db-cAMP can mimic the loss of LH/CG receptors caused by LH. Figure 6.3.7 shows the effect of protease inhibitors on LH (3.3nM), db-cAMP (1mM), cholera toxin (11.9nM) and forskolin (80μM) regulation of LH/CG receptors. All the above reagents caused a loss in LH/CG receptors over a 2h incubation period. In the presence of the protease inhibitors no loss of LH/CG receptors was observed.
FIG. 6.3.6 Scatchard analysis of the effect of protease inhibitors on MA10 cells. MA10 cells were plated at 1x10^6 cells per well in 6 well plates and used 2 days latter. Cells were incubated in the absence (A) or presence (B) of protease inhibitors (leupeptin 100μM, PMSF 10μM, aprotinin 900 units/ml) overnight at 4°C with 1.4nM [125I]-hCG and increasing concentration of unlabelled hCG (0-2.8nM). Non-specific binding was assessed with 14nM hCG. After the overnight incubation, cells were washed with 0.01M PBS-BSA to remove excess label and were solubilized with 0.5M NaOH. Results were interpreted using the McPherson EBDA-ligand radioligand binding analysis program. Values are the mean ± S.D., n = 2.
**B: Protease inhibitor treated MA10 cells**

- $K_D = 3.2 \pm 0.98 \text{ nM}$
- $B_{\text{max}} = 19,270 \pm 2,456$ Binding sites/cell

---

- $K_D = 4.0 \pm 1.2 \text{ nM}$
- $B_{\text{max}} = 24,077 \pm 1,253$ Binding sites/cell
FIG. 6.3.7 The effect of protease inhibitors on the regulation of $[^{125}I]$-hCG binding sites in MA10 Leydig cells. MA10 cells were plated at $2 \times 10^5$ in 1ml of medium and preincubated ± protease inhibitors (leupeptin 100μM, PMSF 10μM, aprotinin 900 units/ml) in 1ml fresh medium, for 30min. at 37°C. The cells were further incubated in the absence of protease inhibitors for 2h (either without hormone; with 3.3nM LH; with 1mM db-cAMP; 11.9nM cholera toxin; or with 80μM forskolin) or in the presence of protease inhibitors for 2h; (either without hormone; with 3.3nM LH; with 1mM db-cAMP; 11.9nM cholera toxin; or with 80μM forskolin). After 2h the cells were placed on ice and washed twice with 50mM glycine-buffered saline (pH 3.0) before $[^{125}I]$-hCG binding was determined as described in Materials and Methods (2.2.10). Values given are the mean, ± S.E.M., replicates = 6, n = 3, *P<0.05 - student t-test.
FIG. 6.3.8 Autoradiograph and densitometric trace of proteins derived from culture medium of rat testis Leydig cells prelabelled with $[^{125}\text{I}]-\text{hCG}$. The figure shows an autoradiograph (i), of two experiments and densitometric trace (ii), obtained from SDS-PAGE of culture medium from rat testis Leydig cells. Rat testis Leydig cells were plated at a density of $5 \times 10^6$/plate and treated with 2.8nM $[^{125}\text{I}]-\text{hCG} + \text{NaN}_3$ at 34°C for 3h (lanes: (i) 3, 5, 8 and 11; (ii) B), 2.8nM $[^{125}\text{I}]-\text{hCG}$ at 34°C (lanes: (i) 2, 6, 7 and 9; (ii) C), 2.8nM $[^{125}\text{I}]-\text{hCG}$ overnight at 4°C (lanes: (i) 4, 12 and 13; (ii) D) and 2.8nM $[^{125}\text{I}]-\text{hCG} + \text{NaN}_3$ in the presence of 1500 IU crude hCG at 34°C for 3h (lane: (i) 10). Cells were further incubated at 4°C or 34°C for one hour with DSS (final conc. 1mM). The medium from these incubations was removed and treated with 24% (w/v) TCA to precipitate the proteins. The proteins were solubilized and run on a denaturing SDS-PAGE 10% (w/v) gel. An autoradiograph of the gel was developed 1 week later. Standard $[^{125}\text{I}]-\text{hCG}$ incubated in the presence of DSS (final conc. 1mM) for 1h was run in lane (i) 1 and (ii) A.
116 KDa 88 KDa 42 KDa

D:

C:

B:

A:

Fragment - $^{125}\text{I}$-hCG

Intact $^{125}\text{I}$-hCG
FIG. 6.3.9 Autoradiograph and densitometric trace of proteins derived from culture medium of MA10 cells prelabelled with $[^{125}\text{I}]-\text{hCG}$. The figure shows an autoradiograph (i), of two experiments and densitometric trace (ii), obtained from SDS-PAGE of culture medium from MA10 cells. MA10 cells were grown to sub-confluency in 75cm$^2$ culture flasks (approx. 2x10$^7$/flask). Three series of 4 flasks were treated with 0.28nM $[^{125}\text{I}]-\text{hCG} + 5\text{mM NaN}_3$ at 37°C for 3h (lanes: (i) 2 and 6; (ii) B), 0.28nM $[^{125}\text{I}]-\text{hCG}$ at 34°C (lanes: (i) 1 and 5; (ii) C) and 0.28nM $[^{125}\text{I}]-\text{hCG}$ overnight at 4°C (lanes: (i) 3 and 7; (ii) D). Cells were further incubated for one hour with DSS (final conc. 1mM) at 4°C or 37°C. The medium from these incubations was concentrated by centrifugation using concentrators with a molecular size cut-off of 30,000 and treated with 24% (w/v) TCA to precipitate the proteins. The proteins were solubilized and run on a denaturing SDS-PAGE 10% (w/v) gel. An autoradiograph of the gel was developed 1 and 3 weeks later for the MA10 derived gel (this was to identify the fragment). Standard $[^{125}\text{I}]-\text{hCG}$ incubated in the presence of DSS (final conc. 1mM) for 1h is shown in lanes: (i) 4 and 8; (ii) A.

(i)

A = 1 week exposure
B = 3 week exposure
(ii)

DEVELOPED FOR 3 WEEKS

116 kDa

80 kDa

DEVELOPED FOR 1 WEEK

38 kDa

Overlap

FRAGMENT • [125I] hCG

INTACT • [125I] hCG

α1 [125I] hCG

232
FIG. 6.3.10  Autoradiographs of Leydig cell culture medium incubated with $[^{125}\text{I}]$-hCG. The figure shows an autoradiograph, obtained from SDS-PAGE of rat testis Leydig (lane 1-4) and MA10 (lanes 5-8) cells conditioned culture medium. Rat testis Leydig cells and MA10 cells were grown as for figure 6.3.8 and 6.3.9 respectively. Cells were treated in the presence (lanes 4 and 7) or absence (lanes 3 and 8) of NaN$_3$ (5mM) for 3h at 34°C or at 4°C, overnight, (lanes 2 and 6). The culture media was removed and further incubated for 3h with 1.4nM $[^{125}\text{I}]$-hCG and DSS (final conc. 1mM). The media was then treated as before. An autoradiograph of the gel was developed 2 weeks later. Standard $[^{125}\text{I}]$-hCG incubated in the presence of DSS (final conc. 1mM) for 1h was run in lanes 1 and 5.
6.3.2  *Detection of LH/CG receptor proteolytic fragment.*

Under the various conditions in the above studies there was an apparent loss of receptors at the cell surface: Because these "lost" receptors may have been released into the incubation media, we attempted to detect them by covalently cross-linking $^{[125]}$I-hCG to the receptor. MA10 and rat testis Leydig cells were incubated under conditions where internalization of LH/CG receptors was inhibited, i.e. overnight in the presence of $^{[125]}$I-hCG at 4°C or with $^{[125]}$I-hCG + NaN$_3$ for 3h at 37°C, and compared to incubations where internalization occurred i.e. $^{[125]}$I-hCG at 37°C. The cross-linking agent disuccinimidyl suberate (DSS) was then added and cells were incubated for 1h at 4°C or 37°C. The medium was removed and the proteins were precipitated, solubilized and subjected to SDS-PAGE as described in the Materials and Methods (2.2.15). Figure 6.3.8 and 6.3.9 show an autoradiograph and densitometric trace of an autoradiograph, of the proteins derived from the medium of rat testis Leydig (figure 6.3.8) and MA10 (figure 6.3.9) cells separated by SDS-PAGE. A band of approximate $M_r$ of 80,000-90,000 was detected when the cells were incubated overnight at 4°C (figure 6.3.8 lanes 4,12,13 and D; figure 6.3.9 lanes 3,7 and D) and with NaN$_3$ at 37°C (figure 6.3.8 lanes 3,5,8,11 and B; figure 6.3.9 lanes 2,6 and B), and to a lesser extent with $^{[125]}$I-hCG at 37°C (figure 6.3.8 lanes 2,6,7,9 and C; figure 6.3.9 lanes 1,5 and C). Incubating the rat testis Leydig cells with 2.8nM $^{[125]}$I-hCG + 5mM NaN$_3$ in the presence of crude hCG (1500 IU) (figure 6.3.8 lane 10) resulted in an absence of a band at $M_r$ 90,000. The relative molecular weight of hCG is 36,000 and an appropriate band of $M_r$ 38,000-42,000 was detected on the autoradiographs of $^{[125]}$I-hCG (figure
The effect of protease inhibitors on cyclic AMP production in rat testis and MA10 Leydig cells. Rat testis (RTL) (A) and MA10 (B) Leydig cells were plated at a density of $1 \times 10^5$ cells/well in 24 well plates and preincubated for 2h. During the final 30min the protease inhibitors were added. The cells were then treated as follows with LH (0.033nM), LH (0.33nM), cholera toxin (1.19nM) or forskolin (8μM) in the absence and presence of the protease inhibitors for 2h. Reactions were stopped by the addition of perchloric acid and assayed for cyclic AMP as stated in the Materials and Methods (2.2.17). Values are the mean ± SEM, n=3, replicates=3 - paired student t-test (*P<0.001).
A: RTL

B: MA10

CYCLIC AMP (pmol/10^6 cells/2h)

NORMAL PROTEASE INHIBITORS

NORMAL PROTEASE INHIBITORS
FIG. 6.3.12  The effect of protease inhibitors on pregnenolone production in Leydig cells. Rat Leydig (A) and MA10 (B) cells were plated at $1 \times 10^5$/well with 1ml incubation medium. The cells were preincubated for 30min with or without protease inhibitors before the addition of reagents. Protease inhibitors were present throughout the incubations. Cells were incubated for 2h in the absence or presence of 0.033nM, 0.33nM LH, 5μM 22R(OH) cholesterol, 1mM dibutyryl cyclic AMP or 8μM forskolin. Data represents the mean ± SD of 2 experiments. Significant differences are shown for inhibition only (*P< 0.001).
BASAL
LH (0.033nM)
LH (0.33nM)
22R(OH) CHOLESTEROL (5μM)
FORSKOLIN (8μM)
db-cAMP (1mM)

A: Rat testis

B: MA10

PREGNENOLONE (ng/10^6 cells/2h)

NORMAL
PROTEASE INHIBITORS

NORMAL
PROTEASE INHIBITORS
FIG. 6.3.13 The effect of protease inhibitors on testosterone production in rat testis Leydig cells. Cells were plated at 1x10^5/well with 1ml incubation medium. The cells were preincubated for 30min with or without protease inhibitors before the addition of reagents. Protease inhibitors were present throughout the incubations. Cells were incubated for 2h in the absence or presence of 0.033nM, 0.33nM LH, 5µM pregnenolone, 5µM 22R(OH) cholesterol, 8µM forskolin or 1mM dibutylryl cyclic AMP. Data represents the mean ± SD of 2 experiments. Significant differences are shown for inhibition only (*P< 0.05).
6.3.8 lanes 1 and A; figure 6.3.9 lanes 4,8 and A), this therefore gives a $M_r$ of 40,000-50,000 for the proteolytic fragment. In order to determine if $[^{125}\text{I}]-\text{hCG}$ could be cross-linked to the proteolytic fragment after release from the Leydig cells, the following experiment was carried out. Rat testis Leydig and MA10 cells were incubated in the presence or absence of $\text{NaN}_3$ (5mM) for 3h or at 4°C overnight, the medium was removed and then incubated with 1.4nM $[^{125}\text{I}]-\text{hCG}$ and DSS (final concentration 1mM) for 3h at 37°C. As can be seen from the autoradiograph (figure 6.3.10) no band was detected at $M_r$ 90,000 in lanes 2, 4, 6 and 8 - and only partially in lanes 3 and 7 - after a 2 week exposure. This is in contrast to the results shown in chapter 7, where LH/CG binding proteins were detected. However, incubation times were increased to 24h and 48h, so that these proteins could be detected.

6.3.3 The effect of protease inhibitors on cyclic AMP production and steroidogenesis.

MA10 and rat testis Leydig cells were preincubated for 30 min with leupeptin (100$\mu$M), PMSF (10$\mu$M), and aprotinin (900 units/ml) in 1ml of medium at 37°C. LH (0.033nM and 0.33nM), 22R(OH)-cholesterol (5$\mu$M), cholera toxin (1.19nM), forskolin (8$\mu$M) or dibutyryl cyclic AMP (1mM) were then added for 2h. The reactions were stopped and measured for pregnenolone, testosterone and cyclic AMP production. Figure 6.3.11 shows the effect of incubating rat testis Leydig (fig. 6.3.11 A) and MA10 (fig. 6.3.11 B) cells in the presence and absence of
protease inhibitors on cyclic AMP production. In rat testis Leydig cells, under the same conditions where we see an increase in LH/CG receptors we see a concurrent fold increase in LH (0.033 and 0.33 nM)-stimulated cyclic AMP production (figure 6.3.11 A). Neither cholera toxin- nor forskolin-stimulated cyclic AMP production was affected. In MA10 cells (figure 6.3.11 B) both 0.33nM and 3.3nM LH-stimulated cyclic AMP production was inhibited by 70%, but forskolin- and cholera toxin-stimulated cyclic AMP production were unaffected.

The effect of the protease inhibitors on steroid production is shown in figures 6.3.12 and 6.3.13. LH, forskolin and dibutyryl cyclic AMP stimulated pregnenolone were all inhibited in rat testis Leydig (figure 6.3.12 A) and MA10 (figure 6.3.12 B) cells. There was no effect on the conversion of 22R(OH)-cholesterol to pregnenolone. Figure 6.3.13 shows the effect of protease inhibitors on testosterone production in rat Leydig cells. In these experiments LH, db-cAMP, and forskolin stimulated testosterone were inhibited. Pregnenolone and 22R(OH)-cholesterol conversion to testosterone were inhibited but to a lesser extent.

6.3.4 The effect of soybean trypsin inhibitor on cyclic AMP and testosterone production.

Soybean trypsin inhibitor is used in the isolation of rat testis Leydig cells. It is added at a concentration of 10μM and is present to inhibit the protease contaminants in the collagenase (Ascoli, and Segaloff, 1986). Figure 6.3.14 and 6.3.15 show the dose response curves for LH-stimulated cyclic AMP and
FIG. 6.3.14 The effect of soybean trypsin inhibitor on LH-stimulated cyclic AMP production in rat testis Leydig cells. Rat testis Leydig cells were plated at 1.5x10^5 in 1ml of medium and incubated ± 0.02% (v/v) (equivalent to 10μM) soybean trypsin inhibitor for 2h at 34°C. The cells were then incubated in the presence of increasing concentration of LH (0 -3.3 nM) for 2h. Reactions were stopped by the addition of perchloric acid and assayed for cyclic AMP as stated in Materials and Methods (2.2.17). Values are the mean ± SEM, n=3, replicates=3 - paired student t-test (*P<0.001).
FIG. 6.3.15  The effect of soybean trypsin inhibitor on LH-stimulated testosterone production in rat testis Leydig cells. Rat testis Leydig cells were plated at 1.5x10^5 in 1ml of medium and incubated ± 0.02% (v/v) (equivalent to 10μM) soybean trypsin inhibitor for 2h at 34°C. The cells were then incubated in the presence of increasing concentration of LH (0 - 3.3 nM) for 2h. Reactions were stopped by the addition of perchloric acid and assayed for testosterone as stated in Materials and Methods (2.2.18). Values are the mean ± SEM, n=3, replicates=3 - paired student t-test (*P<0.001).
FIG. 6.3.16 The effect of soybean trypsin inhibitor on db-cAMP-stimulated testosterone production in rat testis Leydig cells. Rat testis Leydig cells were plated at 1.5x10^5 in 1ml of medium and incubated ± 0.02% (v/v) (equivalent to 10μM) soybean trypsin inhibitor for 2h at 34°C. The cells were then incubated in the presence of increasing concentration of db-cAMP (0 - 1mM) for 2h. Reactions were stopped by the addition of perchloric acid and assayed for testosterone as stated in Materials and Methods (2.2.18). Values are the mean ± SEM, n=3, replicates=3 - paired student t-test (*P<0.001).
testosterone production in rat testis Leydig cells treated with 10µM soybean trypsin inhibitor. At all doses of LH used there was a one fold increase in cyclic AMP production (figure 6.3.14) as indicated above. In contrast, testosterone production at all doses of LH was inhibited (figure 6.3.15). The dose response curve for db-cAMP stimulated testosterone in the presence of 10µM soybean trypsin inhibitor is shown in figure 6.3.16. No effect was seen on testosterone production.

6.3.5 The effect of protease inhibitors on LH/CG action in mouse testis Leydig cells.

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 therefore repeated our studies on normal mouse testis Leydig cells. Figure 6.3.17 demonstrates the effect of LH (3.3nM), db-cAMP (1mM), PMA (10⁻⁷M) and forskolin (80µM) on receptor regulation in mouse testis Leydig cells. As observed in the mouse tumour Leydig cells LH, db-cAMP and forskolin caused a >50% loss in binding sites. This decrease was inhibited in the presence of the protease inhibitors. LH-stimulated cyclic AMP production was inhibited from 205.8±58 to 21.2±5.7 pmol/10⁶ cells/2h whereas stimulation of cyclic AMP by forskolin was unaffected (figure 6.3.18). LH- and db-cAMP-stimulated testosterone production was also inhibited in the presence of the protease inhibitors (figure 6.3.19). There was partial inhibition of 22R(OH)-cholesterol conversion to testosterone - this was similar to the results for 22R(OH)-cholesterol conversion to testosterone in rat Leydig cells (figure 6.3.13).
FIG. 6.3.17 The effect of protease inhibitors on the regulation of $[^{125}\text{I}]-\text{hCG}$ binding sites in mouse testis Leydig cells. Cells were plated at $2 \times 10^5$ in 1ml of medium and preincubated ± protease inhibitors (leupeptin 100μM, PMSF 10μM, aprotinin 900 units/ml) in 1ml fresh medium, for 30min. at 34°C. The cells were further incubated in the absence of protease inhibitors for 2h (either without hormone; with 3.3nM LH; with 1mM db-cAMP; $10^{-7}$M PMA; or with 80μM forskolin) or in the presence of protease inhibitors for 2h; (either without hormone; with 3.3nM LH; with 1mM db-cAMP; $10^{-7}$M PMA; or with 80μM forskolin). After 2h the cells were placed on ice and washed twice with 50mM glycine-buffered saline (pH 3.0) before $[^{125}\text{I}]-\text{hCG}$ binding was determined as described in Materials and Methods (2.2.10). Values given are the mean, ± S.E.M., replicates = 6, n = 3, *P<0.05 - student t-test.
FIG. 6.3.18  The effect of protease inhibitors on cyclic AMP production in mouse testis Leydig cells. Cells were plated at 1x10^5/well with 1ml incubation medium. The cells were preincubated for 30min with or without protease inhibitors before the addition of reagents. Protease inhibitors were present throughout the incubations. Cells were incubated for 2h in the absence or presence of 3.3nM LH, 10^{-6} M PMA or 8μM forskolin. Data represents the mean ± SD of 2 experiments. Significant differences are shown for inhibition only (*P<0.001).
**FIG. 6.3.19** The effect of protease inhibitors on testosterone production in mouse testis Leydig cells. Cells were plated at $1 \times 10^5$/well with 1ml incubation medium. The cells were preincubated for 30min with or without protease inhibitors before the addition of reagents. Protease inhibitors were present throughout the incubations. Cells were incubated for 2h in the absence or presence of 3.3nM LH, $10^{-6}$M PMA, 8μM forskolin or 5μM 22R(OH) cholesterol. Data represents the mean ± SD of 2 experiments. Significant differences are shown for inhibition only (*P < 0.05).
The results in this chapter establish that serine proteases are involved in all the stages of LH/CG action in the mouse and rat Leydig cells. In agreement with previous experiments carried out with intact luteal cells and isolated plasma membranes from luteal cells we have found that LH/CG receptors are cleaved at the plasma membrane in intact Leydig cells by endogenous proteases, releasing a water soluble component of approximate Mr of 90,000 (Metsikko et al., 1990). We have demonstrated that in mouse tumour Leydig cells this process is involved in down-regulation and also occurs under conditions where internalization of LH/CG-receptor complexes was inhibited. In contrast, in the rat Leydig cell we demonstrated that proteolysis of the receptor is a continuous process over the first four hours after isolation and participated in the normal regulation of the hormonal response of the cell in vitro.

The size of the fragment detected in this study (Mr=40,000-50,000) is comparable to the size of the extracellular domain of the LH/CG receptor in the rat ovary. The rat ovarian receptor was reported to be Mr=93,000, and to have 7 transmembrane domains, with an extracellular component of Mr=50,000-60,000 (McFarland et al., 1989). It was also demonstrated that isolated rat ovarian LH/CG receptors could be cleaved by endoproteinase Glu-C at a single glutamyl residue to give an extracellular component of Mr=36,000 (Sojar and Bahl, 1989).

The effect of protease inhibitors on down-regulation in the MA10 and MLTC-1 cells and the increase in binding in the rat testis Leydig and R2C cells, clearly indicates the involvement of proteolytic cleavage in the regulation of the
LH/CG receptors. The fact that LH-stimulated cyclic AMP production was increased by the same magnitude as the receptor number in the presence of protease inhibitors, enforced the concept of continual proteolysis in the rat testis Leydig cells. This, however, only occurred over the first 4h of the incubation. The fact that an increase in LH/CG receptors was also observed in the R2C cells discounted that this rapid loss in receptors after isolation was due to contaminating enzymes within the collagenase. The increase in receptors in the presence of protease inhibitors also indicated that in vivo in the rat this process was inhibited. It is known that the peritubular myoid cells synthesize plasminogen activator inhibitor (Nargolwalla et al., 1990), which may be involved in this process. The nature of the protease has not been characterized, but as the inhibitors used inhibit serine proteases, it could be a member of the plasminogen or kallikrein families, which are glycoprotein - serine proteases. The involvement of plasminogen activator has been implicated in the down-regulation of acetylcholine receptors (Hatzfeld et al., 1982) and EGF receptors (Gross et al., 1983). Roche and Ryan have also reported that ovarian membranes contain plasminogen activators and numerous plasminogen-independent proteases (Roche and Ryan, 1986). It has also been reported that hCG has primary sequence analogies to the serine protease chymotrypsin (Willey and Leidenberger, 1989), which could indicate that hCG may cleave the receptor itself, i.e. during desensitization hCG saturates the available receptors resulting in a decrease in internalization allowing the proteolytic activity of hCG to occur at the plasma membrane.

No $[^{125}I]$-hCG-labelled fragment was detected when the medium from
treated cells was incubated with $[^{125}\text{I}]$-hCG. Therefore it would appear that to
detect the fragment, $[^{125}\text{I}]$-hCG must be bound to the receptor before proteolysis
occurred. This implied that the fragment would not act as a serum binding protein
for LH \textit{in vivo} and was therefore unlike the growth hormone derived serum binding
protein (Leung \textit{et al.}, 1987; Spencer \textit{et al.}, 1988). However, we show in chapter 7
that LH/CG binding proteins are synthesized by MA10 cells, but the synthesis of
these proteins is slow and requires longer incubation times than 3h to detect them.

The present experiments indicated that serine proteases were 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. The results
obtained in this study were in contrast to those published (Ascoli and Puett, 1978;
Ascoli, 1978), in which there was no effect on progesterone production with three
different trypsin inhibitors ($p$-tosyl-L-arginine, $p$-aminobenzamidine, lima bean
trypsin inhibitor). However, our results for the inhibition of testosterone with
10\text{$\mu$}M soybean trypsin inhibitor were in agreement with those of Willey and
Leidenberger (1989). In addition, in the mouse Leydig cells, proteases were
required for the stimulation of cyclic AMP production by LH.

The evidence for the location of the action of the proteases on
steroidogenesis was 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
(Gower, 1988). 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 (Freeman, 1989; Nagy and Freeman, 1990) and that cyclic AMP regulates its transport in MA10 cells (Freeman, 1987). Our experiments indicate, therefore, that the transport of cholesterol from either the plasma membrane and/or from the outer to inner mitochondrial membrane requires 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 under the same conditions. As no effect of the protease inhibitors was seen on forskolin- or cholera toxin-stimulated cyclic AMP, the protease inhibitors most probably modulate the LH/CG receptor. This indicates that in mouse Leydig cells proteolysis of the LH/CG receptor was required for activation or that the inhibitors were affecting the binding of LH. The latter is unlikely however, because protease inhibitors have no effect on the binding characteristics of [125I]-hCG. Serine proteases have also been shown to activate adenylate cyclase (McIlroy et al., 1980), but no effect was seen on cholera toxin or forskolin stimulated cyclic AMP.

It may be concluded that in mouse Leydig cells proteolytic cleavage of the receptor at the plasma membrane occurred during down-regulation and was a result of an inhibition of internalization. However, in the rat Leydig cell proteolysis of the LH/CG receptor occurred for the first 4h after isolation, when this was inhibited there was an increase in LH-stimulated cyclic AMP production. This
indicated that the isolation procedure was removing an inhibitory factor that prevented proteolysis occurring in vivo. Proteases also have an important role in steroidogenesis by regulating cholesterol transport. In the mouse Leydig cell LH-stimulated cyclic AMP production requires proteolysis of the LH/CG receptor for activation.
CHAPTER 7

DETERMINATION OF STRUCTURE-FUNCTION-RELATIONSHIPS OF THE LH/CG RECEPTOR USING ANTISENSE OLIGODEOXYNUCLEOTIDES
A novel method to study the mechanisms of LH/CG receptor desensitization and truncation, using antisense oligodeoxynucleotides has been investigated. Characterization of the method was carried out using an antisense oligodeoxynucleotide coding for amino acids 8-14 (Oligo 1) of the NH$_2$-terminal sequence of the LH/CG receptor. Mouse tumour (MA10) Leydig cells were incubated for 48h with the addition of 2.5μM of Oligo 1 at time 0 and 24h. It was found that Oligo 1 completely inhibited synthesis of the LH/CG receptor. We have also localised sites involved in the regulation of the LH/CG receptor using antisense oligodeoxynucleotides coding for the C-terminal sequences for the following amino acid sequences 576-582 (Oligo 2), 611-617 (Oligo 3), 649-655 (Oligo 4) and 666-672 (Oligo 5). Incubation with Oligo 2 and 3 did not alter LH-stimulated cyclic AMP and pregnenolone production, but Oligo 4 resulted in a 50% decrease in cyclic AMP production. Phorbol 12-myristate-13-acetate (PMA) (10$^{-7}$M) and dibutyryl-cyclic AMP (db-cAMP) (1μM) induced desensitization of pregnenolone was prevented in cells incubated with Oligo 2, 3 and 4. LH (3.3nM) and PMA induced cyclic AMP desensitization was prevented with Oligo 2, 3 and 4, but desensitization still occurred with cells treated with Oligo 5. LH induced desensitization of pregnenolone production was not prevented with any of the antisense oligodeoxynucleotides used. All the antisense oligodeoxynucleotides prevented LH and db-cAMP induced loss of surface binding over 2h.

We have also investigated whether high affinity binding proteins can be detected in culture medium of MA10 cells. Proteins detected had molecular
weights of 53,000 (53K), 43,000 (43K) and 33,000 (33K). Incubation of MA10 cells with antisense oligodeoxynucleotides coding for amino acids 8-14 (Oligo 1) and 335-341 (Oligo 6) of the LH/CG receptor N-terminus resulted in a decrease in the expression of these binding proteins, as detected by [125I]-hCG binding. Treating cells with Oligo 6, also resulted in the appearance of a smaller binding protein of molecular weight 23,000 (23K). Incubation with an irrelevant oligodeoxynucleotide and an antisense oligodeoxynucleotide coding for the C-terminus 666-672 (Oligo 5) of the LH/CG receptor had no effect on the expression of the 53K, 43K and 33K binding protein. The rate of expression of these binding proteins was double that derived for the intact receptor. Oligo 1 and Oligo 6 also inhibited the expression of an intact LH/CG receptor at the surface of the MA10 cells. An autoradiograph of [125I]-hCG cross-linked to proteins in solubilized control cells demonstrated that the 43K binding protein could be detected within the cell. A similar experiment with Oligo 6 treated cells demonstrated a decrease in [125I]hCG binding to the intact receptor and 43K binding protein. The 53K and 33K proteins were not detected.

The results clearly showed that desensitization and loss of surface LH/CG receptors was prevented in MA10 cells by the presence of C-terminal antisense oligodeoxynucleotides. Furthermore, the latter did not inhibit the stimulatory effects of LH on cyclic AMP production or steroidogenesis. The results also indicated that the phosphorylation sites and a conformational change in the C-terminal tail and the 7th transmembrane domain were required for the mechanism of LH/CG receptor desensitization. High affinity LH/CG binding proteins were also secreted from the MA10 cell and these proteins were derived from i)
proteolytic cleavage of the intact receptor (43K) or ii) from the mRNA transcript coding for the intact receptor or the mRNA transcript coding only the extracellular domain (i.e 53K and 33K protein).

7.2 Introduction

In this chapter an adaptation of the inhibitory antisense oligodeoxynucleotide strategy is reported. The inhibitory antisense oligodeoxynucleotide strategy has been used to study the effects c-myc (Heikkila et al., 1987), cyclin (Jaskulski et al., 1988) and type IIß regulatory subunit mRNA for cyclic AMP dependent protein kinase (Tortora et al., 1990). The strategy involves incubating cells with the antisense oligodeoxynucleotides. Antisense oligodeoxynucleotides are readily taken up by cells and bound to either the DNA or mRNA. The antisense oligodeoxynucleotides then either prevent the export of mRNA from the nucleus, inhibit the translation of mRNA or the mRNA was cleaved by cellular nucleases at the point of binding of the antisense oligodeoxynucleotide (Weintraub, 1990). These processes may therefore result in the complete inhibition of synthesis of specific proteins and/or generation of truncated proteins.

Continued exposure of Leydig cells to LH leads to a desensitization of cyclic AMP production and steroidogenesis (Chapter 3). In chapter 3 we showed that there were marked species differences in the mechanisms of desensitization and that in addition to desensitization, truncation of the LH/CG receptor occurred,
which resulted in the release of the extracellular fragment of the LH/CG receptor (Chapter 6).

Consensus PKC sites have been reported within the cytoplasmic C-terminal tail of the LH/CG receptor (amino acids 668-670 and 675-677) (Loosfelt et al., 1989), there are also other possible phosphorylation sites at 638-640, 659-661, 674-675 and 679-680, where PKA could act. There is also a consensus PKC site on the third intracellular loop at 560-561 (Loosfelt et al., 1989). Site-directed mutagenesis studies on βAR have shown that deletion of the C-terminal phosphorylation sites lead to a decrease in desensitization of β-adrenergic receptors (for a review see Sibley et al., 1988).

The 1.2kb LH/CG receptor mRNA transcript (Segaloff et al., 1990) codes for the extracellular domain of the LH/CG receptor and could possibly generate a truncated LH/CG binding protein (Xie et al., 1990; Tsai-Morris et al., 1990). Truncated forms of the LH/CG receptor conform to alternative splicing patterns that are consistent either with the deletion of complete exons or alternate acceptor sites within exons. All the donor sites corresponded to known donor and acceptor consensus sequences (Tsai-Morris et al., 1991).

In this chapter we have used antisense oligodeoxynucleotides coding for different regions of the LH/CG receptor to localise regions within the LH/CG receptor that are involved in the mechanisms of desensitization and down-regulation. We have also used the antisense oligodeoxynucleotides to investigate the existence of secreted high affinity LH/CG binding proteins.
7.3 Results.

7.3.1 Characterization of a protocol for the addition of LH/CG receptor antisense oligodeoxynucleotides.

MA10 cells were depleted of surface LH/CG receptors by treating with trypsin, then incubated with and without the antisense oligodeoxynucleotides for 48h in culture. We have previously shown that the LH/CG receptor in MA10 cells were replenished during this time (Chapter 4). Loss of binding sites and desensitization was induced by incubating the cells with LH, PMA or db-cAMP (Chapter 3 and 5). Characterization of the method was carried out using an antisense oligodeoxynucleotide coding for amino acids 8-14 (5′ TGC CCC GAC CCC TGC GAC TGC 3′ - Oligo 1) of the NH₂-terminal sequence of the LH/CG receptor (figure 7.3.1). For all the studies an irrelevant oligo (5′ TCG ATG ATC ATC GTC GAC GAT 3′) with a mixture of all four nucleotides was used as a control.

Oligo 1 was added to MA10 cells over a range of concentration 0-15µM at the time the cells were plated after trypsin treatment (figure 7.3.2) for 48h. At the end of the 48h incubation period the treated cells were assessed for [125I]-hCG binding. The irrelevant oligo was added at a concentration of 20µM. One addition of antisense oligodeoxynucleotide resulted in a 50% loss of binding sites. The irrelevant oligo had no effect. Tables 7.3.1 and 7.3.2 show the effect of the above treatment on cyclic AMP and pregnenolone production. After incubation with the oligos the cells were then either stimulated in the absence or presence of LH.
FIG 7.3.1 The amino acid sequence of the LH/CG receptor. Shows the amino acid sequence derived from the rat ovary LH/CG receptor (McFarland et al., 1989). Filled in circles indicate the sequences coding for the antisense oligodeoxynucleotides used to study the regulation and action of the LH/CG receptor. Also indicated are potential phosphorylation sites, PKC consensus phosphorylation sites, glycosylation sites and the point of divergence between the full length LH/CG receptor mRNA and shorter forms. The alternative amino acids shown in the phosphorylation areas are the differences between the rat (McFarland et al., 1989) and pig (Loosfelt et al., 1989) LH/CG receptors sequences.
LH receptor Structure:

Regions encoded for by the Antisense Oligodeoxynucleotides.
FIG. 7.3.2 The effect of one addition of Oligo 1 at time zero on the level of \([^{125}\text{I}]-\text{hCG}\) binding sites. MA10 cells were plated at a concentration of 2x10^5 cells/well/500μl in 24 well plates and incubated with Oligo 1 or the irrelevant oligo at the concentrations indicated over a 48h incubation period. At 48h the cells were washed with 0.1M PBS and then the level of \([^{125}\text{I}]-\text{hCG}\) binding sites determined as described in Materials and Methods (2.2.10). Values are the mean ± SD of two experiments, replicates = 3.
### TABLE 7.3.1

The effect of one addition of Oligo 1 at time zero on LH-stimulated cyclic AMP. MA10 cells were plated at a concentration of $2 \times 10^5$ cells/well/500µl in 24 well plates and incubated with Oligo 1 or the irrelevant oligo at the concentrations indicated over a 48h incubation period. At 48h the cells were washed with 0.1M PBS and then incubated for a further 2h in the absence or presence of LH (3.3nM). Cyclic AMP was determined as described in Materials and Methods (2.2.17). Values are the mean ± SD of two experiments, replicates = 3.

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>Basal</th>
<th>LH (3.3nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irrelevant 20</td>
<td>12.4 ± 6.1</td>
<td>372 ± 43.0</td>
</tr>
<tr>
<td>0</td>
<td>13.9 ± 0</td>
<td>428 ± 31.0</td>
</tr>
<tr>
<td>2</td>
<td>1.8 ± 1.9</td>
<td>400 ± 38.9</td>
</tr>
<tr>
<td>5</td>
<td>6.7 ± 3.4</td>
<td>453 ± 26.8</td>
</tr>
<tr>
<td>10</td>
<td>3.4 ± 2.4</td>
<td>437 ± 31.0</td>
</tr>
<tr>
<td>15</td>
<td>4.6 ± 1.9</td>
<td>411 ± 29.0</td>
</tr>
</tbody>
</table>
TABLE 7.3.2  The effect of one addition of Oligo 1 at time zero on LH-stimulated pregnenolone production. MA10 cells were plated at a concentration of $2\times10^5$ cells/well/500μl in 24 well plates and incubated with Oligo 1 or the irrelevant oligo at the concentrations indicated over a 48h incubation period. At 48h the cells were washed with 0.1M PBS and then incubated for a further 2h in the absence or presence of LH (3.3nM). Pregnenolone was determined as described in Materials and Methods (2.2.18). Values are the mean ± SD of two experiments, replicates = 3.
FIG. 7.3.3  The effect of two additions of Oligo 1 added at time zero and 24h on the level of [125I]-hCG binding sites. MA10 cells were plated at a concentration of 2x10^5 cells/well/500μl in 24 well plates and incubated with Oligo 1 or the irrelevant oligo at the concentrations indicated over a 48h incubation period added at time 0 and 24h. At 48h the cells were washed with 0.1M PBS and then the level of [125I]-hCG binding sites determined as described in Materials and Methods (2.2.10). Values are the mean ± SD of two experiments, replicates = 3.
FIG. 7.3.4  The effect of two additions of Oligo 1 added at time zero and 24h on LH-stimulated cyclic AMP. MA10 cells were plated at a concentration of $2 \times 10^5$ cells/well/500μl in 24 well plates and incubated with Oligo 1 or the irrelevant oligo at the concentrations indicated over a 48h incubation period added at time 0 and 24h. At 48h the cells were washed with 0.1M PBS and then incubated for a further 2h in the absence or presence of LH (3.3nM). Cyclic AMP was determined as described in Materials and Methods (2.2.17). Values are the mean ± SD of two experiments, replicates = 3.
FIG. 7.3.5  The effect of two additions of Oligo 1 added at time zero and 24h on LH-stimulated pregnenolone. MA10 cells were plated at a concentration of $2 \times 10^5$ cells/well/500 µl in 24 well plates and incubated with Oligo 1 or the irrelevant oligo at the concentrations indicated over a 48h incubation period added at time 0 and 24h. At 48h the cells were washed with 0.1M PBS and then incubated for a further 2h in the absence or presence of LH (3.3nM). Pregnenolone was determined as described in Materials and Methods (2.2.18). Values are the mean ± SD of two experiments, replicates = 3.
(3.3nM). No effect on LH-stimulated cyclic AMP (table 7.3.1) or pregnenolone (table 7.3.2) production was observed.

The effect of adding two doses of Oligo 1 or the irrelevant oligo at time 0 or 24h was also investigated. Figure 7.3.3 shows the profile obtained for $[^{125}I]$-hCG binding. Oligo 1 was added at the concentrations 2.5μM, 5μM and 10μM and the irrelevant oligo was added at 10μM. The addition of Oligo 1 at all concentrations caused >98% loss of $[^{125}I]$-hCG binding. The irrelevant oligo had no effect. Cells treated as above were also assessed for cyclic AMP (figure 7.3.4) and pregnenolone production (figure 7.3.5). A complete loss of LH-stimulated cyclic AMP and pregnenolone production was observed. Incubations with the irrelevant oligo had no effect on LH-stimulated cyclic AMP or pregnenolone.

7.3.2 The effect of antisense oligodeoxynucleotides on the level of LH/CGR receptors and LH-stimulated cyclic AMP and pregnenolone production.

Having established a procedure that deletes >98% of LH/CGR receptors the effect of an antisense oligodeoxynucleotide which would interact with the LH/CGR receptor mRNA sequences encoding LH/CGR receptor was investigated. The sites involved in the regulation of the LH/CGR receptor were localized using antisense oligodeoxynucleotides coding for the C-terminal sequences for the following amino acid sequences 576-582 (5' CTT ATC ACT GTC ACC AAC TCG 3' - Oligo 2), 611-617 (5'GAT TTC CTT CTG CTG CTG AGC 3' - Oligo 3), 649-655 (5' AGT AAG CCG TCC CAG GCT ACC 3' - Oligo 4) and 666-672 (5' CCC ATA CCA CCG AGA
FIG 7.3.6 The theoretical deletions caused by the antisense oligodeoxynucleotides encoding regions of the C-terminus of the LH/CG receptor. Filled in circles indicate the sequences coding for the antisense oligodeoxynucleotides used to study the regulation and action of the LH/CG receptor. Also indicated are potential phosphorylation sites, PKC consensus phosphorylation sites, glycosylation sites and the point of divergence between the full length LH/CG receptor mRNA and shorter forms.
LH receptor Structure:
Deletion caused by OLIGO 2
**LH receptor Structure:**
Deletion caused by OLIGO 4

![Diagram of LH receptor Structure: Deletion caused by OLIGO 4](image)

**LH receptor Structure:**
Deletion caused by OLIGO 5

![Diagram of LH receptor Structure: Deletion caused by OLIGO 5](image)
GCG TTA 3' - Oligo 5) (figure 7.3.1). The theoretical deletions that would be caused by these antisense oligodeoxynucleotides are shown in figure 7.3.6. Oligo 2 would delete the 7th transmembrane domain and intracellular tail; Oligo 3 would delete only the intracellular tail; Oligo 4 would delete the consensus protein kinase C sites and other potential phosphorylation sites (Loosfelt et al., 1989); Oligo 5 would delete the final nine amino acids of the C-terminal intracellular tail.

Tables 7.3.3, 7.3.4 and 7.3.5 show the effect incubating cells with the above antisense oligos on [125I]-hCG binding sites (table 7.3.3), LH-stimulated cyclic AMP production (table 7.3.4) and LH-stimulated pregnenolone production (table 7.3.5). There was no effect on the levels of binding sites observed in cells treated with the oligos. Oligos 2 and 3 had no effect on LH-stimulated cyclic AMP production, however, cells treated with Oligo 4 demonstrated a 50% inhibition of LH-stimulated cyclic AMP production. Cells treated with the oligos 2, 3, 4 and 5 demonstrated no change in LH- or db-cAMP-stimulated pregnenolone production.

7.3.3  

The effect of antisense oligodeoxynucleotides on LH/CG receptor desensitization and down-regulation.

To cause desensitization of cyclic AMP and pregnenolone in MA10 cells the cells were incubated for 2h in the absence or presence of either LH (3.3nM), db-cAMP (1mM) or PMA (10^-7). Cells were acid washed with 50mM glycine buffered saline (pH 3.0) (Ascoli, 1982) to remove bound LH and 3 times with media to
TABLE 7.3.3  The effect of adding the LH/CG receptor antisence oligodeoxynucleotides on the concentration of $[^{125}\text{I}]-\text{hCG}$ binding sites. MA10 cells were plated at a concentration of $2 \times 10^5$ cells/well/500μl in 24 well plates and incubated with 2.5μM antisense oligodeoxynucleotide over a 48h incubation period. Cells were either incubated in the absence of oligodeoxynucleotides or in the presence of Oligo 2, 3, 4 and 5 added at time 0 and 24h. At 48h the concentration of $[^{125}\text{I}]-\text{hCG}$ binding sites was determined. Values are the mean ± SD of two experiments, replicates = 3.
<table>
<thead>
<tr>
<th>Oligo</th>
<th>Basal</th>
<th>LH (0.33nM)</th>
<th>LH (3.3nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10.65 ± 1.6</td>
<td>153 ± 12.6</td>
<td>215.7 ± 21.2</td>
</tr>
<tr>
<td>2</td>
<td>15.6 ± 3.6</td>
<td>150 ± 8.1</td>
<td>243.0 ± 43.9</td>
</tr>
<tr>
<td>3</td>
<td>18.9 ± 8.6</td>
<td>147 ± 13.6</td>
<td>238.5 ± 21.9</td>
</tr>
<tr>
<td>4</td>
<td>12.85 ± 0.9</td>
<td>77.9 ± 4.7 *</td>
<td>124.6 ± 16.8 *</td>
</tr>
</tbody>
</table>

**TABLE 7.3.4** The effect of adding the LH/CG receptor antisense oligodeoxynucleotides on LH-stimulated cyclic AMP production. MA10 cells were plated at a concentration of 2x10⁵ cells/well/500μl in 24 well plates and incubated with 2.5μM antisense oligodeoxynucleotide over a 48h incubation period. Cells were either incubated in the absence of oligodeoxynucleotides or in the presence of Oligos 2, 3, and 4 added at time 0 and 24h. At 48h the cells were washed with 0.1M PBS and then incubated in the presence of LH (0.33nM and 3.3nM) and cyclic AMP was measured as described in Materials and Methods (2.2.17). Values are the mean ± SD of two experiments, replicates = 3, *p<0.05.
<table>
<thead>
<tr>
<th>Oligo</th>
<th>Basal</th>
<th>LH (0.33)</th>
<th>LH (3.3nM)</th>
<th>db-cAMP (1mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>75.4 ± 9.2</td>
<td>825 ± 9.8</td>
<td>1103 ± 21.9</td>
<td>1050 ± 56.9</td>
</tr>
<tr>
<td>2</td>
<td>88.6 ± 9.2</td>
<td>1018 ± 9.4</td>
<td>1242 ± 97</td>
<td>1094 ± 97</td>
</tr>
<tr>
<td>3</td>
<td>85.0 ± 0.7</td>
<td>986 ± 26.3</td>
<td>1179 ± 97</td>
<td>1124 ± 150</td>
</tr>
<tr>
<td>4</td>
<td>69.5 ± 6.3</td>
<td>1072 ± 77</td>
<td>1140 ± 65</td>
<td>1053 ± 100</td>
</tr>
<tr>
<td>5</td>
<td>92.5 ± 16.2</td>
<td>900 ± 12.5</td>
<td>1066 ± 69.2</td>
<td>1071 ± 11.3</td>
</tr>
</tbody>
</table>

**TABLE 7.3.5** *The effect of adding the LH/CG receptor antisense oligodeoxynucleotides on LH-stimulated pregnenolone production.*

MA10 cells were plated at a concentration of 2x10⁵ cells/well/500μl in 24 well plates and incubated with 2.5μM antisense oligodeoxynucleotide over a 48h incubation period. Cells were either incubated in the absence of oligodeoxynucleotides or in the presence of Oligos 2, 3, and 4 added at time 0 and 24h. At 48h the cells were washed with 0.1M PBS and then incubated in the presence of LH (0.33nM and 3.3nM) and pregnenolone was measured as described in Materials and Methods (2.2.18). Values are the mean ± SD of two experiments, replicates = 3.
remove excess db-cAMP and PMA. The cells were further incubated for 2h in the absence or presence of LH (3.3nM).

Figure 7.3.6 shows the profiles obtained for cyclic AMP production in control cells (figure 7.3.7 A) and cells pretreated with Oligo 2 (figure 7.3.7 B), Oligo 3 (figure 7.3.7 C), and Oligo 4 (figure 7.3.7 D). In control cells preincubations with LH (3.3nM) and PMA (10^-7 M) caused a complete loss of LH (3.3nM) stimulated cyclic AMP (figure 7.3.7 A). In cells treated with Oligo 2 and 3 there was a complete recovery of LH and PMA induced desensitization of cyclic AMP. Preincubation with Oligo 4 resulted in a 50% reduction in LH-stimulated cyclic AMP as was reported above. However, no further loss of stimulated cyclic AMP production was observed after pretreatment with LH or PMA (figure 7.3.7 D).

Figure 7.3.8 shows the profiles obtained for pregnenolone production in control cells (figure 7.3.8 A), and cells pretreated with Oligo 2 (figure 7.3.8 B), Oligo 3 (figure 7.3.8 C), and Oligo 4 (figure 7.3.8 D). In control cells preincubations with LH (3.3nM), db-cAMP (1mM) or PMA (10^-7 M) caused a loss of LH (3.3nM) stimulated pregnenolone (figure 7.3.8 A). In cells treated with Oligo 2, 3 and 4, PMA and db-cAMP induced desensitization was prevented whereas LH-induced desensitization was not (figure 7.3.8 B, C and D).

To demonstrate that the above observations were due to a direct interaction of the antisense oligodeoxynucleotide with a functional region of the LH receptor nucleotide sequence, MA10 cells were also treated with an antisense oligodeoxynucleotide encoding for 7 amino acids at the end of the C-terminus of the LH/CG receptor. The results are shown in figure 7.3.9. The desensitization caused by LH, db-cAMP or PMA was not prevented by this oligodeoxynucleotide.
FIG. 7.3.7  *The effect of antisense oligodeoxynucleotides 2, 3 and 4 on cyclic AMP desensitization.* MA10 cells were incubated for 48h in the absence and presence of either 2.5μM Oligo 2, 3 or 4 added at time zero and 24h. After treatment with antisense oligodeoxynucleotides, MA10 cells were then further incubated for 2h without or with LH (3.3nM) and PMA (10^-7M). PMA was dissolved in DMSO at a concentration of 10^-2M and diluted to the concentration required in medium. After 2h the cells were placed on ice and washed twice with 50mM glycine-buffered saline (pH 3.0) and three times with medium. The cells were then incubated in fresh medium for 2h in the absence or presence of 3.3nM LH. The reactions were stopped using perchloric acid. Cyclic AMP was determined as described in Materials and Methods (2.2.17). Results are the mean ± S.D. Statistical analysis was by the paired student t-test (*P<0.01), n=2, replicates = 3.
Cyclic AMP (pmol/10^6 cells/2h)

A: CONTROL

- CONTROL
- LH (3.3 nM)
- PMA (10^-7 M)

B: OLIGO 2

- CONTROL
- LH (3.3 nM)
- PMA (10^-7 M)
C: OLIGO 3

D: OLIGO 4
FIG. 7.3.8  The effect of antisense oligodeoxynucleotides 2, 3 and 4 on desensitization of pregnenolone production. MA10 cells were incubated for 48h in the absence and presence of either 2.5µM Oligo 2, 3 or 4 added at time zero and 24h. After treatment with antisense oligodeoxynucleotides, MA10 cells were then further incubated for 30min with inhibitors of pregnenolone metabolism before being incubated for 2h either without or with LH (3.3nM), db-cAMP (1mM) or PMA (10⁻⁷M). PMA was dissolved in DMSO at a concentration of 10⁻²M and diluted to the concentration required in medium. After 2h the cells were placed on ice and washed twice with 50mM glycine-buffered saline (pH 3.0) and three times with media. The cells were then incubated in fresh medium for 2h in the absence or presence of 3.3nM LH. The reactions were stopped using perchloric acid. Pregnenolone was determined as described in Materials and Methods (2.2.18). Results are the mean ± S.D.  Statistical analysis was by the paired student t-test (*P<0.01), n=2, replicates = 3.
A: CONTROL

B: OLIGO 2

PREGNENOLONE (ng/10^6 cells/2h)
C: OLIGO 3

PREGNENOLONE (ng/10^6 cells/2h)

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>LH (3.3nM)</th>
<th>db-cAMP (1mM)</th>
<th>PMA (10^-7 M)</th>
</tr>
</thead>
</table>

D: OLIGO 4

PREGNENOLONE (ng/10^6 cells/2h)

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>LH (3.3nM)</th>
<th>db-cAMP (1mM)</th>
<th>PMA (10^-7 M)</th>
</tr>
</thead>
</table>

BASAL
LH (3.3nM)
FIG. 7.3.9  The effect of antisense oligodeoxynucleotide 5 on desensitization of pregnenolone production. MA10 cells were incubated for 48h in the absence and presence of 2.5μM oligo 5 added at time zero and 24h. After treatment with the antisense oligodeoxynucleotide, MA10 cells were then further incubated for 30min with inhibitors of pregnenolone metabolism before being incubated for 2h either without or with LH (3.3nM), db-cAMP (1mM) or PMA (10^{-7}M). PMA was dissolved in DMSO at a concentration of 10^{-2}M and diluted to the concentration required in medium. After 2h the cells were placed on ice and washed twice with 50mM glycine-buffered saline (pH 3.0) and three times with medium. The cells were then incubated in fresh medium for 2h in the absence or presence of 3.3nM LH. The reactions were stopped using perchloric acid. Pregnenolone was determined as described in Materials and Methods (2.2.18). Results are the mean ± S.D. Statistical analysis was by the paired student t-test (*P<0.01), n=2, replicates = 3.
A: CONTROL

B: ANTISENSE
FIG. 7.3.10  The effect of antisense oligodeoxynucleotides 2, 3, 4, and 5 on receptor regulation by LH and db-cAMP. MA10 cells were plated at a concentration of $2 \times 10^5$ cells/well/500μl in 24 well plates and incubated in the absence or presence of 2.5μM antisense oligodeoxynucleotide 2, 3, 4 or 5 over a 48h incubation period with additions at time 0 and 24h. At 48h the cells were washed with medium and further incubated for 2h in the absence or presence of either 3.3nM LH, or 1mM db-cAMP. Before the concentration of $[^{125}]$-hCG binding sites was determined, cells were washed with 50mM glycine buffered saline (pH 3.0) to remove bound LH and 3 times with media. Statistical analysis was by the student t-test, *$P<0.01$. Values are the mean ± SD of two experiments, replicates = 6.
Previously we have reported in chapter 5, that LH (3.3nM) and db-cAMP (1mM) can cause a loss of surface LH/CG receptors over 2h. MA10 cells were pretreated for 48h in the absence or presence of oligos 2, 3, 4, and 5. The cells were then incubated for 2h with LH (3.3nM) or db-cAMP (1mM) before $[^{125}\text{I}]-hCG$ binding sites were assessed at 4°C (figure 7.3.10). In control cells there was a 50% loss in surface binding. All the antisense oligodeoxynucleotides prevented the loss of binding when added to the MA10 cells.

7.3.4 *Demonstration of secreted high affinity LH/CG binding sites using antisense oligodeoxynucleotides.*

Data for binding of $[^{125}\text{I}]-hCG$ in concentrated medium or solubilized cells is expressed with respect to the initial number of cells from which they were derived. This is to enable a comparison to surface binding.

Figure 7.3.11 shows control cell Scatchard plots for $[^{125}\text{I}]-hCG$ binding derived from culture medium of MA10 cells collected during the last 24h of the 48h incubation (figure 7.3.11 A) and cell surface binding (figure 7.3.11 B). In the medium (figure 7.3.11 A) of MA10 cells we detected binding for $[^{125}\text{I}]-hCG$ with a dissociation constant ($K_D$) of 3.66 ± 0.47 nM - suggesting the existence of a high affinity binding protein. The Hill coefficient (0.999) showed that this was a single binding site. The Bmax obtained for this protein (16,259 ± 1,089 binding sites/cell/24h) indicated that it appears in the medium at a rate of approximately
600 binding sites/cell/h. The surface binding detected was comparable to results previously reported (Chapter 5) with a $K_D$ of $3.75 \pm 0.96$ nM.

We have further investigated the existence of binding proteins released into the culture medium of MA10 cells, using antisense oligodeoxynucleotides encoding for different regions of the LH/CG receptor. We have used 3 antisense oligodeoxynucleotides: Oligo 1 coding for amino acids 8-14 of the NH$_2$-terminal of the LH/CG receptor extracellular domain; Oligo 5 coding for the amino acids 666-672 of the C-terminal intracellular tail; and Oligo 6 coding for amino acids 335-341 (5' ATG GGC TAT GCC TTC CTT AGG 3') of the extracellular domain prior to the first transmembrane domain (figure 7.3.12). The irrelevant oligo with a mixture of the 4 nucleotides was used as a control. MA10 cells were treated with 0.05% (w/v) trypsin/0.53mM EDTA for 2 min to remove cell surface LH/CG receptors. The cells were then incubated over a 48h incubation period in the absence or presence of 2.5μM antisense oligodeoxynucleotide added at time zero and 24h.

Table 7.3.6 shows the Scatchard analysis for surface binding of cells treated with the antisense oligos. We found that the antisense oligos encoding for regions in the extracellular domain (Oligo 1 and Oligo 6) prevented the expression of binding at the surface of MA10 cells. The irrelevant oligo and Oligo 5 had no effect on surface binding. The $K_D$ for these binding sites was comparable to control cells (figure 7.3.11 B).

Medium collected over the final 24h of the 48h incubation with antisense oligos was also assessed for binding of $[^{125}\text{I}]$-hCG (table 7.3.7). No effect on the level of binding sites in the culture medium obtained from cells treated with Oligo 5 or the irrelevant oligo was observed. However, a decrease was observed in
FIG. 7.3.11 Scatchard analysis of $[^{125}I]$-hCG binding in the culture medium and on the surface of MA10 cells. MA10 cells were plated at a density of $3 \times 10^6$ cells/flask and used 48h later. The medium was changed every 24h. The final collection of medium was retained for analysis of $[^{125}I]$-hCG binding (A). The medium was concentrated and an equivalent volume of medium derived from $5 \times 10^5$ cells was used in each incubation. Medium was incubated in 1ml solubilization buffer in the presence of 0.28nM $[^{125}I]$-hCG and increasing concentrations of cold hCG (0-0.7nM). Non-specific binding was determined using 11.2nM pure hCG. Surface binding (B) was assessed by incubating $1 \times 10^6$ cells/well/1ml of medium with 0.28nM $[^{125}I]$hCG and increasing concentrations of pure hCG (0-0.7nM). Non-specific binding was determined with 11.2nM pure hCG and incubated overnight at 4°C. Cells were then washed twice with 0.01M PBS before washing for 2min. with 50mM glycine-buffered saline (pH 3.0) on ice. The acid wash was then counted in a gamma counter. Results are the mean ± SD of two determinations.
A: Control cell medium binding

\[ K_D = 3.66 \pm 0.47 \text{ nM} \]

\[ B_{\text{max}} = 16,259 \pm 1,089 \]

Binding sites/cell/24h

B: Control cell surface binding

\[ K_D = 3.75 \pm 0.96 \text{ nM} \]

\[ B_{\text{max}} = 18,066 \pm 357 \]

Binding sites/cell
FIG. 7.3.12  The amino acid sequence of the LH/CG receptor. Shows the amino acid sequence derived from the rat ovary LH/CG receptor (McFarland et al., 1989). Filled in circles indicate the sequences coding for the antisense oligodeoxynucleotides used to study the regulation and action of the LH/CG receptor. Also indicated are glycosylation sites and the point of divergence between the full length LH/CG receptor mRNA and shorter forms.
the $K_D$ for medium binding with Oligo 5 treated cells. The $K_D$ still indicated the presence of a high affinity binding site, therefore no difference in biological activity would be expected. Treatment of cells with Oligo 1 or 6 resulted in a loss of binding in the medium of these cells.

Figure 7.3.13 shows an autoradiograph of $[^{125}\text{I}]-\text{hCG}$ cross-linked to proteins in concentrated medium derived from cells treated as above with the antisense oligos. Aliquots with equivalent amounts of protein were incubated with 0.14nM $[^{125}\text{I}]-\text{hCG}$ and DSS (final conc. 1mM) overnight at 4°C. 30μl of medium was then added to 30μl of Laemmli's buffer (Laemmli, 1970) and run on a 10% polyacrylamide gel. Gels were exposed for 2 days with autoradiographic film and intensifying screens at -70°C. In control cells (figure 7.3.13 lane 1) and irrelevant oligo treated cells (figure 7.3.13 lane 2) we observed bands of approx. $M_r$ 200,000, 90,000 and 80,000. $[^{125}\text{I}]-\text{hCG}$ incubated in the presence of DSS, overnight at 4°C, in 100μl of medium ran as a band of $M_r$ 47,000 (figure 7.3.13 lane 6). Taking $[^{125}\text{I}]-\text{hCG}$ as having a molecular weight of 47,000, therefore, indicated that the 90,000 and 80,000 $M_r$ bands would generate proteins of $M_r$ 43,000 (43K) and 33,000 (33K) respectively. The 200,000 $M_r$ band would involve a protein of $M_r$ of 153,000. This protein could therefore be a dimerized form of the binding proteins, i.e. a protein of $M_r$ 53,000 (53K). Treatment with Oligo 1 (figure 7.3.13 lane 3) or 6 (figure 7.3.13 lane 5) caused a decrease in the intensity of the above bands as compared to control cells. An additional band of $M_r$ 70,000 was observed in the medium of cells treated with Oligo 6 (figure 7.3.13 lane 5), i.e. involving a protein of $M_r$ 23,000. The cut off limit for the protein concentrator used was 30,000 $M_r$, therefore the loss of binding may be due to a loss in the 23K protein, rather than
TABLE 7.3.6  

<table>
<thead>
<tr>
<th></th>
<th>K_D (nM)</th>
<th>Bmax</th>
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<tbody>
<tr>
<td></td>
<td>(binding sites/10^6 cells)</td>
<td></td>
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<tr>
<td>Irrelevant</td>
<td>2.56 ± 0.87</td>
<td>18,453 ± 4,132</td>
</tr>
<tr>
<td>Oligo 1</td>
<td>no fit</td>
<td>no fit</td>
</tr>
<tr>
<td>Oligo 5</td>
<td>3.98 ± 0.34</td>
<td>19,143 ± 2,457</td>
</tr>
<tr>
<td>Oligo 6</td>
<td>no fit</td>
<td>no fit</td>
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The effect of antisense oligodeoxynucleotides on the surface expression of LH/CG receptors. MA10 cells were plated at a density of 1x10^6 cells/well and incubated for 48h either in the presence of Oligo 1, 5, 6 or the irrelevant oligo. Surface binding was assessed by incubating cells in 1ml of medium with 0.28nM [¹²⁵I]-hCG and increasing concentrations of pure hCG (0-0.7nM). Non-specific binding was determined with 11.2nM pure hCG and incubated overnight at 4°C. Cells were then washed twice with 0.01M PBS before washing for 2min with 50mM glycine-buffered saline (pH 3.0) on ice. The acid wash was then counted in a gamma counter. Results are the mean ± SD of two determinations.
TABLE 7.3.7 Scatchard analysis of [125I]-hCG binding in the culture medium of antisense treated MA10 cells. MA10 cells were plated at a density of 3x10^6 cells/flask and incubated for 48h either in the presence of Oligo 1, 5, 6 or the irrelevant oligo. The medium was changed every 24h. The final collection of medium was retained for analysis of [125I]-hCG binding. The medium was concentrated and an equivalent volume of medium derived from 5x10^5 cells was used in each incubation. Medium was incubated in 1ml solubilization buffer in the presence of 0.28nM [125I]-hCG and increasing concentrations of pure hCG (0-0.7nM). Non-specific binding was determined using 11.2nM pure hCG. Scatchard analysis was assessed as described in Materials and Methods (2.2.11). Results are the mean ± SD of two determinations.
FIG. 7.3.13 An autoradiograph of $[^{125}\text{I}]$-hCG cross-linked to proteins derived from the culture medium of MA10 cells. MA10 cells at a density of $1 \times 10^7$ cells/flask were incubated for 48h either in the absence (lane 1) or presence of Oligo 1 (lane 3), 5 (lane 4), 6 (lane 5) or the irrelevant oligo (lane 2). The medium was changed after 24h. The medium collected in the second 24h was retained and concentrated. The resultant concentrates were then incubated with $[^{125}\text{I}]$-hCG and DSS and run on a 10% polyacrylamide gel. Autoradiographic exposure was for 2 days with intensifying screens. $[^{125}\text{I}]$-hCG (lane 6) was incubated with DSS overnight at 4°C in $100\mu\text{l}$ of medium.

Equal concentrations of concentrated sample protein was run under reducing conditions on SDS-Page.
an inhibition of synthesis. There was no effect on the appearance of the three larger molecular weight bands in the medium of cells treated with Oligo 5 (figure 7.3.13 lane 4).

We also investigated the effect of Oligo 6 on the formation of the LH/CG receptor, by studying $[^{125}\text{I}]-\text{hCG}$ binding profiles in solubilized cells. Figure 7.3.14 shows the Scatchard analysis of $[^{125}\text{I}]-\text{hCG}$ binding in control solubilized cells (figure 7.3.14 A) and Oligo 6 treated solubilized cells (figure 7.3.14 B). Cells were solubilized at the end of the 48h incubation period with solubilization buffer. High affinity binding sites were detected in both control and Oligo 6 treated cells, however, the Bmax for cells treated with Oligo 6 was 10 fold lower than those for control cells. Solubilized cells were also incubated with $[^{125}\text{I}]-\text{hCG}$ and DSS (figure 7.3.15). In control cells (figure 7.3.15 lane 3) we detected three bands of $M_r$ 300,000, 140,000 and 90,000. The 140,000 $M_r$ band corresponds to the intact receptor with $[^{125}\text{I}]-\text{hCG}$ bound ($M_r$ 47,000), i.e. 93,000 $M_r$. The 300,000 $M_r$ band may be due to dimerized LH/CG receptors. The 90,000 $M_r$ band would generate a binding protein of the same $M_r$ as the 43K protein detected in the culture medium of MA10 cells (figure 7.3.13). The intensity of the 90,000 $M_r$ band is far greater than the other two. Oligo 6 treated cells (figure 7.3.15 lane 4) expressed the 300,000, 140,000 and 90,000 $M_r$ bands with a lower intensity to those from control cells. The autoradiograph also shows $[^{125}\text{I}]-\text{hCG}$ cross-linking to proteins in medium collected over 48h. In lane 1 (control) and 2 (oligo 6) we see that a band of approx. $M_r$ 90,000 is present with equal intensity. This is in contrast to the results shown in figure 7.3.13. The lack of detection of the 53K and 33K proteins may indicate that they are synthesized after the cells have been in culture.
FIG. 7.3.14  Scatchard analysis of $[^{125}\text{i}]$-hCG binding in solubilized MA10 cells.

MA10 cells at a density of $1 \times 10^7$ cells/flask were incubated for 48h in the absence (A) or presence of Oligo 6 (B). Cells were solubilized and an equivalent volume of medium derived from $5 \times 10^5$ cells was used in each incubation. Solubilized cells were incubated in 1ml solubilization buffer in the presence of 0.28nM $[^{125}\text{i}]$-hCG and increasing concentrations of pure hCG (0-0.7nM). Non-specific binding was determined using 11.2nM pure hCG. Scatchard analysis was assessed as described in Materials and Methods (2.2.11). Results are the mean ± SD of two determinations.
A: Control solubilized cell binding

\[ K_D = 0.1 \pm 0.02 \text{ nM} \]
\[ B_{\text{max}} = 32,518 \pm 1,535 \text{ Binding sites/cell} \]

B: Oligo 6 treated solubilized cell binding

\[ K_D = 0.33 \pm 0.02 \text{ nM} \]
\[ B_{\text{max}} = 3,613 \pm 338 \text{ Binding sites/cell} \]
FIG. 7.3.15 An autoradiograph of $[^{125}\text{I}]$-hCG cross-linked to proteins of solubilized cells and culture medium derived from cells treated with Oligo 6. MA10 cells at a density of $3 \times 10^6$ cells/flask were incubated for 48h either in the absence (lanes 1 and 3) or presence of Oligo 6 (lanes 2 and 4). Medium collected over 48h was retained and concentrated. The cells were then scrape from the culture flasks in 1ml of solubilization buffer. The resultant solubilized cells (lanes 3 and 4) and medium concentrates (lanes 1 and 2) were then incubated with $[^{125}\text{I}]$-hCG and DSS as indicated and run on a 7% polyacrylamide gel. Autoradiographic exposure was for 2 days with intensifying screens. Standard $[^{125}\text{I}]$-hCG (lane 5) was incubated with DSS overnight at 4°C in 100μl of medium.
for 24h. The concentration of the 43K protein may therefore, be in excess in the 48h collection of medium. Detection of the 43K protein in medium collected over 48h from cells treated with Oligo 6, indicated that the effect of antisense oligodeoxynucleotides is not apparent till after 24h of incubation.

7.4 Discussion

We have shown that the synthesis of LH/CG receptors was inhibited by >98% using an antisense oligodeoxynucleotide coding for the NH2-terminus (Oligo 1) of the LH/CG receptor. We also clearly showed that desensitization and loss of surface LH/CG receptors was prevented using MA10 cells that have been incubated in the presence of the C-terminal antisense oligodeoxynucleotides. Furthermore, we show that the latter did not inhibit the stimulatory effects of LH on cyclic AMP production and steroidogenesis. We have also established that the MA10 cell secretes high affinity LH/CG binding proteins that are derived from the translation of the mRNA coding for the intact LH/CG receptor protein or the mRNA coding for the extracellular domain of the LH/CG receptor (Segaloff et al., 1990). The size of the proteins detected were of approx. Mr of 53,000 (53K), 43,000 (43K) and 33,000 (33K).

Studies using Oligo 1 demonstrated that though the addition of one dose resulted in a 50% loss of LH/CG receptors, LH-stimulated cyclic AMP still occurred. This supported early observations that LH dose not have to occupy all
the available LH/CG receptors to give maximal stimulation (Catt and Dufau, 1973).

An irrelevant oligodeoxynucleotide containing a mixture of all four nucleotides at
every position had no effect on the regulation or synthesis of LH/CG receptors.

We have used 4 antisense oligodeoxynucleotides encoding the amino acids
576-582 (oligo 2), 611-617 (oligo 3), 649-655 (oligo 4) and 666-672 (oligo 5) to
localise the regions of the LH/CG receptor involved in desensitization and down-
regulation. Deletion of the 7th transmembrane domain and C-terminal
intracellular tail with oligo 2 or 3 (figure 7.3.12) resulted in no effect on the
expression of the LH/CG receptor at the surface of the MA10 cells or on LH-
stimulated cyclic AMP and pregnenolone production. This indicated that the 7th
transmembrane domain and intracellular tail of the LH/CG receptor are not
required for the activation of the $G_s$-protein. However, treatment with oligo 2 and
3 prevented desensitization of cyclic AMP and pregnenolone, indicating that the
7th transmembrane and intracellular tail are involved in the mechanism of
desensitization. Deletion of only the PKC consensus sites in the C-terminal
intracellular tail, using oligo 4 (figure 7.3.12) again had no effect on the expression
of surface receptors, but resulted in a 50% decrease in LH-stimulated cyclic AMP
production. This indicated that receptors were being expressed in a desensitized
state and that the conformation of the 7th transmembrane and intracellular tail
was important in the mechanism of desensitization. Further desensitization of
cyclic AMP was not observed in cells pretreated with oligo 4.

LH-induced desensitization of pregnenolone production was not prevented by
treatment with any of the antisense oligodeoxynucleotides, indicating that
desensitization of steroidogenesis by LH involves further modification of the

301
LH/CG receptor and/or lesions in post receptor events; these are apparently not regulated by either cyclic AMP or PKC.

Pretreatment with oligo 5 did not affect LH-, db-cAMP- or PMA-induced desensitization of pregnenolone. Receptors in these cells contained the potential PKC phosphorylation sites and other phosphorylation sites in this area (figure 7.3.12). This indicates that the phosphorylation sites may be involved in generating the conformational changes in the receptor required for the cyclic AMP- and PMA-dependent mechanisms of desensitization of cyclic AMP production and steroidogenesis.

All of the oligodeoxynucleotides used resulted in an inhibition of LH- and db-cAMP-induced loss of surface LH/CG receptors. This indicated that the final nine amino acids in the C-terminus intracellular tail were crucial for this mechanism. The final nine amino acids may code for an internalization sequence or interact with another protein which may be involved in inhibiting internalization to allow for LH-induced loss of receptors by proteolytic cleavage (Chapter 5 and 6). These results also showed that desensitization of LH/CG receptors is not linked to the down-regulation of LH/CG receptors as previously proposed (Freeman and Ascoli, 1978).

The extracellular domain of the LH/CG receptor has a $M_r$ of 56,000, of which 18,000 is due to glycosylation. Deglycosylation of the extracellular domain would result in a $M_r$ of 38,000. Identified cDNA for potential soluble LH/CG binding proteins are derived from exons 1-9 of the LH/CG receptor gene (Tsai-Morris et al., 1991) and contain 307-316 amino acids. The potential soluble LH/CG binding proteins, would therefore have a $M_r$ of 43,000-44,000. This estimation of the
molecular weight assumes that all the potential glycosylation sites are occupied. Exon 10, which is not present in the secreted forms, contains three sites for glycosylation, therefore the degree of glycosylation would be equivalent to $M_r = 9,000$ (assuming 18,000 $M_r$ for the native LH/CG receptor). Our present data therefore suggested that the 43K and 33K secreted binding proteins may be derived from one of these isolated cDNA sequences.

The appearance of a band of $M_r$ 200,000 may indicate that in the presence of $[^{125}I]$-hCG and the cross-linking reagent DSS, dimerization may have occurred. Dimerized proteins may involve interaction between two binding protein molecules and either 1 or 2 hCG molecules. This would generate proteins of approx. $M_r$ 76,000 (76K) or 53,000 (53K).

The use of antisense oligodeoxynucleotides encoding for amino acids 8-14 (Oligo 1) of the extracellular domain inhibited the expression of both the intact receptor and the binding proteins, indicating that they have the same NH$_2$-terminal sequences. Oligo 3 (335-341) also partially affected the expression of the binding proteins and synthesis of the intact receptor. Oligo 6 treatment of MA10 cells also resulted in the generation of a protein of $M_r$ of 23,000 (23K). The 23K protein would be generated by premature truncation of the intact receptor or binding proteins. The decrease in intensity of the radioactive bands detected in medium of cells treated with Oligo 6 may be due to deletions within the binding region for LH/CG, thus decreasing the ability of LH/CG to bind to the intact receptor.

The binding data for the LH/CG binding proteins showed that they were produced in excess as compared to surface located LH/CG receptors, with a synthesis rate of approximately 600-700 molecules/cell/h. Intact surface LH/CG
receptors are expressed at a rate of 300-400 molecules/cell/h (Chapter 4). The 43K and 33K binding proteins were therefore derived from a different mRNA transcript to the intact LH/CG receptor. This supported the concept that the 1.2kb mRNA transcript (Segaloff et al., 1990) may code for a secreted LH/CG binding protein. Expression of the 1.2kb mRNA transcript was greater than for the other transcripts (6.7, 4.3 and 2.6 kb (Segaloff et al., 1990)), and it was not under the control of LH/CG (LaPolt et al., 1991). Continual translation of the 1.2kb transcript may be occurring.

Only the 43K binding protein was detected in the solubilized cells, suggesting that it may also be generated due to breakdown of the intact LH/CG receptor by proteolysis. Treatment with Oligo 6 resulted in a decrease in the levels of the 43K protein, indicating that it was probably derived from the intact LH/CG receptor.

We previously reported in chapter 6 that a protein was proteolytically cleaved from the LH/CG receptor at the plasma membrane. This protein had a molecular weight of 40,000-45,000.

In contrast, expression of a mutated construct of the rat luteal LH/CG receptor (Xie et al., 1990), demonstrated that high affinity binding sites for LH/CG could only be detected in the solubilized cells of a eukaryotic cell expression system. However, expression of a LH/CG receptor cDNA containing a 266 bp deletion resulting in truncation of the open reading frame, and omission of the 1st transmembrane domain, demonstrated secretion of binding sites into the medium of a eukaryotic cell expression system (Tsai-Morris et al., 1990). In chapter 6 we reported that we could not detect hCG binding sites in medium collected from rat testis Leydig cells or MA10 cells after 3h. The discrepancies observed, could
therefore be due to the length of time required to express the LH/CG binding protein and the detection limits of \[^{125}\text{I} \text{-hCG}\] binding studies.

We concluded that the C-terminal sequences containing the potential phosphorylation sites for PKA and PKC were required for LH-induced desensitization of cyclic AMP. The mechanism required a conformational change in the C-terminal intracellular tail that affected the 7th transmembrane domain, as was observed with the effects of having only two thirds of this region present, i.e. a 50% reduction in LH-stimulated cyclic AMP. Three-dimensional modelling of G protein-linked receptors (Findlay and Eliopoulos, 1990) has indicated that there is a highly conserved region within the 7th transmembrane domain that undergoes a conformation change during activation of the receptor by ligand.

MA10 cells also secreted high affinity binding proteins that were expressed at a faster rate than that of the intact LH/CG receptor. The 53K and 43K protein LH/CG binding protein may be derived from proteolytic cleavage of the intact receptor. The 43K and 33K proteins could also be derived from the 1.2kb mRNA transcript of the LH/CG receptor.

We have also established a novel method that has distinct advantages over other methods such as site directed mutagenesis, because the functional activity of the modified receptors can be investigated without the need for expression systems.
CHAPTER 8

GENERAL DISCUSSION
8.1 Discussion

The aim of this thesis was to study the structure-function relationships and regulation of the LH/CGR receptor. The importance of these studies in relation to the control of steroidogenesis must also be addressed. The control of spermatogenesis is dependent on the sensitivity of the peritubular, Sertoli and germ cells to androgens (Skinner, 1991). Our results showed that LH/CGR receptors were present in Leydig cells in excess, therefore dramatic reductions in receptor numbers or sensitivity would be required to affect testosterone production. The role of the LH/CGR receptor would therefore appear to be involved in the negative modulation of steroid production. In our studies maximal stimulation of cyclic AMP production and steroidogenesis was maintained when only 15% of the receptors were occupied and when there was a 50% decrease in receptor numbers, caused by NH$_2$-terminal LH/CGR receptor antisense oligodeoxynucleotide treatment.

The regulation of LH/CGR receptors was found to be different between the rat and mouse Leydig cells, under chronic treatment with LH (3.3nM). Our results indicated, that similar to the GnRH-receptor complex (Handelsman and Swerdloff, 1986), proteolytic processing of the LH/CGR-receptor complex occurs at the plasma membrane. In mouse Leydig cells proteolytic cleavage of the LH/CGR receptor at the plasma membrane occurred when the LH/CGR receptors were saturated (figure 8.1.1). LH/CGR action was mimicked by NaN$_3$ treatment, which inhibits internalization (figure 8.1.2), suggesting that proteolysis occurred at the plasma membrane. The loss of LH/CGR receptors was mediated by cyclic AMP (figure 8.1.2), possibly by phosphorylating the receptor. Using LH/CGR receptor
Proteolytic Cleavage 1

Receptors Saturated by LH

Inhibited by serine protease inhibitors
Inhibition of internalization
Using energy depleting agents e.g. NaN₃

Incubations in the presence of high concentrations of cyclic AMP
LH action mimicked by db-cAMP, cholera toxin and forskolin
antisense oligodeoxynucleotides we showed that the final nine amino acids in the cytoplasmic C-terminal tail were crucial for down-regulation to occur. Cycloheximide also inhibited down-regulation of LH/CG receptors. These two pieces of evidence suggested that a cyclic AMP regulated protein interacts with the LH/CG receptor to inhibit internalization. These results were in contrast to those of Wang et al. (1991a), where 8-bromo-cAMP did not cause an acute loss of LH/CG receptors. In the rat Leydig cells both db-cAMP and PMA had no effect on the levels of LH/CG receptors during 2-4h. In rat tumour Leydig (R2C) cells, proteolytic cleavage of the LH/CG receptor was continuous, whereas in rat testis Leydig cells proteolytic cleavage only occurred over the first four hours of the incubation. Isolation of the rat testis Leydig cells may have removed an unknown factor that was involved in this inhibition. Inhibition of the proteolytic activity, using serine protease inhibitors, added directly after isolation, resulted in an increase in receptor number and subsequent LH-stimulated cyclic AMP production.

Proteolytic cleavage of the LH/CG receptor at the plasma membrane was confirmed by the detection of a LH/CG binding protein of 40,000-45,000 Mr. To detect the LH/CG receptor fragment the binding protein required radiolabelled hCG to be cross-linked to the receptor before cleavage occurred. Preliminary data was obtained supporting the concept of the existence of LH/CG binding proteins which are secreted from MA10 cells. These binding proteins may not be derived from proteolytic cleavage of the LH/CG receptor. The high affinity LH/CG binding proteins were related to the LH/CG receptor, but may be derived from different LH/CG receptor mRNA transcripts. The rate of synthesis of the binding proteins was greater than for the intact LH/CG receptor. The role of these
proteins is unknown. The suspected mRNA transcript (1.2kb (Segaloff et al. 1990))
for the truncated LH/CG receptor binding proteins is in excess of the other mRNA
transcripts in the testis, but is not under the control of LH/CG (LaPolt et al.
1991). One possible role for these proteins is to prevent desensitization or down-
regulation of LH/CG receptors, by decreasing the concentration of available LH
in the interstitium. These proteins may be directly secreted from the cells or
reside in the plasma membrane and be released by proteolytic cleavage at the
plasma membrane (figure 8.1.3). The released proteins would then bind LH in the
interstitial fluid. The role of proteolysis in the release of these binding proteins
from the plasma membrane, as opposed to cleavage from the intact LH/CG
receptor, may explain the discrepancies between published reports on the secretion
of LH/CG receptor extracellular domain from eukaryotic expression systems (Xie
et al., 1990; Tsai-Morris et al., 1990). The plasma membrane proteases may not
be present in the eukaryotic cells used in the expression systems.

The internalization of the LH/CG-receptor complex in rat and mouse was
also different. In the mouse there are two phases of LH/CG-receptor complex
internalization, when the receptors are saturated. Initially there is a rapid phase
which resulted in a 50% loss of surface receptors. The loss is reflected by a
similar increase in intracellular associated radioactivity. The second phase
involves a slower loss of surface receptors, during which time intracellular levels
of radioactivity also decreased due to the release of degradation products from the
cell. In the rat Leydig cells there was a slow loss of surface binding, with very low
levels of intracellular radioactivity detected. In the rat Leydig cells LH/CG
receptors were reported to be rapidly internalized and recycled (Habberfield et al.
Release of the LH Receptor Extracellular Domain
1986;1987). The results obtained would therefore indicate that in rat Leydig cells, the LH/CG-receptor complex is recycled several times before LH/CG dissociates and is degraded.

A further difference between the rat and mouse Leydig cell is in the mechanism of desensitization. Studies using LH/CG receptor antisense oligodeoxynucleotides showed that in MA10 cells, desensitization occurs at two sites. LH-induced desensitization is due to a change in the responsiveness of the LH/CG receptor and a change in the enzymes of the steroidogenic pathway. The phorbol ester PMA and the cyclic AMP analogue db-cAMP induced desensitization is due to changes in the responsiveness of the LH/CG receptor. LH/CG receptor antisense oligodeoxynucleotides encoding regions of the C-terminal region, demonstrated that the PKC consensus phosphorylation sites (Loosfelt et al., 1989) and other potential phosphorylation sites are essential for desensitization to occur in the MA10 cells. The results also suggest that the conformation of the 7th transmembrane domain and cytoplasmic C-terminal tail may play a role in desensitization. Desensitization in the mouse results in a lack of both cyclic AMP production and steroidogenesis. Desensitization in the mouse Leydig cells does not require the loss of LH/CG receptors. In contrast in the rat Leydig cell desensitization of cyclic AMP results in a decrease in cyclic AMP production with an increase in basal cyclic AMP levels. There was no effect on testosterone stimulation. PMA was able to mimic the decrease in cyclic AMP, but there was no increase in basal levels. PMA has been proposed to act on the G-protein $G_i$ (Platts et al. 1988). The cyclic AMP analogue db-cAMP had no effect (Dix et al. 1982;1987). An explanation for the difference between the rat and mouse Leydig
cells may involve differences in phosphorylation of the LH/CG receptor.

The observation that 60% of the LH/CG receptors were lost during the isolation procedure of rat testis Leydig cells, and that these receptors were directly coupled to adenylate cyclase, raises several points; i) the differences in down-regulation and desensitization, between mouse and rat Leydig cells, may be only due to the loss of LH/CG receptors and ii) this observation would suggest, in the rat Leydig cells, the existence of subtypes of the LH/CG receptor.

The observation that LH/CG receptors could only be up-regulated in the MA10 cell, indicated that this was not a normal phenomenon. These results are in contrast to the effect of cyclic AMP observed by Wang et al. (1991a), where cyclic AMP over a similar time period caused a loss of LH/CG receptor mRNA and thus a loss of LH/CG receptors.

The results obtained from the stimulation studies showed that interaction with a G-protein does not involve the 7th transmembrane domain. This supports data obtained for the β-adrenergic receptor, where the intracellular loop between the 5th and 6th transmembrane where important in the activation of G-protein coupling (Strader et al., 1989). Cyclic AMP production and steroidogenesis was similar between all the cells. Phosphodiesterase activity, however, was greater in the rat Leydig cell. The discrepancies reported between the dose response curves for LH-stimulated cyclic AMP and steroid (Rommerts and Cooke, 1988) in the rat testis Leydig cell was not evident.

In conclusion a sequence of events can be postulated for the activation and desensitization of the LH/CG receptor. The work of Ji and Ji (1991) suggested that LH/CG binds to its receptor with a two step mechanism. LH/CG binds to the
Binding of LH to its Receptor

FIG. 8.1.4
Activation of LH Receptors

FIG. 8.1.5

Adenylate Cyclase

Gα

ATP

G Protein

LH Receptor

β

PKA

316
Desensitization of LH Receptors

Phosphorylation

Desensitized Receptor

Internalization
Degradation of Hormone
Recycling of Receptor
Acute loss of LH Receptors

1. Desensitized Receptor

Synthesis or Phosphorylation of \( ? \) Protein (dependent on cAMP conc.)

Acute loss of LH Receptors

2. Active Receptor

Internalization
large extracellular domain of the LH/CG receptor via its β-subunit (Ryan et al., 1988). This is by high affinity binding. Binding of the β-subunit causes a conformational change in the extracellular domain which then "flips" the α-subunit into a position where it can interact with the transmembrane domains. This is by low affinity binding (figure 8.1.4).

Interaction of the α-subunit with the seven transmembrane domains causes a conformational change in the 3rd intracellular loop (Strader et al., 1989), the 7th transmembrane domain (Findlay and Eliopoulos, 1990) and cytoplasmic C-terminal tail. This allows interaction and activation of the G-protein Gs (figure 8.1.5). The α-subunit of Gs intern activates adenylate cyclase. Cyclic AMP is synthesized and the cyclic AMP-dependent protein kinase (PKA) activated. Activation of PKA results in phosphorylation of the LH/CG receptor cytoplasmic C-terminal tail. This causes a conformational change in the cytoplasmic C-terminal tail and 7th transmembrane domain. Interaction and activation of Gs is thus terminated, resulting in an uncoupling of the LH/CG receptor from Gs/adenylate cyclase system - desensitization (figure 8.1.6). The LH/CG receptor is then internalized, degraded or recycled. If saturation of LH/CG receptors occurs with a rapid increase in cyclic AMP synthesis, activation of PKA results in either the initiation of synthesis or phosphorylation of an unknown protein. This protein then interacts with either desensitized or active receptors to terminate activation of Gs and inhibit internalization and allow proteolytic cleavage of the LH/CG extracellular domain to occur (figure 8.1.7).
Results derived from the different cell types clearly show that the mechanisms of desensitization and down-regulation of LH/CG receptor cannot be studied in only one cell type. Further characterization of the nature of the protease enzymes associated with the plasma membrane of Leydig cells, is required to characterize the proteolytic cleavage of the LH/CG receptor. The role of this mechanism should also be investigated \textit{in vivo}, as it appears that it is inhibited \textit{in vivo} with rat testis Leydig cells. Clarification of the role of cyclic AMP in the regulation of LH/CG receptors in up/down-regulation and desensitization is required, in respect of the discrepancies observed between these studies and others (Freeman and Ascoli, 1982; Dix \textit{et al.}, 1982; Lefèvre \textit{et al.}, 1985; Wang \textit{et al.}, 1991a). The preliminary studies on the existence of high affinity LH/CG binding proteins should be extended, to separate the different sizes of protein detected (53K, 43K, and 33K). The rate of appearance of each protein should be studied and there relation to the intact LH/CG receptor characterized. The existence of these proteins \textit{in vivo} should also be assessed.

The adaptation of the antisense inhibitory strategy, provides a method to study different functional regions of the LH/CG receptor. The results observed with this strategy were based on a theoretical generation of truncated proteins. No direct evidence was provided to support this hypothesis, however preliminary evidence from the study of LH/CG binding proteins may provide evidence. Treatment with the antisense oligodeoxynucleotide 6 (encodes for a region prior to the first transmembrane domain) resulted in the generation of a 4th binding
protein of 23k as well as inhibiting synthesis. The antisense oligodeoxynucleotides could therefore have more than one effect. Clarification of these effects on the LH/CG receptor gene, mRNA and protein are therefore required to give an accurate interpretation of the results. However, the results obtained demonstrated that the above strategy is very powerful and allows the study of proteins within their native cell. This has many advantages over expression systems. The use of antisense oligodeoxynucleotides may also provide a means of generating mutant MA10 cells that have modified LH/CG receptors.
References


Freeman D. A. (1987a) Constitutive steroidogenesis in the R2C Leydig tumor cell line is maintained by the adenosine 3',5'-cyclic monophosphate-independent production of a cycloheximide-sensitive factor that enhances mitochondrial pregnenolone biosynthesis. Endocrinology 120, 124-132.


List of publications

Papers


Communications


ABSTRACT. Regulation of the truncation of LH receptors was investigated in two types of mouse tumor Leydig cells (MA10 and MLTC-1), rat testis Leydig cells (RTL), and a rat tumor Leydig cell (R2C). Receptor numbers were measured by binding [125I]hCG to the cells cultured in monolayers. Addition of 3.3 nM LH for 2 h at 34°C had no detectable effect on binding sites in RTL or R2C cells, but in MA10 and MLTC-1 cells it caused a loss in binding sites. The effect on MA10 and MLTC-1 cells could be mimicked by inhibiting receptor internalization with 5 mM NaN3 and prevented by the addition of protease inhibitors. Incubating RTL and R2C cells with protease inhibitors caused a 2- to 3-fold increase in binding sites and a 2- to 3-fold decrease in LH (0.033 and 0.33 nM)-stimulated cAMP production. RTL and MA10 cells were incubated in the presence of hCG, a radioactive protein complex with an approximate molecular mass of 80,000-90,000 was released into the incubation medium. We conclude that LH receptors are regulated by proteolytic cleavage in the plasma membrane in both mouse and rat Leydig cells. Furthermore, truncation of the LH receptor in the mouse cell line (MLTC-1) and rat Leydig cells is involved in down-regulation, whereas in the rat testis Leydig cell (RTL) cell recycle, but do not down-regulate, the receptor.

LH AND hCG are polypeptide hormones which interact with a specific cell surface receptor to stimulate steroidogenesis in the ovary and testis via the second messenger cAMP. Like other receptors for polypeptide hormones, the LH receptor exhibits ligand-induced loss or down-regulation of cell surface LH/hCG-binding capacity (1). This negative modulation of receptor levels in vitro is time and hormone concentration dependent (1). Decreased binding capacity is the result of a lowered LH receptor level rather than an altered affinity of the receptor for the ligand (1).

Several reports have shown that proteolytic cleavage of hormone receptors at the plasma membrane is involved in down-regulation, e.g. epidermal growth factor receptors (2), acetylcholine receptors (3), and β-adrenergic receptors (4). There are also reports indicating that binding of polypeptide hormones to their receptors causes a conformational change, which exposes, on the outer surface of the plasma membrane, a trypsin-sensitive site on the receptor, e.g. insulin (5) and glucagon (6).

The possible involvement of a membrane protease(s) as a modulator of LH/hCG receptor function has also been suggested. Protease inhibitors have been shown to inhibit the activation of adenylate cyclase and hepatic membrane preparations at concentrations that inhibit proteolytic activity (7), and a serine protease inhibitor markedly reduced the association of hCG to its receptor in ovarian membrane preparations, but not in solubilized preparations of the LH receptor (8). Kellokumpu and Rajamaki demonstrated that when rat ovarian membranes were isolated and incubated with [125I]hCG, proteolytic cleavage of the LH/hCG receptor occurred. This cleavage resulted in the release of two distinct [125I]hCG fragment complexes with molecular masses of 74,000 (9). Susceptibility to this endogenous membrane protease activity was increased by LH binding (10). Kellokumpu (11) also investigated the persistence of this process in a cultured mouse Leydig cell line (MLTC-1). He concluded that the internalization in the MLTC-1 cells (or luteal cells) meant that this process of proteolysis was not evident.

In the present study in order to investigate proteolysis in the regulation of LH receptor binding sites in cells, two model systems were chosen that means of regulation of the LH receptor in testis Leydig (RTL) cell (12) and rat tumor Leydig cell recycle, but do not down-regulate, the receptor. The mouse tumor Leydig cells, MA10 (13...
Ovine LH (batch oLH 26; potency, 2.3 U/mg) and hCG (batch CR-127; potency, 14,900 IU/mg) were obtained from the NICHD (Bethesda, MD). Crude hCG (5,000 IU) was obtained from Serono Laboratories Ltd. (Hertfordshire, England). Forskolin, cholera toxin, leupeptin, phenylmethylsulfonylfluoride (PMSF), and aprotinin were purchased from Sigma Chemical Co. (Dorset, England). Powdered media and sera were obtained from Gibco Ltd. (Middlesex, England). Disuccinimidyl suberate (DSS) was purchased from Pierce Chemicals (Cheshire, England). Carrier-free Na$^{125}$I was obtained from the Radiochemical Centre, Amersham (Amersham, England). Centriprep 30 concentrators were purchased from Amicon Division, W. R. Grace and Co. (Danvers, MA). All other reagents were purchased from Sigma or BDH (Dorset, England).

Methods

Cell propagation. Stock cultures of MA10, MLTC-1, and R2C cells were maintained, according to the method of Ascoli (14), in Waymouth's MB752/1 medium, modified to contain 20 mM HEPES (pH 7.7), 1.12 g/liter NaHCO$_3$, 40 $\mu$g/ml gentamycin, and 15% horse serum (growth medium) in a humid atmosphere of 2.5% CO$_2$-97.5% O$_2$ at 34 C. The cells were subcultured by trypsinization [0.05% (wt/vol) trypsin], and experimental cultures were plated in growth medium and used 2-3 days after subculture. The growth medium was replaced on alternate days in culture. Cell number was determined by using a hemocytometer, and viability of the cells by the trypan blue exclusion method (15) and diaphorase histochemistry (16).

Preparation of RTL cells. RTL cells were isolated and purified from adult male Sprague-Dawley rats (200-250 g) under non-sterile conditions, as described by Platts et al. (17). After plating out (conditions described in the legends), the cells were always preincubated at 34 C for 2 h to recover from the preparation and attach to the culture wells. Cell number and viability were determined as described above, and purity was assessed by the 3$\beta$-hydroxysteroid dehydrogenase assay (18).

Measurement of cAMP production. Cells were preincubated with protease inhibitors for 30 min and then incubated with LH, cholera toxin, or forskolin for 2 h at 34 C. cAMP was extracted from the cells and medium by the addition of HClO$_4$ (final concentration, 0.5 mol/liter), which was neutralized by adding K$_2$PO$_4$ (final concentration, 0.23 mol/liter). The levels of cAMP were determined by RIA by the method of Steiner et al. (PBS-BSA). If the cells had been incubated in the presence of LH/hCG, they were further washed with 50 mM buffered saline (pH 3) for 2 min on ice to remove LH/hCG (22). After washing again with 0.01 M PBS, cells were incubated overnight at 4 C in the presence of hCG (0.56 nM) and 0, 0.14, or 0.28 nM hCG. The level of binding was determined by aspirating the culture medium, washing twice with 0.01 M PBS-BSA to remove hormone, and dissolving the cells in 0.5 M NaOH. Nonspecific binding, approximately 10% of total binding, was determined in the presence of 400 IU unlabeled hCG.

Detection of proteolytic fragment. Cross-linking of receptor complex: Cells were incubated in the absence of [$^{125}$I]hCG for the times and conditions described in the legends. Cells treated with [$^{125}$I]hCG were further incubated with DSS (final concentration, 1 mM). Medium from cells incubated with [$^{125}$I]hCG was removed and incubated with hCG and DSS for 3 h. The incubation medium was then concentrated using Centriprep 30 concentrators (cut-off limit, 30,000 mol wt) before protein precipitation with 24% trichloroacetic acid (final concentration, 8%, wt/vol) and centrifuged in an Eppendorf microcentrifuge for 2 min (13,000 X g). The supernatant was refreshed with 2 M Tris base (20 m M Tris-HCl buffer, pH 6.8, 0.05% (wt/vol) bromophenol blue, and 5% (vol/to ethanol), boiled for 5 min, and centrifuged for 1 min (13,000 X g) before SDS-polyacrylamide gel electrophoresis (SDS-PAGE; gels contained 10% polyacrylamide). Radioactive bands were visualized by autoradiography or by OsO$_4$ staining.

Electrophoresis and autoradiography: Nonspecific binding, approximately 10% of total binding, was determined in the presence of 400 IU unlabeled hCG.

Results

To desensitize the MA10, MLTC-1, and R2C cells with respect to steroid production (i.e., receptor from its transducing system), cells were incubated with Na$_2$N$_3$ (5 mM; with LH) to inhibit internalization. Incubating the cells with Na$_2$N$_3$ (5 mM) caused a small decrease in the cells from 99% to 86% and would, therefore,
Fig. 1. Regulation of \([^{125}\text{I}]\)hCG binding sites. The effect of inhibiting receptor internalization and protease activity. MA10 and MLTC-1 cells (A) and R2C and RTL (B) cells were plated at 150 \(\times\) 10^3 in 1 ml medium and preincubated with or without protease inhibitors (100 \(\mu\)M leupeptin, 10 \(\mu\)M PMSF, and 900 U/ml aprotinin) in 1 ml fresh medium for 30 min at 34°C. The cells were further incubated in the absence of protease inhibitors for 2 h [without hormone (■), with 5 \(\text{mM}\) NaN₃ (□), or with 3.3 \(\text{nM}\) LH (△)], or in the presence of protease inhibitors for 2 h [without hormone (■), with 5 \(\text{mM}\) NaN₃ (□), with 3.3 \(\text{nM}\) LH (△), or with 3.3 \(\text{nM}\) LH plus 5 \(\text{mM}\) NaN₃ (■)]. After 2 h the cells were placed on ice and washed twice with 50 \(\text{mM}\) glycine-buffered saline (pH 3.0) before \([^{125}\text{I}]\)hCG binding was determined. Values given are the mean ± SEM (replicates = 6; n = 2). *, \(P < 0.05\); **, \(P < 0.01\) (by Student's t test). Due to the variance in control binding of cultured Leydig cells (31), only experiments where control binding was approximately the same have been represented. Experiments were repeated four times with similar results for the MA10, MLTC-1, and RTL cells and twice for the R2C cells.

Fig. 2. The effect of protease inhibitors on cAMP production in cells. RTL cells were plated at a density of 100 \(\times\) 10^3 in 24-well plates and preincubated for 2 h. During this time, the protease inhibitors were added. The cells were then incubated in basal (■), LH (0.033 nM; □), LH (0.33 nM; ■), chol. (△), and forskolin (8 \(\mu\)M; ◆), in the absence and presence of protease inhibitors for 2 h. Reactions were stopped by the addition of ice-cold 1 N HCl and assayed for cAMP as stated in Materials and Methods. Values are given as the mean ± SEM (n = 3; replicates = 3). *, \(P < 0.05\); **, \(P < 0.01\) (by Student's t test).

Table 1. The effect of preincubating Leydig cells with 5 mM NaN₃ on the binding characteristics of \([^{125}\text{I}]\)hCG.

<table>
<thead>
<tr>
<th>Dissociation constant (nM)</th>
<th>MA10 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td>Sodium azide (5 mM)</td>
<td>3.5 ± 0.5</td>
</tr>
<tr>
<td>LH (3.3 nM)</td>
<td>2.3 ± 0.25</td>
</tr>
</tbody>
</table>

MA10 cells were plated at a density of 500 \(\times\) 10^3 in 24-well plates and used 2 days later. RTL cells were isolaed in Materials and Methods, plated at a density of 200 \(\times\) 10^3 in 24-well plates, and incubated for 2 h before being either incubated with or without LH (3.3 nM) or NaN₃. The cells were washed with 50 \(\text{mM}\) glycine-buffered PBS-BSA before being used for Scatchard analysis. MA10 cells were incubated in a final volume of 1 ml with 1.4 \(\text{nM}\) \([^{125}\text{I}]\)hCG and concentrations of pure hCG (CR-127; 0–14 nM hCG). Nonspecific binding was determined with 400 IU chymotrypsin. After overnight incubation, cells were washed with 0.5 M HCl to remove excess label and were solubilized with 0.5 M NaOH. Scatchard analysis was determined using the McPherson EB program for Scatchard binding analysis program. Values are the mean ± SEM. The effect of the latter was found on the apparent number of receptors per cell (control, 17,018 receptors/well washed, 21,126 ± 4,924) or on the K_d (control, 1.25 nM; acid-washed, 5.9 ± 2.1 nM).
A 2-h incubation of MA10 and MLTC-1 cells (Fig. 1A) in the presence of LH (3.3 nM) caused a 50% loss in binding of $^{[125]I}$hCG. The loss in binding caused by LH was mimicked when NaN$_3$ (5 mM) was present either with or without LH. The loss of binding that occurred with NaN$_3$ treatment alone in the MA10 and MLTC-1 cells indicated that in the mouse tumor cells loss of receptors had occurred at the cell surface rather than by internalization, since NaN$_3$ inhibits internalization. Therefore, the possible involvement of surface proteases was investigated. Preincubating the MA10 and MLTC-1 cells with a cocktail of protease inhibitors (leupeptin, PMSF, and aprotinin) for 30 min before the addition of LH or NaN$_3$ prevented the observed loss of receptors over a 2-h incubation period at 34 C, as determined by subsequent $^{[125]I}$hCG binding at 4 C (in the absence of protease inhibitors). Similar results were obtained for the MA10 cells when horse serum was replaced by 0.1% (wt/vol) BSA (data not shown), thus demonstrating that the protease activity was not derived from the serum.

Similar experiments were carried out on the rat Leydig cells. A 2-h incubation of RTL and R2C cells (Fig. 1B) with either NaN$_3$ or LH did not cause a loss in binding sites. However, preincubating these cells with the protease inhibitors resulted in a 2-fold increase (R2C) and a 3-fold increase (RTL) in binding sites (determined by $^{[125]I}$hCG binding at 4 C in the absence of protease inhibitors) under all of the conditions used, thus suggesting that there is a continual process of proteolysis of the LH receptor in the RTL and R2C cells in binding sites in the RTL cells was comparable to a change in the affinity of the LH receptor. Analysis was carried out on cells treated as above. The effect of NaN$_3$ (5 mM) and LH treatment on the $K_d$ for $^{[125]I}$hCG in the MA10 cells is shown in Table 1. LH and NaN$_3$ had no effect on the $K_d$.

The above results indicated that in mouse Leydig cells there was a loss in binding sites in the presence of NaN$_3$ (5 mM) and LH and that the LH receptor was inhibited to a change in the affinity of the LH receptor. Analysis was carried out on cells treated as above. The effect of NaN$_3$ (5 mM) and LH treatment on the $K_d$ for $^{[125]I}$hCG in the MA10 cells is shown in Table 1. LH and NaN$_3$ had no effect on the $K_d$.

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These results demonstrate that at 4°C [i.e. conditions that prevent internalization of receptors (25)] and in the presence of protease inhibitors the level of detectable binding was increased. The effect of the protease inhibitors was not as great as at 34°C, but this can be explained at 34°C. The cross-linking agent DSS was removed, and the proteins were precipitated, and subjected to SDS-PAGE, as described in Materials and Methods. Figure 3 shows an autoradiograph of the proteins derived from MA10 cells prelabeled with [125I]hCG. MA10 cells were grown to subconfluency in 75-cm² culture flasks (~20 × 10⁶ cells/flask). Three series of flasks were treated with 0.28 nM [125I]hCG plus 5 mM NaN₃ at 34°C for 3 h (B), 0.28 nM [125I]hCG at 34°C (C), and 0.28 nM [¹²⁵I]hCG overnight at 4°C (D). Cells were further incubated for 1 h with DSS (final concentration, 1 mM) at 4 or 34°C. The medium from these incubations was concentrated by centrifugation using concentrators with a molecular size cut-off of 30,000 and treated with 24% (wt/vol) trichloroacetic acid to precipitate the proteins. The proteins were solubilized and run on a denaturing SDS-PAGE 10% (wt/vol) gel. An autoradiograph of the gel was developed 1 and 3 weeks later for the MA10-derived gel (this was to identify the fragment). Standard [¹²⁵I]hCG incubated in the presence of DSS (final concentration, 1 mM) for 1 h is shown in A. The trace is a representation of one experiment of two that required similar processing for the autoradiograph.

Discussion

The results of this study establish that LH receptors during desensitization of mouse and rat Leydig cells. In agreement with experiments carried out with intact luteinated plasma membranes from luteal cells, that LH receptors are cleaved at the plasma membrane.
in intact Leydig cells by endogenous proteases, releasing a water-soluble component of approximately 90,000 mol wt (for a review, see Ref. 26). We have demonstrated that in mouse tumor Leydig cells this process is involved in down-regulation and also occurs under conditions where internalization of LH/hCG-receptor complexes is inhibited. In contrast, in the rat Leydig cell we have demonstrated that proteolysis of the receptor is a continuous process and participates in the normal regulation of the hormonal response of the cell.

The size of the fragment detected in this study (mol wt, 40,000-50,000) is comparable to the size of the extracellular domain of the LH receptor in the rat ovary. McFarland et al. (27) reported the rat ovarian receptor to have a 93,000 mol wt, 7 transmembrane domains, with an extracellular component of 50,000-60,000 mol wt. Sojar and Bahl (28) also demonstrated that isolated rat ovarian LH receptors could be cleaved by endoproteinase Glu-C at a single glutamyl residue to give an extracellular component of 36,000 mol wt. Cloning and sequencing of the porcine LH/hCG testis receptor cDNA demonstrated the existence of three other types of clones, corresponding to shorter proteins where the putative transmembrane domain was absent (29). The point of divergence of the shorter forms from the full-length porcine LH/hCG receptor may coincide with the point of proteolytic cleavage of the extracellular domain. This may depend on the tertiary structure of the domain and its proximity to the plasma membrane.

The effect of protease inhibitors on down-regulation in the MA10 and MLTC-1 cells and the loss of binding in the RTL and R2C cells, clearly indicates involvement of proteolytic cleavage in the regulation of LH receptors. The fact that LH-stimulated LHCanaconduction is increased by the same magnitude in number in the presence of protease inhibitors indicates the concept of continual proteolysis in LH-sensitive cells. The results obtained for the mouse tumor Leydig cells indicate that LH at desensitizing concentrations of LH internalization by causing a conformational change in the receptor (perhaps by phosphorylation), which is internalized and, therefore, allows proteolysis of the receptor. In the RTL and R2C cells, internalization and recycling are concurrent with proteolytic cleavage of the receptor at the plasma membrane and, therefore, allows proteolysis of the receptor. In the results obtained for rat Leydig cells also demonstrate species difference in the regulation of LH receptors, but the results obtained for rat Leydig cells also demonstrate difference in the regulation of LH receptors derived from one species. The nature of the protease that facilitates proteolytic cleavage of the receptor at the plasma membrane and, therefore, allows proteolysis of the receptor cannot be made. The nature of the protease that is characterized, but as the inhibitors used in the presence of proteases, it could be a member of the plasminogen and kallikrein families, which are glycoproteinases. The involvement of plasminogen has been implicated in the down-regulation of
medium from treated cells was incubated with $^{125}$I-hCG. Therefore, it would appear that to detect the fragment $^{125}$I-hCG must be bound to the receptor before proteolysis occurs. This implies that the fragment would not act as a serum binding protein for LH in vivo and is, therefore, unlike the GH-derived serum binding protein (33).

It may be concluded that the regulation of LH receptors during desensitization in rat and mouse Leydig cells is totally different. In mouse Leydig cells proteolytic cleavage of the receptor at the plasma membrane only occurs during down-regulation and is the result of an inhibition of internalization. In the rat Leydig cell proteolysis of the LH receptor is occurring all the time, and when this is inhibited there is an increase in LH-stimulated cAMP production. It can also be concluded that the cleaved receptor is not acting as a serum binding protein.

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We are grateful to Prof. E. Barnard FRS (Medical Research Council Molecular Neurobiology Unit, Cambridge, England) for helpful discussions, to Dr. M. Ascoli (The Population Council, New York, NY) for the gift of the MA10 cells, and to Dr. I. Mason (University of Texas, Southwestern Medical School, Dallas, TX) for the gift of the MLTC-1 and R2C cells.

**References**

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of MA10 cells, with a concomitant 2-fold increase in the level of binding sites. Under conditions where receptor increased by 3–8-fold there was also a significant increase in pregnenolone production. It is concluded that LH AMP have positive regulatory effects on LH receptors in MA10 cells by inducing the synthesis of new receptors. Induced receptors are functionally coupled to steroidogenesis.

INTRODUCTION

Lutropin (luteinizing hormone, LH) and human chorionic gonadotropin (hCG) are polypeptide hormones that interact with the same cell surface receptor to stimulate steroidogenesis in Leydig cells in the testis via the second messenger cyclic AMP, and also by other mechanisms involving chloride channels (Choi & Cooke, 1990), Ca\(^{2+}\) and arachidonic acid and/or its metabolites (Abayasekara et al., 1990). The LH receptor also exhibits ligand-induced loss, i.e. down-regulation of cell surface LH binding capacity (Freeman & Ascoli, 1981).

The mechanism(s) involved in the synthesis of LH receptors in Leydig cells is not well understood. Increases in LH receptor concentrations have been demonstrated in vivo (Huhtaniemi et al., 1981) and in vitro (Baranao & Dufau, 1983), but the increases were transient and the receptors were shown not to be functionally coupled.

Cyclic AMP has been demonstrated in other cell systems to have a role in the regulation of plasma membrane receptor synthesis and growth of cultured cells. However, both positive and negative effects have been found. For example, the growth hormone receptor is down-regulated in the presence of cyclic AMP (Gorin et al., 1988), whereas the expression of thyroid-stimulating hormone receptors (Lissitzky et al., 1975) and insulin receptors (Thomopoulos et al., 1977) is increased by cyclic AMP. Cyclic AMP also has effects on the growth of cells, either stimulating (Dumont et al., 1989) or inhibiting (Pastan et al., 1975) growth.

In granulosa cells, up-regulation of LH receptors occurs in vitro when the cells are stimulated with follicle-stimulating hormone, acting via cyclic AMP (Knecht & Catt, 1982) and oestrogens (Knecht et al., 1984). The increase in receptor numbers is derived from the synthesis of new receptors and not from a pool of receptors (Segaloff & Limbird, 1983).

In this study, we present evidence to show that there is a cyclic AMP-dependent mechanism for the up-regulation of LH receptors in cultured mouse tumour (MA10) cells, furthermore that these new receptors are functionally coupled to steroidogenesis.

MATERIALS AND METHODS

Materials

Ovine LH (batch oLH-26, potency 2.3 unit/mg) and hCG (batch CR-127, potency 14900 units/mg) were obtained from National Institute of Child Health and Human Development (Bethesda, MD, U.S.A.). MA10 cells were a donation from M. Ascoli, The Population Council (New York, U.S.A.). Crude hCG (5000 units) was obtained from Serono (Welwyn Garden City, Herts., U.K.). Forskolin and dibutyryl cyclic AMP [(Bu)\(_2\)cAMP] were obtained from Sigma (Poole, Dorset, U.K.). Powered methyl cellulose filters (GF/C) were purchased from Maidstone, Kent, U.K.). Carrier-free Na\(^{25}\) from Amersham International (Amersham, Herts, U.K.). [\(^{3}\)H]Methionine was purchased from NEN-DuPont (Boston, MA, U.S.A.). Cyanoketone and SU 10603 (inhibitor of cholesterol metabolism) were obtained from Sigma Group Europe (Guildford, Surrey, U.K.). All other chemicals were purchased from Sigma or BDH (Poole, England).

MA10 cell propagation

Stock cultures of MA10 cells (Ascoli, 1981) were maintained in Waymouth MB752/1 medium, containing (pH 7.7), 1.12 g of NaHCO\(_3\)/l, 40 \(\mu\)g of gentamicin, 15% horse serum (growth medium), in a humidified CO\(_2\)/O\(_2\) (1:39) at 34 °C. The cells were subcultured after treatment [0.05 % (w/v) trypsin] and experimentally plated in growth medium or serum-free media containing 0.5% (w/v) BSA, 4 mg of insulin/l and 10 mg of triphosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium.
Cultured cells were placed on ice, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS: 0.01 M-phosphate/0.9% NaCl, pH 7.4) containing 0.1% BSA (PBS/BSA). If the cells had been incubated in the presence of LH/hCG, they were further washed with 50 mM-glycine-buffered 0.9% NaCl (pH 3) for 2 min on ice to remove surface-bound LH/hCG (Ascoli, 1982). After washing again with 0.01 M-PBS/BSA, the cells were reincubated overnight in 0.1% BSA-containing Waymouth MB752/1 medium at 4 °C with 125I-hCG (0.56 nM) and with 0.014 or 0.28 nM highly purified hCG. The level of binding was determined by aspirating the medium, washing twice with 0.01 M-PBS/BSA to remove unbound hormone and dissolving the cells in 0.5 M-NaOH before determination of radioactivity by γ-counting. Non-specific binding, which was approx. 10% of the total binding, was determined in the presence of 400 units of unlabelled crude hCG.

Measurement of pregnenolone and cyclic AMP production

Cells were preincubated with inhibitors of pregnenolone metabolism [cyanoketone (5 μM) or SU10603 (20 μM) dissolved in ethanol] for 30 min and then incubated with LH, hCG, cholera toxin or forskolin for 2 h at 34 °C. Pregnenolone and cyclic AMP were extracted from the cells by the addition of HClO₄ (final concentration 0.5 mol/l), which was neutralized by adding K₂PO₄ (final concentration 0.23 mol/l). The levels of cyclic AMP were determined by radioimmunoassay by the method of Steiner et al. (1972), as modified by the acetylation procedure described by Harper & Brooker (1975). Pregnenolone production was determined by radioimmunoassay by the method of van der Vusse et al. (1975). Each determination was carried out in triplicate.

Measurement of protein synthesis using the incorporation of [3H]methionine

Cells were preincubated for 14 h in the presence or absence of LH (0.033 nM) and (Bu₂)cAMP (1 mM) in 3 ml of growth medium at 34 °C. Following this incubation the cells were washed twice with 1 ml of methionine-free medium [Dulbecco’s modified Eagle’s medium (DMEM)/F12 plus 15% horse serum] and recultured in 1 ml of the same medium in the presence or absence of LH/(Bu₂)cAMP or (Bu₂)cAMP plus 5 μCi of [3H]methionine (200 mCi/mmol). After 6 h the cells were washed twice with DMEM/F12 containing 10 mm-methionine, scraped from the dish in 1 ml of PBS containing 0.1% BSA and 10 mm-methionine and homogenized. Portions of 100 μl were pipetted on to Whatman GF/C filters (prewashed in PBS/BSA/methionine) and the protein was precipitated and washed with ice-cold 30% (w/v) trichloroacetic acid/10 mm-methionine by a vacuum filtration method.

![Fig. 1. The effects of (Bu₂)cAMP, cholera toxin growth of MA10 cells](image)
Fig. 2. Time courses of the effects of LH, cholera toxin, forskolin and cyclic AMP on the levels of binding sites in MA10 cells

Cells were plated at a density of 150 x 10^3/well in 24-well plates and used 2 days later. (a) Cells were incubated at 34 °C, with LH [0.033 (□), 0.33 (○) and 3.3 (△) nM]. At the indicated times the cells were placed on ice and washed and ^125I-hCG binding was determined. (b) Cells were treated as above with 1 mM-(Bu)₂cAMP (□), 11.9 nM-cholera toxin (○) or 1 μM-forskolin (△). Results are the means ± S.D. Statistical analysis was by Student's paired t test; n = 6 (* P < 0.05, **P < 0.02, ***P < 0.01). The results are representative of two similar experiments.

only a 30% loss with 0.33 nM-LH (Fig. 2a) on its own at the same time point. Incubating cells with 1 mM-(Bu)₂cAMP and cholera toxin (Fig. 3b) enhanced the induced levels of binding sites from 4-6-fold. (Bu)₂cAMP (1 mM) had no effect on forskolin (1 μM)-stimulated induction of binding sites (Fig. 3b) and inhibited the increase observed with 10 μM-forskolin (results not shown).

In order to determine the mechanism involved in the observed increase in receptors, the effects of transcription and translation inhibitors on binding sites were investigated (Fig. 4). Parallel protein synthesis assays were also undertaken on cells derived from the same population. Cells were preincubated for 14 h in the absence or the presence of LH (0.033 nM) and (Bu)₂cAMP (1 mM) and then further incubated for 6 h after the addition of cycloheximide (3.5 and 35 mM) or actinomycin D (3.98 mM). It was found that incorporation of the labelled amino acid into proteins during the final 6 h of incubation was increased when LH and (Bu)₂cAMP were present together [control (3.2 ± 0.6) x 10⁴ c.p.m./10⁶ cells; stimulated (5.2 ± 0.3) x 10⁴ c.p.m./10⁶ cells; P < 0.05]. When LH was removed for the last 6 h [i.e. cells incubated in the presence of (Bu)₂cAMP only] this increase was not detectable. The effects of cycloheximide and actinomycin D on receptor levels in the MA10 6 h incubation period are shown in Fig. 4. Both a loss of binding sites in control cells. In cells pretreated for 14 h with LH and (Bu)₂cAMP, an the presence of the inhibitors, a 50% decrease induced binding sites was observed. At the concent inhibitors used there was only a 5% decrease the cells, which would not account for the binding sites.

The effect of (Bu)₂cAMP on the rate of receptor recovery of the receptors was observed by monitoring receptor levels in MA10 cells was also studied. Cells were incubated for 14 min with 0.05% trypsin which resulted in a detectable binding sites. Trypsin-treated cells were then further incubated for 6 h in the presence of (Bu)₂cAMP (1 mM) and recovery of the receptors was observed by monitoring binding sites at various time points (Fig. 5). In contrast, MA10 cells returned to pre-trypsin-treated levels by 48 h. In (Bu)₂cAMP, receptor levels reached a maximum level was maintained for 72 h and was 2-fold higher than the levels. (Bu)₂cAMP also prevented the loss of binding sites observed when MA10 cells are incubated in serum-free medium for longer than 24 h (results not shown).
Cells were plated at a density of $150 \times 10^3$/well in 24-well plates in a volume of 1 ml at 34°C and used 2 days later. Cells were preincubated as for Fig. 3. At the end of 14 h the inhibitors were added (□, basal; □, 3.98 mM-actinomycin D; ■, 3.5 mM-cycloheximide; and □, 35 mM-cycloheximide), and incubated for a further 6 h. At the end of the total incubation time cells were placed on ice and washed, and $^{125}$I-hCG binding was determined. Results are means ± s.d. Statistical analysis was by Student's paired $t$ test; $n = 6$ ($^*P < 0.05$, **$P < 0.01$, ***$P < 0.001$). The data represent one of two similar experiments.

The effect of preincubating MA10 cells for 24 h with LH (0.033 nM) and/or (Bu)$_2$ cAMP (1 mM) on the subsequent LH-stimulated pregnenolone production of these cells when incubated with 3.3 nM-LH is shown in Fig. 6. The results show that in the cells treated with (Bu)$_2$ cAMP alone or with LH there was a marked increase in pregnenolone production both in the presence and the absence of LH in the second incubation (Fig. 6). These increases correlated with the increases in receptor numbers (Figs. 2b and 3a). In order to remove residual bound LH the cells were acid-washed before the second incubation; this was found to decrease pregnenolone production in the control cells by 50% (Fig. 6). Table 1 shows the production of cyclic AMP in pretreated cells on stimulation with LH (3.3 nM). The washing procedure used appears to have inhibited the production of cyclic AMP, though there is still a 50% maximal stimulation of pregnenolone observed in these cells.

### DISCUSSION

In this study we present evidence for the postulation of the synthesis of LH receptors by LH and cultured mouse tumour (MA10) Leydig cells. Prior regulation of LH receptors in rat testis Leydig cells may occur over a short period of time and to not be on LH on its receptor, but an effect on the phosphate (Huhtaniemi et al., 1981). LH in our studies did not have a short-term effect, and all concentrations of LH resulted in receptor down-regulation. This is in contrast to the situation in rat testis Leydig cells where it is established that induced desensitization and eventual down-regulation of the LH receptor (Cooke & Rommerts, 1988; Cooke, 1988). These events are not thought to

system during a further 6 h incubation with a labelled amino acid. The constant presence of LH was required for this increase to occur. The effect of the translation and transcription inhibitors on the levels of LH receptors indicated that, for the maintenance of receptors, continual protein synthesis is required. The initiation of this process may occur at the gene level, as was demonstrated in porcine granulosa cells (Segaloff & Limbird, 1983), not from a pool of mRNA. The stimulatory effect of cyclic AMP on the induction of binding sites was also shown in cells which had been trypsin-treated; cyclic AMP increased the rate of recovery of binding sites in these cells, as well as raising the number of detectable binding sites.

The results obtained by pretreating the cells for 24 h with LH and (Bu)_2cAMP and then adding LH (3.3 nM) show that there is an increase in functionally coupled LH receptors. The results also indicated that there was no detectable production of cyclic AMP in cells that have been acid-washed. The maximal pregnenolone production in control cells could therefore be the result of two pathways acting together; one of these pathways may not be cyclic AMP-dependent. Previously it was shown that intracellular levels of cyclic AMP could not be restimulated after exposure to 1.12 nM-hCG for 15, 30 or 120 min using an acid wash to remove the hCG (Segaloff & Ascoli, 1981).

To conclude, the results of the present study support the concept of a role for cyclic AMP in the maintenance and regulation of LH receptors in MA10 cells. These cells are similar to granulosa cells in that factors acting via cyclic AMP maintain the LH receptor. In the MA10 cells, LH also appears to have two effects on its receptor. In the presence of LH, down-regulation occurs; this is probably due to an increased rate of degradation of the receptor upon binding of LH. When cyclic AMP is added in the presence of low doses of LH (< 0.033 nM) up-regulation occurs, which is probably due to an increased rate of synthesis of the LH receptor.

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Key words: Antisense; Oligodeoxynucleotide; Receptor; Desensitization; Luteinizing hormone; Leydig cell

Summary

We report a novel method to study the mechanisms of luteinizing hormone (LH) receptor desensitization and truncation, using antisense oligodeoxynucleotides that code for regions of the NH$_2$-terminus of the third extracellular loop and the C-terminus of the LH receptor. Mouse tumour (MA10) Leydig cells were incubated for 48 h with the addition of 2.5 $\mu$M antisense oligodeoxynucleotides at time 0. It was found that the NH$_2$-terminus oligodeoxynucleotide completely inhibited synthesis of LH receptors. Pretreatment with the third extracellular loop oligodeoxynucleotide inhibited LH-, cyclic AMP (db-cAMP)- and phorbol 12-myristate 13-acetate (PMA)-induced desensitization and truncation of LH receptors. Truncation, but not desensitization, of the LH receptor was prevented if pretreated with the C-terminus oligodeoxynucleotide. These results indicate that different sites of the C-terminal intracellular tail of the LH receptor are involved in the regulation of desensitization and truncation of the LH receptor.

Introduction

Luteinizing hormone (LH) stimulates testosterone synthesis in the testis Leydig cell via cyclic AMP and other second messengers (review: Rommerts and Cooke, 1988). Continued exposure of the Leydig cells to LH leads to a desensitization of these same responses and eventually to down-regulation of LH receptors (review: Cooke and Rommerts, 1988). Previously we demonstrated that desensitization of the rat Leydig cell involves an uncoupling of the LH receptor from its transducing system, i.e. a uncoupled to adenylate cyclase (Dix et al., 1989). We have recently shown that stimulation of protein kinase C (PKC) and cyclic-AMP-dependent protein kinase (PKA) leads to desensitation in the mouse Leydig cell (West et al., 1989) and that in addition to desensitization, truncation of the LH receptor occurs, which results in release of the extracellular fragment of the receptor (West and Cooke, 1991). We report the adaptation of the inhibitory antisense oligodeoxynucleotide strategy to investigate further mechanisms involved in desensitization and truncation. The inhibitory antisense oligodeoxynucleotide strategy has been used to study effects of c-myc (Heikkila et al., 1987), c-jun (Kulski et al., 1988) and type II$\beta$ regul
Materials and methods

Materials. Antisense oligodeoxynucleotides were purchased from British Biotechnology (Oxford, U.K.). Ovine luteinizing hormone (batch oLH-26, potency: 2.3 U/mg) and human chorionic gonadotropin (hCG) (batch CR-127, potency: 14,900 IU/mg) were obtained from the National Institute of Child Health and Human Development (Bethesda, MD, U.S.A.). Crude hCG (5000 IU) was obtained from Serono Laboratories (U.K.) (Hertfordshire, U.K.). Powdered media and sera were obtained from Gibco (Middlesex, U.K.). Carrier-free Na$^{125}$I was obtained from the Amersham Radiochemical Centre (Amersham, U.K.). All other reagents were purchased from Sigma (Dorset, U.K.) or BDH (Dorset, U.K.).

Methods. Iodination of hCG, measurement of $[^{125}\text{I}]$hCG binding sites and the maintenance of stock cultures of MA10 cells were carried out as previously described (West and Cooke, 1991).

Addition of antisense oligodeoxynucleotides. To deplete surface LH receptors, MA10 cells were treated with 0.05% trypsin/0.53 mM EDTA for 2 min at 34°C and then cultured in medium (West and Cooke, 1991). The cells were then plated at either $2 \times 10^5$ cells/well/500 $\mu$l in 24-well plates for $[^{125}\text{I}]$hCG binding studies or at $5 \times 10^4$ cells/well/200 $\mu$l in 96-well plates for desensitization studies. Antisense oligodeoxynucleotides, at concentrations of 2.5 $\mu$M, were added during plating down of the cells and 24 h afterwards. Incubations with LH, db-cAMP, or PMA were carried out after a 48 h preincubation with antisense oligodeoxynucleotides. Three anti-

Results

MA10 cells were depleted of surface LH receptors by treating with trypsin (West and Cooke, 1991) and then incubated with and without the antisense oligodeoxynucleotides for 48 h in medium. We have previously shown that the LH receptors on MA10 cells are replenished during this process (West et al., 1990). Loss of binding sites and receptor function were induced by incubating the cells with LH, PMA or db-cAMP (West et al., 1990).

The NH$_2$-terminal antisense oligodeoxynucleotide was added to MA10 cells either at time 0 (at the time the cells were plated for treatment), at 24 h or at time 0 and 24 h, and Table 1 shows the results obtained for $[^{125}\text{I}]$hCG binding sites. Figure 1 and Table 1 shows the results obtained for the effects of LH-stimulated AMP and pregnenolone when MA10 cells were treated with 2.5 $\mu$M NH$_2$-terminal antisense oligodeoxynucleotide. The addition of the antisense oligodeoxynucleotide either at time 0 resulted in a 75% loss of $[^{125}\text{I}]$hCG binding (Fig. 1) and no effect on LH-stimulated AMP or pregnenolone production was observed. When the NH$_2$-terminal oligodeoxynucleotide was added at time 0 and 24 h, there was no loss of $[^{125}\text{I}]$hCG binding (Fig. 1) and no effect on LH-stimulated AMP or pregnenolone production was observed.
Fig. 1. The effect of adding the LH receptor NH₂-terminal antisense oligodeoxynucleotide at different times on the concentration of [¹²⁵I]hCG binding sites. MA10 cells were plated at a concentration of 2×10⁵ cells/well/500 µl in 24-well plates and incubated with 2.5 µM antisense oligodeoxynucleotide over a 48 h incubation period. Cells were either incubated in the absence (1) of oligodeoxynucleotides or in the presence of NH₂-terminal oligodeoxynucleotide added at time 0 (2), 24 h (3) or 0 and 24 h (4). Cells were also incubated in the presence of the irrelevant oligodeoxynucleotide added at time 0 (5) or 0 and 24 h (6). At 48 h the concentration of [¹²⁵I]hCG binding sites was determined (values are the mean ± SD of two experiments, replicates = 6).

loss of LH-stimulated cyclic AMP or pregnenolone production (Table 1). Incubations with the irrelevant oligodeoxynucleotide added at time 0 or 0 and 24 h showed no effect on receptor numbers (Fig. 1) or stimulation of cyclic AMP or pregnenolone (Table 1).

Having established a procedure that deletes

> 98% of LH receptors we investigated the effect of an antisense oligodeoxynucleotide which interact with the mRNA sequence encoding

Fig. 2. The effect of an antisense oligodeoxynucleotide for the third extracellular loop of the LH receptor regulation by LH and db-cAMP. MA10 cells were plated at a concentration of 2×10⁵ cells/well/500 µl in 24-well plates and incubated in the absence or presence of 3.3 nM LH and 1 µM antisense oligodeoxynucleotide over a 48 h period with additions at time 0 and 24 h. At 48 h cells were washed with medium and further incubated in the absence or presence of either 3.3 nM LH and 1 µM db-cAMP. Before the concentration of [¹²⁵I]hCG was determined, cells were washed with 50 ml PBS (pH 3.0) to remove bound LH with medium. Statistical analysis was by the Student's t-test. *P < 0.01 (values are the mean ± SD of two experiments, replicates = 6).

TABLE 1
THE EFFECT OF ADDING THE LH RECEPTOR NH₂-TERMINAL ANTISENSE OLIGODEOXYNUCLEOTIDE AT DIFFERENT TIMES ON LH-STIMULATED CYCLIC AMP AND PREGNENOLONE

MA10 cells were treated as for Fig. 1. The cells were then incubated in the absence or presence of 3.3 nM LH. The LH was removed from the cells and cyclic AMP and pregnenolone concentrations were measured (values are the mean ± SD of two experiments, replicates = 3).

<table>
<thead>
<tr>
<th>Antisense</th>
<th>Time</th>
<th>Cyclic AMP (pmol/10⁶ cells/2 h)</th>
<th>Pregnenolone (ng/10⁶ cells/2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Basal</td>
<td>LH (3.3 nM) stim.</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>11.9 ± 0.1</td>
<td>362 ± 31.1</td>
</tr>
<tr>
<td>NH₂-terminal</td>
<td>0</td>
<td>6.2 ± 1.9</td>
<td>350 ± 32.5</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>8.2 ± 3.1</td>
<td>328 ± 14.1</td>
</tr>
<tr>
<td></td>
<td>0/24 h</td>
<td>12.6 ± 6.0</td>
<td>16.1 ± 0.6 *</td>
</tr>
<tr>
<td>Irrelevant</td>
<td>0</td>
<td>8.5 ± 2.3</td>
<td>314 ± 9.9</td>
</tr>
<tr>
<td></td>
<td>0/24 h</td>
<td>7.4 ± 3.2</td>
<td>320.7 ± 6.8</td>
</tr>
</tbody>
</table>

*P < 0.01.
Fig. 3. The effect of an antisense oligodeoxynucleotide encoding for the third extracellular loop of the LH receptor on desensitization of cyclic AMP production. MA10 cells were plated in 96-well plates at a concentration of $5 \times 10^4$ cells/well/200 μl. Cells were incubated in the absence or presence of 2.5 μM antisense oligodeoxynucleotide over a 48 h incubation period with additions at time 0 and 24 h. At 48 h the cells were washed and further incubated for 2 h in the absence or presence of either 3.3 nM LH, or $10^{-7}$ M PMA. PMA was dissolved dimethyl sulfoxide (DMSO) at $10^{-2}$ M and diluted to the required concentration with medium. Before a further incubation in the absence or presence of 3.3 nM LH, cells were washed with 50 mM glycine-buffered saline and 3 times with medium. Reactions were stopped after 2 h and cyclic AMP was measured (values are the mean ± SD of two experiments, replicates = 3).

Fig. 4. The effect of an antisense oligodeoxynucleotide encoding for the third extracellular loop of the LH receptor on desensitization of pregnenolone production. MA10 cells were treated as for Fig. 3 with a 2 h incubation with 3.3 nM mM db-cAMP or $10^{-7}$ M PMA, before restimulating with 3.3 nM LH. Reactions were stopped after 2 h and pregnenolone was measured (values are the mean ± SD of two experiments, replicates = 3).

PMA in control cells (Fig. 4A) was also prevented by the antisense oligodeoxynucleotide. However, LH-induced desensitization of steroidogenesis was not reversed (Fig. 4B) — this indicates that LH action involves additional mechanisms compared to the other two ligands. Desensitization by db-cAMP and PMA of steroidogenesis is apparently due to an interaction with the receptor and is linked to desensitization of cyclic AMP production. The mechanism may involve phosphorylation on the intracellular sequences, where two consensus sites for PKA phosphorylation exist.

To demonstrate that the above observations were due to a direct interaction of the antisense oligodeoxynucleotide with a functional region of the LH receptor nucleotide sequence, MA10 cells were also treated with an antisense oligodeoxynucleotide encoding for the third extracellular loop of the LH receptor.
cleotide encoding for 7 amino acids at the end of the C-terminus of the LH receptor. The results are shown in Table 2. The desensitization caused by LH, db-cAMP or PMA was not prevented by this oligodeoxynucleotide. However, it did prevent the loss of binding sites induced by LH and db-cAMP. This indicates that the loss of hCG binding sites is not linked to LH-induced desensitization. It also indicates that the mechanism involved in the loss of binding sites is not a result of phosphorylation of the LH receptor, because there are no phosphorylation sites present in this region of the cloned LH receptors.

Discussion

We have shown that the synthesis of LH receptors can be completely prevented by incubating MA10 cells with an antisense oligodeoxynucleotide coding for the NH$_2$-terminus of the LH receptor. Incubating MA10 cells with an antisense oligodeoxynucleotide coding for the third extracellular loop indicated that a truncated, but functional, receptor is synthesized lacking potential phosphorylation sites and that these phosphorylation sites are necessary for LH-induced desensitization. However, results from incubations with an antisense oligodeoxynucleotide coding for the amino acids at the end of the C-terminus of the LH receptor indicate that these phosphorylation sites are necessary for the loss of binding sites. Results indicate that this procedure to delete C-terminal phosphorylation sites from the LH receptor and may be applicable to other receptors. This novel site directed mutagenesis, because the activity of the modified receptors can be gated without the need for expression of the receptor.

Acknowledgements

We are grateful to the Science and Engineering Research Council and the Peter Siemon Ascoli (University of Iowa College of Medicine, Iowa, IA, U.S.A.) for the gift of the antisense oligodeoxynucleotide.

References


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 disruption using a 50 ml syringe, followed by Percoll density gradient [7] and incubated as for the rat Leydig cells. MA10 cells were established by 3β-hydroxysteroid dehydrogenase [8].

The addition of cyanoketone (5 μM) and SUX (pregnenolone metabolism inhibitors) (Sterling Reagents) with protease inhibitors was made 30 min prior to the reagents. The latter inhibitors (leupeptin (100 μM), PMSF and aprotinin (900 units/ml) (Sigma) were added as stored aliquots to the incubation medium. LH (NIH-LH, 2.3 IU/mg, Bethesda, MD, USA), dibutyryl cyclic AMP and cholera toxin (Sigma) were dissolved in incubation medium. Incubations were stopped and measured for testosterone and cyclic AMP production using radioimmunoassay of testosterone [9], pregnenolone [10], cyclic AMP [11,12]. Statistical analysis was by the Student’s t-test.

3. RESULTS

MA10 and rat Leydig cells were preincubated with leupeptin (100 μM), PMSF and aprotinin (900 units/ml) in 1 ml of medium (0.033 nM and 0.33 nM), 22R(OH)-cholesterol, cholera toxin (1.19 nM), forskolin (8 μM) and cyclic AMP (1 mM) were then added for 30 min. Incubations were stopped and measured for testosterone and cyclic AMP production. There was no effect on testosterone and cyclic AMP production of the effect of the protease inhibitors on steroidogenesis in rat Leydig (Fig. 1A) and MA10 cells. LH, forskolin and dibutyryl cyclic AMP-stimulated pregnenolone were all inhibited in two types. There was no effect on the conversion of 22R(OH)-cholesterol to pregnenolone. These results suggest that steroidogenesis in Leydig cells requires proteolysis before the conversion to pregnenolone. In the mouse but not rat Leydig cells, LH-stimulated cyclic AMP production is also dependent on proteolysis.
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 follow-
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

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

Acknowledgements: We are grateful to the Medical Research Council and the Wellcome Trust for financial support and to the University of Iowa College of Medicine, Iowa, USA, for use of the MA10 cells.

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NORMAL PROTEASE INHIBITORS

Fig. 1. The effect of protease inhibitors on pregnenolone production in rat 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 (□) LH, 5 µM (■) pregnenolone, 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 inhibition only (*P<0.001).

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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

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

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REFERENCES

Summary

The role of cyclic AMP and phorbol esters in luteinizing hormone (LH) receptor down-regulation in Leydig cells has been studied. Dibutyryl cyclic AMP (db-cAMP) (0.01, 0.1 and 1 mM), forskolin, and cholera toxin (1.19 nM) caused a 30–50% loss of $[^{125}\text{I}]$hCG binding sites and an inhibition of hormone-receptor-$[^{125}\text{I}]$hCG complex internalization in mouse tumour Leydig (MA10, MLTC-1) cells during 6–8 h. In contrast, db-cAMP had no effect on the level of binding sites or internalization of the hormone-receptor complex in rat testis Leydig cells or a rat tumour (R2C) Leydig cell. Phorbol 12-myristate 13-acetate (PMA) at concentrations from $10^{-9}$ to $10^{-5}$ M had no effect on hormone binding or hormone-receptor complex internalization in any of the Leydig cells. In contrast a 2 h preincubation of MLTC-1 cells with $10^{-7}$ M PMA caused a loss of subsequent LH-stimulated cyclic AMP and pregnenolone production. These results indicate that LH receptor down-regulation is mediated by cyclic AMP dependent kinase A and not protein kinase C, in mouse Leydig cells. No down-regulation of rat Leydig cell LH receptor occurred with either kinase.

Introduction

The luteinizing hormone (LH) receptor exists in a dynamic state exhibiting recycling in the rat testis Leydig cell (Habberfield et al., 1987) and continual receptor synthesis in the cultured mouse tumour (MA10) Leydig cell (West et al., 1990). In mouse Leydig cells (Freeman and Ascoli, 1981; Lefèvre et al., 1985) the LH receptor exhibits ligand-induced loss, i.e. down-regulation, of cell surface binding capacity when the cells are desensitized. This negative modulation of LH receptors is not observed in rat Leydig cell desensitization. At concentrations of LH which down-regulate the LH receptor in the Leydig cell, proteolytic cleavage of the hormone-receptor complex occurs at the plasma membrane which is due to an inhibition of the internalization mechanism (West and Cooke, 1991). In this report we present evidence to show that the down-regulation of LH receptors is dependent on cyclic AMP and not PMA in mouse Leydig cells.

Materials and methods

Ovine LH (batch oLH-26, potency: 2,400 IU/mg), and hCG (batch CR-127, potency: 14,900 IU/mg) were obtained...
Stock cultures of MA10, MLTC-1 and R2C cells were maintained according to the method of Ascoli (1981). Rat testis Leydig (RTL) cells were isolated and purified from adult male Sprague-Dawley rats (200–250 g) under non-sterile conditions, as described by Platts et al. (1988). Viability and purity of the cells were determined by the trypan blue exclusion method (Jeejeebhoy et al., 1975), diaphorase histochemistry (Aldred and Cooke, 1983) and by the 3β-hydroxysteroid dehydrogenase assay (Cooke et al., 1983). Iodination of hCG and [125I]hCG binding were performed as previously described (West and Cooke, 1991). Internalization of [125I]hCG was performed as indicated in the legend for Fig. 2. Pregnenolone and cyclic AMP were measured according to the methods of Van der Vusse et al. (1975), Steiner et al. (1972) and Harper and Brooker (1975).

Results

Dix et al. (1982, 1987) reported that PMA, but not cyclic AMP can cause desensitization in rat Leydig cells. Fig. 1 shows the effect of preincubating MLTC-1 cells (similar results were obtained for MA10 cells) for 2 h with either LH (3.3 nM), db-cAMP (1 mM) or PMA (10^{-7} M) on subsequent LH-stimulated pregnenolone and cyclic AMP production. After preincubation, the cells were stimulated for 2 h with or without LH (3.3 nM). Pretreatment with LH or PMA markedly decreased the ability of LH to stimulate cyclic AMP production (Fig. 1A). Further stimulation of pregnenolone production was inhibited by pre-treatment with LH, db-cAMP or PMA (Fig. 1B). Basal pregnenolone production in cells pretreated with LH or db-cAMP was significantly lower than in control cells.

To study the levels of LH receptors, desensitization, mouse and rat Leydig cells were incubated for 2 h at 34°C, with db-cAMP (0.1 and 1 mM) or PMA (10^{-9} to 10^{-5} M). cAMP caused a 30–50% loss in des
Fig. 2. The effect of db-cAMP on the levels of $[^{125}I]$hCG binding sites in Leydig cells. Leydig cells were plated at $150 \times 10^3$ cells/well in 1 ml medium. The cells were incubated for 2 h in the absence (1) or presence of 0.01 mM (2), 0.1 mM (3), 1 mM db-cAMP (4) or 3.3 nM (5) LH. At the times indicated cells were placed on ice and washed twice with 0.01 M PBS-BSA before $[^{125}I]$hCG binding was determined (values given are the mean ± SD, $n = 2$, replicates = 6).

$[^{125}I]$hCG binding sites by 120 min in MA10 and MLTC-1 cells, with no effect on binding sites in rat Leydig and R2C cells (Fig. 2). PMA had no significant effect on binding sites, by 120 min, in

### TABLE 1

THE EFFECT OF PMA ON THE LEVELS OF $[^{125}I]$hCG BINDING SITES IN LEYDIG CELLS

Leydig cells were plated at $150 \times 10^3$ cells/well in 1 ml growth medium. Rat Leydig cells were preincubated for 2 h. The cells were incubated for 2 h with PMA at concentrations from $10^{-9}$ to $10^{-5}$ M. After 2 h the cells were placed on ice and washed twice with 0.01 M PBS-BSA before $[^{125}I]$hCG binding was determined (values given are the mean ± SD, $n = 2$, replicates = 6).

<table>
<thead>
<tr>
<th>PMA (M)</th>
<th>Binding sites (fmol/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MA10</td>
</tr>
<tr>
<td>0</td>
<td>10.7 ± 2.11</td>
</tr>
<tr>
<td>$10^{-9}$</td>
<td>7.9 ± 2.11</td>
</tr>
<tr>
<td>$10^{-8}$</td>
<td>6.3 ± 1.3</td>
</tr>
<tr>
<td>$10^{-7}$</td>
<td>6.6 ± 2.0</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>12.6 ± 0.6</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>9.2 ± 1.3</td>
</tr>
</tbody>
</table>

Fig. 3. The effect of db-cAMP and PMA on the internalization of $[^{125}I]$hCG in MA10 cells. Cells were plated at $150 \times 10^3$ cells/well in 6-well plates. Two days later the cells were incubated at 10°C, to inhibit internalization (Habberfield et al., 1982). Two days later the cells were incubated for 2 h in 1 ml fresh growth medium with 1.4 nM $[^{125}I]$hCG in the presence of either 0.1 (v) or 1 mM (□) db-cAMP for 1 h. The cells were then placed on ice and washed with 0.01 M PBS-BSA to remove unbound hormone. The cells were then redissolved in 1 ml fresh growth medium at 34°C with the same additions. The cells were then placed on ice, medium removed and washed with 0.01 M PBS-BSA. The surface bound $[^{125}I]$hCG was determined by washing the cells twice with 50 ml cold buffered saline (pH3) (Ascoli, 1982) and counting the combined washings. To measure the amount of $[^{125}I]$hCG bound (B), cells were dissolved in 1 ml of 0.5 M NaOH and counted in a γ-counter (values represent the mean ± SD, $n = 2$, replicates = 6).
associated radioactivity (Fig. 3A) in the presence of db-cAMP demonstrated a more rapid decline than in control cells, though there was no increase in internalized levels (Fig. 3B). A similar profile was obtained for MLTC-1 cells. Incubating MA10 and MLTC-1 cells with PMA or rat Leydig cells with db-cAMP or PMA showed no significant effect on the internalization profile of $[^{125}]$hCG

A similar profile was obtained for MLTC-1 cells. Incubating MA10 and MLTC-1 cells with PMA or rat Leydig cells with db-cAMP or PMA showed no significant effect on the internalization profile of $[^{125}]$hCG

**Discussion**

Previously, we have reported that down-regulation in mouse Leydig cells is followed by protease internalization followed by protease degradation of the extracellular fragment of the receptor (West and Cooke, 1991). The figure demonstrates that this is a cyclic AMP process because db-cAMP, but not PMA, was able to mimic the effects of LH. Forskolin and cholera toxin were also able to bring down-regulation of the LH receptor to a state of internalization followed by degradation. MA10 and MLTC-1 cells. In contrast, PMA or db-cAMP was observed on internalization of hormone in rat Leydig cells.

It has been reported that in mouse LH-induced desensitization and down-regulation are concurrent (Freeman and Ascoli, 1987). LH-induced desensitization was mimicked by forskolin and PMA (Lefèvre et al., 1985). Previously though, no report of LH binding sites was reported with either forskolin. In our studies forskolin, PMA, and db-cAMP caused a loss in binding in the same conditions that cause down-regulation in rat Leydig cells, PMA can cause desensitization (Ascoli, 1987), but cyclic AMP does not (Dixon et al., 1987). These results indicate that there are differences in the sites and mechanisms of phosphorylation of the LH receptor in the rat Leydig cells.

![Fig. 4. The effect of protease inhibitors on the levels of $[^{125}]$hCG binding sites in Leydig cells. MA10 cells were plated at $150 \times 10^3$ in 1 ml medium and preincubated ± protease inhibitors (leupeptin 100 $\mu$M, PMSF 10 $\mu$M, aprotinin 900 units/ml) in 1 ml fresh medium, for 30 min at 34°C. The cells were further incubated ± protease inhibitors for 2 h either in the absence (1) or presence of 3.3 nM LH (2), 1.19 nM cholera toxin (3), or 80 $\mu$M forskolin (4). After 2 h the cells were placed on ice and washed twice with 50 mM glycine-buffered saline (pH 3.0) before $[^{125}]$hCG binding was determined (values given are the mean ± SD, n = 2, replicates = 6, *P < 0.05, paired Student t-test).]