THE ROLE OF ADENOSINE TRIPHOSPHATE IN HUMAN GRANULOSA-LUTEAL CELLS

BY

CHEN-JEI TAI
M.D., KAOHSIUNG MEDICAL COLLEGE, TAIWAN, 1990

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

IN

THE FACULTY OF GRADUATE STUDIES
DEPARTMENT OF OBSTETRICS AND GYNAECOLOGY
PROGRAM OF REPRODUCTIVE AND DEVELOPMENTAL SCIENCES

WE ACCEPT THIS THESIS AS CONFORMING TO THE REQUIRED STANDARD.

THE UNIVERSITY OF BRITISH COLUMBIA
APRIL 2001
©CHEN-JEI TAI, 2001
In presenting this thesis in partial fulfilment of the requirements for an advanced
degree at the University of British Columbia, I agree that the Library shall make it
freely available for reference and study. I further agree that permission for extensive
copying of this thesis for scholarly purposes may be granted by the head of my
department or by his or her representatives. It is understood that copying or
publication of this thesis for financial gain shall not be allowed without my written
permission.

Department of **OB/ GYN**

The University of British Columbia
Vancouver, Canada

Date **April 12, 2001**
ABSTRACT

Adenosine triphosphate (ATP) is released from cells such as platelets and co-released with neurotransmitter granules from autonomic nerves by exocytosis. Extracellular ATP binds to a G protein-coupled P2 purinoceptor that activates phospholipase C and phosphatidylinositol hydrolysis, generating diacylglycerol and inositol 1,4,5-trisphosphate, which stimulate protein kinase C (PKC) and cytosolic calcium mobilization, respectively.

Autonomic nerves have been shown to innervate the ovary and may be involved in regulating steroidogenesis. It is tempting to speculate that the co-released ATP from autonomic nerve endings in the ovary may play a role in regulating ovarian function.

A series of experiments has been performed in this study to examine (1) the expression and regulation of P2U purinergic receptor (P2UR) in human granulosa-luteal cells (hGLCs), (2) the role of PKC in ATP-induced calcium oscillations, (3) the action and mechanism of antigonadotropic effect of ATP on hGLCs, (4) the functional role of extracellular ATP in the ovary, and (5) the effect of ATP on the activation of the mitogen-activated protein kinase (MAPK) signaling pathway and its physiological role in hGLCs.

This study demonstrated for the first time the expression of P2UR mRNA in hGLCs, and the regulation of P2UR mRNA by hCG, cAMP and forskolin. The P2UR was functional in hGLCs, since activation of the P2UR by ATP or UTP resulted in rapid and transient mobilization of cytosolic calcium at the single cell level.

It appears that PKC may have dual actions by providing positive forward actions as well as negative feedback in controlling various signaling steps. As shown in this
study, ATP was capable of inducing calcium mobilization, which was negatively regulated by PKC, from both intracellular stores and extracellular influx in cultured hGLCs. The antigonadotropic effect of extracellular ATP was revealed as it significantly reduced hCG-stimulated cAMP production. The inhibitory effect of ATP was reversed by PKC inhibitors, staurosporin and bisindolylmaleimide I, indicating the involvement of PKC in mediating the antigonadotropic action of ATP in hGLCs.

Further, our data demonstrated that ATP was able to activate the MAPK signaling pathway in hGLCs. After binding to P2-purinoceptor, ATP activated MAPK subsequent to PLC and PKC activation through a PTX-insensitive G-protein in hGLCs. MAPK mediated the anti-gonadotropic action of ATP in steroidogenesis by reducing hCG-stimulated progesterone production.

These findings support a potential role of ATP in regulating ovarian function.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xi</td>
</tr>
<tr>
<td>PUBLICATION LIST</td>
<td>xiv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>xvii</td>
</tr>
</tbody>
</table>

## PART 1. BACKGROUND

1. INTRODUCTION

1.1 INTRODUCTION

1.2 HORMONAL REGULATION OF THE OVARY

1.3 INNERVATION OF THE OVARY

1.4 ADENOSINE 5'-TRIPHOSPHATE (ATP) AND PURINERGIC RECEPTOR

1.5 SIGNAL TRANSDUCTION VIA P2U-PURINERGIC RECEPTOR (G-PROTEIN-COUPLED SYSTEM)

1.5.1 GTP-BINDING PROTEINS (G-PROTEINS)

1.5.2 PHOSPHOLIPASE C

1.5.3 CALCIUM

1.5.4 PROTEIN KINASE C

1.5.5 MITOGEN-ACTIVATED PROTEIN KINASE (MAPK)

1.5.6 CYCLIC AMP/PKA SIGNAL TRANSDUCTION

HYPOTHESIS

SPECIFIC OBJECTIVES

iv
LIST OF TABLES

Table 1 Pharmacological classification of P2-purinergic receptors. 23
Table 2 Characteristics of IP3 receptors. 36
LIST OF FIGURES

Fig.1. Hypothalamus-pituitary-ovary axis and feedback loop. 4
Fig.2. The structure of pre-ovulatory follicle. 7
Fig.3. Hormone-stimulated steroidogenesis. 11
Fig.4. Steroidogenesis in the ovary. 12
Fig.5. Chemical structure of adenosine triphosphate (ATP). 16
Fig.6. Schematic representation of synthesis, storage, release and inactivation of ATP in purinergic nerve. 21
Fig.7. Scheme of the P2U purinoceptor. 25
Fig.8. The molecular domain of PLC isozymes. 32
Fig.9. Summary of two major receptor-mediated pathways for the formation of IP3 and DAG. 34
Fig.10. PKC structure. 41
Fig.11. Signal transduction pathway for the activation of PKC 44
Fig.12. Regulation of sequential kinase pathways that activate MAPKs. 47
Fig.13. Signal-transduction pathways of receptors or stress-activated MAPKs. 48
Fig.14. The cAMP signal transduction pathway. 51
Fig.15. The apparatus for DNA transferring. 58
Fig.16. Standard curve for protein assay. 66
Fig.17. Standard curves for radioimmunoassay. 70
Fig.18. Standard curve for cAMP assay. 72
Fig.19. Expression of P2UR mRNA in human granulosa-luteal cells (hGLCs). 83
Fig.20. Validation of semiquantitative PCR for P2UR in hGLCs. 87
Fig.21. The effect of different reagents on the regulation of P2UR mRNA in cultured hGLCs. 89
Fig.22. The dose effect of human chorionic gonadotropin (hCG) on the regulation of P2UR mRNA in cultured hGLCs. 90
Fig.23. The time effect of human chorionic gonadotropin (hCG) on the regulation of P2UR mRNA in cultured hGLCs. 91
Fig.24. The effects of 8-bromo-cAMP (cAMP) and forskolin on the regulation of P2UR.
mRNA in cultured hGLCs.

Fig.25. Effects of ATP and UTP on inducing cytosolic calcium mobilization in cultured hGLCs using microspectrofluorimetry.

Fig.26. Dose effects of ATP and UTP on inducing cytosolic calcium mobilization in cultured hGLCs using microspectrofluorimetry.

Fig.27. A. Effects of ATP on inducing cytosolic calcium oscillations in cultured hGLCs.
   B. Effects of 10 µM ATP on hGLCs cultured for various days (Day 3-Day 7).

Fig.28. Dose-dependent effects of PMA on ATP-evoked cytosolic calcium oscillations in cultured hGLCs.

Fig.29. The role of PKC in ATP induced-calcium oscillations in cultured hGLCs.

Fig.30. The effect of PMA on ATP-evoked cytosolic calcium oscillations in cultured hGLCs.

Fig.31. A. The role of PKC in homologous desensitization of ATP induced-calcium oscillations in cultured hGLCs.
   B. and C. The effect of PKC inhibitor.

Fig.32. A proposed model of the potential cross-talk between ATP-activated protein kinase C (PKC) and cytosolic calcium oscillations in hGLCs.

Fig.33. The effect of ATP on hCG-stimulated intracellular cAMP production in human granulosa-luteal cells (hGLCs).

Fig.34. The effect of PMA on hCG-stimulated intracellular cAMP production in human granulosa-luteal cells (hGLCs).

Fig.35. The role of staurosporin (ST) in inhibitory effect of ATP on hCG-stimulated cAMP production.

Fig.36. The role of Bisindolylmaleimide I (Bis) in inhibitory effect of ATP on hCG-stimulated cAMP production.

Fig.37. The presence of PKC isoforms in hGLCs.

Fig.38. PCR product showing the absence of PKCγ in hGLCs in three different patients.

Fig.39. Translocation of PKCα from cytosolic to membrane fraction after ATP
treatment in hGLCs.

Fig. 40. Down-regulation of PKCα in hGLCs achieved by prolonged treatment with 1 μM PMA for 18 hours.

Fig. 41. A proposed model of the potential cross-talk between ATP-activated protein kinase Cα (PKCα) and hCG-induced cAMP production in hGLCs.

Fig. 42. The dose-response of ATP on MAPK activation in human granulosa-luteal cells (hGLCs).

Fig. 43. The time course of ATP on MAPK activation in human granulosa-luteal cells (hGLCs).

Fig. 44. MAP kinase activity in hGLCs detected using a MAP kinase assay kit.

Fig. 45. The effect of suramin, a P2 purinoceptor inhibitor, on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs).

Fig. 46. The effect of pertussis toxin (PTX), a Gi protein inhibitor, on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs).

Fig. 47. The effect of neomycin, a PLC inhibitor, on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs).

Fig. 48. The effect of staurosporin, a PKC inhibitor (PKCI), on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs).

Fig. 49. The effect of PD98059, a MEK inhibitor (MEKI), on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs).

Fig. 50. The effect of ATP on intracellular cAMP production in human granulosa-luteal cells (hGLCs).

Fig. 51. The effect of MAPK on progesterone production in human granulosa-luteal cells (hGLCs).

Fig. 52. A. The effect of hCG on MAPK activation in human granulosa-luteal cells (hGLCs).

B. The effect of PD98059, a MEK inhibitor (MEKI), on hCG-induced progesterone production in human granulosa-luteal cells (hGLCs).

Fig. 53. The effect of MEKI in inhibitory effect of ATP on hCG-stimulated cAMP production.

Fig. 54. Proposed intracellular signaling cascades of ATP in hGLCs.
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>°C</td>
<td>Degree celsius</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Ci</td>
<td>Curie</td>
</tr>
<tr>
<td>cpm</td>
<td>Counts per minute</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DDT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>dNTP</td>
<td>Deoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diaminetetraacetic acid</td>
</tr>
<tr>
<td>E2</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular signal-regulated kinase 1/2</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>g (as in xg)</td>
<td>Acceleration of gravity</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>G-protein</td>
<td>GTP-binding protein</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotropin</td>
</tr>
<tr>
<td>hGLCs</td>
<td>Human granulosa-luteal cells</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-trisphosphate</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>IVF-ET</td>
<td>In vitro fertilization-embryo transfer</td>
</tr>
<tr>
<td>JNK/SAPK</td>
<td>c-jun terminal kinase/stress-activated protein kinase</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodaltons</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing hormone</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------------------------------</td>
</tr>
<tr>
<td>MAPKKks (=MKK)</td>
<td>MAPK kinases</td>
</tr>
<tr>
<td>MAPKKks</td>
<td>MAPK kinase</td>
</tr>
<tr>
<td>MAPKKKks</td>
<td>MAPKKK kinase</td>
</tr>
<tr>
<td>MEK 1/2</td>
<td>MAPK/ERK kinase 1/2</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>n (as in nM)</td>
<td>NM</td>
</tr>
<tr>
<td>P2UR</td>
<td>P2U purinergic receptor</td>
</tr>
<tr>
<td>P4</td>
<td>Progesterone</td>
</tr>
<tr>
<td>P450arom</td>
<td>Cytochrome P450 aromatase</td>
</tr>
<tr>
<td>P450c17</td>
<td>Cytochrome P450 17alpha hydroxylase/C17-20 lyase</td>
</tr>
<tr>
<td>P450sec</td>
<td>Cytochrome 450 side chain cleavage enzyme</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBS-G</td>
<td>Phosphate buffered saline-gelatin</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PDK-1</td>
<td>Phosphoinositide-dependent-kinase-1</td>
</tr>
<tr>
<td>PGF2α</td>
<td>Prostaglandin F2α</td>
</tr>
<tr>
<td>PIP2</td>
<td>Phosphatidylinositol 4,5-bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3,4,5-triphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>aPKC</td>
<td>Atypical protein kinase C</td>
</tr>
<tr>
<td>cPKC</td>
<td>Conventional protein kinase C</td>
</tr>
<tr>
<td>nPKC</td>
<td>Novel protein kinase C</td>
</tr>
<tr>
<td>PLA</td>
<td>Phospholipase A</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PTX</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>Reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>sec</td>
<td>Second</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SE</td>
<td>Standard error</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>StAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>Taq</td>
<td>Thermus aquaticus, source of a DNA polymerase</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethlenediamine</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris(hydroxyl methyl) aminomethane</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>v/v</td>
<td>Volume per volume</td>
</tr>
<tr>
<td>w/v</td>
<td>Weight per volume</td>
</tr>
</tbody>
</table>
**PUBLICATION LIST**

**PAPERS IN REFERRED JOURNALS**


mitogen-activated protein kinase by gonadotropin-releasing hormone in human granulosa-uteal cells. Endocrinology (In press)


ABSTRACTS AND PRESENTATIONS

granulosa-luteal cells. 32nd Annual meeting of the Society for the Study of Reproduction. Pullman, Washington, USA 60 (1): 131


ACKNOWLEDGEMENTS

I would like to express my most sincere appreciation to my supervisor Dr. Peter C.K. Leung. Only under his guidance and assistance was this thesis made possible.

I am also extremely indebted to my supervisory committee members, Dr. Rurak, Dr. Auersperg, Dr. Buchan and Dr. Fluker.

Sincerely, I would like to express my gratitude to all my colleagues, K.W. Cheng, S.K. Kang, K-C Choi, P.S. Nathwani, M. Woo, CS Chou and XM Zhu, and members of Dr. Auersperg's lab for their invaluable advice and whole-hearted support.

Grateful acknowledgement must be extended to Dr. C.R. Tzeng, Taipei Medical University Hospital, for introducing me into this field of reproductive sciences.

I sincerely thank the staff of the Genesis Fertility Centre, Vancouver, Canada for the generous provision of human granulosa-luteal cells, which are indispensable for this study. I also thank the Medical Research Council of Canada and the Canadian Institutes of Health Research for supporting my research.

A special note of appreciation is extended to my parents and parent-in-laws for their countless support and backing throughout my study.

Over the past several years in Canada, I have been blessed to have my dear wife and beloved daughters accompanying me, with their smiling faces, and providing me the source of strength and motivation required for fulfilling this study. Thank you, Lynn, Felicia and Eunice.
1.1 INTRODUCTION

Granulosa cells exhibit increasing secretory levels of estrogen during folliculogenesis. After the endogenous LH surge or subsequent to the administration of LH/HCG, granulosa cells of the Graafian follicle alter their main steroidogenic secretory products from estrogens to progestin [Channing et al., 1980]. Evidence suggests that growth factors [Pully and Marone, 1986; Veldhuis and Gwynne, 1989; Kamada et al., 1992], cytokines [Fukuoka et al, 1988], and vasoconstrictive peptides [Pucell et al., 1991] play a role in regulating the differentiation and proliferation of ovarian granulosa cells in an autocrine and/or paracrine manner. In addition, adrenergic and cholinergic nerves innervating the ovary may also be involved in regulating steroidogenesis [Mohsin and Pennefather, 1979; Burden and Lawrence, 1978; Stefenson et al, 1981]. Neurotransmitters such as epinephrine and norepinephrine have been shown to stimulate progesterone (P4) secretion in human granulosa cells via interaction with adrenergic receptors [Webley et al., 1988].

Intracellularly, ATP is a major energy source for diverse reactions and does not cross the plasma membrane of viable cells. ATP and its metabolites are released from cells such as platelets and purinergic nerves, and are coreleased with neurotransmitter granules from autocrine nerves by exocytosis [Gordon, 1986, Burnstock, 1977]. Extracellular ATP binds to a G protein-coupled P2 purinoceptor that activates
phospholipase C (PLC) and phosphatidylinositiode hydrolysis, generating diacylglycerols and inositol 1,4,5-triphosphate, which stimulate protein kinase C (PKC) and intracellular calcium mobilization, respectively [Berridge, 1984]. Thereafter, ATP may participate in various types of physiological responses including secretion, change in membrane potential, cell proliferation, platelet aggregation, neurotransmission, cardiac function, and muscle contraction [el-Moatassim et al, 1992; Burnstock, 1990].

Pharmacologically, the P2U purinoceptor has been identified in human granulosa-luteal cells (hGLCs) using microspectrofluorimetry [Lee et al, 1996; Kamada et al, 1994]. Furthermore, ATP has also been shown to regulate basal progesterone and estradiol secretion from luteinized human granulosa cells [Kamada et al, 1994]. It is tempting to speculate that the coreleased ATP from nerve endings innervating the ovary may play a role in regulating ovarian function. The following studies are designed to examine the expression and regulation of ATP receptor, the signaling pathway, and functional role of ATP in hGLCs.
1.2 Hormonal regulation of the ovary

1.2.1 Hypothalamo-pituitary-gonadal axis (Fig. 1)

The hypothalamo-pituitary-gonadal axis plays an important role in the regulation of sexual maturation and reproductive functions. Gonadotropin-releasing hormone (GnRH), synthesized in the hypothalamic neurons and secreted in a pulsatile manner, acts as a key regulator of the hormonal cascade [Braden and Conn, 1993; Conn, 1994]. Through the hypothalamo-hypophyseal portal system, GnRH is transported to the anterior lobe of the pituitary gland, where it stimulates the release of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) [Braden and Conn, 1993; Leung and Steele, 1992]. FSH and LH then regulate the production of steroid hormones from the ovary. Steroid hormones such as progesterone and estrogen negatively or positively regulate the secretion of GnRH and/or gonadotropins.
Fig. 1 Hypothalamus-pituitary-ovary axis and feedback loop.
1.2.2 Hormonal regulation of menstrual cycle

The understanding of the regulation of the ovarian cycle can be divided into three phases: the follicular phase, ovulation and the luteal phase. During the follicular phase a series of events takes place to ensure the recruitment and selection of a dominant follicle destined for ovulation. The reduced steroidogenesis and inhibin A secretion during the luteal phase give rise to an increase of FSH level, beginning several days before menses [Vermesh and Kletzky, 1987; Welt et al., 1997]. Aromatization, which converts androgens to estrogens, is induced or activated through the action of FSH. Thus, FSH initiates steroidogenesis in granulosa cells and stimulates granulosa cell growth [Yong et al., 1992]. Under the synergistic effect of estrogen and FSH, there is an increased production of follicular fluid, which accumulates in the intercellular spaces between granulosa cells. In the presence of FSH, estrogen becomes the dominant substance in the follicular fluid, whereas androgens predominate in the absence of FSH [McNatty et al., 1979; McNatty et al., 1980].

The two-cell/ two-gonadotropin system is an integrative process, between granulosa and theca cells, required for the production of steroid hormones in the follicular phase. The aromatase activity of granulosa cells is much higher than that of theca cells. In human preantral and antral follicles (Fig. 2), LH receptors are present only on theca cells and FSH receptors are present only on granulosa cells. Stimulated by LH, theca cells produce androgens which will pass through basement membrane and enter granulosa cells. Androgens are converted to estrogens under the effect of FSH-induced aromatization [Kobayashi et al., 1990; Yamoto et al., 1992]. The successful conversion to an estrogen dominant follicle marks the selection of a follicle destined to ovulate, and
only a single follicle succeeds. The negative feedback of estrogen on FSH secretion from the pituitary gland inhibits the development of all but the dominant follicle [Goodman and Hodgen, 1983]. The combination of FSH and a high local estrogen level in the dominant follicle provides the optimal environment for LH receptor development [Jia XC and Hsueh AJW, 1984; Kessel et al., 1985]. During the late follicular phase, estrogens rise rapidly to reach a peak approximately 24-36 hours prior to ovulation. The high level of estrogens positively feedback to induce the LH surge from anterior pituitary gland [Pauerstein et al., 1978; Fritz et al., 1992].

The LH surge initiates the continuation of meiosis in the oocyte, luteinization of granulosa cells, the rise of progesterone production, expansion of the cumulus and the synthesis of prostaglandins, which are required for ovulation [Tedeschi et al., 1992; O’Grady et al., 1972; Killick et al., 1987; Miyazaki et al., 1991]. FSH, LH and progesterone stimulate the activity of proteolytic enzymes responsible, together with prostaglandins, for digestion and rupture of the follicular wall [Yoshimura et al., 1987; Peng et al., 1992].

Normal luteal function requires optimal preovulatory follicular development and continuous LH support. Insufficient FSH during the follicular phase is related with lower preovulatory estrogens level, reduced midluteal progesterone production and a decreased luteal cell mass [Smith et al., 1986]. Progesterone exerts actions both centrally and locally to suppress the growth of any new follicle [diZerega and Hodgen, 1982; Gougeon and Lefevre, 1984]. Regression of the corpus luteum may be the action of luteolysis, mediated by estrogen, prostaglandin and endothelin-1 [Girsh et al., 1996a;
Girsh et al., 1996b; Auletta and Flint, 1988].

Fig. 2 The structure of pre-ovulatory follicle.
1.2.3 Human granulosa cells

After ovulation, the ruptured follicle reorganizes to become the corpus luteum [Drews, 1995]. Collectively, granulosa-lutein cells, theca interna cells, and the cells of the microvasculature interact with one another to give rise to this transient endocrine organ [Drews, 1995]. Concurrently, the granulosa cells begin to undergo the process of luteinization, which is characterized by both morphologic and functional differentiation [Amsterdam and Rotmensh, 1987; Zelznik, 1991]. Morphological luteinization is expressed histologically by an increase in cell size, and ultrastructurally by well-developed Golgi apparatus, endoplasmic reticulum and mitochondria [Rotmensh et al., 1986]. Functionally, luteinization is associated with an increase in the production and secretion of progesterone [Schipper et al, 1993]. The differential expression of the enzymes involved in the production of gonadal steroids is regulated by the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). If implantation does not occur, the corpus luteum regresses rapidly, allowing resumption of the next cycle of folliculogenesis [Drews, 1995].

1.2.4 Steroidogenesis in the ovary

The overall steroid biosynthesis pathway shown here is based on the findings of Kenneth J Ryan et al. [Ryan, 1959; Ryan and Smith, 1965]. The human ovary produces all 3 classes of sex steroids: estrogens, progestins and androgens.

The gonadotropic hormones from the anterior pituitary bind to G-protein coupled receptors, activate adenylate cyclase and increase intracellular cAMP level. Cyclic AMP activates protein kinase A and leads to gene transcription that encodes the steroidogenic
enzymes and accessory proteins. Additionally, cAMP stimulates the hydrolysis of cholesterol esters, resulting in the release of free cholesterol [Stocco, 1999]. Most of the cholesterol used for steroid synthesis is derived from the mobilization and transport of intracellular stores [Liscum and Dahl, 1992; Reaven et al., 1995]. Indeed, the rate-limiting step in steroidogenesis is the transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane where fully active cytochrome P450 side chain cleavage enzyme (P450scc) waits for the substrate. In response to hormone stimulation, steroidogenic acute regulatory (StAR) protein was shown to stimulate the translocation of cholesterol from the sterol-rich outer mitochondrial membranes to the cholesterol-poor inner mitochondrial membrane [Stocco, 1998] (Fig. 3).

During steroidogenesis, the number of carbon atoms in cholesterol is always reduced, never increased (Fig. 4). Thus, the biosynthesis begins with the cleavage of a 6-carbon side-chain of the 27-carbon cholesterol molecule to form the first steroid, the 21-carbon-containing molecule pregnenolone. This reaction is catalyzed by P450scc, which is located on the matrix side of the inner mitochondrial membrane [Simpson and Boyd, 1966, 1967; Yago and Ichii, 1969; Churchill and Kimura, 1979]. Once pregnenolone is formed, it may be metabolized within the mitochondria to progesterone by the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD) [Sulimovici et al., 1973; Chapman et al., 1992; Cherradi et al., 1994, 1995, 1997; Sauer et al., 1994] (Fig. 3).

Progesterone is then hydroxylated, and the side chain is cleaved subsequently by
cytochrome P450 17alpha hydroxylase/C 17-20-lyase (P450c17) to form androstenedione (19 carbons, C-19). Androstenedione (ketone) may be reduced to testosterone (hydroxyl) by the 17β-hydroxysteroid dehydrogenase reaction. Both C-19 steroids (androstenedione and testosterone) are rapidly converted to the corresponding C-18 phenolic steroid estrogens (estrone and estradiol) by aromatization reaction. This process involves hydroxylation of the 19-methyl group, followed by oxidation, loss of the 19-carbon, and ring A aromatization (dehydrogenation). Aromatization is mediated by cytochrome P450 aromatase (P450arom) found in the endoplasmic reticulum [Simpson et al., 1994].
Fig. 3 Hormone-stimulated steroidogenesis.
StAR: steroidogenic acute regulatory protein
P450scc: cytochrome P450 side chain cleavage enzyme
3β-HSD: 3β-hydroxysteroid dehydrogenase
Fig. 4 Steroidogenesis in the ovary.
P450scc: cytochrome P450 side chain cleavage enzyme
P450c17: cytochrome P450 17alpha hydroxylase/C 17-20-lyase
P450arom: cytochrome P450 aromatase
1.3 Innervation of the ovary

According to Mitchell, the ovarian nerves originate from three sources [Mitchell, 1938]. The superior ovarian nerves come from the intermesenteric nerves and from the renal plexus and descend along the outer side of the ovarian blood vessels to the ovary. The middle ovarian nerves from the superior hypogastric plexus (pre-sacral nerve) or from the hypogastric nerve are usually paired and also supply the fallopian tube. The inferior ovarian nerves may be three or four in number and arise from the inferior hypogastric plexus or the lower end of the hypogastric nerve. About half of the ovarian nerves are post-ganglionic with their cell bodies in the inferior mesenteric or spinal ganglia. The remainders are preganglionic and synapse in ganglia located in or near the ovaries and oviduct [Marshall, 1970; Langley and Anderson, 1895a, 1895b; Brundin, 1965].

The presence of intraovarian nerves is reported on the basis of histological studies [Frankenhauser, 1867; Waldeyer, 1870]. Thereafter, many studies have been performed to examine the pattern of the intrinsic innervation of the ovary. Not only the vascular bed but also other components, such as the follicle or its wall, are innervated by autonomic nerves [Guttmacher and Guttmacher, 1921]. Ovaries from cow, sheep, cat and guinea pig are richly supplied with adrenergic nerves in the cortical stroma, particularly enclosing follicles in different stages of development. In the follicular wall the nerve terminals are located in the theca externa, running parallelly to the follicular surface. Numerous adrenergic terminals also surround ovarian blood vessels. The adrenergic innervation is of intermediary density in the human ovary as well as the pig,
dog and cat ovaries. The cholinergic innervation is generally less developed, but has the same distribution as the adrenergic system around blood vessels and in the ovarian stroma, including follicular walls [Stefenson et al., 1981]. Some of the locations of adrenergic nerves in the ovary in all species investigated correspond to the distribution of the vascular system, but varicose nerve terminals are also found to run along various structures of the ovarian parenchyma [Owman et al., 1967].

The findings of a well-developed autonomic innervation of the ovary and its follicles, together with the notion that sympathetic activation contracts the follicular wall and increases intrafollicular pressure, have led to an hypothesis that peripheral neurogenic factors may also be involved in regulating follicular development, ovulation and ovarian function [Owman et al., 1975, 1979].

Evidence shows that neurotransmitters play a role in regulating ovarian steroidogenesis. Catacholamines have been demonstrated to modulate basal and gonadotropin-stimulated steroid secretion in human granulosa cells [Webley et al., 1988; Papenfuss et al., 1993; Bodis et al., 1993]. Further, cholingeric effects are demonstrated using acetylcholine and carbachol in human granulosa cells, suggesting that cholingeric neurotransmission may have a physiological significance in the ovary [Bodis et al., 1993].

Considering that the ovary is a well-innervated organ, co-release of ATP and noradrenaline from neurons suggests a potential role of ATP in regulating ovarian
functions [von Kugelgen et al., 1994].

1.4 Adenosine 5'-triphosphate (ATP) and purinergic receptor

1.4.1 Chemical structure of ATP

The molecule of ATP contains adenine, ribose and triphosphate [Albert et al, 1994] (Fig. 5).
Fig. 5 Chemical structure of Adenosine Triphosphate (ATP).
1.4.2 The sources of ATP

Intracellularly, ATP is produced during glycolysis, the citric acid cycle and via the electron transport chain and oxidative phosphorylation. Two molecules of ATP are formed from per molecule of glucose under anaerobic conditions. In contrast, 38 molecules of ATP are formed per molecule of glucose under aerobic conditions [Mayes, 1993].

The sources of extracellular ATP are mainly neuronal in origin; either released from purinergic nerve endings, or coreleased with traditional neurotransmitter granules such as acetylcholine and noradrenaline during neurotransmission [Morel and Meunier, 1981; Poisner and Trifaro, 1982]. The concentration of ATP in adrenergic granules of the sympathetic nervous system and in acetylcholine-containing granules of the parasympathetic nervous system can be as high as 150mM [Poisner and Trifaro, 1982]. Exocytotic release of ATP has also been found in nonneuronal cells, including platelets [Born and Kratzer, 1984], adrenal chromaffin cells [Cena and Rojas, 1990], mast cells [Osipchuk and Cahalan, 1992], and basophilic leukocytes [Osipchuk and Cahalan, 1992]. Another way to release cytosolic ATP is via intrinsic plasma membrane channels in the absence of irreversible cytolysis. The multidrug resistance (mdr1) gene product (or P glycoprotein) can function as a channel for ATP [Abraham et al, 1993]. Overexpression of P glycoprotein in transfected Chinese hamster ovary cells was accompanied by a three-fold increase in the steady-state release of ATP to the extracellular medium [Abraham et al, 1993].

Although ATP is present in millimolar level in the cytosol of all cell types,
extracellular levels of the nucleotide are normally maintained at extremely low levels by ubiquitous ecto-ATPase and ectophosphatase [Dubyak and el-Moatassim, 1993].

Extracellular ATP is quickly degraded by the ubiquitous ecto-ATPase and ecto-ATP diphosphohydrolase (Dombrowski et al., 1998; Dubyak and el-Moatassim, 1993; Zimmermann et al., 1998), there is no in vivo report about physiologically relevant time or condition with the release of ATP. Considering that ATP is co-released with neurotransmitter such as norepinephrine and acetylcholine from nerve endings (Gordon, 1986), and stress or acute exercise may induce the release of norepinephrine (De Cree et al., 1997; Paredes et al., 1998), it leads us to speculate that stress or exercise may induce the release of ATP and affect the ovarian function.

1.4.3 Biological roles of ATP

Intracellular ATP plays fundamental and ubiquitous roles in energy metabolism, nucleic acid synthesis, and enzyme regulation [Albert et al, 1994]. ATP is therefore called the energy currency.

Biological responses to extracellular ATP have been shown in virtually every organ and/or tissue system. Due to its ability to modulate contractility of most vascular smooth muscles and cardiac myocytes, extracellular ATP can exert significant effects on cardiovascular function and regional blood flow [Dubyak and el-Moatssim, 1993; Olsson and Pearson, 1990].
In the nervous system, extracellular ATP can potentiate acetylcholine release at the neuromuscular synapses that develop during in vitro culture of Xenopus-derived neurons and muscle cells [Fu and Poo, 1991].

In the lung, ATP is a particularly efficacious agonist for stimulating surfactant release from type II alveolar pneumocytes [Rice et al, 1990]. In vitro studies with tissue-cultured lymphocytes suggest that extracellular ATP might modulate DNA synthesis, cell-mediated killing, and apoptosis [Ikehara et al, 1981; Fillipini et al, 1990; Zheng et al., 1991].

In addition, extracellular ATP had been shown to act as a secretagogue in endocrine tissues or organs. ATP stimulates the secretion of pancreatic hormones (insulin and glucagon) from intact pancreas [Bertrand et al., 1990]. In porcine thyroid cells, ATP induces production of hydrogen peroxide, which is an essential process of iodide organization and a key reaction of Thyroid Stimulating Hormone (TSH)-induced thyroid hormone synthesis [Nakamura and Ohtaki, 1990]. Steroidogenesis in adrenocortical fasciculata cells is stimulated when P2 receptors are occupied [Kawamura et al., 1991].

In the reproductive system, extracellular ATP has been shown to activate contraction in intact myometrium [Osa and Maruta, 1987]. In addition, amnion cells isolated from human placenta express ATP receptors that are coupled to inositol phospholipid breakdown and Ca\(^{2+}\) mobilization [Vander Kooy et al, 1989]. ATP can trigger the acrosome reaction in vitro in human sperm [Foresta et al, 1992]. After binding to P2-purinergic receptors, ATP increases the secretion of testosterone in rat Leydig cells.
[Foresta et al., 1996]. Furthermore, extracellular ATP has been shown to regulate steroidogenesis in human granulosa cells and porcine granulosa cells [Kamada et al., 1994].

1.4.4 Purinergic receptor

1.4.4.1 Introduction

The term “purinergic” was introduced by Burnstock [Burnstock, 1971] to represent nerves that use ATP or other purine nucleotides as transmitters (Fig. 6). The notion that one type of nerve may use ATP or a related purine as the transmitter is supported by an electrophysiological observation that a response to neuronal stimulation is demonstrated following blockade of adrenergic and cholinergic nerve transmission [Burnstock, 1981]. Evidence has been accumulated that ATP is the principal active substance released from purinergic nerves. Such evidence includes (1) synthesis and storage of ATP in nerves; (2) release of ATP from nerve endings while they are stimulated; (3) action of exogenous ATP close to the responses to non-adrenergic, non-cholinergic nerve stimulation; (4) the presence of Mg$^{2+}$-activated ATPase, 5'-nucleotidase and adenosine deaminase, enzymes which inactivate ATP; (5) antagonists block the response to exogenous ATP; and (6) the identification of purinergic receptors [Burnstock, 1977; Su, 1983].
Fig. 6 Schematic representation of synthesis, storage, release and inactivation of ATP in purinergic nerve. (Modified from Burnstock, 1972)
1.4.4.2 Classification

The purinergic receptors have been classified as the P1 receptors and P2 receptors. Pharmacologically, the P1 receptors have a high affinity for extracellular adenosine and AMP (Adenosine > AMP > ADP > ATP), whereas P2 receptors have high affinity for ATP and ADP (ATP > ADP > AMP > Adenosine) [Gordon, 1986; el-Moatassim et al., 1992; Burnstock, 1990].

The P1 purinergic receptors have been subclassified into A1, A2a, A2b and A3 receptors on the basis of different effects of adenosine on adenylate cyclase [Gordon, 1986; el-Moatassim et al., 1992; Burnstock, 1990; Fredholm et al., 1994]. Six subtypes of P2 purinergic receptors, P2X, P2Y, P2Z, P2U, P2T and P2D have been identified in pharmacological and functional studies and supported by cloning data [Fredholm et al., 1994]. These include four ATP receptor types, termed P2X, P2Y, P2Z, and P2U. The ADP-selective receptor expressed in platelets has been denoted as the P2T (for thrombocyte) purinergic receptor [Dubyak and el-Moatassim, 1993]. P2D purinoceptors can be activated by the diadenosine polyphosphates, Ap4A and Ap5A (diadenosine tetraphosphate and diadenosine pentaphosphate) [Miras-Portugal, 1996].

Functionally, P2X-type receptors act as ligand-gated ion channels. P2Y-receptors and P2T function as G-protein-coupled calcium mobilizing ATP receptors, and P2Z receptors are associated with ATP-induced nonselective pore formation [Dubyak and el-Moatassim, 1993; Fredholm et al., 1994]. UTP is a particularly potent and efficacious agonist for P2U receptors. P2U also acts via a G-protein-coupled system; i.e., P2U receptors are functionally similar to but pharmacologically distinct from the P2Y-receptor.
class [Seifert and Schulz, 1989] (Table 1).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Agonist Selective</th>
<th>Antagonist</th>
<th>Signal Transduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X</td>
<td>AMP-C-PP=ATP=ADP&gt;AMP</td>
<td>Suramin</td>
<td>Intrinsic ion channel</td>
</tr>
<tr>
<td>P2Y</td>
<td>2-MeSATP&gt;ATP&gt;ADP&gt;&gt;=UTP</td>
<td></td>
<td>G-protein activation</td>
</tr>
<tr>
<td>P2Z</td>
<td>BzATP&gt;ATP&gt;&gt;ADP,AMP</td>
<td></td>
<td>Intrinsic channel/pore</td>
</tr>
<tr>
<td>P2U</td>
<td>UTP&gt;ATP=ATPγS&gt;ADP</td>
<td>Suramin</td>
<td>G-protein activation</td>
</tr>
<tr>
<td>P2T</td>
<td>2-MeSADP&gt;ADP</td>
<td></td>
<td>Intrinsic ion channel ?</td>
</tr>
<tr>
<td>P2D</td>
<td>Ap4A&gt;ADPβS&gt;Ap5A&gt;2-MeSATP</td>
<td></td>
<td>increase calcium</td>
</tr>
</tbody>
</table>

2-MeSATP: 2-Methylthio ATP
BzATP: Benzoylbenzoyl-ATP
ATPγS: Adenosine 5’-O-(3-thiotriphosphate)
2-MeSADP: 2-Methylthio ADP
ADPβS: Adenosine 5’-O-(3-thiodiphasphate)
1.4.4.3 The structure of P2-purinergic receptor (G protein-coupled receptors)

Reported cDNA and amino acid sequences of P2U receptors [Lustig et al., 1993; Parr et al., 1994] and P2Y receptors [Webb et al, 1993; Filtz et al., 1994] have shown that these receptors are members of the superfamily of G protein-coupled receptors. This superfamily contains seven putative transmembrane segments, with their amino termini located on the extracellular side and their carboxy termini located on the intracellular side of the cell membrane [Harden et al., 1995] (Fig. 7).

The P2-purinoreceptors are the only known G protein-coupled receptors that have a histidine residue at the cytoplasmic region of the third transmembrane segment, instead of the usual aspartic acid. This residue may be involved in transmission of ligand binding to the activation of the G protein in this G protein-coupled receptors [Fraser et al., 1988].
Fig. 7 Scheme of the P2U purinoceptor. P2U purinoceptor contains seven transmembrane domains with an extracellular N-terminus and intracellular C-terminus. Stars (*) denote the locations of potential phosphorylation sites by protein kinases such PKA or PKC. A near consensus ATP-binding site is located close to the N-terminus.
1.4.4.4 Cloning of P2 purinoceptors

The cDNA of P2Y and P2U purinoceptors have been cloned. Hydropathicity analyses of the predicted amino acid structures reveal that these receptors belong to the superfamily of seven transmembrane domain (TM), G-protein-coupled receptors. P2Y purinoceptors cloned from chick and turkey have about 30% homology with cloned murine and human P2U purinoceptors, and they share less identity with cloned P1 purinoceptors [Webb et al., 1993; Filtz et al., 1994; Lustig et al., 1993; Par et al., 1994]. In addition, the cloning, structural prediction and functional studies of rat P2X purinoceptors confirm that these receptors constitute another subfamily of P2 purinoceptors, the ligand-gated ion channel receptors [Valera et al., 1994; Brake et al., 1994].

Functional expression of cloned murine P2U purinoceptors in K562 human erythroleukemic cells has identified the P2U purinoceptors as a 53 kDa plasma membrane protein [Erb et al., 1993]. Considering that the predicted amino acid sequence of P2U purinoceptor indicates a molecular weight of approximately 42 kDa, this receptor appears to be glycosylated on one or both Asn (Asparagine) residues in its N-terminal extracellular domain [Boarder et al., 1995].

Sequence comparisons among P2Y and P2U purinoceptors with the adenosine A1 receptor reveal that several positively charged amino acid residues in TM3, TM6 and TM7 are present in the two P2 purinoceptors but not in the adenosine A1 receptor, suggesting that these positively charged residues are associated with the binding of the
negatively charged phosphate moieties of P2 purinoceptor agonists [Erb et al., 1995]. This suggestion is supported by site-directed mutagenesis of murine P2U purinoceptor cDNA followed by functional expression of the mutated receptors [Erb et al., 1995]. Neutralization of the positively charged amino acids Arg 262 and Arg 292 by substitution with Leu markedly reduced nucleotide potencies, indicating an inhibition of the binding of the negatively charged phosphates of P2U purinoceptor agonists.

Northern blot analysis demonstrates the existence of P2Y purinoceptor mRNA in chicken brain, spinal cord, gastrointestinal tract, spleen and leg muscle [Webb, 1993]. P2U purinoceptor mRNA is presented in mouse spleen, testis, kidney, liver, lung, heart and brain [Lustig et al., 1993] and in human heart, liver, lung, kidney, placenta and skeletal muscle [Parr et al., 1994].

1.4.5 Clinical application and development

As an extracellular signaling molecule, ATP binds to P2 receptor, a G-protein coupled receptor, to regulate physiological functions. While eight possible members of the P2X receptor and more than 11 P2Y receptors have been reported, an array of studies are undergoing to explore the possible clinical applications [Burnstock et al., 1998; Williams, 1999; Williams and Jarvis, 2000].

ATP is able to stimulate pancreatic insulin release via a glucose-dependent mechanism through activation of a P2Y receptor [Loubatrieres-Mariani et al., 1997]. Based on these studies, it has been suggested that a P2Y receptor agonist may improve
Exogenous ATP and UTP are potent stimulants of chloride secretion in airway epithelium [Manson et al., 1993] and mucin glycoprotein production from epithelial goblet cells [Lethem et al., 1993] acting through the P2Y2 receptor. While both ATP and UTP are equipotent, UTP is being developed as an inhalation formulation to enhance mucociliary clearance, chloride secretion and sputum expectoration for the treatment of cystic fibrosis and chronic bronchitis [Connolly and Duley, 1999; Donaldson and Boucher, 1998].

Extracellular ATP may act as a trigger of apoptosis or programmed cell death [Zheng et al., 1991]. The cytolytic action of ATP provides a potential approach to treatment of cancers [Papaport, 1997].

glucose tolerance and act as a potential antidiabetic drug.
1.5 Signal transduction via P2U-purinergic receptor (G protein-coupled system)

After binding to a G-protein coupled P2 purinergic receptor, ATP stimulates phospholipase C. The resultant production of diacylglycerol and inositol triphosphate activate protein kinase C (PKC) and intracellular calcium \([\text{Ca}^{2+}]_i\) mobilization, respectively.

1.5.1 GTP-binding proteins (G-proteins)

1.5.1.1 Composition and classification of G-proteins

Hormones and neurotransmitters interact with seven-transmembrane spanning receptors to regulate cellular functions through G-protein's [Post and Brown, 1996]. G-proteins are heterotrimeric in nature and are composed of \(\alpha,\beta\) and \(\gamma\) subunits encoded by distinct genes. Molecular cloning revealed the presence of at least 17 \(G\alpha\) isoforms, which can be divided into two subgroups- pertussis toxin (PTX) sensitive and PTX insensitive. PTX sensitive \(G\alpha\) proteins include \(Gt1\) (\(t=\)transducin), \(Gt2\), \(Gii\) (\(i=\) inhibitory), \(Gi2\), \(Gi3\), \(Go\), \(G_{\text{gust}}\) (\(gust=\) gustducin), \(Gz\), whereas \(Gs\) (\(s=\) stimulatory), \(Golf\) (\(olf=\) olfactory), \(Gq\), \(G11\), \(G12\), \(G13\), \(G14\) and \(G15/16\) belong to PTX-insensitive group. In addition, five subtypes of \(\beta-\) subunit (\(\beta1, \beta2, \beta3, \beta4\) and \(\beta5\)) and 11 subtypes of \(\gamma-\)subunit are identified [Downes and Gautam, 1999].
1.5.1.2 Activation of G-proteins

Under basal conditions, G-proteins exist as heterotrimers with GDP bound to the \( \alpha \)-subunit. Union of agonist to G-protein coupled receptor induces the release of GDP, the binding of GTP and the dissociation of GTP-\( \alpha \) complex from \( G\beta\gamma \) dimer. GTP-\( \alpha \) and \( G\beta\gamma \) then interact with their effectors such as ion channels, adenylate cyclase and phospholipase C (PLC). Endogenous GTPase hydrolyses GTP to GDP and leads to the re-association of GDP-\( \alpha \) and \( G\beta\gamma \), thus terminating signal transduction [Lopez-Ilasac, 1998].

1.5.1.3 Expression of G-proteins in the human ovary

It is demonstrated that human granulosa-luteal cells express PTX-insensitive G proteins, \( \alpha \)q and \( \alpha \)11, which are believed to exert biological function through PLC-\( \beta \) activation [Carrasco et al., 1997].
1.5.2 Phospholipase C

1.5.2.1 Introduction

An array of extracellular signals stimulates hydrolysis of phosphatidylinositol 4,5-bisphosphate by phosphoinositide specific phospholipase C (PLC) to yield two important second messengers, diacylglycerol and inositol 1,4,5-trisphosphate. A large number of studies provided an insight into structures and regulation of PLC isozymes [Williams, 1999; Katan, 1998].

1.5.2.2 Classification and structure

Ten mammalian PLC isozymes have been reported and classified into three subtypes: PLCβ1-4, PLCγ1-2, PLCδ1-4. The basic structure of PLC includes PH domain, EF-hand, catalytic domain and C2 domain. It is thought that PH (pleckstrin homology) domain is associated with membrane binding [Lemmon et al., 1995]. The EF-hand domain is a region of the enzyme that forms a flexible link to the PH domain, and there is no evidence that this domain of PLC binds calcium [Grobler and Hurley, 1998]. The catalytic domain hydrolyzes the substrate through a two-step mechanism: phosphotransferase reaction and phosphohydrolase reaction [Williams, 1999]. The C2 domain, which is similar to the second conserved domain of protein kinase C, is present in all of the isozymes. The function of C2 domain, with three calcium-binding sites per domain, has been studied intensively. They suggest that the C2 domain may act as a calcium-dependent lipid membrane binding module [Nalefski and Falke, 1996] (Fig. 8).
Fig. 8 The molecular domain of PLC isozymes. The β–isozymes have C-terminal extensions of about 400 residues. The γ–class of isozyme has an insertion of about 500 residues in catalytic domain.
1.5.2.3 Activation of PLC (Fig. 9)

The regulation of four mammalian PLCβ isozymes (β1–β4) has been extensively studied. Activation of PLCβ by many agonists such as histamine, vasopressin, GnRH and ATP is induced through G-protein coupled receptors, and the reaction is mediated by the α-subunit and/or by βγ-subunits [Rhee and Bae, 1997; Morris and Scarlata, 1997; Jiang et al., 1997; Kim et al., 1997].

It is generally accepted that stimulation of PLCγ involves receptor or non-receptor tyrosine kinases. Almost all polypeptide growth factor receptors, containing an intrinsic tyrosine kinase activity, have been linked to activation of PLCγ. Some receptors such as T-cell antigen receptor and IgE receptor with no tyrosine kinase activity themselves are able to activate PLCγ through non-receptor tyrosine kinase such as Src or Syk [Rhee and Bae, 1997, Kamat and Carpenter, 1997].

Regulation of the δ-class of isozymes has not been fully characterized. Unlike PLCβ and PLCγ, the activation of PLCδ is induced by an increase in calcium concentration in the absence of any other stimuli [Allen et al., 1997].
Fig. 9 Summary of two major receptor-mediated pathways for the formation of IP3 and DAG.
1.5.3 Calcium

Calcium ions play a major role in transmitting extracellular stimuli into regulating a wide range of biological events such as muscle contraction, fertilization, neurotransmitter release, secretory processes, cell proliferation, gene expression and apoptosis [Evenaset al., 1998; Berridge, 1993].

1.5.3.1 Ca$^{2+}$ store and mobilization

In eukaryotic cells the cytoplasmic calcium concentration ranges from 100 nM in a resting (baseline) level to 1-10 μM, whereas the extracellular free Ca$^{2+}$ concentration is around 1.2 mM. The flow of Ca$^{2+}$ can be regulated by voltage, receptor or store-operated channels [Barritt, 1999].

Intracellularly, calcium is stored in the endoplasmic reticulum (ER) and/or sarcoplasmic reticulum (SR). Two major intracellular Ca$^{2+}$-release channels in the ER and SR are identified- the inositol-1,4,5-trisphosphate receptor (IP3R) and the ryanodine receptor (RyR). Calcium release through IP3R is induced by IP3, while cyclic ADP-ribose regulates the RyR [Mikoshiba, 1997]. Muscle tissue, especially skeletal and cardiac, provides the richest source of RyR, whereas IP3Rs are more evenly distributed.

At least three isoforms of both IP3R and RyR have been characterized [Shoshan-Barmatz and Ashley, 1998; Joseph, 1996]. RyR is almost twice as large as the IP3R. The skeletal RyR1 and cardiac RyR2 share 66% homology. RyR3 has been
demonstrated in mink lung epithelium [Takeshima et al., 1989; Zorzato et al, 1990; Otsu et al, 1990]. Characteristics of IP3 receptors are shown in Table 2. Various pharmacological agents have been shown to modulate IP3R activity but not specific. For example, heparin acts as a competitive inhibitor, but it may also inhibit the generation of IP3. Caffeine is a potent inhibitor of the IP3R by abolishing IP3-induced calcium release, but it may activate RyRs [Parker and Ivorra, 1991; Missiaen et al., 1992].

<table>
<thead>
<tr>
<th>Table 2 Characteristics of IP3 receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Type-I</td>
</tr>
<tr>
<td>Type-II</td>
</tr>
<tr>
<td>Type-III</td>
</tr>
</tbody>
</table>

(Modified from Joseph, 1996)
1.5.3.2 Intracellular Ca²⁺-binding proteins

The main targets of Ca²⁺ released from intracellular stores are Ca²⁺-binding proteins, many of which play important roles in regulating cellular functions. These proteins include calmodulin, troponin C, calpain and protein kinase C [James and Putney, 1998; Spyracopoulous et al., 1997; Blanchard et al., 1997; Essen et al., 1996].

1.5.3.3 Calcium channels

The entry of calcium from extracellular source is regulated by calcium channels. There are three main channels classified on the basis of their regulatory mechanism including voltage-operated channels, receptor-operated channels and store-operated channels [Berridge, 1997]. Physiological and molecular studies have identified several different voltage-dependent calcium channels such as L-, N-, P-, Q-, R- and T-type [Jones, 1998]. Pharmacologically, L-type channels are highly sensitive to dihydropyridines (DHPs), and N-type channels are blocked potently by ω-conotoxin [Plummer et al., 1989]. P channels, originally characterized in Purkinje neurons of the cerebellum, are now defined by rapid block by the spider toxin ω-Aga IVA [Mintz et al., 1992].

1.5.3.4 Intercellular calcium waves

Calcium waves are not only confined to single cells but can diffuse from one cell to another via two separate mechanisms. One mode of transmission depends on the diffusion of either calcium itself or IP3 through gap junctions [Boitano et al., 1992]. In
cells lacking gap junctions, waves spread by means of a secreted intermediate [Osipchuk and Cahalan, 1992].
1.5.4 Protein kinase C

1.5.4.1 Introduction

The PKC family, a group of widely distributed serine/threonine kinases, mediates intracellular signaling of numerous cellular regulators including hormones, neurotransmitters and growth factors [Nishizuka, 1984; Berridge, 1993; Dekker LV, 1997]. There are currently 13 known PKC isozymes that are divided into four groups based on their requirements for activation and structures. Conventional PKCs (cPKC) (α, βI, βII and γ) are regulated by diacylglycerol (DAG), phosphatidylerine and Ca\textsuperscript{2+}. βI and βII are alternative mRNA splicing products of the same gene, differing by fewer than 50 amino acids in the V5 region. Novel PKCs (δ, ε, θ and η) (nPKC) require diacylglycerol and phosphatidyserine, but are calcium-independent. Atypical protein kinase Cs (ζ, ι and λ) (aPKC) require neither calcium nor DAG. PKCμ and ν, a fourth subfamily, are activated by DAG but not structurally related to PKC. PKCν has 77.3% similarity to human PKCμ. [Newton, 1997; Jaken, 1996; Hayashi et al, 1999].

1.5.4.2 Structure (Fig. 10)

Protein kinase C, including four conserved (C1-C4) and five variable (V1-V5) regions, consists of a regulatory domain attached by a hinge region to a conserved kinase catalytic domain. The regulatory domain contains two domains, C1 and C2, and an autoinhibitory pseudosubstrate sequence within the C1 domain. The C1 region of the cPKCs and nPKCs contains two copies of a Cys-rich zinc finger motif that constitutes the
DAG/phorbol ester binding site. The aPKCs have only one copy in C1 region that does not bind DAG or phorbol ester. The C2 region of the cPKCs contains an Asp-rich calcium and phospholipid-binding site, which is absent in nPKCs and aPKCs. The catalytic domain contains the ATP-binding site, C3, and the substrate-binding site, C4 [Mollor and Parker, 1998]. PKCμ is also referred to as PKD in mouse, which is characterized by lack of the typical pseudosubstrate site as well as the presence of unique amino-terminal hydrophobic domains together with its unusually large molecular size. Furthermore, a pleckstrin homology (PH) domain is identified in the regulatory region. Functional studies reveal that DAG and phorbol ester promote PKCμ kinase activity [Johannes et al., 1994; Dieterich et al., 1996.
Fig. 10. PKC structure. The classical group (cPKC) contains all four conserved amino acid regions. The novel group (nPKC) lacks a calcium binding C2 region. The atypical group (aPKC) also lacks the C2 region as well as half of the C1 region. PKC\(\mu\) contains a pleckstrin homology (PH) domain.
1.5.4.3 Distribution of PKC isozymes

PKCα, δ, and ζ seem to be the most widely distributed isozymes of the PKC family. In contrast, PKCγ is expressed mainly in the central nervous system, while PKCθ is expressed in skeletal muscle and haemopoetic cells. Further, PKCη is highly expressed in skin and lung tissue, with low levels detected in the brain and spleen [Wetsel et al., 1992; Nishizuka, 1988; Osada et al., 1992].

Various PKC isoforms are present in the ovary of different species. In the rabbit corpus luteum, α, β and δ isoforms of PKC are identified [Maizels et al., 1992], while porcine corpora lutea contain α and β [DeManno et al., 1992]. Western blot analysis reveals that bovine corpus luteum expresses α and δ [Orwig et al., 1994].

1.5.4.4 Signal transduction

After binding to G-protein coupled receptors, an array of hormones, neurotransmitters and growth factors activate phospholipase C (PLC) resulting in the generation of IP3 and DAG, which releases Ca^{2+} from stores in the endoplasmic reticulum and subsequently activates PKC. Alternatively, phospholipase D, which can be activated by a receptor, by PKC or by kinases such as mitogen activated protein kinase (MAPK), cleaves phosphatidylcholine (PC) to phosphatidic acid (PA). PA either activates PKC directly or is hydrolysed to DAG for PKC activation [Nishizuka, 1995]. Atypical PKCζ is activated via a different transduction pathway - through phosphatidylinositol-3,4,5—triphosphate (PIP3) generated by activation of
phosphatidylinositol-4,5-diphosphate-3-kinase (PI-3-kinase). It is believed that phosphoinositide-dependent-kinase-1 (PDK-1) mediates the activation of PKCζ [Zhou et al., 1994; Toker, 2000] (Fig. 11).

1.5.4.5 Localization of PKC isozymes

Active PKC may translocate to specific cellular compartments and bind to certain proteins, which direct PKCs to second messenger activators and position PKCs in the proximity of appropriate substrate proteins. There are two general categories of proteins that are associated with active PKC binding. The first includes substrates that interact with C-kinases (STICKs) and the second includes nonsubstrate protein (receptors for activated C-kinase; RACKs) that place PKCs in close contact with substrate proteins [Jaken and Parker, 2000].

1.5.4.6 The role of PKC

A wide range of cellular function is known to be regulated via the PKC signaling pathway such as cell differentiation and proliferation, secretion, cytoskeleton function, cell-cell contacts, gene expression, cell survival and the modulation of membrane ion channels or receptors. In addition, there is evidence that PKC has a dual action by providing positive forward actions as well as negative feedback, controlling various signaling steps [Nishizuka, 1989; Nishizuka, 1986].
Fig. 11 Signal transduction pathway for the activation of PKC

PKC: protein kinase C
PLC: phospholipase C
PLD: phospholipase D
PME: phosphomonoesterase
DAG: diacylglycerol
PI4,5P2: phosphatidylinositol 4,5 bisphosphate
PI3,4,5P3: phosphatidylinositol 3,4,5 Trisphosphate
IP3: inositol triphosphate
PC: phosphatidylcholine
PA: phosphatidic acid

PI3-K: phosphoinositide-3-kinase
PDK-1: phosphoinositide-dependent-kinase-1

PKC: protein kinase C
PLC: phospholipase C
PLD: phospholipase D
PME: phosphomonoesterase
DAG: diacylglycerol
PI4,5P2: phosphatidylinositol 4,5 bisphosphate
PI3,4,5P3: phosphatidylinositol 3,4,5 Trisphosphate
IP3: inositol triphosphate
PC: phosphatidylcholine
PA: phosphatidic acid

Fig. 11 Signal transduction pathway for the activation of PKC

44
1.5.5 Mitogen-activated protein kinase (MAPK)

1.5.5.1 Introduction

The ubiquitous MAP kinases comprise a family of serine/threonine kinases that are involved in the transduction of externally derived signals regulating cellular proliferation, differentiation and division. Activated MAPK may mediate various cellular functions in the membrane, cytoplasm, nucleus, or cytoskeleton. Upon activation, MAP kinases translocate to the nucleus, resulting in the phosphorylation and activation of nuclear transcription factors involved in DNA synthesis and cell division [van Biesen et al., 1996; Blenis, 1993].

1.5.5.2 Classification and activation cascades

MAP kinases have been classified into three subfamilies: extracellular-signal regulated kinases (ERKs), stress-activated protein kinases/ c-jun N-terminal kinases (SAPKs/JNKs) and p38 kinase [Lopez-Ilasaca, 1998]. Both tyrosine kinase receptors and G-protein-coupled receptors have been demonstrated to activate ERKs. The JNK and p38 MAPK are activated by environmental stresses (osmotic shock, heat shock, UV radiation) or cytokines (interleukin-1 and tumor necrosis factor-α) [Raingeaud et al., 1995]. The discovery of the first mammalian MAPK was based on the identification of p42 and p44 MAPKs (ERK1 and ERK2, respectively) in 1987 [Ray and Sturgill, 1987]. These two MAPK isoforms are activated by dual phosphorylation on a TEY (Threonine-Glutamic acid-Tyrosine) motif by the MEK1 (MAPK/ERK-activating kinase,
MAPK kinases) and MEK2 isoforms. Both MEK1 and 2 are themselves activated by phosphorylation of two serine residues by the protein kinase Raf (MAPKK kinase) [Ahn et al, 1992; Brunet and Pouyssegur, 1997].

There are currently seven different MAPK kinases (MAPKK) identified. The first MAPKKs to be cloned were MEK1/2 which activate ERK1/2. MKK4 and MKK7 phosphorylate and activate JNK. MKK3 and MKK6 specifically phosphorylate and activate p38, whereas MKK5 activates ERK5 [Fanger GR, 1999] (Fig. 12).

Translocation of activated MAPK to the nucleus and subsequent phosphorylation of a variety of transcription factors including c-Myc, Elk-1 and ATF2 support the involvement of MAPK in transducing cytoplasmic signals to nucleus. Specifically, ERK1 targets ELK-1, whereas ERK2 prefers c-Myc. SAPK/JNK is able to effect c-jun, ATF2 and Elk, and p38 targets ATF2, Elk and Max [Lopez-Ilasaca, 1998] (Fig. 13).
Fig. 12 Regulation of sequential kinase pathways that activate MAPKs. MAPKs such as ERK, JNK, p38 are activated by tyrosine and threonine phosphorylation by MAPKK, MEKs or MKKs. MAPKKs are activated by serine and threonine phosphorylation by the MAPKKKS such as the Rafs and MEKKs.

MAPK: mitogen-activated protein kinase
MAPKK: MAPK kinase
MAPKKK: MAPKK kinase
MEKK: MEK kinase
ERK: extracellular-signal regulated kinase
JNK: c-Jun N-terminal kinase
Stress/cytokine | Growth factor, hormone, Neurotransmitter
---|---
Heat shock, Osmotic shock, UV | TNFα, IL1α,β, G-protein-coupled

Fig. 13 Signal-transduction pathways of receptors or stress-activated MAPKs.
1.5.6 Cyclic AMP /PKA signal transduction

The cAMP signaling system is a second messenger-dependent pathway that converts extracellular signal into intracellular responses and regulates many vital functions such as gene transcription, proliferation, differentiation, reproductive function and secretion. ATP has been demonstrated to increase intracellular cAMP production by activating adenylate cyclase in several cell systems [Communi et al., 1997; Conigrave et al., 1998], indicating the stimulation of protein kinase A (PKA).

Many neurotransmitters and hormones transduce their signals into cells through the cAMP-dependent PKA pathway. These agonists include neurotransmitters such as acetylcholine, dopamine, norepinephrine, serotonin and histamine as well as peptide ligands such as somatostatin, corticotropin-releasing factor, growth-hormone-releasing hormone, follicle-stimulating hormone and luteinizing hormone [Brandon et al., 1997].

The cAMP signaling pathway is composed of a cascade of regulatory proteins. The cascade elements consist of a hormone or neurotransmitter (first messenger)-specific transmembrane receptor that is coupled to a heterotrimeric G-protein complex (Gα and Gβγ), resulting in the activation of an intracellular effector adenylate cyclase. Adenylate cyclase generates cAMP (second messenger) which activates PKA responsible for the phosphorylation of appropriate substrates [Spiegel et al., 1992; Tang and Gilman, 1991; Clapham and Neer, 1993]. The cAMP signal is terminated by cyclic nucleotide phosphodiesterase, which hydrolyzes cAMP to 5'-AMP, and by phosphoprotein
phosphatases, which dephosphorylate the phosphoproteins [Ishikawa, 1998; Beavo and Reifsnyder, 1990; Shenolikar and Nairn, 1991].

The elevation of cAMP leads to the activation of PKA, a tetrameric enzyme, which is composed of a dimeric regulatory (R) subunit and two monomeric catalytic (C) subunits. The R-subunit, a pseudosubstrate to inhibit phosphotransferase activity of C-subunit, binds with cAMP resulting in the release of active C-subunit [Beebe, 1994]. Catalytic subunits migrate into the nucleus where they phosphorylate a single serine residue on CREB protein (cyclic AMP response element-binding protein, a transcriptional activator) and thereby activate it. The active CREB then interact with the cAMP response element (CRE) found in the promoters of cAMP-responsive genes to initiate transcription [Sassone-Corsi, 1995] (Fig. 14).
Ligands

Extracellular

Neurotransmitters:
acetylcholine, dopamine, norepinephrine, serotonin and histamine

Peptide ligands:
somatostatin, corticotropin-releasing factor, growth-hormone-releasing hormone, follicle-stimulating hormone, luteinizing hormone and human chorionic gonadotropin

Intracellular

G-protein

Adenylate cyclase

ATP

cAMP

PKA

Nucleus

CRE Binding protein (CREB)

cAMP Response Element (CRE)

Activate transcription of cAMP-responsive gene

Fig. 14 The cAMP signal transduction pathway.
HYPOTHESIS

The ovary is a well-innervated endocrine organ, drawing our attention to investigate the role of neurotransmitters in modulating ovarian function. Considering that ATP is released from nerve endings and evokes cytosolic calcium oscillations in human granulosa cells (hGLCs), it is hypothesized that ATP plays a role in regulating ovarian function such as steroidogenesis. To address this hypothesis, a series of studies are performed to examine the expression of P2 purinergic receptor, signaling pathways and functional role of ATP in hGLCs.

SPECIFIC OBJECTIVES

1. To examine the expression and regulation of P2 purinergic receptors in hGLCs.

2. To examine the role of protein kinase C in regulating ATP-induced cytosolic calcium oscillations in hGLCs.

3. To examine the mechanism of antigonadotropic action of ATP in hGLCs: the involvement of protein kinase C.

4. To examine the effect of ATP on activation of MAPK, its intracellular signaling pathway and functional role in hGLCs.
PART 2 GENERAL MATERIALS AND METHODS

2.1 Human granulosa-luteal cells culture

Human GLCs were collected from patients undergoing an In Vitro Fertilization - Embryo Transfer program. The use of human GLCs was approved by UBC Clinical Screening Committee for Research and Other Studies Involving Human Subjects. The follicular fluid collected during oocyte retrieval was centrifuged at 1000 xg for 10 min. The pellet containing granulosa cells and red blood cells was resuspended with Dulbecco's Modified Eagle Medium (DMEM). Granulosa cells were then separated from red blood cells in follicular aspirates by centrifugation through equal volume of Ficoll Paque at 1000 xg for 15 min. Granulosa cells sitting on interphase were collected and washed twice with DMEM. After brief centrifugation, cell pellet of granulosa cells were suspended in DMEM containing 100 U penicillin G sodium/ ml, 100 μg streptomycin/ml and 10% fetal bovine serum. The cells were plated at a density of approximately 200,000 cells per dish in 35-mm culture dishes. The dishes were incubated at 37 °C under a water-saturated atmosphere of 5% CO2 in air for 3 days.

Human GLCs were collected from patients undergoing in vitro fertilization treatment who ranged in age from 23 to 43 years. Forty-nine percent had severe male factor infertility while the remainder had various female factors or long-standing unexplained infertility. Ovarian stimulation entailed a long luteal phase down-regulation protocol for women under 40 years or a follicular phase flare protocol for women >40
years, as previously described (Yuzpe et al., 2000). Human granulosa-luteal cells obtained from 105 patients were used in this study.

2.2 Isolation of total RNA

Total RNA was prepared from the cultured hGLCs by the phenol-chloroform method of Chromczynski and Sacchi [Chromczynski and Sacchi, 1987]. Human GLCs were washed twice with phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.3), and cells were lysed in 500 µl of solution D (4 M guanidine thiocyanate, 25 mM sodium citrate (pH 7.0), 0.5% N-lauroyl sarcosine, and 0.1 M β-mercaptoethanol). The lysate was then transferred to a 1.7 ml microcentrifuge tube, 50 µl of sodium acetate (pH 4.0), 500 µl of DEPC (diethylpyrocarbonate)-saturated phenol (pH 6.0) and 100 µl of chloroform: isopropanol mixture (24:1) was added, and the tube was vortexed briefly and cooled on ice for 20 min. After incubation, the tube was centrifuged at 4 °C at 10,000 xg for 20 min, and supernatant was collected into a new tube without disturbing the interphase. For the second extraction, the supernatant was mixed with 400 µl of chloroform: isopropanol mixture, vortexed briefly and centrifuged for 5 min. The supernatant was collected and 500 µl of isopropanol was added. The RNA was then precipitated at −70 °C for 1 h.

Following precipitation, the tube was centrifuged at 10,000 xg for 20 min. The supernatant was removed and the pellet was washed twice with 500 µl of 70% ethanol.
Finally, the pellet containing some residual ethanol was dried by speedvac. The pellet was resuspended in 20 µl of DEPC-water.

The RNA concentration was determined based on absorbance at 260 nm. To examine the integrity, extracted RNA was checked by separation in 1% formaldehyde-agarose gel. RNA samples were loaded along with ethidium bromide containing gel loading buffer (6X loading buffer= 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol), and run at 70 V for 3 h. The demonstration of two RNA bands (18S and 28S) revealed the integrity of extracted RNA.

2.3 Reverse transcription of RNA to first-strand cDNA

RNA obtained from human granulosa-luteal cells was reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Oakville, Canada). One microgram of total RNA dissolved in DEPC-water (8 µl in total) was heated at 65 °C for 10 min and cooled on ice for 5 min. DTT (1 µl), oligo-dT (1 µl) and bulk mixture (5 µl) was added to the sample, and the mixture was incubated at 37 °C for 1h. After incubation, the sample was boiled for 10 min to inactivate reverse transcriptase and stored at −20 °C until use.
2.4 Southern blot analysis

Nine microliter of PCR products were mixed with 1 μl of 10X gel loading buffer and loaded on 1% agarose gel containing ethidium bromide (200 μg/ 100 ml gel). The gel was run at 100 Volts for 1 h in 1X TBE running buffer (90 mM Tris-borate, 2 mM EDTA, pH 8.0). The gel was then incubated in denaturing solution (1.5 M NaCl, 0.5 M NaOH) with agitation for 30 min and neutralized in neutralizing solution (1.5 M NaCl, 1.0 M Tris, pH 8.0) for 15 min x 2. The samples in the gel were transferred overnight to a Nylon membrane (Hybond N, Amersham Pharmacia Biotech) using an apparatus (Fig. 15) containing 10X SSC (1X SSC= 0.15 M NaCl, 0.015 Sodium citrate(2H2O), pH 7.0). The membrane was wrapped in Saran Wrap and exposed to UV light for 5 min to crosslink DNA with the membrane. The membrane was stored at 4 °C until the probing steps.

Digoxigenin-labeled cDNA probes for a targeted molecule were prepared using DIG DNA labeling kit following manufacturer’s protocol (Roche Molecular Biochemicals). Briefly, template cDNA (1 μg) was denatured by boiling for 10 min and quickly chilled on ice. The cDNA was labeled in 20 μl reaction containing 2 μl of hexanucleotide mix, 2 μl of dNTP mix, 1 μl of Klenow enzyme at 37 °C for 3 h. After incubation, the reaction was stopped by adding 2μl of 0.2 M EDTA (pH 8.0). Labeling efficiency was determined by comparing signal intensity with DIG-labeled control-DNA provided by the manufacturer.
The membrane was prehybridized for 2 h in hybridization buffer (50% deionized formamide, 5X SSC, 0.1% w/v N-lauroylsarcosine, 0.02% SDS, 2% blocking reagent) and hybridized with denatured DIG-labeled cDNA probe (boiled for 10 min) at 42 °C for 20 h. The membrane was then washed twice with 2 X SSC, 0.1% SDS for 10 min at room temperature (RT), followed by twice high stringency washing with 0.1 X SSC, 0.1 % SDS at 65 °C for 15 min. After washing, the membrane was incubated for 30 min in buffer solution (Buffer 2, 1% blocking reagent in Buffer 1 [0.1 M Maleic acid, 0.15 M MaCl, pH 7.5]) and incubated with anti-DIG-AP connjugate (1: 10, 000) for 30 min. The membrane was washed twice in washing buffer ( 0.3% Tween 20 in Buffer 1 ) and equilibrated in Buffer 3 (0.1M Tris-HCl, 0.1 M NaCl, 50 mM MgCl2, pH 9.5) for 5 min, followed by incubation with CSPD® (1:200) at room temperature (RT) for 10 min. Finally, the membrane was wrapped with saran wrap and incubated at 37 °C for 15 min prior to exposure to Kodak Omat X-ray film (Eastman Kodak Co., Rochester, NY).
Fig. 15 The apparatus for DNA transferring.
2.5 Subcloning and plasmid isolation

PCR products were fractioned in 1% agarose gel and eluted. Briefly, PCR products of correct size were cut from the gel and boiled in 500 μl solution containing 1X TBE and 0.5 M NaCl for 3 min, followed by heated at 65 °C until completely dissolved. Samples were added with prewarmed (65 °C) TE-saturated phenol (pH7.5, 500 μl), vortexed and centrifuged at RT for 5 min. The supernatant was collected and centrifuged for another 5 min to remove residual gel. The collected supernatant was added with phenol/chloroform/isoamyl alcohol (25: 24:1), vortexed and centrifuged for 5 min. The supernatant was collected, added with equal volume of isopropanol and 0.1 volume of 3 M sodium acetate (pH 5.2), vortexed briefly and stored at −20 °C for 2 h, followed by centrifugation at 4 °C at 14,000 rpm for 20 min. The pellet was rinsed with 70% ethanol, centrifuged and dried with speedvac. The pellet was then dissolved in 15 μl of TE and stored at −20 °C until use.

PCR products isolated from gel were cloned into pCR vector using TA Cloning Kit (Invitrogen, San Diego, CA). Briefly, purified PCR products (10 ng) was ligated in 10 μl ligation reaction containing 25 ng pCR II vector, 1 X ligation buffer, 20 IU T4 DNA ligase at 4 °C for 16 h. The ligated plasmid was incubated with competent E. Coli (One Shot™) on ice for 30 min, transformed into E. Coli by heat shock at 42 °C for 1 min and incubated on ice for 5 min. Two hundred and fifty microliter of LB broth (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl in 1000 ml distilled H2O) was added to the mixture and incubated at 37 °C for 1 h. E. Coli mixture was then plated on
LB-ampicillin plates (50 mg/ml LB) pre-coated with X-Gal (20 μl of 20 mg/ml stock solution) and IPTG (10 μg/plate) and incubated at 37 °C for 18 h. White and blue colonies represent positive (cDNA-incorporated vector) and negative (vector only) results, respectively.

Positive colonies were selected and cultured 2 ml LB and plasmid was isolated using the alkaline lysis procedure. Briefly, bacteria culture (1.5 ml of LB) was transferred to microcentrifuge tube and centrifuged at 10,000 xg for 1 min. The pellet was resuspended with 200 μl of ice-cold Solution I (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 100 μg/ml RNase), lysed with 200 μl of Solution II (200 mM NaOH, 1% SDS) for 5 min and neutralized with 200 μl of ice-cold Solution III (3.0 M Potassium acetate, pH 5.5). The mixture was centrifuged at 10,000 xg for 20 min, and supernatant was transferred to a new tube. The supernatant was added with equal volume of mixture of phenol: chloroform: isoamylalcohol (25: 24: 1) and centrifuged at 4 °C for 20 min. The plasmid-containing supernatant was added with equal volume of isopropanol for precipitation and stored at -20 °C for 1 h. Plasmid pellet was obtained after centrifugation at 4 °C for 20 min, washed with 70 % ethanol, dried with speedvac and dissolved in 20 μl of TE. The cDNA-containing plasmid was stored at -20 °C until use.

2.6 Sequence analysis

The cDNA-containing plasmid was sequenced by the dideoxy nucleotide chain
termination method using the T7 DNA polymerase sequencing kit (Amersham Pharmacia Biotech). Sequencing analysis was performed using universal M13 forward and reverse primers. Double-strain cDNA-containing plasmid (2 μg in 32 μl TE) was denatured by adding 8 μl of 2 M NaOH to a final volume of 40 μl. DNA was then precipitated with sodium acetate/ethanol (7 μl of 3 M sodium acetate, pH 4.8, 4 μl of distilled water, and 120 μl of 100% ethanol), collected by centrifugation at 10,000 xg for 20 min, washed with 70% ethanol, and dried with speedvac. The denatured DNA template was resuspended in 10 μl of distilled water, followed by adding 2 μl of primer (forward or reverse) and 2 μl of annealing buffer (1 M Tris-HCl, pH 7.6, 100 mM MgCl2, 160 mM DTT) to a final volume of 14 μl. The reaction was performed at 65 °C for 5 min and 37 °C for 10 min, followed by RT for 15 min to promote annealing. After the annealing reaction, 3 μl of Labeling Mix (including dCTP, dTTP and dGTP), 1 μl of [α-35S]dATP (10 μCi, Ammersham Pharmacia Biotech.), and 2 μl of diluted T7 DNA polymerase (3 U) were added to a final volume of 20 μl and incubated for 5 min at RT. Meanwhile, four tubes of 2.5 μl dNTP mix (A,C,G or T mix, A-mix including ddATP with dNTP) were prepared and prewarmed at 37 °C for 5 min prior to adding 4.5 μl (out of 20 μl) of the prepared labeling reaction mixture. After incubation at 37 °C for 5 min, the reaction was terminated by adding 5 μl of Stop solution (0.3% bromphenol blue and xylene cyanol, 10 mM EDTA, pH 7.5, and 97.5 % deionized formamide) and stored at -20 °C until use.

Polyacrylamide 6%/7 M urea sequencing gels was prepared and prerun at 45 Walts for 1 h before loading the samples. Samples (2.5 μl) were boiled for 5 min prior to
loading into the gel and then run for 3 to 4 h at 45 W constant power. Gel was dried at 80 °C for 2 h using a gel dryer (Model 583, Biorad Laboratories, Richmond, CA) and exposed to Kodak Omat X-ray film at -70 °C for 24 h. The sequence of PCR product was sent to GeneBank at NCBI (National Center for Biotechnology Information) through the internet (www.ncbi.nlm.gov) to compare the identity with published sequences.

2.7 Northern blot analysis

Approximately 15 µg of total RNA was dissolved in denaturing solution: (50% formamide, 2.2 M formaldehyde) and incubated at 60 °C for 15 min. Samples were added with 1X gel loading buffer and separated by electrophoresis in a 1% agarose-formaldehyde gel (20 mM MOPS, 2.2 M formaldehyde, 8 mM sodium acetate, 1 mM EDTA, pH 8.0) at 70 V for 3 h. RNA was transferred onto a nylon membrane (Amersham, Hybond-N) in 2X SSPE (20X SSPE= 3M NaCl, 0.2 M NaH2PO4-H2O, 0.025 M EDTA) for 18 h. Membrane was then incubated under UV light for 5 min to crosslink the RNA to the membrane. The membrane was pre-wetted in 5X SSPE for 30 min. They were then incubated in a prehybridization solution of 5X SSPE containing 50% deionized formamide, 5X Denhardt's, 1% SDS and 100 µg/ml heat-denatured salmon sperm DNA at 42 °C for 3 hours.

Meanwhile, radiolabeled cDNA probe was prepared using the Random Labeling Kit
According to manufacturer’s protocol, DNA template (100 ng) in 23 μl was denatured at 100 °C for 5 min and placed on ice for 5 min. Two μl of each dNTP (dCTP, dGTP and dTTP) with 15 μl of random priming buffer, 5 μl of [α-32P]dATP (50 μCi, Amersham Pharmacia Biotech) and 1 μl of Klenow fragment were added into denatured DNA sample and incubated at RT for 3h. The labeled DNA was purified using G-50 Sephadex column (Amersham Pharmacia Biotech.).

The radiolabeled probe was denatured by boiling for 5 min and then added to the prehybridization solution. The blot was incubated in the presence of the radiolabeled probe at 42 °C for 16 hours, then washed twice with 2X SSPE at room temperature (5 min / wash), twice with 2X SSPE containing 1% SDS at 55 °C (30 min / wash), twice with 0.2X SSPE at room temperature (30 min / wash) and finally exposed to Kodak Omat X-ray film.

### 2.8 Western blot analysis

The hGLCs were washed twice with ice-cold PBS and lysed with 100 μL of cell lysis buffer (RIPA, 150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1 % Nonidet P-40, 0.5 % deoxycholate, 0.1% SDS, 1.0 mM PMSF, 10 μg/mL leupeptin and 100 μg/mL aprotinin) at 4 °C for 30 min. The cell lysate was centrifuged at 10,000 x g for 5 min and the supernatant was collected for Western blot analysis.
The amount of protein was quantified using Bio-Rad DC Protein Assay kit (Bio-Rad Laboratories, Richmond, CA). Cellular extract (10 μl) was incubated with 25 μl of reagent A (an alkaline copper tartrate solution) and 200 μl of reagent B (a dilute Folin Reagent) for 15 min with gentle shaking at room temperature. This assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. As with the Lowry assay, there are two steps which lead to color development: the reaction between protein and copper in an alkaline medium, and the subsequent reduction of Folin reagent by the copper-treated protein [Lowry et al., 1951]. Color development is mainly due to the amino acids tyrosine and tryptophan, and to a lesser extent, cystine, cysteine and histidine [Lowry et al., 1951; Peterson, 1979]. Proteins induce a reduction of the Folin reagent by loss of 1, 2, or 3 oxygen atoms so as to produce one or more of several possible reduced species that have a characteristic blue color with maximum absorbance at 750 nm and minimum absorbance at 450 nm [Peterson, 1979]. Absorbance of the sample was measured using microplate reader (Model EL311, Bio-Tek instruments, Vermont, CA) at 630 nm. A standard curve for protein assay is constructed in each assay using standard solution of bovine serum albumin (Fig. 16).

Aliquots (30 μg) of protein were mixed with 1X sample buffer (8X loading buffer= 100 mM Tris-HCl, pH 6.8, 4 % SDS, 0.2 % bromphenol blue, 20 % glycerol, 8 % 2-mecaptoethanol) and boiled for 10 min. The samples were then subjected to 10 % SDS-polyacrylamide gel electrophoresis [Laemmli, 1970] in 1X gel running buffer (25 mM Tris, pH 8.0, 250 mM glycine, 0.1% SDS) at 30 miniamper for 3 h. The proteins
were then electrophoretically transferred from the gels onto nitrocellulose membranes (Amersham Pharmacia Biotech, Oakville, Canada) [Towbin et al., 1979] in transfer buffer (200 mM Glycin, 25 mM Trizma base, 20% methanol) at 100 Volts for 90 min. These nitrocellulose membranes were then pre-incubated with blocking solution (5% skim milk, 0.05% Tween 20 in TBS (Tris-buffered saline, pH 8.0, 5 mM Tris base, 100 mM sodium chloride)) for 2 h and incubated with primary antibody for 16 h at 4 °C with gently shaking in sample bag (VWR). After washing three times with TBS-T (0.1% Tween 20 in TBS) and two times with TBS, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1: 1000 dilution in blocking solution), and the signal was visualized using ECL system (enhanced chemiluminescent system, Amersham Pharmacia Biotech, Oakville, Canada) followed by autoradiography. The autoradiograms were quantified using a laser densitometer (BIO-RAD, Model 620, Video Densitometer).
Fig. 16 Standard curve for protein assay. Absorbance of the samples was measured using ELISA reader at 630 nm.

\[ y = 3.1487x - 0.155 \]
2.9 Microspectrofluorimetry

Fluorescence ratio imaging is a widely used technique for the detection of calcium in living cells. The Fura-2 is a calcium chelator that emits quantitatively different fluorescence (510 nm) at different excitation wavelengths. In the presence of high concentrations of calcium, Fura-2 fluoresces brightly when excited at 340 nm and dimly when excited at 380 nm. In conditions of low calcium, the fluorescence intensity at 340 nm and 380 nm is reversed with bright at 380 nm and dim at 340 nm. Thus, Fura-2 is a dual-excitation/single-emission dye [Gryniewicz et al., 1985]. The reversal of fluorescence intensity in response to alterations of calcium level is the key for use of fluorescence ratio imaging to detect cytosolic calcium oscillations.

Human GLCs were seeded onto 25-mm circular glass cover slips (5,000 /slip) and incubated for 3 days at 37 °C in humidified air with 5% CO2 prior to microfluorimetric experiments. Cytosolic calcium concentrations were measured using the dual-excitation single-emission fluorimetric technique, as described previously [Squires et al., 1997]. Briefly, the cells were incubated with 5-10 μM fura-2 AM acetoxymethyl ester (Molecular Probes, Eugene, OR) for 30 min at 37 °C in humidified air with 5% CO2. The cover slip was mounted onto the perifusion chamber and equilibrated for 10 min with balanced salt buffer (NaCl 137 mM, KCl 5.36 mM, CaCl2 1.26 mM, MgSO4·7H2O 0.81 mM, Na2HPO4·7H2O 0.34 mM, KH2PO4 0.44 mM, NaHCO3 4.17 mM, HEPES 10 mM, glucose 2.02 mM, pH 7.4) in humidified air with 5% CO2. The fura-2 ratio measurements were performed using the Attoflour Digital Fluorescence Microscopy
System (Atto Instruments, Rockville, MD). The perifusion chamber was connected to a multiunit six-channel perifusion system with a flow rate of 1-2 ml/min. Fura-2 loaded cells were observed through a 40X fluorescent objective lens and were illuminated alternatively with light at 340 nm and 380 nm. Emitted light was filtered using a 510 nm long-pass filter and detected using a low light sensitive camera. Measurements of cytosolic calcium were performed at 1-2 sec intervals. All records were corrected for background fluorescence (determined from cell-free region of cover slip). Changes in the fluorescence ratio recorded at 340 and 380 nm correspond to changes in cytosolic calcium.
2.10 Radioimmunoassay for progesterone

Progesterone levels in the culture medium were measured by established radioimmunoassay [Vaaninen et al, 1997]. Anti-progesterone antibody was kindly provided by Dr. D. T. Armstrong (University of Western Ontario). Briefly, samples were diluted 200 times with PBS-G (phosphate buffered saline-gelatin, 0.1 M PBS, 0.1% gelatin; 1X PBS-G = 5.18 g/ L Na2HPO4•7H2O, 16.65 g/ L NaH2PO4•H2O, 9g/L NaCl, 0.1% gelatin, 0.1g/L Thimerosal).

Samples (100 μl) were incubated with antibody (100 μl) and tracer (100 μl), with a final concentration of 7,000 cpm/ml of [1,2,6,7,16,17-3H]Progesterone (Amersham Pharmacia Biotech). A standard curve was constructed in each assay with progesterone ranging between 0.39-25 ng/ ml (Fig. 17). After incubation at 4 °C for 16-24 hours, 500 μl charcoal/dextran solution (0.5g/ 0.05g in 200 ml of PBS-G) was added to each tube to remove unbound progesterone or tracer. After centrifugation at 4 °C at 4500 rpm for 30 min, 700 μl of the supernatant was collected and added with 3 ml of scintillation cocktail (Amersham Pharmacia Biotech), and the vials were counted with a β–counter (LKB Wallac, Turku, Finland). The cells in each dish were harvested for quantifying protein amount using Bio-Rad Protein Assay kit. Samples were assayed in triplicate and progesterone concentrations were standardized against total protein content.
Fig. 17 Standard curves for radioimmunoassay.  A. Progesterone v.s. % Bound.  
B. Log[Progesterone] v.s. % Bound.
2.11 Radioimmunoassay for cAMP

Prior to scheduled treatments, hGLCs were incubated in serum-free medium containing 0.1% BSA and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich Corp., phosphodiesterase inhibitor) for 30 min. After treatment, the medium was removed and ice-cold 100% ethanol was added, followed by incubation on ice for 20 min. Cyclic AMP-containing ethanol was collected and centrifuged at 4 °C at 10,000 xg for 20 min. The supernatant was collected and dried using a speedvac. The pellet was dissolved in 120 µl of Reagent 1 (0.05 M Tris, pH 7.5, 4 mM EDTA). Intracellular cAMP levels were measured using the [3-H]-cAMP assay system, following the protocol provided by manufacturer (Amersham Pharmacia Biotech). A standard curve was constructed in each assay with cAMP concentrations between 1-16 pmole (Fig. 18). The sample (50 µl) was incubated with 100 µl of binding protein (purified from bovine muscle) and 50 µl of [8-3H]adenosine 3',5'-cyclic phosphate. After incubation at 4 °C for 16 h, 100 µl of charcoal was added to remove unbound antigen. After centrifugation at 4500 rpm for 30 min, 200 µl of the supernatant was collected and added with 3 ml of scintillation cocktail, and the vials were counted with a β-counter. All samples were assayed in duplicate.
Fig. 18 Standard curve for cAMP assay.
Co: the cpm bound in the absence of unlabelled cAMP.
Cx: the cpm bound in the presence of standard or unknown unlabelled cAMP.

$c = 2.5631 \times - 3.2104$

$R^2 = 0.9949$
PART 3

EXPRESSION AND REGULATION OF P2U-PURINERGIC RECEPTOR IN HUMAN GRANULOSA-LUTEAL CELLS

3.1 Abstract

The P2U purinoceptor (P2UR) has been identified pharmacologically in the ovary. However, the expression and regulation of the P2UR messenger ribonucleic acid (mRNA) in human ovarian cells are still poorly characterized. The present study was designed to examine the expression and regulation of the P2UR in human granulosa-luteal cells (hGLCs) by reverse transcription - polymerase chain reaction (RT-PCR) and Northern blot analysis. A PCR product corresponding to the expected 599 bp P2UR cDNA was obtained from hGLCs. Molecular cloning and sequencing of the PCR product revealed an identical sequence to the reported P2UR cDNA. Two mRNA transcripts of 2.0 kb and 4.6 kb were identified in hGLCs using Northern blot analysis. The expression of the P2UR mRNA was down-regulated by hCG in a dose- and time-dependent manner. Treatment with 8-bromo-cAMP and forskolin also attenuated P2UR mRNA levels. Calcium signaling following the activation of the P2UR in single hGLCs was studied using microspectrofluorimetry. It revealed that, like adenosine triphosphate (ATP), uridine triphosphate (UTP) also induced cytosolic calcium mobilization in a dose-dependent manner. These results demonstrate for the first time that the P2UR mRNA is expressed in hGLCs, and that P2UR mRNA is regulated by hCG, cAMP and forskolin. The P2UR expressed in hGLCs is functional, since activation of the P2UR by
ATP or UTP resulted in rapid and transient mobilization of cytosolic calcium at the single
cell level. These findings further support a potential role of this neurotransmitter
receptor in the human ovary.

3.2 Introduction

Adenosine triphosphate (ATP) is released from cells such as platelets and
co-released with neurotransmitter granules from autonomic nerves by exocytosis [Gordon,
1986]. Extracellular ATP binds to a G protein-coupled P2 purinoceptor that activates
phospholipase C and phosphatidylinositol hydrolysis, generating diacylglycerol and
inositol 1,4,5-triphosphate, which stimulate protein kinase C and cytosolic calcium
([Ca2+]i) mobilization, respectively [Berridge, 1984; el-Moatassim et al., 1992].
Thereafter, ATP may participate in various types of physiological responses, including
secretion, membrane potential, cell proliferation, platelet aggregation, neurotransmission,
cardiac function, and muscle contraction [el-Moatassim et al., 1992; Burnstock, 1990].

Purinergic receptors have been classified as P1 receptors and P2 receptors.
Pharmacologically, the P1 receptors have a high affinity for extracellular adenosine and
AMP (Adenosine > AMP > ADP > ATP), whereas P2 receptors have high affinity for
ATP and ADP (ATP > ADP > AMP > adenosine) [Gordon, 1986; el-Moatassim et al.,
1992; Burnstock, 1990]. Six subtypes of P2 purinergic receptors, P2X, P2Y, P2D, P2T,
P2Z, and P2U, have been identified in pharmacological and molecular cloning studies
Functionally, a P2U purinoceptor (P2UR) has been detected in human granulosa-luteal cells (hGLCs) using microspectrofluorimetry [Kamada et al., 1994]. Autonomic nerves have been shown to innervate the ovary and may be involved in regulating steroidogenesis [Owman et al., 1967; Bodis et al., 1993a; Bodis et al., 1993b]. It is tempting to speculate that the co-released ATP from autonomic nerve endings in the ovary may play a role in regulating ovarian function. ATP has been shown to regulate the production of progesterone and estradiol in hGLCs [Kamada et al., 1994]. These findings provide further evidence that ATP is able to regulate ovarian function through binding to ATP receptors.

Although P2UR has been identified pharmacologically in human ovary [Kamada et al., 1994, Lee PSN et al., 1996], its expression and regulation at the messenger RNA (mRNA) levels have not as yet been characterized. To understand further the potential role of ATP and the receptor of this neurotransmitter in the ovary, the present study was designed to detect the expression of the P2UR in hGLCs and to examine the regulation and signaling of this receptor in vitro.
3.3 Materials and Methods

Reagents and Materials

Prostaglandin F2α, gonadotropin-releasing hormone (GnRH), human chorionic gonadotropin (hCG), estradiol, progesterone, 8-bromo-adenosine-3',5'-cyclic monophosphate (8-bromo-cAMP), forskolin, ATP and uridine triphosphate (UTP) were obtained from Sigma Chemical Co. (St. Louis, MO) Dulbecco's Modified Eagle Medium (DMEM, phenol red free), penicillin-streptomycin, were obtained from GIBCO-BRL (Burlington, Ontario, Canada). Fura-2 AM was purchased from Molecular Probes (Eugene, OR).

Human granulosa-luteal cells culture and treatments

Human GLCs were collected from patients undergoing an In Vitro Fertilization - Embryo Transfer program and processed as mentioned in PART 2. Human GLCs were then cultured in phenol-red free DMEM containing 100 U penicillin G sodium/ml, 100 μg streptomycin/ml and 10% fetal bovine serum at a density of approximately 200,000 cells per dish in 35-mm culture dishes. The dishes were incubated at 37 °C under a water-saturated atmosphere of 5% CO2 in air for 3 days. To examine the regulation of the P2UR mRNA, hGLCs were incubated in serum-free medium for 24 hours prior to
treatment with estradiol (10^{-7} M), progesterone (10^{-7} M), prostaglandin F2\alpha (10^{-7} M), GnRH, (10^{-7} M), hCG, (5 IU/ml) or ATP (10 \mu M) for 24 hours. For dose-response experiments, hGLCs were treated with different concentrations of hCG (0.1, 1, 5, 10 IU/ml) for 24 hours. For time-course analysis, hGLCs were treated with 5 IU/ml of hCG for 0, 3, 6, 12, 24 or 48 hours. To further delineate the underlying mechanism, by which the expression of P2UR mRNA was regulated, cells were treated with 8-bromo-cAMP (1mM) or forskolin (10 \mu M) for 24 hours prior to the determination of P2UR mRNA levels.

Total RNA isolation and RT-PCR

Total RNA was prepared from the cultured hGLCs by the phenol-chloroform method of Chromczynski and Sacchi [Chromczynski and Sacchi, 1987] as mentioned in PART 2. One microgram of total RNA obtained from human granulosa-luteal cells was reverse transcribed into cDNA using the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Morgan, Canada). One set of oligonucleotide primers (5'-CCTGGAAATGCGTCCACCACATAT-3 and 5'-GACGTGGAATGGCAGGAAGCAGA -3) based on the published human P2U receptor sequence [Parr et al., 1994] was designed for polymerase chain reaction (PCR) to amplify the P2UR from hGLCs. PCR reactions were performed in the presence of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 400 \mu M dNTPs, 0.25 U Taq DNA polymerase, 2\mu M primers, and 1\mu l cDNA template per 25 \mu l reaction. Amplification was carried out for 33 cycles with a condition of denaturation at 94\degree C for 60 seconds, annealing at 64\degree C for 35 seconds and extension at
72°C for 90 seconds, and a final extension at 72°C for 15 minutes. The same amount of cDNA of each sample was used for amplification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers for GAPDH (5'-ATGTTCTGTCATGGGTGTGAACCA-3' and 5'-TGGCAGGTTTTTCTAGACGGCAG-3') were designed based on published sequence [Tokunaga et al., 1987]. Amplification was carried out for 18 cycles with a condition of denaturation at 94°C for 60 seconds, annealing at 55°C for 35 seconds and extension at 72°C for 90 seconds, and a final extension at 72°C for 15 minutes.

Cloning and sequencing of RT-PCR product

Ten μl of PCR products of P2UR were fractionated in a 1% agarose gel stained with ethidium bromide. The expected PCR products (599 bp) were isolated from gel, cloned using the TA cloning kit (Invitrogen) and sequenced by the dideoxy chain termination method using a T7 DNA polymerase sequencing kit (Pharmacia Biotech, Morgan, Canada). The sequence of the cDNA was sent to GenBank at NCBI (National Center for Biotechnology Information) through internet (www.ncbi.nlm.nih.gov) to compare the identity with published human P2UR. This cDNA was then used as the template for making probes for Northern and Southern blot analysis.

Northern blot analysis
Approximately 15 µg total RNA was separated by electrophoresis in a 1% agarose-formaldehyde gel and transferred onto a charged nylon membrane (Amersham, Hybond-N) as described in PART 2. The Northern blots were incubated in 5X SSPE for 30 min. They were then transferred to a prehybridization solution of 5X SSPE containing 50% deionized formamide, 5X Denhardt's, 1% SDS, water and heat-denatured salmon sperm DNA (final concentration, 0.2 mg/ml). The blots were prehybridized at 42 °C for 3 hours. The radiolabeled P2UR probe was then added to the prehybridization solution. The blots were incubated in the presence of the radiolabeled probe at 42 °C for 16 hours, then washed twice with 2X SSPE at room temperature (5 min / wash), twice with 2X SSPE containing 1% SDS at 55 °C (30 min / wash), twice with 0.2X SSPE at room temperature (30 min / wash) and finally exposed to Kodak Omat X-ray film.

Southern blot analysis

After sequencing, the cloned cDNA of P2U receptor was used as the template to make digoxigenin (DIG)-labeled probe using a DIG DNA Labeling Kit following the protocol provided by manufacturer (Boehringer Mannheim Laval, Canada). The PCR products in 1% agarose gel were transferred to Hybond-N nylon membranes (Amersham Inc., Canada) and hybridized with DIG-labeled P2U receptor cDNA. The membranes were processed as per the manufacturer’s protocol. Finally, the membranes were exposed for 10 min at room temperature to X-ray film. The autoradiograms were
scanned with a laser densitometer (BIO-RAD, Model 620, Video Densitometer) and P2UR mRNA levels were standardized against GAPDH.

Quantification of P2UR mRNA

To compare the expression and regulation of P2UR mRNA, semiquantitative PCR was performed. For validation, various cycles of (27-38) PCR were performed to amplify P2UR mRNA. GAPDH was used in the present study to normalize the PCR product of P2UR mRNA. For GAPDH, 15-27 cycles of PCR were performed for validation. Southern blot analysis was carried out using equal amount of amplified PCR products (10 µg), and the results were quantified using a laser densitometer.

Microspectrofluorimetry

Human GLCs were seeded onto 25-mm circular glass cover slips (5,000 /slip) and incubated for 3 days at 37 °C in humidified air with 5% CO2 prior to microfluorimetric experiments. Cytosolic calcium concentrations were measured using the dual-excitation single-emission fluorimetric technique, as described in PART 2. To confirm the presence of functional P2UR in hGLCs, cells were treated with 100 µM of ATP or UTP (Sigma Chemical Co.). For dose-response experiments, cells were treated with various concentrations of ATP or UTP (1, 10, and 100 µM), prior to cytosolic calcium determinations.
Data analysis

Relative P2UR mRNA levels were expressed as the ratio of P2UR to GAPDH. For each patient, the data are represented as the percent change relative to the control. Data of the same treatment groups are represented as means ± standard errors (SE). Statistical analysis was performed by one-way analysis of variance followed by Tukey test. Differences were considered significant at p < 0.05.

3.4 Results

Expression of the P2U receptor mRNA in human granulosa-luteal cells

The expression of P2U receptor mRNA in hGLCs was examined by RT-PCR using one set of primers designed on the basis of the published human P2UR expressed in airway epithelium. The positions and sequences of primers were shown as Fig. 19A. An expected 599-bp DNA fragment was observed in ethidium bromide-stained gel from hGLCs isolated from 3 different patients (Fig. 19B). No product was obtained from the negative control (without first strain cDNA template in PCR reaction). The PCR products from hGLCs were subcloned and sequenced. Sequence analysis revealed that
the cloned cDNA is identical to nucleotide position 436-1034 of the published human
P2U receptor [Parr, et al., 1994]. This cDNA was then used as a template for making
probes for Northern and Southern blot analyses. Using Northern blot analysis, two
P2UR transcripts of 2.0 kb and 4.6 kb were detected in hGLCs as shown in Fig. 19C.
Fig. 19A. Expression of P2UR mRNA in human granulosa-luteal cells (hGLCs). Demonstration of positions and sequences of primers designed on the basis of published human P2U purinoceptor (P2UR). This set of primers was used in polymerase chain reaction (PCR) to amplify P2UR mRNA isolated from hGLCs.
Fig 19B. Ethidium bromide-stained DNA gel showing the PCR products of three patients. One μg of total mRNA of hGLCs from each patient was reverse transcribed into cDNA, and aliquots were amplified using PCR with primers shown in Fig. 1A. A 599 bp product was obtained in PCR from three patients. Control represented without cDNA in PCR.
Fig. 19C. Demonstration of P2UR mRNA in hGLCs by Northern blot analysis.

Fifteen μg of total mRNA was loaded and separated by electrophoresis in 1% agarose-formaldehyde gel and transferred onto a charged nylon membrane. Radio-labeled cDNA of P2UR was used as the probe to detect the presence of P2UR mRNA in hGLCs. Finally, the blot was subjected to radioautography. Two transcripts of 2.0 kb and 4.6 kb mRNA were detected in this study.
Validation of PCR for P2UR transcript

To determine the condition under which the amplification of P2UR was in the logarithmic phase, various cycles of PCR were performed. Equal aliquots (1μl) of cDNA were amplified by different PCR cycles. Ten μl of the PCR products were fractionated, transferred onto a charged nylon membrane, detected by DIG-labeled cDNA and finally subjected to radioautography. The results were quantified using a laser densitometer. A linear relationship between PCR products and amplification cycles was observed in both P2UR (Fig. 20) and GAPDH (data not shown). Thirty-three cycles for P2UR and 18 cycles for GAPDH were employed for quantification in subsequent regulation studies.
Fig. 20 Validation of semiquantitative PCR for P2UR in hGLCs. Total RNA of hGLCs was isolated and reverse transcribed in the first strain cDNA. Equal aliquots (1μl) of cDNA were amplified by different PCR cycles (27-38) as described in Materials and Methods. Ten μl of the PCR products were fractionated by electrophoresis in 1% agarose gel and transferred onto a charged nylon membrane. DIG-labeled cDNA of P2UR was used as the probe to detect the expression of P2UR in hGLCs. After washing, the membrane was exposed to Kodak Omat X-ray film, and the result was quantified using a laser densitometer. A linear relationship was observed between PCR products and amplification cycles.
Regulation of the P2UR mRNA in hGLCs

To examine the regulation of P2UR mRNA, hGLCs were treated with hCG (5 IU/ml), estradiol (10^-7 M), progesterone (10^-7 M), ATP(10μM), prostaglandin F2 α (10^-7 M), or GnRH (10^-7 M), respectively. As shown in Fig. 21, no significant change of P2UR mRNA levels was observed in the groups treated with estradiol, progesterone, ATP, prostaglandin F2α, or GnRH. In contrast, about 30% decrease of P2UR mRNA (P<0.05) was noted in the hCG-treated group. To further examine the effect of hCG on P2UR expression, hGLCs were treated with increasing concentrations of hCG for 24 hours. As shown in Fig. 22, hCG down-regulated the level of P2UR mRNA in a dose-dependent manner. Further, a time-course analysis revealed that hCG down-regulated P2UR mRNA in a time-dependent manner (Fig. 23). It is well established that hCG activates adenylate cyclase and increases the production of cAMP in ovarian cells. To examine the possible mechanism by which P2UR mRNA is regulated by hCG, hGLCs were treated with 8-bromo-cAMP and forskolin, an activator of adenylate cyclase. As shown in Fig. 24, both cAMP and forskolin significantly down-regulated the expression of P2UR mRNA.
Fig. 21 The effect of different reagents on the regulation of P2UR mRNA in cultured hGLCs. Cells were treated with human chorionic gonadotropin (hCG, 5 IU/ml), prostaglandin F2α (PGF, 10^{-7} M), GnRH (10^{-7} M), progesterone (P4, 10^{-7} M), estradiol (E2, 10^{-7} M) or ATP (10 μM) for 24 hours in serum-free condition as described in Materials and Methods. The PCR products (upper panel) were normalized by GAPDH (middle panel). The size of PCR product is shown on the right hand side of upper panel. Data represent the means ± standard error for four separate experiments with samples from four patients. *, Significantly different from control (p<0.05)
Fig. 22 The dose effect of human chorionic gonadotropin (hCG) on the regulation of P2UR mRNA in cultured hGLCs. Cells were treated with various concentrations of hCG (0.1-10 IU/ml) for 24 hours in serum-free condition as described in Materials and Methods. The PCR products (upper panel) were normalized by GAPDH (middle panel). The size of PCR product is shown on the right hand side of upper panel. Data represent the means ± standard error for four separate experiments with samples from four patients. *, Significantly different from control (p<0.05)
Fig. 23 The time effect of human chorionic gonadotropin (hCG) on the regulation of P2UR mRNA in cultured hGLCs. Cells were treated with 5 IU/ml of hCG for 0-48 hours in serum-free condition as described in Materials and Methods. The PCR products (upper panel) were normalized by GAPDH (middle panel). The size of PCR product is shown on the right hand side of upper panel. Data represent the means ± standard error for three separate experiments with samples from four patients. *, Significantly different from control (p<0.05)
Fig. 24 The effects of 8-bromo-cAMP (cAMP) and forskolin on the regulation of P2UR mRNA in cultured hGLCs. Cells were treated with cAMP (1 mM) and forskolin (10 μM) for 24 hours in serum free condition as described in Materials and Methods. The PCR products (upper panel) were normalized by GAPDH (middle panel). The size of PCR product is shown on the right hand side of upper panel. Data represent the means ± standard error for four separate experiments with samples from four patients. *, Significantly different from control (p<0.05).
Effects of ATP and UTP on intracellular calcium mobilization in single cells

The P2UR expressed in hGLCs was tested functionally and pharmacologically using microspectrofluorimetry in single cell studies. As shown in Fig. 25, hGLCs responded equally well to 100 μM ATP and UTP, indicating the expression of a functional P2UR in these cells at the level of calcium signaling. The cytosolic calcium mobilization was characterized by a spike and a marked increase in cytosolic calcium, followed by numerous oscillations with decreasing amplitudes. To examine further the dose-response relationship, hGLCs were treated with increasing concentrations of ATP or UTP (1 - 100 μM). It has been demonstrated that submicromolar concentrations of ATP were incapable of mobilizing cytosolic calcium [Lee PSN et al., 1996]. As shown in Fig. 26, both ATP and UTP were able to induce cytosolic mobilization in micromolar levels with maximal responses reached when treated with 10 μM of ATP or UTP, and no difference was noted between cells treated with 10 μM and 100 μM.
Fig. 25 Effects of ATP and UTP on inducing cytosolic calcium mobilization in cultured hGLCs using microspectrofluorimetry. Fura-2 loaded human granulosa-luteal cells were treated with 100 μM of ATP and UTP. Data of calcium oscillations were presented as ratio (340:380 nm). Both ATP and UTP were able to induce calcium oscillations, and no significant difference was noted between these two treatments in hGLCs.
Fig. 26 Dose effects of ATP and UTP on inducing cytosolic calcium mobilization in cultured hGLCs using microspectrofluorimetry. A. Fura-2 loaded human granulosa-luteal cells were treated with various concentrations of ATP (1-100 μM). B. Fura-2 loaded human granulosa-luteal cells were treated with various concentrations of UTP (1-100 μM). Data of calcium oscillations were presented as ratio (340:380 nm). The results demonstrated that both ATP and UTP were able to induce calcium oscillations in a dose-dependent manner.
3.5 Discussion

Sources of extracellular ATP are mainly neuronal in origin. ATP is either released from purinergic nerve endings, or co-released with other neurotransmitter granules such as acetylcholine and noradrenaline during neurotransmission [Morel and Meunier, 1981; Winkler and Carmichael, 1982]. The concentration of ATP in adrenergic granules of sympathetic nerves and in acetylcholine-containing granules of parasympathetic nerves can be as high as 150mM [Winkler and Carmichael, 1982]. Exocytotic release of ATP has also been found in non-neuronal cells, including platelets [Born and Kratzer, 1984], adrenal chromaffin cells [Cena and Rojas, 1990], mast cells, and basophilic leukocytes [Osipchuk and Cahalan, 1992]. Although ATP is present in millimolar concentrations in the cytosol, extracellular levels of the nucleotide will normally be maintained at very low levels by the ubiquitous ecto-ATPase and ecto-ATP diphosphohydrolase [Dombrowski et al., 1998; Dubyak and el-Moatassim, 1993; Zimmermann et al., 1998].

Adrenergic and cholinergic nerves have been shown to innervate the ovary and may be involved in the regulation of steroidogenesis [Mohsin and Pennefather, 1979; Burden and Lawrence, 1978; Stefenson et al., 1981]. In human granulosa cells, epinephrine and norepinephrine have been shown to stimulate progesterone secretion via interaction with the β-adrenergic receptor [Webley, 1988]. In other reproductive tissues, extracellular ATP has been shown to activate contraction in the intact myometrium [Osa and Maruta, 1987]. Amnion cells isolated from the human placenta express ATP receptors that are coupled to inositol phospholipid breakdown and Ca2+ mobilization.
[Vander Kooy et al., 1989]. ATP can trigger the acrosome reaction in human sperm in vitro [Foresta et al., 1992]. Following binding to P2-purinergic receptors, ATP can increase the secretion of testosterone in rat Leydig cell [Foresta et al., 1996].

The human P2UR gene has been mapped to chromosome 11q 13.5-14.1 [Dasari et al., 1996]. The human P2UR cDNA was cloned and sequenced from airway epithelium [Parr et al., 1994]. The P2UR in the human ovary has not as yet been characterized. The present study demonstrates for the first time the expression of P2UR in human ovarian cells. Northern blot analysis revealed that two species of mRNA, 2.0kb and 4.6 kb, were expressed in hGLCs. Interestingly, human uterine cervical cells express at least four distinct transcripts, 2.0, 2.2, 3.0 and 4.6 kb [Gorodeski et al., 1998], while human nasal and proximal-tubule epithelia and liver express only a single 2.1 kb mRNA [Parr et al., 1994]. The expression of P2UR in hGLCs supports the hypothesis that extracellular ATP might play a role in the regulation of ovarian function.

Relatively few studies have focused on the regulation of P2UR mRNA in response to hormone treatments. For example, retinoids have been shown to regulate the expression of P2UR mRNA in human uterine cervical cells [Gorodeski et al., 1998]. In the present study, hGLCs were treated with estradiol, progesterone, PGF2α, GnRH, ATP and hCG. The result shows that only hCG attenuated the expression of P2UR mRNA in these cells, suggesting that LH/hCG may play a role in regulating the expression of P2UR in the human ovary. It is well established that activation of LH/CG receptor activates adenylate cyclase and PKA [Lustbader et al., 1998]. To further elaborate the mechanism
by which hCG regulates the expression of P2UR mRNA, hGLCs were treated with exogenous 8-bromo-cAMP and forskolin, an activator of adenylate cyclase. Our results show that both 8-bromo-cAMP and forskolin markedly down-regulated the expression of P2UR mRNA levels, supporting the notion that hCG down-regulation of the expression of P2UR mRNA may be mediated by adenylate cyclase and cAMP. Recently, we and others have shown that hCG can alter the mRNA levels of GnRH receptor and PGF2α receptor [Peng et al., 1994; Ristmaki et al., 1997; Vaananen et al., 1998] in hGLCs. There appears to be a complex interaction of GnRH and PGF2α on steroid hormone production in hGLCs [Vaaninen et al., 1997]. In the present study, hCG, but not GnRH or PGF2α, has been demonstrated to down-regulate the expression of P2UR in dose- and time-dependent manners. The physiological significance of the hCG effect on P2UR remains to be determined. It has been reported that ATP may act as a trigger for apoptosis or programmed cell death [Zheng et al. 1991], and that ATP at a concentration of 2.0 mM causes cell necrosis and death in the ovary [Channing, 1970]. It is conceivable that hCG is capable of minimizing the detrimental effect of ATP, at least in part, by down-regulation of P2UR expression in hGLCs.

ATP has been shown to induce cytosolic calcium oscillations in human granulosa-luteal cells [Fredholm et al., 1994; Lee PSN et al., 1996; Squires et al., 1997]. Pharmacologically, the order of agonist potency for P2UR is ATP=UTP>ATP γ S>>2MeSATP [Kamada et al., 1994; Harden et al., 1995]. It also has been demonstrated that the cytosolic calcium oscillations evoked by ATP are initiated by the release of calcium from cytosolic stores and maintained by extracellular calcium.
influx [Lee PSN et al., 1996]. In the present study, we confirmed the presence of a functional P2UR in hGLCs. Further, our results clearly indicate that, like ATP, UTP is also capable of evoking cytosolic calcium mobilization in a dose-dependent manner. These data provide further evidence that the P2UR expressed in hGLCs is functional following receptor activation by the ligand, at the level of signal transduction.

In summary, our results demonstrate for the first time the expression of P2UR in the human ovary at the mRNA level. We have determined that the level of P2UR mRNA is down-regulated by hCG, presumably via a cAMP-mediated mechanism. The P2UR expressed in hGLCs is functional, in terms of calcium signaling. Taken together, these findings further support a role for ATP and the P2UR in the regulation of human ovarian function.
PART 4

ADENOSINE TRIPHOSPHATE-EVOKED CYTOSOLIC CALCIUM OSCILLATIONS IN HUMAN GRANULOSA-LUTEAL CELLS: ROLE OF PROTEIN KINASE C

4.1 Abstract

Adenosine triphosphate (ATP) has been shown to modulate progesterone production in human granulosa-luteal cells (hGLCs) in vitro. After binding to a G-protein coupled P2 purinergic receptor, ATP stimulates phospholipase C. The resultant production of diacylglycerol and inositol triphosphate activates protein kinase C (PKC) and intracellular calcium \([\text{Ca}^{2+}]_i\) mobilization, respectively. In the present study, we examined the potential cross-talk between the PKC and \(\text{Ca}^{2+}\) pathway in ATP signal transduction. Specifically, the effect of PKC on regulating ATP-evoked \([\text{Ca}^{2+}]_i\) oscillations were examined in hGLCs. Using microspectrofluorimetry, \([\text{Ca}^{2+}]_i\) oscillations were detected in Fura-2 loaded hGLCs in primary culture. The amplitudes of the ATP-triggered \([\text{Ca}^{2+}]_i\) oscillations were reduced in a dose-dependent manner by pretreating the cells with various concentrations (1 nM to 10 μM) of the PKC activator, phorbol-12-myristate-13-acetate (PMA). Ten μM of PMA completely suppressed 10
μM ATP-induced oscillations. The inhibitory effect occurred even when PMA was given during the plateau phase of ATP evoked [Ca^{2+}]_i oscillations, suggesting that extracellular calcium influx was inhibited. The role of PKC was further substantiated by the observation that, in the presence of a PKC inhibitor, Bisindolylmaleimide I, ATP-induced [Ca^{2+}]_i oscillations were not completely suppressed by PMA. Furthermore, homologous desensitization of ATP-induced calcium oscillations was partially reversed by Bisindolylmaleimide I, suggesting that activated PKC may be involved in the mechanism of desensitization. These results demonstrate that PKC negatively regulates the ATP-evoked [Ca^{2+}]_i mobilization from both intracellular stores and extracellular influx in hGLCs and further support a modulatory role of ATP and P2 purinoceptor in ovarian steroidogenesis.
4.2 Introduction

Adenosine triphosphate (ATP), released from autocrine nerves by exocytosis, activates phospholipase C (PLC) through binding to a G protein-coupled P2 purinoceptors. This activation leads to the production of diacylglycerol and inositol 1,4,5-triphosphate, which in turn activates protein kinase C (PKC) and mobilizes intracellular calcium ([Ca$^{2+}$])$_i$, respectively [Berridge, 1984; Gordon 1986]. Through this signaling pathway, ATP may participate in various types of physiological responses, including secretion, membrane potential, cell proliferation, platelet aggregation, neurotransmission, cardiac function and muscle contraction [el-Moatassim et al., 1992; Burnstock, 1990].

Protein kinase C, a serine-threonine kinase which can be activated by tumor-promoting phorbol esters, has been shown to play a key role in intracellular signaling and regulate a wide range of cell functions [Hug and Sarre, 1993; Nishizuka, 1992]. In many systems, PKC has been shown to regulate calcium channel activity and modulate calcium signaling pathway [Nishizuka, 1992; Berridge, 1991; Tsien and Tsien, 1990]. In the ovary, activated PKC has been reported to alter ATP-triggered intracellular calcium oscillations in chicken granulosa cells [Morley et al., 1996] and inhibit steroidogenesis in swine granulosa cells [Veldhuis and Demers, 1986]. Recently, ATP has been shown to evoke calcium oscillations and regulate steroidogenesis in human granulosa cells [Kamada et al., 1994; Lee et al., 1996; Squires et al., 1997]. However, the cross-talk between the ATP-triggered PKC and Ca$^{2+}$ signaling pathways in the
human ovary is not understood. The present study was designed to examine the potential effect of PKC in the regulation of ATP-trigger calcium oscillations in human granulosa-luteal cells (hGLCs). As well, the role of PKC in the homologous desensitization of ATP-triggered calcium oscillations was investigated.

4.3 Materials and Methods

Reagents and Materials

ATP and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin and fetal bovine serum (FBS) were purchased from GIBCO-BRL (Burlington, Ontario, Canada). Fura-2 AM was purchased from Molecular Probes (Eugene, OR). Bisindolylmaleimide I, a PKC inhibitor, was obtained from CALBIOCHEM (Cedarlane, Ontario, Canada).

Human granulosa-luteal cells (hGLCs) in culture

Human GLCs were collected from patients undergoing In Vitro Fertilization - Embryo Transfer (IVF-ET) program and processed as mentioned in PART 2. Human
GLCs were seeded onto 25-mm circular glass cover slips (5,000 cells /slip) and incubated for 3 days at 37 °C in humidified air with 5% CO2 prior to microfluorimetric experiments [Lee PS et al., 1996].

**Microspectrofluorimetry**

Cytosolic calcium concentrations were measured using the dual-excitation single-emission fluorimetric technique, as described previously.

**Treatments**

To examine the effect of ATP on inducing intracellular calcium oscillations, hGLCs were treated with various concentrations of ATP (1, 10, or 100 µM) prior to cytosolic calcium determinations on day 3. Further, hGLCs were cultured for various days (3,5 or 7 days) prior to 10 µM ATP treatment.

To investigate the effect of PKC on regulating ATP-triggered calcium oscillations, hGLCs were treated with various concentrations of PKC activator, phorbol-12-myristate-13-acetate (PMA) (1, 10, 100 nM, 1 or 10 µM) for 5 min, followed by treatment with 10 µM ATP for 3 min.
To further investigate the role of PKC in the regulation of ATP-induced calcium oscillations, hGLCs were pretreated with 1 μM Bisindolylmaleimide I, a PKC inhibitor [Toullec et al., 1991], for 2 minutes prior to PMA and ATP stimulation as performed in the previous experiments.

It has been demonstrated that the intracellular calcium changes are initiated by the release of calcium from cytosolic stores and followed by extracellular calcium influx. To examine whether PKC affects the calcium influx in ATP-evoked calcium mobilization, cells were treated with PMA during the plateau phase of calcium oscillations.

To examine the role of PKC in homologous desensitization of ATP-evoked calcium oscillations, PMA was administered between two ATP treatments. In addition, hGLCs were treated repeatedly with ATP in the absence or presence of Bisindolylmaleimide I.

Data analysis

Data were shown as means of three individual experiments and presented as the mean ±SD. The data were analyzed by one way ANOVA followed by Tukey test. Data were considered significant when P< 0.05.
4.4 Results

*Induction of cytosolic calcium oscillation by ATP in hGLCs*

Human GLCs were treated with various concentrations of ATP (1, 10, or 100 μM) for 3 min. Our results showed that ATP triggered calcium oscillations in these cells (Fig. 27A). The response to ATP was characterized by a spike and a marked increase in cytosolic calcium, followed by numerous oscillations with decreasing amplitudes to pre-activated levels. As shown in Fig. 27A, ATP induced cytosolic mobilization in a dose-dependent manner, with maximal response reached when treated with 10μM of ATP, and no difference was noted between cells treated with 10 μM and 100 μM. Fig. 27B demonstrated the effects of 10 μM ATP on inducing calcium mobilization in hGLCs with various culturing days. There were no significant difference in both of the patterns and amplitudes of ATP-evoked calcium oscillations.
Fig. 27A Effects of ATP on inducing cytosolic calcium oscillations in cultured hGLCs. Fura-2 loaded hGLCs were treated with various concentrations of ATP (1-100 µM). Data of calcium oscillations were presented as ratio (340:380 nm).
Fig. 27B. Effects of 10 μM ATP on hGLCs cultured for various days (Day 3-Day 7). Data of calcium oscillations were presented as ratio (340:380 nm).
The role of PKC in ATP-triggered calcium oscillations in hGLCs

To determine the role of activated PKC in ATP-triggered calcium oscillations, hGLCs were pre-treated with increasing concentrations of PKC activator, PMA (1 nM, 10nM, 100nM, 1 μM or 10 μM) for 5 min, and then stimulated with 10 μM ATP. As shown in Fig. 28, PMA pretreatment reduced the amplitudes of ATP-induced calcium oscillations in a dose-dependent manner. Complete inhibition of initial [Ca^{2+}]_i spike was noted when cells were pretreated with 10 μM PMA.

To further examine the role of PKC in the regulation of ATP-triggered calcium oscillations, Fura-2 loaded hGLCs were pretreated in sequence with 1 μM Bisindolylmaleimide I for 2 min, and Bisindolylmaleimide I plus 10 μM PMA for 5 min, prior to treatment of 10 μM ATP. The results revealed that, in contrast to pretreatment with PMA alone (Fig. 28F), ATP induced calcium oscillations when the cells were pretreated with both PMA and the PKC inhibitor (Fig. 29).

To examine if PMA affects the calcium influx in ATP-evoked calcium mobilization, cells were treated with 10 μM PMA during the plateau phase of ATP-triggered calcium oscillations. The result, when compared with control (Fig. 30A), demonstrated that the amplitudes of calcium oscillations of PMA-treated cells declined to baseline level abruptly (Fig. 30B), suggesting that calcium influx was inhibited by activated PKC.
Fig. 28 Dose-dependent effects of PMA on ATP-evoked cytosolic calcium oscillations in cultured hGLCs. Fura-2 loaded hGLCs were pre-treated with various concentrations of PMA (1 nM - 10 μM, B-F) for 5 min prior to treatment with 10 μM ATP. Data of calcium oscillations were presented as ratio (340:380 nm).
Fig. 29 The role of PKC in ATP induced-calcium oscillations in cultured hGLCs. Fura-2 loaded hGLCs were pretreated in sequence with 1 μM Bisindolylmaleimide I (PKCI) for 2 min, and Bisindolylmaleimide I plus 10 μM PMA for 5 min, prior to treatment with 10 μM ATP. Data of calcium oscillations were presented as ratio (340:380 nm).
Fig. 30 The effect of PMA on ATP-evoked cytosolic calcium oscillations in cultured hGLCs. A. The biphasic pattern of ATP-induced cytosolic calcium oscillations in cultured hGLCs, which was initiated by the release of calcium from cytosolic store and followed by extracellular calcium influx. B. Fura-2 loaded hGLCs were treated with 10 μM PMA during the plateau phase of calcium oscillations. Data of calcium oscillations were presented as ratio (340:380 nm).
Calcium replacement is required to maintain cytosolic calcium oscillations during repeated ATP treatments [Squires et al., 1997]. In the present study, treatment of hGLCs with PMA completely suppressed the subsequent ATP-induced calcium oscillations (Fig. 31A), suggesting that activated PKC may play a role in mediating homologous desensitization. In addition, calcium oscillations were partially reversed during subsequent exposures of hGLCs to ATP in the presence of Bisindolylmaleimide I (Fig. 31C), when compared with repeated ATP exposures in the absence of Bisindolylmaleimide I (Fig. 31B), supporting the proposal that PKC may be involved in homologous desensitization of ATP-triggered calcium oscillations.
Fig. 31 A The role of PKC in homologous desensitization of ATP induced-calcium oscillations in cultured hGLCs. Fura-2 loaded hGLCs were treated with 10 μM PMA for 5 min following exposure to ATP. No cytosolic calcium oscillations were induced by subsequent ATP treatment.
Fig. 31B The role of PKC in homologous desensitization of ATP induced-calcium oscillations in cultured hGLCs. B. and C. The effect of PKC was observed in the absence or presence of PKC inhibitor, Bisindolylmaleimide (PKCI) during repeated treatment of ATP in hGLCs. Data of calcium oscillations were presented as ratio (340:380 nm). Data represent the means ± standard error. *, Significantly different from control (p<0.05)
4.5 Discussion

ATP, released from nerve endings, has been shown to participate in various types of physiological responses [el-Moatassim et al., 1992; Burnstock, 1990; Owman et al., 1967; Bodis et al., 1993a; Bodis et al., 1993b]. It is tempting to speculate that the co-released ATP from autonomic nerve endings in the ovary may play a role in regulating ovarian function. ATP has been shown to regulate the production of progesterone and estradiol in hGLCs [Kamada et al., 1994]. We have reported previously that the P2U purinoceptor is expressed in hGLCs [Tai et al., 2000], further supporting a physiological role of ATP in the human ovary.

Calcium, a second messenger, has been shown to mediate several physiological activities including fertilization, embryo development, cell proliferation and cell death [Berridge et al., 1998]. As demonstrated in this study, ATP is able to mobilize cytosolic calcium, implicating a role of ATP in the control of ovarian function. This finding leads us to postulate that several calcium dependent kinases such as PKC or Ca$^{2+}$/calmodulin-dependent protein kinase [James and Putney, 1998] may be involved in regulating cellular function. However, the precise role of calcium oscillations is not clear yet [Tsien and Tsien, 1990]. PKC has been reported to modulate the activities of ion channels including calcium channels and potassium channels [Shearman et al., 1989]. In addition, PKC has been shown to modulate cytosolic calcium and cAMP levels induced by activation of P2U-purinergic receptor on rat glioma cells [Munshi et al., 1993]. In the ovary, PKC has been reported to modulate ATP-evoked calcium...
oscillation in chicken granulosa cells, supporting the notion that the calcium oscillations were reduced by either activation or inhibition of PKC activity [Morley et al., 1996]. In the present study, a role of PKC in regulating ATP-induced calcium oscillations was revealed in hGLCs. Our results demonstrate that the activation of PKC activity negatively regulated the ATP-evoked cytosolic calcium mobilization from both intracellular stores and extracellular influx in cultured hGLCs. Pretreatment with a PKC inhibitor reversed the inhibitory effect of activated PKC, further supporting the role of PKC in ATP-evoked calcium oscillations in the human ovary.

ATP has been shown to effect a homologous desensitization of ATP-receptors [Dickenson and Hill, 1993]. Homologous desensitization is characterized by a reduced response to an agonist due to repeated treatments with the same agonist. It has been suggested that PKC activated by agonists may be involved in the mechanism of desensitization in several studies [Dickenson and Hill, 1993; Brown et al., 1987; Jones et al., 1990]. ATP-evoked calcium oscillations are dependent upon calcium mobilization from both cytosolic stores and extracellular influx. Calcium replacement is required to maintain cytosolic calcium oscillations during repeated ATP treatments [Squires et al., 1997]. However, the calcium replacement still cannot prevent the down-regulation of the amplitudes of oscillations during repeated ATP treatments, implying that another regulator exists. Several other studies have linked this type of desensitization with activated PKC [Munshi et al., 1993; Wilkinson et al., 1994]. In many systems, PKC has been shown to regulate calcium channel activity and modulate calcium signaling pathway [Nishizuka, 1992; Berridge, 1991; Tsien and Tsien, 1990]. In the present study, repeated treatment of ATP decreased the amplitude of the initial spike of calcium oscillations,
which can be partially reversed by pretreatment with PKC inhibitor. This result indicates that ATP induced homologous desensitization in calcium oscillations in hGLCs.

The mechanism of PKC in regulating calcium oscillations is not clear. Several proteins in the ATP signal transduction pathway can be proposed to act as potential targets of activated PKC. Considering several potential phosphorylation sites in P2U purinoceptor [Lustig et al., 1996], the P2U purinoceptor function may be affected by activated PKC (Fig. 32-③). This proposal is supported by the finding that phorbol ester, a PKC activator, can inhibit the function of G-protein-coupled receptor [Leeb-Lundberg et al., 1985; Thomopoulos et al., 1982]. With respect to receptor-coupled G-proteins, several studies have shown that phorbol ester can regulate G-protein-mediated responses Orellana et al., 1987; Sagi-Eisenberg, 1989; Krishnamurthi et al., 1989], indicating that P2UR-coupled G-protein may be inhibited by activated PKC in hGLCs (Fig. 32-②). In addition, activated PKC has been identified to attenuate agonist-induced inositol phospholipid hydrolysis [Zavoico et al., 1985; Dubyak, 1986; Ryu et al., 1990], suggesting that agonist-stimulated phospholipase C may be desensitized through a negative feedback involving the activation of PKC (Fig. 32-③). Inositol triphosphate (IP3), a product of inositol phospholipid hydrolysis, binds to IP3 receptors on endoplasmic reticulum and induces the release of calcium from the intracellular stores. PKC has been shown to phosphorylate a serine site on IP3 receptors [Ferris et al., 1991; Ferris et al., 1992], implying that activated PKC may shut down cytosolic calcium mobilization through inactivation of the function of IP3 receptors (Fig. 32-③). Calcium influx from the extracellular environment plays a critical role in maintaining the plateau phase following an initial peak of cytosolic calcium oscillations [Lee et al., 1996].
Protein kinase C has been demonstrated to down-regulate or alter calcium influx in agonist-induced calcium mobilization in different systems [Shearman et al., 1989; Di Virgilio et al., 1986; Rane and Dunlap, 1986; Clunes and Kemp, 1996] (Fig. 32-©). Based on above findings, it can be proposed that the ATP-activated PKC may feedback at different levels intracellularly, including the P2U purinoceptor, G-protein, phospholipase C, IP3 receptor or calcium channel, culminating in a shutdown of the calcium signaling pathway in hGLCs.
Fig. 32 A proposed model of the potential cross-talk between ATP-activated protein kinase C (PKC) and cytosolic calcium oscillations in hGLCs. P2UR = P2U purinoceptor on cell membrane; G = G-protein; PLC = phospholipase C; PIP2 = phosphatidyl-inositol 4,5-bisphosphate; DAG = diacylglycerol; IP3 = inositol 1,4,5-triphosphate; IP3R = IP3 receptor.
In conclusion, our results demonstrated that (1) ATP was capable of inducing calcium oscillation in human granulosa-luteal cells in a dose-dependent manner, (2) PKC negatively regulated the ATP-evoked \([Ca^{2+}]_i\) mobilization from both intracellular stores and extracellular influx in cultured hGLCs, and (3) PKC was involved in ATP-induced homologous desensitization in hGLCs. Taken together, these results indicate that ATP may exert a feedback regulation on its own signaling pathway through activation of PKC in the human ovary.
PART 5

ANTIGONADOTROPIC ACTION OF ATP IN HUMAN GRANULOSA-LUTEAL CELLS: INVOLVEMENT OF PKCα

5.1 Abstract

The presence of P2U purinoceptor in human granulosa-luteal cells (hGLCs) indicates the potential role of ATP in regulating ovarian function. Human chorionic gonadotropin (hCG) exerts its action via increasing the accumulation of intracellular cAMP. In this study, the inhibitory effect of ATP on hCG-induced cAMP production was observed. Extracellular ATP has been shown to activate protein kinase C (PKC) after binding to a purinoceptor. To understand the role of PKC in mediating ATP action, hCG-stimulated cAMP level was examined in the presence of PKC activator, 1 μM phorbol-12-myristate-13-acetate (PMA), or PKC inhibitor, 1 μM staurosporin or 1 μM bisindolylmaleimide I. PMA, like 10 μM ATP, significantly reduced hCG-evoked cAMP production. In addition, the inhibitory effect of ATP was reversed by staurosporin and bisindolylmaleimide I. To further investigate the involvement of PKC isoforms in mediating inhibitory effect of ATP, the presence of PKC isoforms in cultured hGLCs was examined by Western blot using monoclonal antibodies against specific isoforms. As well, translocation of PKC isoform from cytosolic fraction to membrane
fraction was studied to identify the active PKC isozyme subsequent to ATP treatment, and the change of PKC isoform in PKC-depleted cells (achieved by exposure to PMA for 18 h) was examined. Our results demonstrated the presence of PKCα, δ, ε and λ isoforms in hGLCs and the translocation of PKCα subsequent to ATP treatment. In PKC-depleted cells, PKCα level was reduced, and no significant effect of ATP on hCG-stimulated cAMP production was noted. To our knowledge, this is the first demonstration of PKC isoforms in hGLCs and the involvement of activated PKC in mediating the antigonadotropic effect of extracellular ATP. Taken together, these results further support a role of this neurotransmitter in regulating ovarian function.
5.2 Introduction

Binding of human chorionic gonadotropin (hCG) with the LH/CG receptor activates adenylate cyclase, leading to the production of cAMP which is the intracellular messenger mediating several cellular functions [Lustbader et al., 1998; Marsh, 1976; Marsh, 1975; Furger et al., 1996].

Adenosine triphosphate (ATP) is released from cells such as platelets or co-released with neurotransmitter granules from autocrine nerves by exocytosis [Gordon, 1986]. ATP has been shown to participate in various types of physiological responses, including secretion, membrane potential, cell proliferation, platelet aggregation, neurotransmission, cardiac function, and muscle contraction [el-Moatassim et al., 1992; Burnstock, 1990; Owman et al., 1967; Bodis et al., 1993a; Bodis et al., 1993b]. It is tempting to speculate that the co-released ATP from autonomic nerve endings in the ovary may play a role in regulating ovarian function. We have reported previously that the P2U purinoceptor is expressed in hGLCs [Tai et al., 2000a], further supporting a physiological role of ATP in the human ovary.

After binding to a G protein-coupled P2 purinoceptor, extracellular ATP activates phospholipase C and phosphatidylinositol hydrolysis, generating diacylglycerol and inositol 1,4,5-triphosphate, which stimulate protein kinase C (PKC) and cytosolic calcium mobilization, respectively [el-Moatassim et al., 1992; Tai et al., 2000a; Berridge, 1984]. The PKC family, a group of widely distributed serine/threonine kinases,
mediates intracellular signaling of numerous cellular regulators including hormones, neurotransmitters and growth factors [Nishizuka, 1984; Berridge, 1993]. Thirteen isozymes have been identified and categorized into four subclasses: (a) conventional protein kinase Cs (α, βI, βII and γ) which are regulated by diacylglycerol, phosphatidylserine and Ca\(^{2+}\), (b) novel protein kinase Cs (δ, ε, θ and η) which are regulated by diacylglycerol and phosphatidylserine, (c) atypical protein kinase Cs (ζ, τ and λ) whose regulation have not been clearly established, and (d) a fourth subfamily, μ and ν [Newton, 1997; Jaken, 1996; Hayashi et al., 1999]. It is noteworthy to mention that multiple and various PKC isoforms are present in the ovary of different species. In the rabbit corpus luteum, α, β and δ isoforms of PKC are identified [Maizels et al., 1992], while porcine corpora lutea contain α and β [DeManno et al., 1992]. Western blot analysis reveals that bovine corpus luteum expresses α and δ [Orwig et al., 1994].

In the present study, we demonstrated that ATP reduced hCG-induced cAMP accumulation in human granulosa-luteal cells (hGLCs). To establish the mechanism, we examined the effect of PKC on hCG-induced cAMP production, the expression of PKC isozyymes and the translocation of PKC isozyme subsequent to ATP treatment.
5.3 Materials and Methods

Reagents and Materials

ATP, staurosporin, human chorionic gonadotropin (hCG) and phorbol-12-myristate-13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco’s Modified Eagle Medium (DMEM), penicillin-streptomycin and fetal bovine serum (FBS) were purchased from GIBCO-BRL (Burlington, Ontario, Canada). bisindolylmaleimide I, a PKC inhibitor, was obtained from CALBIOCHEM (Cedarlane, Ontario, Canada).

Human granulosa-luteal cells culture

Human GLCs were collected from patients undergoing an In Vitro Fertilization - Embryo Transfer program and processed as mentioned in PART 2.

Radioimmunoassay for intracellular cAMP

To determine the effect of ATP on hCG-induced intracellular cAMP accumulation, hGLCs were incubated in serum-free medium containing 0.1% BSA and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich Corp.) for 30 min. Human GLCs
were then treated with hCG (1 IU/mL) in the presence or absence of ATP (10 μM) for 20 min. Human GLCs were lysed with 100% ethanol. Intracellular cAMP levels were measured using the [³H]-cAMP assay system, following the protocol provided by manufacturer (Amersham Pharmacia Biotech).

*Treatment for cyclic AMP assay*

To investigate the role of PKC in hCG-evoked cAMP accumulation, hGLCs were treated with 1 IU/ml hCG in the presence or absence of 1 μM PMA, a PKC activator. To understand the involvement of PKC in the effect of ATP on hCG-induced cAMP production, hGLCs were treated with ATP plus hCG in the presence or absence of PKC inhibitor (1 μM staurosporin or 1 μM bisindolylmaleimide I). In this study, hGLCs were treated with staurosporin or bisindolylmaleimide I for 15 min followed by the administration of ATP.

*Western blot analysis*

To establish the expression of PKC isozymes, hGLCs were processed as described in PART 2. Aliquots (30 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis under reducing condition, as previously described [Laemmlli, 1970]. The proteins were then electrophoretically transferred from the gels onto nitrocellulose
membranes (Amersham Pharmacia Biotech, Oakville, Canada) according to the procedures of Towbin et al. [Towbin et al., 1979]. These nitrocellulose membranes were probed with a mouse monoclonal antibody directed against the PKC isozymes (Transduction Laboratories, Lexington, KY) at 4°C for 16 h. After washing, the membranes were incubated with HRP-conjugated goat-anti mouse secondary antibody, and the signal was visualized using ECL system (Amersham Pharmacia Biotech, Oakville, Canada) followed by autoradiography.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

In view of PKCy being expressed mainly in the nervous system [Nishizuka, 1988], and the monoclonal antibody for PKCα may cross-react with PKCy, a set of primers, as reported previously, was used to examine the existence of PKCy in hGLCs [Moore et al, 1999]. Total RNA was isolated from hGLCs as mentioned before [Tai et al., 2000a]. As a positive control, the mRNA from human antral gastrin cells was kindly provided by Dr. Buchan [Moore et al., 1999].

Translocation experiment of PKC isozymes

Human GLCs were incubated in serum-free medium for 4 h prior to treatment. To examine the translocation of activated PKC, hGLCs were treated with 10 μM ATP for 1
or 5 min. Fractionation of cytosolic and membrane proteins was performed as described previously [Tippmer et al., 1994]. In brief, cells were harvested in test buffer (10 mM Tris/HCl pH 7.4, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 2 mM dithiothreitol, 1000 U/ml aprotinin, 0.8 µg/ml leupeptin, 2 mM PMSF), disrupted by three freeze/thaw steps, and centrifuged at 17,000 xg for 30 min. The supernatant was collected as cytosolic fraction. The pellet was redissolved in lysis buffer (20 mM Hepes/NaOH pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 8 mM EGTA, 15 mM MgCl2, 2 mM PMSF) and centrifuged at 17,000 xg at 4 °C for 30 min. The supernatant was collected as membrane fraction. Equal amounts of cytosolic and membrane proteins (20 µg) were loaded for Western blot analysis. The translocation of PKC isoforms was detected using monoclonal antibodies against PKCα, δ, γ or λ.

**PKC depletion**

Long term treatment of PMA (16 h) is associated with PKC depletion [Abayasekara, 1993a]. In the down-regulation experiment, hGLCs were pretreated with 1 µM PMA for 18 h prior to the treatment. Separate studies were performed to examine the expression of PKCα and the effect of ATP on hCG-induced cAMP accumulation.
Statistical Analysis

Intracellular cAMP levels were shown as pmole per $2 \times 10^5$ cells/dish. Data were represented as means ± standard errors (SE). Statistical analysis was performed by one-way analysis of variance followed by Tukey’s multiple comparison test. Differences were considered significant at $p < 0.05$. 
5.4 Results

Effect of ATP on hCG-induced cAMP production

ATP has been demonstrated to increase intracellular cAMP production by activating adenylyl cyclase in several cell systems [Communi et al., 1997; Conigrave et al., 1998]. To examine the effect of ATP on intracellular cAMP production, hGLCs were treated with 10 μM ATP, ATP plus hCG or 1 IU/mL hCG alone for 20 min. As demonstrated in Fig. 33, hCG markedly increased intracellular cAMP level. In contrast, ATP did not increase intracellular cAMP accumulation in hGLCs, when compared with control group. This result indicates that the P2U purinoceptor expressed in hGLCs is not coupled to adenylyl cyclase. Instead, ATP reduced hCG-evoked cAMP production by 40 %, when compared to hCG treatment alone.
Fig. 33 The effect of ATP on hCG-stimulated intracellular cAMP production in human granulosa-luteal cells (hGLCs). Human GLCs were treated with hCG (1 IU/ml) in the presence or absence of ATP (10 μM) for 20 min as described in the Materials and Methods. Samples were assayed in triplicates following manufacturer’s protocol. Values were presented as the Mean ± SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. hCG.
The role of PKC in hCG-induced intracellular cAMP accumulation

Phorbol ester has been shown to activate protein kinase C in human ovarian tissue [Kawai et al., 1985]. When hGLCs were treated with 1 μM PMA, hCG-stimulated cAMP production was reduced by 30% (Fig. 34).

To further investigate the role of PKC, hGLCs were treated with ATP plus hCG in the presence or absence of PKC inhibitor (1 μM staurosporin or 1 μM Bisindolylmaleimide I). As shown in Fig. 35 & 36, the inhibitory effect of ATP on hCG-evoked cAMP production was reversed by PKC inhibitors, supporting the active involvement of PKC in the regulation of cAMP production.
Fig. 34 The effect of PMA on hCG-stimulated intracellular cAMP production in human granulosa-luteal cells (hGLCs). Human GLCs were treated with hCG (1 IU/ml) in the presence or absence of PMA (1 μM) for 20 min as described in the Materials and Methods. Samples were assayed in triplicates following the manufacturer’s protocol. Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. hCG.
Fig. 35 The role of staurosporin (ST) in the inhibitory effect of ATP on hCG-stimulated cAMP production. Human GLCs were treated with hCG plus ATP in the presence or absence of staurosporin (1 μM). Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. hCG
Fig. 36 The role of bisindolylmaleimide I (Bis) in the inhibitory effect of ATP on hCG-stimulated cAMP production. Human GLCs were treated with hCG plus ATP in the presence or absence of bisindolylmaleimide I (1 μM). Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. hCG.
Expression of PKC isozymes in hGLCs

Eight antibodies against various PKC isozymes were used for the Western blot analysis. When compared with the positive control, five (α, γ, δ, i and λ) isoforms were identified by showing bands of the expected sizes (Fig. 37). PKCβ, ε and θ were absent in hGLCs. In the present study, the positive controls were Jurkat cells for PKCθ and rat brain for the rest of PKC isoforms.

Considering that the monoclonal antibody against PKCγ may crossreact with PKCα, and PKCγ is found mainly in the nervous system [Nishizuka, 1988], RT-PCR was performed to examine the presence of PKCγ in hGLCs. Our results demonstrated the absence of PKCγ in hGLCs. Primers specific to PKCγ amplified a band of the expected size from the positive control, human antral gastrin cells, but not from hGLCs (Fig. 38). This observation ruled out the existence of PKCγ in hGLCs.
PKC isoforms in human granulosa-luteal cells

Fig. 37 The presence of PKC isoforms in human granulosa-luteal cells. Monoclonal antibodies against eight different PKC isoforms were used in western blot analysis as described in the Materials and Methods. GLC, human granulosa-luteal cells; Cnt, positive control.
Fig. 38 PCR product showing the absence of PKCγ in hGLCs in three different patients.
Translocation of PKCα from cytosolic to membrane fraction

Activation of PKC is associated with a translocation of the enzyme from the cytosolic fraction to the plasma membrane [Tippmer et al., 1994]. In the present study, hGLCs were treated with 10 μM ATP for 1 or 5 min. Of the 4 PKC isoforms, only PKCα, mainly in cytoplasm, was noted with increased expression in membrane fraction and reduced expression in the cytosolic fraction after treatment (Fig. 39). The translocation of PKCα isoform to the plasma membrane was accompanied by a decrease in the amount of PKCα in the cytosolic fraction.

Effect of PKC depletion

After treatment with PMA for 18 h, the PKCα isozyme in hGLCs was significantly down-regulated, when compared with control (Fig. 40A). As well, there was no significant effect of ATP on hCG-stimulated cAMP production after PMA pretreatment (Fig. 40B).
Fig. 39 Translocation of PKCα from cytosolic to membrane fraction after ATP treatment in hGLCs. Human GLCs was treated with ATP for 1 or 5 min. The PKCα levels in different fractions were detected by Western blot analysis. C, cytosolic fraction; M, membrane fraction.
Fig. 40 A. Down-regulation of PKCα in hGLCs achieved by prolonged treatment with 1 μM PMA for 18 hours. B. The effect of ATP on hCG-induced cAMP accumulation in hGLCs after treatment with PMA for 18 hours. Values were presented as the Mean ± SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control.
5.5 Discussion

The presence of P2U purinoceptor in hGLCs highlights a role of extracellular ATP in the human ovary [Tai et al., 2000a]. In this study, we demonstrated that PKC was associated with the inhibitory effect of ATP on hCG-stimulated cAMP accumulation. The presence of PKCα in hGLCs and its translocation from cytosolic fraction to membrane fraction suggest a role of PKCα in mediating ATP action on hCG-induced cAMP production.

Cyclic AMP is well established in transducing hCG actions such as progesterone production in the ovary. Prostaglandin F2α, an antigonadotropic agent, inhibits gonadotropin-induced progesterone production via reducing gonadotropin-stimulated cAMP accumulation [Abayasekara et al., 1993b]. In this study, we demonstrated that ATP reduced hCG-induced cAMP production, further supporting a role of extracellular ATP in regulating ovarian function.

Evidence reveals that extracellular ATP can regulate cellular function through activation of PKC [Gordon, 1986; el-Moattassim et al., 1992; Burnstock, 1990]. Studies also indicate that PKC has dual actions by providing positive forward actions as well as negative feedback in controlling various signaling steps [Nishizuka, 1989; Nishizuka, 1986]. We reported recently that ATP was able to induce cytosolic calcium oscillations, and activated PKC negatively regulated ATP-evoked calcium mobilization from both intracellular stores and extracellular influx in hGLCs [Tai et al., 2000b]. In this study,
the forward action of PKC in mediating the effect of ATP on hCG-induced cAMP accumulation was demonstrated using a PKC activator and PKC inhibitors. Protein kinase C isozyymes consist of single polypeptide chains, each containing an amino-terminal regulatory region and a carboxy-terminal kinase domain [Nishizuka, 1992]. Phorbol esters cause activation of conventional and novel PKC isozyymes through binding to the regulatory region [Nishizuka, 1989]. In the present study, PMA mimicked the effect of ATP by reducing the hCG-induced cAMP production (Fig. 34). In addition, staurosporin, a potent PKC inhibitor [Watson et al., 1988], and bisindolylmaleimide I [Toullec et al., 1991], a selective PKC inhibitor of PKCa, δ, ε and ε, effectively reversed the inhibitory action of ATP in hCG-evoked cAMP production, supporting the notion that PKC plays a role in mediating ATP action in the human ovary.

Multiple and various PKC isoforms have been demonstrated in the ovary of different species [Maizels et al., 1992; DeManno et al., 1992; Orwig et al., 1994]. In the present study, we identified the presence of PKCα, δ, ε and ε isoforms in hGLCs. PKC subspecies are expressed specifically in certain tissues [Nishizuka, 1988]. PKCγ appears to be present predominantly in the nervous system such as the brain and spinal cord [Coussens et al, 1986]. Outside of the nervous system, PKCγ is identified in human antral gastrin cells [Moore et al., 1999]. Based on RT-PCR results, we ruled out the presence of this isoform in hGLCs. We reported previously that 10 μM ATP induced cytosolic calcium oscillations in hGLCs [Tai, et al., 2000a], implying the activation of a calcium-dependent PKC isoform subsequent to ATP exposure. According to our observations, the PKCα, a calcium-dependent PKC isoform, was translocated from the
cytosolic fraction to membrane fraction after ATP treatment, specifying the PKC isoform involved.

Long term exposure to phorbol esters cause down-regulation of PKC activity that is associated with proteolysis of PKC occurring in the hinge region between regulatory and catalytic domains by proteases such as calpain or serine protease [Solanki et al., 1983; Pontremoli et al., 1990; Chida et al, 1986]. Prolonged exposure of PMA (16 h) down-regulates PKC activity in hGLCs [Abayasekara et al., 1993a]. In this study, long term treatment of hGLCs with PMA downregulated the expression of PKCa (Fig. 40A), which was shown to be activated by ATP (Fig. 39). ATP lost its effect on hCG-stimulation cAMP accumulation in PKC-depleted cells, indicating the involvement of PKCa in reducing hCG-induced cAMP production α (Fig. 40B).

The observation that ATP inhibited intracellular cAMP responses to hCG through activation of PKC leads us to speculate several potential action sites of activated PKC. Considering the presence of potential PKC phosphorylation sites in the third intracellular loop and at the C-terminal of the LH/hCG receptor, this receptor may be affected by activated PKC following ATP treatment (Fig. 41-©). These intracellular regions of LH/hCG receptor are related to the coupling of the Gs protein, indicating the possibility of dissociation between LH/hCG receptor and Gs protein by active PKC [Loosfelt et al, 1989; Macfarland et al., 1989]. With respect to receptor-coupled G-proteins, several studies have shown that phorbol ester can regulate G-protein-mediated responses [Orellana et al., 1987; Sagi-Eisenber, 1989; Krishnamurthi et al, 1989], indicating that
LH/hCG-coupled Gs-protein may be inhibited by activated PKC in hGLCs (Fig. 41-©). Adenylyl cyclase activity is closely related with cAMP accumulation. PKC has been demonstrated to alter the responses of adenylyl cyclase to G-protein [Zimmermann and Tausig, 1996], pointing out another action site for activated PKC subsequent to ATP treatment in hGLCs (Fig. 41-©). Cyclic AMP phosphodiesterase causes degradation of cAMP and affects intracellular cAMP accumulation. Stimulation of cAMP phosphodiesterase via PKC is reported in cultured hGLCs [Michael and Webley, 1991], suggesting one more factor associated with regulating cytosolic cAMP level (Fig. 41-©).

In conclusion, our results demonstrated that (1) extracellular ATP reduced hCG-stimulated cAMP accumulation, (2) the PKCα, δ, ι and λ isoforms were present in hGLCs, (3) PKCα was translocated subsequent to ATP treatment, and (4) PKC was involved in mediating the antigonadotropic action of extracellular ATP. Taken together, these results further support a role of this neurotransmitter in ovarian steroidogenesis.
Fig. 41 A proposed model of the potential cross-talk between ATP-activated protein kinase Cα (PKCα) and hCG-induced cAMP production in hGLCs. P2UR = P2U purinoceptor on cell membrane; G = G-protein; PLC = phospholipase C; PIP2 = phosphatidyl-inositol 4,5-bisphosphate; DAG = diacylglycerol; IP3 = inositol 1,4,5-triphosphate; LH/hCG R = LH/hCG receptor.
PART 6

ATP ACTIVATES MITOGEN-ACTIVATED PROTEIN KINASE IN HUMAN GRANULOSA-LUTEAL CELLS

6.1 Abstract

ATP has been shown to activate the phospholipase C (PLC)/ diacylglycerol/ protein kinase C (PKC) pathway. However, little is known about the downstream signaling events. The present study was designed to examine the effect of ATP on activation of mitogen-activated protein kinase (MAPK) signaling pathway and its physiological role in human granulosa-luteal cells (hGLCs). Western blot analysis, using a monoclonal antibody which detected the phosphorylated forms of ERK1 and ERK2 (p42\textsuperscript{mapk} and p44\textsuperscript{mapk}, respectively), demonstrated that ATP activated MAPK in a dose- and time-dependent manner. Treatment of the cells with suramin (a P2-purinoceptor antagonist), neomycin (a PLC inhibitor), staurosporin (a PKC inhibitor) or PD98059 (a MEK, MAPK/ERK kinase, inhibitor) significantly attenuated the ATP-induced activation of MAPK. In contrast, ATP-induced MAPK activation was not significantly affected by pertussis toxin (a Gi inhibitor). To examine the role of Gs protein, intracellular cAMP level was determined after treatment with ATP or human chorionic gonadotropin (hCG). No significant elevation of intracellular cAMP level was noted after ATP treatment. To determine the role of MAPK in steroidogenesis, hGLCs were treated with ATP, hCG, or ATP plus hCG in the presence or absence of PD98059. Radioimmunoassay revealed
that ATP alone did not significantly affect basal progesterone concentration. However, hCG-induced progesterone production was reduced by ATP treatment. PD98059 reversed the inhibitory effect of ATP on hCG-induced progesterone production. To our knowledge, this is the first demonstration of ATP-induced activation of MAPK signaling pathway in the human ovary. These results support the notion that the MAPK signaling pathway is involved in mediating ATP actions in the human ovary.
6.2 Introduction

Extracellular adenosine triphosphate (ATP) is co-released with neurotransmitter granules from nerve endings by exocytosis [Gordon, 1986]. After binding to a G protein-coupled P2 purinoceptor, ATP activates phosphoinositide hydrolysis, generating diacylglycerol and inositol 1,4,5-trisphosphate, which stimulate protein kinase C and cytosolic calcium mobilization, respectively [Berridge, 1984; el-Moatassim et al., 1992]. Thereafter, ATP may participate in various types of physiological responses, including secretion, membrane potential, cell proliferation, platelet aggregation, neurotransmission, cardiac function and muscle contraction [el-Moatassim et al., 1992; Burnstock, 1990]. Considering that the ovary is a well-innervated organ, it is tempting to speculate that the co-released ATP from nerve endings may play a role in regulating ovarian function. We reported previously the expression of P2U purinoceptor in hGLCs [Tai et al., 2000a], further supporting a physiological role of ATP in the human ovary.

Mitogen-activated protein kinases (MAPKs) are a group of serine-threonine kinases involved in converting extracellular stimulus into intracellular signals. Extracellular signal regulated kinases (ERKs), one of MAPKs subfamilies, have been shown to be activated by extracellular agonists such as cytokines, growth factors and neurotransmitters [Cobb and Goldsmith, 1995; Fanger, 1999]. It is believed that two classes of cell surface receptors, G-protein-coupled receptor and receptor tyrosine kinases are associated with the activation of MAPKs [Fantl et al., 1993; Lopez-Ilasaca, 1998; Chabre, 1995]. When activated, ERK1 and ERK2 (also known as p42mapk and p44
mapk, respectively), phosphorylate a variety of substrates, including transcription factors, which have been implicated in the control of cell proliferation and differentiation [Post and Brown, 1996; Cano and Mahadevan, 1995; Blenis, 1993].

The demonstration of P2U purinoceptor in hGLCs highlights the significance of ATP in regulating ovarian function, but little is known about the signaling events and cellular responses subsequent to the binding of ATP to its receptor in the human ovary. Activation of P2 purinoceptor has been shown to increase MAPK activity [Dickenson et al., 1998]. However, the role of MAPK in ovarian cells is poorly understood. In the present study, the signaling cascade proximal to MAPK activation subsequent to ATP exposure was determined in hGLCs. In addition, the functional role of activated MAPK following ATP treatment was studied.

6.3 Materials and Methods

Reagents and Materials

ATP, suramin, pertussis toxin (PTX), neomycin, staurosporin and human chorionic gonadotropin (hCG) were obtained from Sigma Chemical Co. (St. Louis, MO). PD98059, a MEK inhibitor, was purchased from New England Biolabs Inc., Beverly, MA. Dulbecco's Modified Eagle Medium (DMEM), penicillin-streptomycin and fetal
bovine serum (FBS) were obtained from GIBCO-BRL (Burlington, Ontario, Canada). Staurosporin and PD98059 were dissolved in dimethyl sulfoxide (DMSO) as suggested by manufacturers.

*Human granulosa-luteal cells culture*

Human GLCs were collected from patients undergoing an In Vitro Fertilization - Embryo Transfer program and processed as described in PART 2.

*Treatments*

Human GLCs were incubated in serum-free medium for 4 h prior to treatment. To examine the dose-response relationship, hGLCs were treated with increasing concentrations of ATP (100 nM, 1 μM, 10 μM or 100 μM) for 5 min. For time-course experiments, hGLCs were treated with 10 μM ATP for 1, 5, 10 or 20 min.

To determine the intracellular signaling pathway, hGLCs were treated with suramin (300 μM, an inhibitor of P2 purinergic receptor), PTX (200 ng/mL; a Gi inhibitor), neomycin (10 mM; a PLC inhibitor), staurosporin (1 μM; a PKC inhibitor) or PD98059 (50 μM; a MEK inhibitor) in the presence or absence of 10 μM ATP. Human GLCs were pretreated with suramin for 15 min, PTX for 1 h, neomycin for 15 min, staurosporin
for 15 min and PD98059 for 1 h prior to ATP treatment. The cells were collected 5 min after ATP exposure.

Western blot analysis

The hGLCs were processed as mentioned in PART 2. Aliquots (30 μg) were subjected to 10 % SDS-polyacrylamide gel electrophoresis under reducing condition, as previously described [Laemmli, 1970]. The proteins were then electrophoretically transferred from the gels onto nitrocellulose membranes (Amersham Pharmacia Biotech, Oakville, Canada) according to the procedures of Towbin et al. [Towbin et al., 1979]. These nitrocellulose membranes were probed with a mouse monoclonal antibody (New England Biolabs Inc., Beverly, MA) directed against the phosphorylated forms of ERK1 and ERK2 (P-MAPK, p42mapk and p44 mapk, respectively) at 4 °C for 16 h. Alternatively, the membranes were probed with a rabbit polyclonal antibody for p42/p44 MAPK, which detected total MAPK (T-MAPK) levels (New England Biolabs Inc., Beverly, MA). After washing, the membranes were incubated with HRP-conjugated goat-anti mouse secondary antibody for P-MAPK and sheep-anti rabbit secondary antibody for T-MAPK, and the signal was visualized using ECL system (Amersham Pharmacia Biotech, Oakville, Canada) followed by autoradiography. The autoradiograms were quantified using a laser densitometer (BIO-RAD, Model 620, Video Densitometer).
MAP Kinase Assay

To measure MAP kinase activity, a nonradioactive method was utilized (p44/42 MAP Kinase Assay Kit, New England Biolabs Inc.). The kit contains two phospho-antibodies, one to selectively precipitate active MAP kinase and a second to detect MAPK-induced phosphorylation of Elk-1. Briefly, active MAP kinase from hGLCs lysate (200 µg) treated with 10 µM ATP for 5 min was selectively immunoprecipitated with an immobilized monoclonal antibody to phospho-p44/42 MAP (Thr202 and Tyr204) kinase. For a positive control, active MAP Kinase (provided by the manufacturer) was added to the control cell extract. The resulting precipitate was incubated with an Elk-1 fusion protein in the presence of ATP which allowed immunoprecipitated active MAPK to phosphorylate Elk-1 [Marais et al., 1993; Janknecht et al., 1993; Gille et al., 1995]. Phosphorylation of Elk-1 at Ser383 was measured by western blotting using a phospho-Elk-1 (Ser383) antibody. Ser383 of Elk-1 is a major phosphorylation site by MAP kinase and is required for Elk-1-dependent transcriptional activity [Marais et al., 1993; Janknecht et al., 1993; Gille et al., 1995].
Overview of p44/42 MAP Kinase Assay

Step 1: Prepare cell extracts.
   (a) Treat cells.
   (b) Add cell lysis buffer.
   (c) Collect cell lysates.

Step 2: Selective immunoprecipitation of active (phosphorylated) MAPK using immobilized phospho-antibody.
   (a) Add immobilized phospho MAPK Ab
   (b) Immunoprecipitation of cell extracts using immobilized phospho-MAPK Ab.

Step 3: Incubate immunoprecipitated pellets in buffer containing Elk-1 fusion protein and ATP.

Step 4: Analyze Elk-1 phosphorylation using phospho-antibodies by western blotting.
Radioimmunoassay for intracellular cAMP

Human GLCs (2 x 10^5 cells) were plated onto 35 mm culture dishes and cultured for 4 days. The cells were then incubated in serum-free medium containing 0.1% BSA and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX, Sigma-Aldrich Corp.) for 30 min. To determine ATP or hCG-induced intracellular cAMP accumulation, hGLCs were treated with ATP (10 μM) or hCG (1 IU/mL, a positive control) for 20 min. Intracellular cAMP levels were measured using the [^3-H]-cAMP assay system, following the protocol provided by manufacturer (Amersham Pharmacia Biotech).

Radioimmunoassay for progesterone

After culture in DMEM with 10% FBS for 3 days, hGLCs were incubated in DMEM for 4 h prior to treatment for steroidogenesis experiments. To determine the role of MAPK in steroidogenesis, hGLCs were treated with ATP (10 μM), hCG (1 IU/ml), or ATP plus hCG in the presence or absence of PD98059 for 6 h.

Progesterone levels in the culture medium were measured by an established radioimmunoassay [Vaaninen et al, 1997]. Anti-progesterone antibody was kindly provided by Dr. D. T. Armstrong (University of Western Ontario). Briefly, samples were incubated with antibody and tracer, with final concentration of 7,000 cpm/ml of [1,2,6,7,16,17-3H]Progesterone (Amersham Pharmacia Biotech). After incubation for
16-24 hours, a charcoal/dextran solution was added to remove unbound progesterone or tracer. Scintillation cocktail (Amersham Pharmacia Biotech) was added to each sample, and the vials were counted with a β-counter (LKB Wallac, Turku, Finland). The cells in each dish were harvested for quantifying protein amount using Bio-Rad Protein Assay kit. Samples were assayed in triplicates and progesterone concentrations were standardized against total protein contents.

_Human chorionic gonadotropin and MAPK in hGLCs_

Gonadotropins have been demonstrated to activate MAPK in porcine granulosa cells [Cameron et al., 1996]. To examine the effect of hCG on MAPK activation, hGLCs were treated with 1 IU/ml hCG for 1, 5, 10 or 20 min and cell lysates were collected for western blot analysis. The effect of MAPK on hCG-stimulated progesterone production was studied by treating cells with 1 IU/ml hCG in the presence or absence of PD98059 for 24 h.

_MAPK and antigonadotropic effect of ATP on hCG-induced cAMP production_

To examine the activated MAPK and inhibitory effect of ATP on hCG-induced cAMP accumulation, hGLCs were treated with hCG, ATP plus hCG in the presence or absence of PD98059 for 20 min. Intracellular cAMP accumulation was measured as
Statistical Analysis

MAPK and progesterone levels were expressed as a relative ratio of basal levels. Intracellular cAMP levels were shown as pmole per $2 \times 10^5$ cells. Data were represented as means ± standard errors (SE). Statistical analysis was performed by one-way analysis of variance followed by Tukey's multiple comparison test. Differences were considered significant at $p < 0.05$.

6.4 Results

Effect of ATP on MAPK activation

To demonstrate the ability of ATP in activating MAPK, hGLCs were treated with increasing concentrations (100 nM-100 μM) of ATP for 5 min. For time-course analysis, the cells were treated with 10 μM ATP for varying time intervals (1-20 min). As shown in Fig. 42, ATP activated MAPK in hGLCs in a dose-dependent manner. A significant effect was observed at 1 μM with a maximum effect noted at 10 μM, and there is no statistically significant difference between cells treated with 10 μM and 100 μM ATP.
ATP was capable of rapidly inducing MAPK activity. A significant effect was seen within 5 min after treatment, and the activation of MAPK was sustained for at least 15 min (Fig. 43).

**MAP kinase activity**

In vitro MAP kinase activity was measured using a p44/42 MAP kinase assay kit. As shown in Fig. 44, ATP significantly increased MAPK activity.
Fig. 42 The dose-response of ATP on MAPK activation in human granulosa-luteal cells (hGLCs). Human GLCs were treated with increasing concentrations of ATP (0, 100 nM, 1 μM, 10 μM or 100 μM) for 5 min as described in the Materials and Methods. The MAPK were detected by Western blot analysis. The data were shown as relative ratio to basal levels. Values were presented as the Mean±SE of three individual experiments. Statistical analysis was performed by one-way analysis of variance followed by Tukey test. Differences were considered significant at p < 0.05, *.
Fig. 43 The time course of ATP on MAPK activation in human granulosa-luteal cells (hGLCs). Human GLCs were treated with 10 μM ATP for 0, 1, 5, 10 or 20 min as described in the Materials and Methods. The MAPK were detected by Western blot analysis. The data were shown as relative ratio to basal levels. Values were presented as the Mean ± SE of three individual experiments. Differences were considered significant at p < 0.05, *. 
Fig. 44 MAP kinase activity in hGLCs detected using a MAP kinase assay kit. Human GLCs were treated with 10 μM ATP for 5 min as described in the Materials and Methods.
P2-purinergic receptor and ATP-induced MAPK activation

P2U purinergic receptor has been demonstrated in hGLCs [Tai et al., 2000a; Lee et al., 1996]. To investigate the involvement of P2 purinoceptor in ATP-induced MAPK activation, hGLCs were pretreated with 300 µM suramin, a P2 purinoceptor antagonist [Hertog et al., 1992], for 15 min prior to the administration of ATP. As demonstrated in Fig. 45, ATP activated MAPK to about 230 % of basal (control) level. The co-treatment with suramin and ATP significantly reduced MAPK activity by 85 %, when compared to ATP treatment alone.

Pertussis toxin and ATP-induced MAPK activation

A PTX-insensitive G-protein, Gαq/11, is known to be expressed in hGLCs [Carrasco et al., 1997; Lopez Bernal et al., 1995]. To identify the subclass of G-protein involved in the ATP-induced activation of MAPK, human GLCs were pretreated with PTX for 1 h prior to exposure to ATP. Pretreatment of PTX did not alter ATP-induced MAPK activity, indicating that ATP acts through a PTX-insensitive G-protein (Fig. 46).
Fig. 45 The effect of suramin, a P2 purinoceptor inhibitor, on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs). Human GLCs were treated with 10 μM ATP in the presence or absence of suramin (300 μM) as described in the Materials and Methods. The activated MAPK were detected by Western blot analysis. The data were shown as relative ratio to basal levels. Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. ATP.
Fig. 46 The effect of pertussis toxin (PTX), a Gi protein inhibitor, on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs). Human GLCs were treated with 10 µM ATP in the presence or absence of PTX (200ng/mL) as described in the Materials and Methods. The activated MAPK were detected by Western blot analysis. The data were shown as relative ratio to basal levels. Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. ATP.
**PLC and ATP-induced MAPK activation**

Neomycin, an aminoglycoside antibiotic, has been demonstrated to inhibit PLC [Spath et al., 1991]. In this study, hGLCs were pretreated with 10 mM neomycin for 15 min prior to the stimulation of ATP. As shown in Fig. 47, treatment of hGLCs with neomycin significantly inhibited the ATP-induced activation of MAPK. The combined treatment with neomycin and ATP significantly attenuated MAPK activity by 90 %, when compared to ATP treatment alone.

**PKC and ATP-induced MAPK activation**

Staurosporin, a potent inhibitor of protein kinase C [Watson et al., 1998], significantly attenuated the ATP-induced activation of MAPK (Fig. 48). The concomitant treatment with the PKC inhibitor and ATP attenuated MAPK activation by 70 %, when compared to the level stimulated by ATP alone.

**MEK and ATP-induced MAPK activation**

In MAPK activation cascade, MEK is the immediate activator of MAPK. MEK is also known as MAPK Kinase [Fanger, 1999]. MEK inhibitor, PD98059, significantly decreased the ATP-induced activation of MAPK in hGLCs. Simultaneous treatment
with PD98059 and ATP reduced MAPK activity to about 50% of the level stimulated by ATP alone (Fig. 49).
Fig. 47 The effect of neomycin, a PLC inhibitor, on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs). Human GLCs were treated with 10 μM ATP in the presence or absence of neomycin (10 mM) as described in the Materials and Methods. The activated MAPK were detected by Western blot analysis. The data were shown as relative ratio to basal levels. Values were presented as the Mean ±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. ATP.
Fig. 48 The effect of staurosporin, a PKC inhibitor (PKCI), on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs). Human GLCs were treated with 10 μM ATP in the presence or absence of staurosporin (1 μM) as described in the Materials and Methods. The activated MAPK were detected by Western blot analysis. The data were shown as relative ratio to basal levels. Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. ATP.
Fig. 49 The effect of PD98059, a MEK inhibitor (MEKI), on ATP-induced MAPK activation in human granulosa-luteal cells (hGLCs). Human GLCs were treated with 10 μM ATP in the presence or absence of PD98059 (50 μM) as described in the Materials and Methods. The activated MAPK were detected by Western blot analysis. The data were shown as relative ratio to basal levels. Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. ATP.
Effect of ATP on intracellular cAMP accumulation

ATP has been demonstrated to increase intracellular cAMP production by activating adenylyl cyclase in several cell systems [Communi et al., 1997; Conigrave et al., 1998]. To examine the effect of ATP on intracellular cAMP production, hGLCs were treated with 10 μM ATP for 20 min, while 1 IU/mL hCG was used as a positive control. Human CG markedly increased intracellular cAMP level. In contrast, ATP was not able to increase intracellular cAMP accumulation in hGLCs, when compared with control group (Fig. 50). This result indicates that the P2U purinoceptor expressed in hGLCs is not coupled to adenylyl cyclase.

Effect of ATP-evoked MAPK activation on hCG-induced progesterone production

To determine the role of MAPK in ovarian steroidogenesis, hGLCs were treated with ATP (10 μM), hCG (1 IU/ml), or ATP plus hCG in the presence or absence of PD98059. As shown in Fig. 51, 10 μM ATP had no effect on the basal level of progesterone production, while hCG increased progesterone production to 250 % of control in hGLCs. Co-treatment of hGLCs with ATP and hCG significantly inhibited the progesterone production to 50 % of the level induced by hCG alone. Further, the presence of MEK inhibitor (PD98059) reversed the inhibitory effect of ATP on hCG-induced progesterone production.
Effect of ATP on cAMP production

Fig. 50 The effect of ATP on intracellular cAMP production in human granulosa-luteal cells (hGLCs). Human GLCs were treated with ATP (10 μM) or hCG (1 IU/ml) for 20 min as described in the Materials and Methods. Samples were assayed in triplicates following manufacturer's protocol. Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control.
Fig. 51 The effect of MAPK on progesterone production in human granulosa-luteal cells (hGLCs). Human GLCs were treated with ATP (10 μM), hCG (1 IU/ml), or ATP plus hCG in the presence or absence of PD98059 for 6 hours as described in the Materials and Methods. Samples were assayed in triplicates and progesterone concentrations were standardized against total protein content. Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. ATP plus hCG.
Human chorionic gonadotropin activates MAPK in hGLCs

As shown in Fig. 52A, hCG was capable of activating MAPK in hGLCs in a time-dependent manner. Phosphorylated MAPK increased significantly in 1 min, when compared with the control, and reached a maximum response after treatment with 1 IU/ml hCG for 5 min. To investigate the role of hCG-stimulated MAPK in steroidogenesis, hGLCs were treated with hCG in the presence or absence of MEK inhibitor. Radioimmunoassay demonstrated that there was no significant effect of MEKI on hCG-induced progesterone production (Fig. 52B).

MAPK and antigonadotropic effect of ATP on hCG-induced cAMP production

As shown in Fig. 53, the inhibitory effect of ATP on hCG-stimulated cAMP production was not altered significantly in the presence of MEKI, suggesting that the action site of active MAPK is secondary to cAMP production.
Fig 52. A. The effect of hCG on MAPK activation in human granulosa-luteal cells (hGLCs). Human GLCs were treated with 1 IU/ml hCG for various time (1-20 min) as described in the Materials and Methods. The activated MAPK were detected by Western blot analysis. B. The effect of PD98059, a MEK inhibitor (MEKI), on hCG-induced progesterone production in human granulosa-luteal cells (hGLCs). Samples were assayed in triplicates and progesterone concentrations were standardized against total protein content. Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control.
Fig. 53 The effect of MEKI in inhibitory effect of ATP on hCG-stimulated cAMP production. Human GLCs were treated with hCG plus ATP in the presence or absence of MEKI (50 μM). Values were presented as the Mean±SE of three individual experiments. Differences were considered significant at p < 0.05. a, p < 0.05 vs. control; b, p < 0.05 vs. ATP+hCG.
6.5 Discussion

The MAP kinases have been implicated in the regulation of cell growth and differentiation [Brunet and Pouyssegur, 1997]. MAP kinases are classified into three subfamilies: (I) ERKs (extracellular signal-regulated kinases), including ERK1 and ERK2, (II) SAPKs (stress-activated protein kinase), also called c-jun N-terminus kinases (JNKs), and (III) p38 kinase [Lopez-Ilasaca, 1998]. The MEKs, also known as MAPK kinases (MAPKKs), activate the MAPKs by dual phosphorylation on threonine and tyrosine residues of a TEY (Thr-Glu-Tyr) motif [Ahn et al., 1992]. The first MAPKs to be cloned are MAPK/ERK 1 and 2, which are phosphorylated and activated by MEKs [Boulton et al., 1990; Boulton et al., 1991]. MAP kinases have been identified in several steroidogenic cells [Chabre et al., 1995; McNeill et al., 1998], but little is known about their role(s) in steroidogenesis. PGF2α, an anti-gonadotropic hormone, has been demonstrated to stimulate the MEK1/MAPK signaling cascade in bovine luteal cells [Chen et al., 1998]. Recently, Kang et al. reported that MAPKs mediate the inhibitory effect of gonadotropin-releasing hormone in progesterone production in hGLCs [Kang et al., 2000], indicating the role of MAPKs in steroidogenesis. In the present study, the phospho-specific MAPK antibody, which detected phosphorylated Thr202 and Tyr204 on ERK1/2, was used to measure activated MAPKs by Western blot analysis. The concentration of ATP in adrenergic granules of sympathetic nerves and in acetylcholine-containing granules of parasympathetic nerves can be as high as 150 mM [Winkler and Carmichael, 1982]. Our results demonstrated that MAPKs were activated by 10 μM ATP, and furthermore, MAPKs mediated the anti-gonadotropic action of ATP
The P2U purinoceptor has been identified in hGLCs [Tai et al., 2000a] and may be coupled to PTX-sensitive or insensitive G-proteins [Dubyak and el-Moatassim, 1993; Sternweis and Smrcka, 1992]. It has been reported previously that P2U purinoceptors are coupled to a PTX-insensitive G-protein in hGLCs, using microspectrofluorimetry [Lee et al., 1996]. In the present study, ATP-induced phosphorylation of MAPK was not affected by 200 ng/mL PTX, indicating the involvement of PTX-insensitive G-proteins such as Goq/11 [Carrasco et al., 1997; Lopez Bernal et al., 1995]. P2 purinoceptors have been reported to be coupled to adenylyl cyclase in several systems [Communi et al., 1997; Conigrave et al., 1998; Post et al., 1998]. In this study, ATP failed to increase intracellular cAMP accumulation, indicating that the P2U purinoceptor expressed in hGLCs is not coupled to adenylyl cyclase.

After binding to the G-protein-coupled receptor, ATP has been reported to activate phospholipase C [Dubyak and el-Moatassim, 1993; Dubyak, 1991], resulting in the production of inositol trisphosphate (IP3) and diacylglycerol (DAG), which in turn induces calcium mobilization and activates PKC, respectively. PLC-β and PLC-γ isoforms have been identified in hGLCs [Carrasco et al, 1997]. Neomycin has been demonstrated to inhibit all three isoforms of PLCs [Spath et al., 1991]. In the present study, 10 mM Neomycin significantly reduced the level of the phosphorylated form of MAPK, indicating the role of PLC in ATP-induced MAPK activation. PKC has been shown to exert its effects in the ovary [Morley et al., 1996; Abayasekara, 1993a;
Abayasekara, 1993b; Michael and Webley, 1991]. In this study, ATP-induced MAPK activation was significantly attenuated in hGLCs pretreated with staurosporin, a potent PKC inhibitor [Watson et al., 1988], indicating the involvement of PKC in the MAPK activation cascade. MEK is an immediate activator of MAPK. Our data demonstrated that the MEK inhibitor, PD98059, significantly decreased the ATP-induced activation of MAPK. Taken together, this study delineated the ATP signaling pathway in hGLCs from PTX-insensitive G-protein, PLC, PKC, with a MEK to MAPK activation.

ATP has been demonstrated to induce the production of steroid hormones in steroidogenic cells [Foresta et al, 1996; Niitsu, 1992]. In the ovary, 100 µM ATP, ADP and AMP have been shown to regulate the basal levels of progesterone and estrogen in hGLCs, indicating the effects of ATP metabolites on steroidogenesis. However, UTP has no effect on basal progesterone level in hGLCs, implying that the stimulatory effects of purine nucleotides on progesterone production are not through P2U-purinoceptors, but via A2-adenosine receptors [Kamada et al, 1994]. As shown in the present study, a lower concentration of ATP (10 µM) had no effect on the basal level of progesterone production in hGLCs. However, co-treatment of hGLCs with ATP significantly inhibited the progesterone production induced by hCG, indicating an anti-gonadotropic action of ATP in hGLCs. Furthermore, pretreatment of hGLCs with MEK inhibitor reversed the inhibitory effect of ATP on hCG-induced progesterone production.

Luteinizing hormone has been demonstrated to increase MAPK activity in porcine granulosa cells [Cameron et al., 1996]. In the present study, hCG activated both ERK1
and ERK2 in a time-dependent manner. However, the hCG-induced MAPK did not alter hCG-stimulated progesterone production (Fig. 52). Taken together, these observations support the notion that a diverse array of ligands, including hormones, neurotransmitters and growth factors, are able to activate MAPK and cells may contain several MAPK signaling cascades, potentially regulated independently [Van Biesen et al., 1996].

The precise mechanism by which MAPKs affect ovarian steroidogenesis is not clear. As demonstrated in the present study, activated MAPK did not alter the antigonadotrophic action of ATP in hCG-stimulated cAMP production, suggesting that the potential action site of MAPK is distal to cAMP production. Several steroidogenic enzymes such as steroidogenic acute regulatory protein (StAR), cytochrome P450 cholesterol side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD) have been demonstrated in the human ovary [Duncan et al., 1999; Kiriakidou et al., 1996]. Considering the nuclear translocation of activated MAPKs [Fanger, 1999; Post and Brown, 1996; Cano and Mahadevan, 1995; Blenis, 1993], it can be postulated that MAPK is involved in steroidogenesis through altering the production of steroidogenic enzymes (Fig. 54).

To our knowledge, this is the first demonstration of ATP-induced activation of MAPK signaling pathway in the human ovary. Through a PTX-insensitive G-protein and without affecting intracellular cAMP production, ATP activated MAPK subsequent to PLC and PKC activation in hGLCs. These findings support a role of the MAPK signaling pathway in mediating the ATP modulation of steroidogenesis in the human
ovary.
Fig. 54 Proposed intracellular signaling cascades of ATP in hGLCs. ATP binds to a PTX-insensitive G protein-coupled receptor that activates phospholipase C and phosphatidylinositol 4,5-biphosphate (PIP2) hydrolysis, generating diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3), which stimulate PKC and evoke intracellular calcium (Ca^{2+}) mobilization, respectively. PKC, in turn, activates MEK and MAPK (p42^{mapk} and p44^{mapk}), culminating in inhibition of hCG-induced progesterone production.
PART 7 SUMMARY

Adenosine triphosphate (ATP) is co-released with neurotransmitter granules from autonomic nerves by exocytosis. Extracellular ATP binds to a G protein-coupled P2 purinoceptor that activates phospholipase C and phosphatidylinositol hydrolysis, generating diacylglycerol and inositol 1,4,5-triphosphate, which stimulate protein kinase C (PKC) and cytosolic calcium ([Ca2+]i) mobilization, respectively. Thereafter, ATP may participate in various types of physiological responses, including secretion, membrane potential, cell proliferation, platelet aggregation, neurotransmission, cardiac function, and muscle contraction [el-Moatassim et al., 1992; Burnstock, 1990].

The reproductive tract including ovary, oviduct, uterus, cervix and vagina is innervated by autonomic nerves. However, the effect of neurotransmitters on reproductive function is still poorly understood. Considering that the ovary is a well-innervated organ, it is tempting to speculate that the co-released ATP from autonomic nerve endings in the ovary may play a role in regulating ovarian function. In this study, a series of experiments were performed to examine (1) the expression and regulation of P2U purinergic receptor in human granulosa-luteal cells (hGLCs), (2) the functional role of extracellular ATP in the human ovary, (3) the signaling pathway subsequent to the binding of ATP to purinergic receptor in hGLCs, (4) the action and mechanism of antigonadotropic effect of ATP on hGLCs, (5) the roles of protein kinases including protine kinase C (PKC) and mitogen-activated protein kinase (MAPK) in mediating ATP action in hGLCs.
The present study demonstrates for the first time the expression of P2UR in human ovarian cells. The demonstration of P2UR mRNA in hGLCs provides the basis to explore the signaling pathway and functional role of extracellular ATP in the human ovary. Northern blot analysis revealed that two species of mRNA, 2.0kb and 4.6 kb, were expressed in hGLCs. Interestingly, human uterine cervical cells express at least four distinct transcripts, 2.0, 2.2, 3.0 and 4.6 kb [Gorodeski et al., 1998], while human nasal and proximal-tubule epithelia and liver express only a single 2.1 kb mRNA [Parr et al., 1994]. The P2UR was expressed functionally in hGLCs, since activation of the P2UR by both ATP and uridine triphosphate (UTP) resulted in rapid and transient mobilization of cytosolic calcium at the single cell level.

ATP has been demonstrated to regulate the production of steroid hormones in steroidogenic cells [Foresta et al., 1996; Niitsu, 1992]. In the present study, a lower concentration of ATP (10 μM) had no effect on the basal level of progesterone production in hGLCs. However, co-treatment of hGLCs with ATP significantly inhibited the progesterone production induced by hCG, indicating an anti-gonadotropic action of ATP in hGLCs.

Human CG was shown in our study to reduce the expression of P2U purinergic receptor in a dose- and time-dependent manner. It is well established that activation of LH/CG receptor activates adenylate cyclase and PKA [Lustbader et al., 1998]. To further elaborate the mechanism by which hCG regulates the expression of P2UR mRNA, hGLCs were treated with exogenous 8-bromo-cAMP and forskolin, an activator of
adenylate cyclase. Our results show that both 8-bromo-cAMP and forskolin markedly down-regulated the expression of P2UR mRNA levels, supporting the notion that hCG down-regulation of the expression of P2UR mRNA may be mediated by adenylate cyclase and cAMP. It has been reported that ATP may act as a trigger for apoptosis or programmed cell death [Zheng et al. 1991], and that ATP at a concentration of 2.0 mM causes cell necrosis and death in the ovary [Channing, 1970]. It is conceivable that hCG is capable of minimizing the detrimental effect of ATP, at least in part, by down-regulation of P2UR expression in hGLCs.

The PKC family, a group of widely distributed serine/threonine kinases, mediates intracellular signaling of numerous cellular regulators including hormones, neurotransmitters and growth factors [Nishizuka, 1984; Berridge, 1993]. Multiple and various PKC isoforms are present in the ovary of different species. We demonstrated the presence of PKCα, δ, γ and λ isoforms in hGLCs. It appears that PKC may have dual actions by providing forward actions as well as negative feedback in controlling various signaling steps. In this study, the forward action of active PKC-induced by ATP was demonstrated by reducing hCG-stimulated cAMP production. The inhibitory effect of ATP was reversed by PKC inhibitors, staurosporin and bisindolylmaleimide I, indicating the involvement of PKC in mediating antigonadotropic action of ATP in hGLCs. On the other hand, ATP induced calcium mobilization was negatively regulated by activated PKC from both intracellular stores and extracellular influx in cultured hGLCs, indicating the cross-talk between the PKC and Ca2+ pathway in ATP signal transduction.
Our data also demonstrated the potential cross-talk between protein kinase A (PKA) and PKC signaling pathway in hGLCs. Human CG downregulated the expression of P2U purinergic receptor by activation of cAMP/ PKA pathway, culminating in a reduction of purinergic receptor transcription. On the other hand, ATP significantly attenuated hCG-stimulated cAMP and progesterone production through PKC signaling pathway. These observations indicated the complex interactions between gonadotropin and neurotransmitter-related signaling pathway in the ovary.

Extracellular signal regulated kinases (ERK), one of the MAPK subfamilies, have been shown to be activated by extracellular agonists such as cytokines, growth factors and neurotransmitters [Cobb and Goldsmith, 1995; Fanger, 1999]. When activated, ERK1 and ERK2 (also known as p42mapk and p44 mapk, respectively) phosphorylate a variety of substrates, including transcription factors, which have been implicated in controlling cellular proliferation and differentiation [Post and Brown, 1996; Cano and Mahadevan, 1995; Blenis, 1993]. Our data demonstrated that ATP was able to activate ERK1/2 in hGLCs in a dose- and time-dependent manner. After binding to the P2-purinoceptor, ATP activated MAPK subsequent to PLC and PKC activation through PTX-insensitive G-protein in hGLCs. MAPK mediated the anti-gonadotropic action of ATP in steroidogenesis by reducing hCG-stimulated progesterone production.

Physiologically, the ovarian cycle can be divided into three phases: the follicular phase, ovulation and the luteal phase. The cross-talk between extracellular ATP and the gonadotropin-related cAMP signaling pathway shown in this study encourages us to
explore in the future the potential action of ATP in the follicular phase, which is
dominated by FSH. In the present study, we demonstrated the role of ATP in regulating
hCG-stimulated progesterone secretion through the protein kinase C/mitogen-activated
protein kinase pathway in hGLCs obtained from women undergoing the IVF-ET program,
indicating that ATP plays a role in the early luteal phase. Since the uterus is a target
organ of progesterone, ATP may exert some effects on the regularity of the menstrual
cycle and blastocyst implantation. It was observed that prostaglandin F2α exerted a
similar effect in regulating gonadotropin-stimulated progesterone production [Tai et al,
2001b; Vaananen et al, 1997]. In view of the fact that prostaglandin F2α is a luteolytic
agent, it leads us to speculate that there is a possible luteolytic effect of extracellular ATP,
which remains to be determined in the future.

Our data demonstrated the effect of extracellular ATP on regulating human ovarian
function. Furthermore, the demonstration of active involvement of multiple signaling
molecules such as protein kinase C, cytosolic calcium and mitogen-activated protein
kinase delineates the pathway mediating the functional role of ATP in the ovary. These
findings support the notion that extracellular ATP is a potent regulator of human ovarian
function.
PART 8 FUTURE STUDIES

1. To examine the role of ATP in regulating steroidogenic protein and enzymes in hGLCs.

Steroidogenic acute regulatory protein, P450sc and 3β-HSD control major steps in progesterone production. In a previous study, it was observed that ATP reduced hCG-stimulation by attenuating cAMP accumulation induced by hCG. To explore where other molecules are involved in mediating antigonadotropic effect of ATP, we will examine the effect of ATP on these steroidogenic protein and enzymes.

2. To examine the role of active PKC in regulating steroidogenic protein and enzymes in hGLCs.

PKC has been shown to mediate the antigonadotropic action of ATP in hGLCs by reducing hCG-stimulated cAMP production. Considering the multiple potential action sites of activated PKC, we will examine the role of this serine/threonine kinase in regulating the expression of steroidogenic protein and enzymes.
3. To examine the role of activated MAPK in regulating steroidogenic protein and enzymes in hGLCs.

Our results demonstrated that ATP activated MAPK in a dose- and time-dependent manner. Furthermore, MAPK was shown to mediate the antigonadotrophic action of ATP in hGLCs by reducing hCG-stimulated progesterone production but not cAMP accumulation. Considering the nuclear translocation of activated MAPKs [Fanger, 1999; Post and Brown, 1996; Cano and Mahadevan, 1995; Blenis, 1993], it can be postulated that MAPKs are involved in steroidogenesis through altering the synthesis of steroidogenic enzymes.

4. To examine the effect of ATP on inducing apoptosis (programmed cell death) in hGLCs.

ATP has been reported to induce death of hGLCs at a concentration of 2 mM [Channing, 1970]. However, there is no experiment to elucidate the underlying mechanism. Extracellular ATP may act as a trigger for apoptosis [Zheng et al., 1991]. Exploring the relationship between ATP and apoptosis will reveal the potential role of extracellular ATP in hGLCs.
5. To examine the effects of ATP on other ovarian cells.

The ovary is a well-innervated organ coated by a single layer of ovarian surface epithelium (OSE). Evidence shows that ATP is capable of inducing calcium oscillations and regulating cell proliferation in OSE-originated tumor cell lines such as OVCAR-3 and SKOV-3 [Popper and Batra, 1993; Babra and Fadeel, 1994]. However, limited information is available about the role of extracellular ATP in human OSE. Examining the effect of ATP on OSE will disclose a potential role of ATP in the ovary.


10. Barritt GJ 1999 Receptor-activated Ca\textsuperscript{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca\textsuperscript{2+} signalling requirements. Biochem J 337: 153-169


Structure of a calpain Ca\(^{2+}\)-binding domain reveals a novel EF-hand and Ca\(^{2+}\)-induced conformational changes. Nat Struct Biol 4: 532-538


Cholinergic stimulation of progesterone and estradiol secretion by human granulosa cells cultured in serum-free medium. Gynecol Endocrinol 7:83-7


Cholinergic stimulation of progesterone and estradiol secretion by human granulosa cells cultured in serum-free medium. Gynecol Endocrinol 7: 83-87


News sheet 8 Autonomic Neuroscience Institute, Royal Free Hospital School of
Medicine, London, June, 1998

41. Cameron MR, Foster JS, Bukovsky A, Wimalasena J 1996 Activation of
mitogen-activated protein kinases by gonadotropins and cyclic adenosine

42. Cano E, Mahadevan LC 1995 Parallel signal processing among mammalian
MAPKs. Trends Biochem Sci 20: 117-122

43. Carrasco MP, Asboth G, Phaneuf S, Lopez Bernal A 1997 Activation of the

44. Cena V, Rojas E 1990 Kinetic characterization of calcium dependent, cholinergic
receptor-controlled ATP secretion from adrenal medullary chromaffin cells.
Biochim Biophys Acta 1023:213-222

regulation of mitogen-activated protein kinase activity in bovine adrenocortical
cells: cross-talk between phosphoinositides, adenosine 3’, 5’-monophosphate, and
tyrosine kinase receptor pathways. Endocrinology 136: 956-964

46. Channing CP 1970 Influences of the in vivo and in vitro hormonal environment
upon luteinization of granulosa cells in tissue culture. Recent Prog Horm Res
26:589-622

47. Channing CP, Schaerf FW, Anderson LD, TsafririA 1980 Ovarian follicular and
luteal physiology. Int Rev Physiol 22:117-201

48. Chapman JC, Waterhouse TB, Michael SD 1992 Changes in mitochondrial and
microsomal 3β-hydroxysteroid dehydrogenase activity in mouse ovary over the
course of the estrous cycle. Biol Reprod 47: 992-997

49. Chen DB, Westfall SD, Fong HW, Roberson MS, Davis JS 1998 Prostaglandin
F2α stimulates the Raf/MEK1/mitogen-activated protein kinase signaling cascade
in bovine luteal cells. Endocrinology 139: 3876-3885

dehydrogenase/ isomerase and cytochrome P-450scc into a catalytically active
molecular complex in bovine adrenocortical mitochondria. J Steroid Biochem Mol
Biol 55: 507-514

51. Cherradi N, Dfaye G, Chambaz EM 1994 Characterization of the
3β-hydroxysteroid dehydrogenase activity associated with bovine adrenocortical
mitochondria. Endocrinology 134: 1358-1364

52. Cherradi N, Rossier MF, Vallotton MB, Timberg R, Friedberg I, Orly J, Wang XJ,
Stoccl DMM, Capponi AM 1997 Submitochondrial distribution of three key
steroidogenic proteins (steroidogenic acute regulatory protein, P450
side-chain -cleavage and 3β-hydroxysteroid dehydrogenase isomerase enzymes)
upon stimulation by intracellular calcium in adrenal glomerulosa cells. J Biol Chem
272: 7899-7907

53. Chida K, Kato N, Kuroki T 1986 Downregulation of phorbol diester receptors by
proteolytic degradation of protein kinase C in a cultured cell line of fetal rat skin

guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem. 162:156-9


63. Coussens L, Parker PJ, Rhee L, Yang-Feng TL, Chen E, Waterfield MD, Francke


69. Dickenson JM, Hill SJ 1993 Homologous and heterologous desensitization of histamine H1- and ATP-receptors in the smooth muscle cell line, DDT1MF-2: the role of protein kinase C. Br J Pharmacol 110:1449-56

protien kinase Cμ. FEBS Lett 381: 183-187


85. Fanger GR 1999 Regulation of the MAPK family members: role of subcellular localization and architectural organization. Histol Histopathol 14:887-894


91. Filtz TM, Li Q, Boyer JL, Nicholas RA, Harden TK 1994 Expression of a cloned P2Y-purinergic receptor that couples to phospholipase C. Mol Pharmacol 46:8-14


95. Fraser CM, Chung FZ, Wang CD, Venter JC 1988 Site-directed mutagenesis of human beta-adrenergic receptors: substitution of aspartic acid-130 by asparagine produces a receptor with high-affinity agonist binding that is uncoupled from adenylate cyclase. Proc Natl Acad Sci USA 85:5478--82


108. Grobler JA, Hurley JH 1998 Catalysis by phospholipase C81 requires that Ca$^{2+}$ bind to the catalytic domain, but not to the C2 domain. Biochem 37: 5020-5028


119. James W, Putney Jr 1998 Calcium signaling: up, down, up, down.... What's the point? Science 279: 191-192

120. Janknecht R, Ernst WH, Pingoud V, Nordheim A 1993 Activation of ternary complex factor Elk-1 by MAP kinases. EMBO J 12: 5097-5104


205


142. Langley JN and Anderson HK 1895a The innervation of the pelvic and adjoining viscera. Part IV. The internal generative organs. J Physiol (Lond) 19: 122-130

143. Langley JN and Anderson HK 1895b The innervation of the pelvic and adjoining
viscera. Part V. Position of the nerve cells on the course of the efferent nerve fibres. J Physiol (Lond) 19: 131-139


151. Lopez Bernal A, Bellinger J, Marshall J, Phaneuf S, Europe-Finner GN, Asboth G,


156. Lustig KD, Shiau AK, Brake AJ, Julius D 1993 Expression cloning of an ATP receptor from mouse neuroblastoma cells. Proc Natl Acad Sci USA 90:5113-5117


159. Maizels ET, Miller JB, Cutler RE Jr, Ijackiw V, Carney EM, Mizuno K, Ohno S,


177. Moore EDW, Ring M, Scriven DRL, Smith VC, Meloche RM, Buchan AMJ 1999
The role of protein kinase C isozyymes in bombesin-stimulated gastrin release from

178. Morel N, Meunier FM 1981 Simultaneous release of acetylcholine and ATP from
stimulated cholinergic synaptosomes. J Neurochem 36:1766-1773

179. Morley P, Chakravarthy BR, Mealing GA, Tsang BK, Whitfield JF 1996 Role of
protein kinase C in the regulation of ATP-triggered intracellular Ca2+ oscillations
in chicken granulosa cells. Eur J Endocrinol 134:734-750

gamma-subunits. Recent insights from studies of the phospholipase C-beta
isoenzymes. Biochem Pharmacol 54: 429-435

rat glioma cells: modulation of cytosolic Ca2+ and cAMP levels by protein kinase
C. Mol Pharmacol 44: 1185-1191

peroxide in porcine thyroid cells. J Endocrinol 126:283-287

183. Nalefski EA, Falke JJ 1996 The C2 domain calcium-binding motif: structural and
functional diversity. Protein Sci 5: 2375-2390


185. Niitsu A 1992 Calcium is essential for ATP-induced steroidogenesis in bovine
adrenocortical fasciculata cells. Jpn J Pharmacol 269-274

186. Nishizuka Y 1984 The role of protein kinase C in cell surface signal transduction
of the protein kinase C family, nPKCθ, predominantly expressed in skeletal muscle.
Mol Cell Biol 12: 3930-3938

198. Osipchuk Y, Cahalan M 1992 Cell-to-cell spread of calcium signals mediated by
ATP receptors in mast cells. Nature Lond 359:241-244

Biol Chem 265: 13472-13483

female reproductive organs: a histochemical and chemical investigation. Obstet
Gynecol 30:763-773

201. Owman Ch, Sjoberg NO, Svensson KG, Walles B 1975 Autonomic nerves
mediating contractility in the human Graffian follicle. J Reprod Fertil 45: 553-556

202. Owman Ch, Sjoberg NO, Wallach EE, Walles B, Wright KH 1979 Neuromuscular
mechanisms of ovulation. In: Hafez ESE (ed) Human Ovulation: Mechanism,
Prediction, Detection and Regulation. Elsevier/ North Holland, Amsterdam
pp57-100

(Eds.), purinergic approaches in experimental therapeutics. Wiley-Liss, New York
pp545-553

204. Papenfuß F, Bodis J, Tinneberg HR, Schwarz H 1993 Arch Gynecol Obstet 253:
97-102

1998 Stress promotes development of ovarian cysts in rats: the possible role of
sympathetic nerve activation. Endocrine 8: 309-315


213. Plummer MR, Logothetis DE, Hess P 1989 Elementary properties and pharmacological sensitivities of calcium channels in mammalian peripheral
neurons. Neuron 2: 1453-1463


222. Rane SG, Dunlap K 1986 Kinase C activator 1,2-oleoylacylglycerol attenuates voltage-dependent calcium current in sensory neurons. Proc Natl Acad Sci USA


232. Sagi-Eisenberg R 1989 GTP-binding proteins as possible targets for protein kinase
C action. Trends Biochem Sci 14:355-357


249. Stefenson A, Owman C, Sjoberg NO, Sporrong B, Walles B 1981 Comparative
study of the autonomic innervation of the mammalian ovary, with particular regard to the follicular system. Cell Tissue Res 215:47-62


251. Stocco DM 1998 Recent advances in the role of StAR. Reviews of Reproduction 3: 82-85


265. Toullec D, Pianetti P, Coste H, Bellevergue P, Grand-Perret T, Ajakane M, Baudet

266. Towbin H, Staehelin T, Gordon J 1979 Elcetrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA 76, 4350-4354


273. Veldhuis J, Gwynne JT 1989 Insulin-like growth factor type 1 (somatomedin-C)
stimulates high density lipoprotein (HDL) metabolism and HDL-supported progesterone biosynthesis by swine granulosa cells in vitro. Endocrinology 124:3069-3076


283. Wilkinson GF, Purkiss JR, Boarder MR 1994 Differential heterologous and homologous desensitization of two receptors for ATP (P2Y purinoceptors and nucleotide receptors) coexisting on endothelial cells. Mol Pharmacol 45:731-736


DH 1990 Molecular cloning of cDNA encoding human and rabbit forms of the Ca2+ release channel (ryanodine receptor) of skeletal muscle sarcoplasmic reticulum. J Biol Chem 265: 2244-2256