Single Cell Studies of Calcium as Second Messenger in Human Granulosa-Lutein and Embryonic Kidney 293 Cells

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Abstract

It is well established that LH action is mediated primarily by adenylate cyclase/cAMP. Conversely, the role of inositol phosphate/calcium in LH signalling has only recently been investigated. We examined the effects of gonadotrophins on intracellular calcium mobilisation in HEK293 cells transiently transfected with human wild type or chimeric gonadotrophin receptors (n=3400). Intracellular free calcium concentration was measured using fura-2 microspectrofluorimetric techniques. Human LH (2-4 μg/ml) and CG (10 IU/ml) consistently evoked oscillatory calcium signals in HEK293 cells transfected with hLHr, whereas hFSH (2-4 μg/ml) failed to elicit any calcium responses. Both hLH and hFSH failed to elicit a calcium response from HEK293 cells transfected with hFSHr. Pre-treatment of transfected HEK293 cells with pertussis toxin (100 ng/ml) or with U-73122 (10 μM), a phospholipase C inhibitor, negated all gonadotrophin-evoked calcium mobilisation. Our study of chimeric gonadotrophin receptors show that the carboxy-terminal third of the hLHr is crucial in evoking intracellular calcium changes. Although various subdivisions of this region is capable of stimulating calcium transients, an intact carboxy-terminal third of the receptor is required for normal and sustained intracellular calcium profile. To our knowledge, this is the first demonstration of calcium oscillations in response to the activation of the hLH receptor, and to unequivocally show that the hLH receptor is coupled to the inositol phosphate/calcium signalling pathway via a pertussis toxin-sensitive G protein.

The role of extracellular ATP in the human ovary remains equivocal. We demonstrated that P_2 purinoreceptor agonists evoke oscillatory intracellular calcium responses in hGLCs. The cells were responsive to ATP at concentrations ranging from 1-100 μM. ATP and UTP were more effective in stimulating calcium mobilisation than ADP. Neither adenosine nor AMP were capable of inducing intracellular calcium responses. The positive responses to adenosine thiotriphosphate, a non-hydrolysable ATP analogue, indicate that the calcium responses were not due to by-products from ATP hydrolysis, and that hGLCs possess P_{2U} purinoreceptors. We have also demonstrated that these purinergic-mediated intracellular calcium responses involve both Ca^{2+} influx and Ca^{2+} mobilisation from intracellular stores.

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Abbreviations

adenylate cyclase	AC
adenosine diphosphate	ADP
adenosine monophosphate	AMP
adenosine 5′-o-(3-thiotriphosphate)	ATPγS
adenosine triphosphate	ATP
8-bromoadenosine 3′:5′-cyclic monophosphate	8-Br-cAMP
caffeine	caf
carbon dioxide	CO_2
cyclic adenosine 3′,5′-mononphosphate	cAMP
diacylglycerol	DAG
dimethyl sulphoxide	DMSO
Dulbecco's Modified Eagle's Medium	DMEM
ethylene glycol-bis(β-aminoethylether) N,N,N′,N′-tetraacetic acid	EGTA
foetal bovine serum, heat-inactivated	FBS
follicle-stimulating hormone	FSH
fura-2-acetoxymethyl ester	fura-2-AM
gonadotrophin-releasing hormone	GnRH
granulosa-lutein cells	GLCs
guanosine diphosphate	GDP

guanosine triphosphate ..... GTP

human chorionic gonadotrophin ..... hCG

human granulosa-lutein cells ..... hGLCs

inositol 1,4,5-trisphosphate ..... IP_3

international unit(s) ..... IU

intracellular calcium concentration ..... $[Ca^{2+}]_i$

luteinising hormone ..... LH

oestradiol ..... E_2

pertussis toxin ..... PTX

phosphatidylinositol 4,5-bisphosphate ..... PIP_2

phosphatidylinositol ..... PI

phosphatidylinositol-4-phosphate ..... PIP

phospholipase C ..... PLC

potassium chloride ..... KCl

progesterone ..... P_4

prostaglandin $F_{2\alpha}$ ..... $PGF_{2\alpha}$

protein kinase A ..... PKA

protein kinase C ..... PKC

thapsigargin ..... TPG

uridine triphosphate ..... UTP

volume per volume (ml/100 ml)..... v/v

weight per volume (gm/100 ml)..... w/v

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With deepest gratitude,

PSL

Background

Gonadotrophic Hormones

LH and FSH regulate gonadal function and gametogenesis, and are critical for normal sexual maturation and reproductive function. Both hormones are synthesized in and secreted from pituitary gonadotrophs, under the regulation of GnRH. LH and FSH have approximate molecular weights of 28,000 and 33,000, respectively; the uncertainty in their molecular weights result from the heterogeneity of the attached carbohydrate groups and minor differences in amino acid composition. These two pituitary glycoprotein hormones share chemical and structural similarities; both hormones are heterodimers composed of glycosylated subunits (α and ß) tightly bound in a non-covalent association. The individual subunits appear to have no intrinsic biologic activity, and must be appropriately glycosylated and tightly associated to act as gonadotrophins.

Within a species, the α-subunits of glycoprotein hormones possess the same amino acid sequence. The α-subunit of the LH/CG and FSH molecules, common to pituitary glycoprotein hormones, has a molecular weight of 14,000. The ß-subunit of each glycoprotein hormone has a distinct amino acid sequence, and thus dictates hormone specificity [Pierce and Parsons, 1981]. The human CG ß-subunit, structurally very similar to the LH ß-subunit, shows about 80% similarity in amino acid sequence to the LH ß-subunit, and confers almost identical biologic properties when associated with the α-subunit. The human CG ß-subunit contains an additional 32 amino acids at the carboxy-terminal, however this has no apparent role in the biological activity or

metabolism of the human CG molecule. LH and CG bind to the same receptor to initiate hormone action, but with different kinetics.

The main difference in biological activity between human CG and LH is the more prolonged action of hCG *in vivo*, because of its slower metabolic clearance and its somewhat higher affinity for the LH receptor sites in the testis and ovary. These features largely result from different carbohydrate compositions of the two molecules, in particular the much higher sialic acid content of hCG [Lambert, et al., 1998].

The *N*-linked oligosaccharides of these hormones are necessary for proper folding, assembly, secretion, metabolic clearance and biological activity. The carbohydrate content of FSH is greater than that of LH, but they share a similar structure. Specific chemical features of the LH, CG, and FSH molecules include the locations of the carbohydrate moieties: there are two oligosaccharide groups on the α-subunit common to the glycoprotein hormones, one in the human LH ß-subunit, and two in the human FSH and CG ß-subunits. In addition to the *N*-linked oligosaccharides, the human CG ß-subunit also contains four *O*-linked oligosaccharides [Matzuk, et al., 1990]. Deglycosylation has little effect on hormone binding, but it does markedly attenuate the hormones ability to activate target cells in the gonads [Sairam, 1989]. The carbohydrate moieties of the α-subunit, and not the ß-subunit, are essential for the activation of the LH receptor and its GTP-binding protein (G-protein)-coupled adenylate cyclase system [Matzuk, et al., 1989; Sairam, 1989]. The sialic acid content of the glycoprotein hormones varies from twenty residues per molecule in human CG, five residues in FSH, and only one or two in human LH. Removal of the sialic acid residues drastically shortens the

circulating half-life of the hormones, but has little effect on their ability to act on their respective cellular receptor sites [Lambert, et al., 1998].

Luteinising Hormone/Chorionic Gonadotrophin Receptors

LH and CG bind to, and activate, the same cell surface reporter. This receptor belongs to the large family of G-protein-coupled membrane proteins [Loosfelt, et al., 1989; McFarland, et al., 1989]. The LH/CG receptor is a glycoprotein consisting of a single polypeptide chain with six potential *N*-linked glycosylation sites [Kusuda and Dufau, 1988]. The hydrophilic amino-terminal of the receptor comprises approximately half of the total amino acids. This extracellular domain is necessary for high affinity binding to gonadotrophin. The transmembrane portion of the LH/CG receptor has seven membrane spanning segments which form three extracellular loops and three intracellular loops. The short intracellular carboxy-terminal domain contains serine, threonine, and tyrosine residues, suggesting the potential for modulation of receptor function by the action of serine-threonine protein kinases, and tyrosine kinases [Bousfield, et al., 1994].

Structure-function relationship studies have demonstrated that the truncated extracellular amino-terminal half of the receptor is capable of high affinity ligand binding without cAMP induction, whereas the truncated carboxy-terminal is capable of low affinity binding with cAMP induction [Ji and Ji, 1993; Segaloff, et al., 1990]. Binding of the LH ligand to its receptor results in conformational changes leading to activation of the C-terminal. Point mutation studies [Ji and Ji, 1993; Segaloff and Ascoli, 1993; Shenker, et al.,

1993] have demonstrated that high affinity receptor binding and receptor activation with intracellular signal generation are distinct events.

Receptors for LH/CG have be found on a variety of tissues in reproductive systems, including Leydig cells, granulosa cells, and luteal cells [Akamizu, et al., 1990; Ascoli and Segaloff, 1989]; they have also been detected in non-ovarian cells [Lincoln, et al., 1992]. This glycoprotein receptor consists of a single polypeptide chain, and share the same basic structure as the FSH and TSH receptors: a large amino-terminal domain, seven transmembrane spanning domains, and a short carboxy-terminal domain [Frazier, et al., 1990; Rodriguez and Segaloff, 1990; Strader, et al., 1995]. Receptors for LH/CG, FSH, and TSH belong to the large family of G-protein-coupled membrane receptors, but are unusual in that they have large extracellular domains (300-400 amino acids) and bind large ligands (23-38 kDa) [McFarland, et al., 1989]. Other members of the receptor family have small amino-terminal extracellular domains (30-50 amino acids) and bind small ligands (200-300 Da) [Dohlman, et al., 1991; Jackson, 1991; Savarese and Fraser, 1992]. It is the large extracellular domains of the LH/CG receptors which are responsible for the recognition and high affinity binding of the respective glycoproteins [Braun, et al., 1991; Xie, et al., 1990]. Despite the wide range of ligands that activate these receptors, the receptors themselves share a surprising amount of structural homology.

The LH/CG receptor is highly conserved, with the highest degree of conservation in the transmembrane domains and connecting loops, followed by the extracellular amino-terminal domains. The lowest degree of conservation occurs in the he intracellular carboxy-terminal cytoplasmic tails [Segaloff and Ascoli, 1993]. The human receptor is 85% identical to the rat LH/CG receptor and 87% identical to the porcine LH/CG receptor [Minegishi,

et al., 1990]. Despite the high homology between human, rat, and porcine, the human LH/CG receptor has a high degree of species specificity; it does not bind equine LH and CG, rat LH or ovine LH [Jia, et al., 1991].

The large extracellular hydrophilic domain of the LH/CG receptors comprises about half the total number of amino acids, and contains 6 potential sites for *N*-linked glycosylation [Loosfelt, et al., 1989; McFarland, et al., 1989; Minegishi, et al., 1989]. This extracellular domain contains 14 copies of an imperfectly repeated sequence of about 25 amino acids, similar to a repeated motif called "leucine rich repeat" [Leong, et al., 1992]. These repeats allow for the formation of amphipathetic helices or ß-sheets, which can interact with both hydrophilic and hydrophobic surfaces [Krantz, et al., 1991], thus providing a basis for the possible interaction of the hydrophilic extracellular domain with the hydrophobic transmembrane domain of the LH/CG, and also the FSH, receptors [Segaloff and Ascoli, 1993]. Leucine-rich repeats 1-6 have also been shown to be involved in hormone binding [Thomas, et al., 1996]. Involvement of the carbohydrate moieties of LH/CG receptors, in the recognition and high affinity binding, remains equivocal. While some have indicated that at least one of the carbohydrate chains is required for ligand binding [Minegishi, et al., 1989; Zhang, et al., 1995; Zhang, et al., 1991], others have reported that deglycosylation of the LH/CG receptor does not compromise its binding ability [Davis, et al., 1997; Ji, et al., 1990; Petaja-Repo, et al., 1991].

The seven transmembrane spanning domains of the LH/CG receptor are highly homologous with other receptors belonging to the family of G-protein-coupled membrane receptors [Baldwin, 1994]. The seven hydrophobic transmembrane spanning domains are connected by hydrophilic extracellular and intracellular loops. The transmembrane domain of the LH/CG receptor

has also been implicated in hormone binding. Several studies have shown that this region contains a low affinity hormone binding site [Ji and Ji, 1991a; Roche, et al., 1992], and that it may be important in the activation of the adenylate cyclase [Abell and Segaloff, 1997; Ji and Ji, 1991a; Ji and Ji, 1991b].

The short carboxy-terminal cytoplasmic tail, along with the cytoplasmic loops connecting the transmembrane domains, contain potential phosphorylation sites, and thus may be a further site for the regulation of hormone-receptor function. Two potential kinase C phosphorylation sites have been identified, along with a third domain [Loosfelt, et al., 1989]. Mutations of the carboxy-terminal cytoplasmic tail resulted in the non-expression of rat LH/CG receptors on the plasma membrane, suggesting that the carboxy-terminal cytoplasmic tail is important for the trafficking of receptors to the plasma membrane [Rodriguez, et al., 1992; Sanchez-Yague, et al., 1992], and for receptor desensitisation [Sanchez-Yague, et al., 1992].

Desensitisation of LH/CG Receptors

Ligand binding to the LH/CG receptor results in uncoupling and down-regulation. Uncoupling is defined as the agonist-induced change in the functional properties of the receptor without a change in the number of receptors. This relatively fast phenomenon occurs within minutes of the administration of the agonist, is thought to be due to phosphorylation of intracellular amino acid residues, thereby attenuating its ability to activate the effector system(s) (i.e. adenylate cyclase, phospholipase C) [Segaloff and Ascoli, 1993]. Down regulation is defined as the actual reduction in the density of the receptors at the plasma membrane. This slower phenomenon occurs within

minutes to hours of addition of the agonist, and can be caused by a decrease in the synthesis of the receptors, an increase in the degradation of receptors, or by a combination of both [Segaloff and Ascoli, 1993].

Uncoupling of LH/CG Receptors

Uncoupling of the LH/CG receptor leads to a reduction in hormonal responsiveness without a concomitant reduction in the number of LH/CG receptors [Rebois and Fishman, 1986], and without changes to the functional properties of G_s or the catalytic subunit of adenylate cyclase [Rebois and Fishman, 1986; Sanchez-Yague, et al., 1993]. Uncoupling of the $ß_2$-adrenergic receptor involves phosphorylation of different regions of the receptor catalysed by the cAMP-dependent and $ß_2$-adrenergic receptor kinases [Dohlman, et al., 1991; Lefkowitz, et al., 1990]. As both the $ß_2$-adrenergic and LH/CG receptors belong to the same family of G-protein-coupled membrane receptors, they will undoubtedly possess similarities; however, the cAMP-dependent protein kinase is unlikely to be involved in the phosphorylation and/or uncoupling of LH/CG receptors because: (1) increases in cAMP levels elicited by agents other than LH/CG do not uncouple the LH/CG receptor [Rebois and Fishman, 1986]; and (2) there are only weak consensus sites for the cAMP-dependent protein kinase-catalysed phosphorylation in the intracellular regions of the rat, porcine, mouse, or human LH/CG receptor [Kennelly and Krebs, 1991]. Maximal uncoupling of the LH/CG receptor also requires guanosine triphosphate (GTP) [Ekstrom and Hunzicker-Dunn, 1989a; Ekstrom and Hunzicker-Dunn, 1989b; Ezra and Salomon, 1980].

Studies have demonstrated that the carboxy-terminal cytoplasmic tail is involved in the uncoupling of the LH/CG receptor [Sanchez-Yague, et al., 1992; Wang, et al., 1996]. They have shown that truncation of the carboxy-terminal cytoplasmic tail results in a higher maximal cAMP response than that observed with wild-type receptors. Similarly, the magnitude of hCG-induced uncoupling is more pronounced in cells expressing wild-type LH/CG receptors, than those expressing receptors with truncated cytoplasmic tails. The fact that LH/CG receptors, with truncated cytoplasmic tails, still lose hormonal responsiveness upon prolonged exposure to its ligand, suggests that uncoupling is not the only mechanism involved in desensitisation. Truncation studies [Rodriguez, et al., 1992; Sanchez-Yague, et al., 1992] have shown that receptor uncoupling and receptor internalisation are separate phenomena, with different determinants.

Down-Regulation of LH/CG Receptors

Human CG-induced reduction in the density of LH/CG receptors is elicited by both an increase in receptors internalisation, and by decreased transcription of the receptor gene. Exposure of LH/CG receptors to their ligands results in a time-dependent decrease in the number of membrane receptors, without changes in receptor affinity [Freeman and Ascoli, 1982; Rebois and Fishman, 1984]. There is an actual decrease in the number of receptors, and not a mere redistribution of receptors from the cell surface to an intracellular compartment [Ascoli, 1985]. Studies have shown that the entire ligand-receptor complex is internalised into endocytic vesicles and transferred into lysosomes without ligand dissociation [Ascoli, 1982; Ascoli, 1984; Freeman and Ascoli, 1982]. Although only about 50% of internalised receptors follow

this route, the accumulation of these internalised receptors in lysosomes prevent receptor recycling, promotes receptor degradation and is ultimately responsible for receptor down-regulation [Ascoli, 1982; Ascoli, 1984; Freeman and Ascoli, 1982; Segaloff and Ascoli, 1993].

Wang *et al.* [Wang, et al., 1991] demonstrated that ligand-induced down-regulation of LH/CG receptors, in MA-10 cells, consists of 2 distinct phases:

(1) the first phase, lasting 3-4 hrs following ligand exposure, is characterised by an 80% reduction in the levels on LH/CG receptors with little or no changes in the level of LH/CG mRNA. Quantitatively the most important phase, it involves an increased rate of receptor degradation. This in turn seems to be due to internalisation and lysosomal accumulation of the receptor that occurs during receptor-mediated endocytosis of LH/CG.

(2) a further reduction of LH/CG receptor levels that is accompanied by a 40-60% reduction in LH/CG receptor mRNA levels.

Thus, the process of LH/CG-induced down-regulation of the LH/CG receptor involves an increase in receptor degradation and a decrease in receptor synthesis, that is secondary to a decrease in mRNA [Segaloff and Ascoli, 1993]. It is unknown whether the decrease in receptor synthesis during the first phase, is die to LH/CG-induced changes in the rate of translation of the LH/CG receptor mRNA.

Signal Transduction Pathways of LH/CG Receptors

It is well-established that the LH/CG receptor is coupled to the adenylate cyclase/cAMP pathway [Dufau and Catt, 1978; Hunzicker-Dunn and Bimbaumer, 1985; Leung and Steele, 1992]. Alternatively, it has been reported

that the murine and rat LH receptors are coupled to the phospholipase C/inositol 1,4,5-trisphosphate (IP_3) pathway [Davis, 1994; Gudermann, et al., 1992a; Herrlich, et al., 1996; Hipkin, et al., 1993]. The ability of LH to stimulate phospholipase C activity is not associated with the accumulation of cAMP, indicating that the activation of phospholipase C is not secondary to the activation of adenylate cyclase. It has been reported that LH increases IP_3 and $[Ca^{2+}]_i$ in isolated bovine luteal cells [Davis, et al., 1987]. Likewise, inositol phosphates accumulation are increased in porcine granulosa cells following LH treatment [Dimino, et al., 1987]. In porcine granulosa cells isolated from 5.0 nm and 1.0 mm diameter ovarian follicles, LH induces a rapid and transient $[Ca^{2+}]_i$ increment, which is similar to that induced by endothelin-1 [Flores, et al., 1992b] These data lend support to the notion of a novel signalling pathway in LH action, involving adenylate cyclase and phospholipase C.

Follicle-Stimulating Hormone Receptors

FSH is necessary for gonadal development and maturation at puberty [Chappel and Howles, 1991]. FSH acts by binding to specific receptors, localised exclusively in the gonads. The FSH receptor is synthesized in granulosa [Hsueh, et al., 1984] and Sertoli cells [Reichert and Dattatreyamurty, 1989], and transported to the membrane surface.

Like the LH/CG receptor, the FSH receptor belongs to the large family of G protein-coupled membrane proteins [Abou-Issa and Reichert, 1976]. Unlike the LH/CG and TSH receptors, the FSH receptor has not been comprehensively investigated. Like the LH/CG receptor, the human FSH receptor is a single polypeptide chain [Sprengel, et al., 1990] with four potential

N-linked glycosylation sites [Minegishi, et al., 1991]. Although deglycosylation of the FSH receptor does not seem to affect ligand binding, glycosylation is necessary for the proper folding of the glycoprotein hormone, and for its expression on the plasma membrane [Davis, et al., 1995; Rozzell, et al., 1995]. Mutations that prevent receptor folding and/or transportation result in the retention of the receptor protein in the cell.

As aforementioned, the FSH receptor also comprises a large amino-terminal domain, seven transmembrane spanning domains, and a short carboxy-terminal domain. The extracellular amino-terminal domain contains 14 leucine-rich repeats, similar to those described for the LH receptor [Bousfield, et al., 1994]. Ligand specificity is conferred by the extracellular domain, and not by the transmembrane domain [Braun, et al., 1991]. The structure of the seven transmembrane spanning domains is typical of members belonging to the superfamily of G-protein-coupled membrane receptors [Baldwin, 1994].

Desensitisation of FSH Receptors

As with the LH receptors, desensitisation of the FSH receptors can be distinguished into two phases: uncoupling and down-regulation. Uncoupling of the FSH receptor from the G-protein occurs shortly after ligand-receptor bind.[Grasso and Reichert, 1989]. This process occurs via enzymatic phosphorylation of the carboxy-terminal, intracellular domain of the G-protein-coupled receptors and may be due to receptor-specific kinases or to effector kinases typical of the receptor system (i.e. protein kinase A or protein kinase C) [Simoni, et al., 1998]. The down-regulation of receptors involves a

decrease in receptor number through internalisation and sequestration of hormone receptor complexes in lysosomes or reduced receptor protein synthesis as a result of both decreased transcription and/or reduced mRNA half-life. Themmen *et al.* [Themmen, et al., 1991] have shown that the FSH-induced decrease in FSH receptor mRNA is due to a cAMP-dependent, post-transcriptional mechanism.

Signal Transduction Pathways of FSH Receptors

Unlike the LH receptor, in which dual signalling pathways have been demonstrated [Davis, 1994; Gudermann, et al., 1992a; Herrlich, et al., 1996; Hipkin, et al., 1993], the FSH receptor seems to be almost exclusively mediated by the adenylate cyclase-cAMP pathway [Flores, et al., 1992a; Gorczynska, et al., 1994]. Sertoli cells possess the protein kinase C pathway, and exposure of the cells to stimulators of the protein kinase C pathway inhibits FSH-dependent cAMP production [Monaco, et al., 1988; Monaco and Conti, 1987]. FSH neither activates [Quirk and Reichert, 1988] nor inhibits [Monaco, et al., 1988] the phosphatidyl inositol pathway. Studies with chimeric human LH/FSH receptors in HEK293 cells indicate that inositol production upon activation of FSH receptors is weak [Hirsch, et al., 1996].

FSH increases intracellular calcium concentrations in Sertoli cells [Gorczynska and Handelsman, 1991] and granulosa cells [Flores, et al., 1990]. The possibility that FSH receptors might act as ligand-gated calcium channels was deemed unlikely by Shibata *et al.* [Shibata, et al., 1992]; however, FSH may increase intracellular calcium by stimulating other calcium channels pre-existing on granulosa and Sertoli cells [Grasso, et al., 1991]. FSH-induced

elevations in intracellular calcium concentrations are independent of protein kinase C [Flores, et al., 1992a].

Intracellular Signalling in the Ovary

Normal ovarian function is dependent on diverse hormones acting through endocrine, paracrine, autocrine, and intracrine processes. Hormonal signals are often translated into cellular activities via signal transduction pathways. Ovarian hormones exert their effects through complex signal transduction mechanisms, and some may even stimulate multiple second messenger pathways.

Many signalling pathways comprise a series of proteins, including: specific receptors, GTP-binding proteins, second messenger-generating enzymes, protein kinases, target functional proteins, and regulatory proteins. Molecular cloning analysis has revealed that almost all of these signalling proteins show extensive heterogeneity and differential tissue expression with specific intracellular localisation. However, the biological significance of this heterogeneity has not always been clear. There are diverse interactions between signalling systems. These interactions include potentiation, cooperation, synergism, antagonism, and co-transmission. The regulation of cellular functions by hormones and growth factors are dependent upon the ability of the target cells to differentially recognise and respond to the individual effector molecules. Such responses can be rapid (e.g. contraction, transmission, secretion, etc.) or long-term (e.g. differentiation, proliferation, death, etc.).

GTP-Binding Protein-Coupled Receptors

G-protein-coupled receptors comprise the largest known family of cell surface receptors, and are defined by their similarities in structure and function. These surface receptors mediate cellular responses to a diverse array of signalling molecules, including: peptide [Flanagan, et al., 1997] and glycopeptide [Davis, et al., 1987] hormones , neurotransmitters [Jose, et al., 1990], phospholipids [Onorato, et al., 1995], odorants [Firestein and Shepherd, 1992], and photons [LeVine, et al., 1990]. Despite the myriad ligands with which they interact, G-protein-coupled receptors share a surprising amount of primary and tertiary structural homology [Strader, et al., 1994]. G-protein-coupled receptors may be further classified into three subfamilies: rhodopsin/ß-adrenergic, secretin/vasointestinal, and metabotrophic glutamate receptors [Strader, et al., 1995].

G-protein-coupled receptor signalling comprises three components: the surface membrane receptor which binds the extracellular ligand, the heterotrimeric G-protein, and the effector system. Surface membrane receptors known to function via G-protein mediation are characterised by seven transmembrane spanning domains joined by extracellular and intracellular loops [Dohlman, et al., 1991]. Through their intracellular domains, these receptors interact with heterotrimeric G-proteins, which in turn modulate the activity of various effector systems. These effectors generate the intracellular second messengers which ultimately evoke cellular responses to the initial event of receptor activation by the ligand.

Heterotrimeric G-proteins belong to the superfamily of GTP-binding proteins that includes *ras* and *ras*-like proteins, as well, as elongation and

initiation factors of ribosomal protein synthesis . This trimeric unit consists of: an α-subunit which contains a guanine nucleotide binding site and intrinsic GTPase activity, and a ßγ-subunit complex [Neer, et al., 1990]. The family of G-proteins comprises over 20 isoforms, with four classes of α-subunits, five of the ß-subunits, and at least six of the γ-subunit [Coleman and Sprang, 1996].

G-protein-mediated signal transduction begins with the activation of an ligand-specific surface membrane receptor. Ligand binding to the receptor which results in a conformational change that exposes a high-affinity binding site for the G-protein, in its guanosine diphosphate (GDP)-bound heterotrimeric form. the receptor [Rens-Domiano and Hamm, 1995]. Multi-site interactions between the ligand-receptor complex and G-protein leads to the exchange of the α-subunit-bound GDP for guanosine triphosphate (GTP) [Dratz, et al., 1993; Hamm, 1991]. Once GTP-bound, the α-subunit of the G-protein dissociates from the ligand-receptor-ßγ complex, and regulates the appropriate effector system. The system is inactivated when the intrinsic GTPase activity of the α-subunit hydrolyses GTP back to GDP; the α-subunit reverts to its prior conformation and regains high affinity for the ßγ–complex, and the system returns to its resting state. Formation of the heterotrimer is required for high affinity coupling of G-protein to receptor [Cerione, 1991; Fung, 1983].

G-protein α-subunits interact with a diverse array of second messenger enzymes and ionic channels, including: adenylate cyclase, phosphodiesterase, phospholipase C, and potassium and calcium channels [DeVivo and Iyengar, 1994]. It was once thought that only the α-subunit regulates second messenger effector systems, but studies have demonstrated that the ßγ-complex is also important in the regulation of many second messenger systems, solely, or in conjunction with the α-subunit [Clapham and Neer, 1993; Spiegel, et al., 1992;

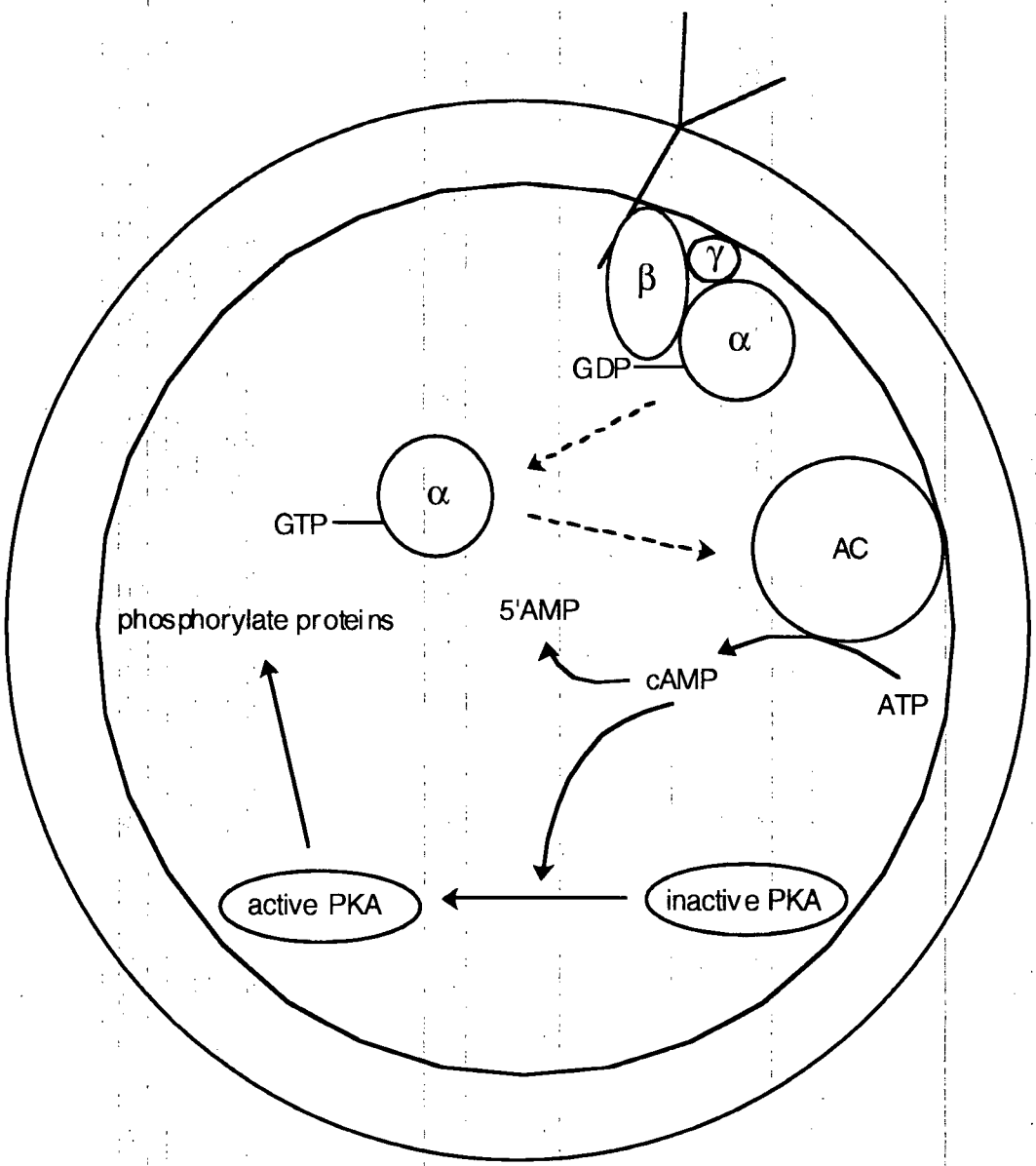
Tang and Gilman, 1991]. The ßγ-complex regulates the yeast mating response; both the α-subunit and the ßγ-complex act independently on muscarine-gated potassium channels, phospholipase C-α isoforms, type I adenylate cyclase, *ras*-mediated extracellular signal-regulated kinases activation, and PI-3-kinase in platelet cytosol; and synergistically in activating adenylate cyclase types II and IV [Clapham and Neer, 1993; Crespo, et al., 1994; Thomason, et al., 1994].

Adenylate Cyclase-Cyclic Adenosine Monophosphate Pathway

Intracellular signalling via the adenylate cyclase-cAMP pathway (Figure 1) is ubiquitous in eukaryotic cells regulating myriad vital functions: energy metabolism, gene transcription, proliferation, differentiation, reproductive functions, secretion, neuronal activity, memory, contractility, and motility. The adenylate cyclase-cAMP signal transduction pathway comprises a cascade of regulatory proteins, and many of them have the potential to modulate the magnitude and/or the duration of signalling events.

Activation of the agonist-specific plasma membrane receptor, which coupled to a heterotrimeric G-protein, elicits a conformational change in the receptor. Two classes of G-protein may be associated with plasma membrane receptors: G_s, a stimulatory G-protein responsible for the activation of adenylate cyclase; G_i, an inhibitory G-protein responsible for the inhibition of the enzyme. Agonist-induced conformational changes to the receptor catalyses the exchange of bound guanosine diphosphate (GDP) for guanosine trisphosphate (GTP). Once GTP-bound, the α-subunit of the G-protein dissociates from the ßγ-complex, and activates the catalytic unit of the adenylate cyclase. The

Figure 1: Adenylate Cyclase - cAMP Pathway



enzyme hydrolyses ATP to cAMP, which then either activates the cAMP-dependent protein kinase A, or is degraded to 5'AMP by phosphodiesterases. The activated protein kinase A can then phosphorylate other proteins [Hanley and Steiner, 1989].

Phospholipase C Pathway

The association of a calcium-mobilising agonist with its receptor activates a phosphodiesterase, phospholipase C. This enzyme preferentially hydrolyses inositol-containing phospholipids. Phosphoinositides present in membranes include phosphatidylinositol and its phosphorylated derivatives, polyphosphoinositides such as phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol 4,5-bisphosphate (PIP_2). The polyphosphoinositides result from the phosphorylation of phosphatidylinositol by ATP in the presence of specific kinases at the plasma membrane to form PIP and subsequently, PIP_2. These reactions are reversible through the hydrolytic activities of specific phosphatases. These polyphosphoinositides are the preferred substrates of the phospholipase C enzyme. Phosphoinositol is also hydrolysed by phospholipase A_2 to form phosphatidic acid and free fatty acid, usually arachidonic acid. Arachidonic acid is the precursor for the biosynthesis of various eicosanoids.

The two isoforms of phospholipase C trigger different pathways (Figure 2). While phospholipase C-ß1 hydrolyses membrane-bound PIP_2 to generate IP_3 and diacylglycerol, phospholipase C-γ1 appears to act exclusively on phosphatidylcholine, the most abundant phospholipid in mammalian membrane, to produce diacylglycerol and phosphocholine [Berridge, 1993].

Figure 2: Phospholipase C-ß1 and Phospholipase C-γ1 Pathways

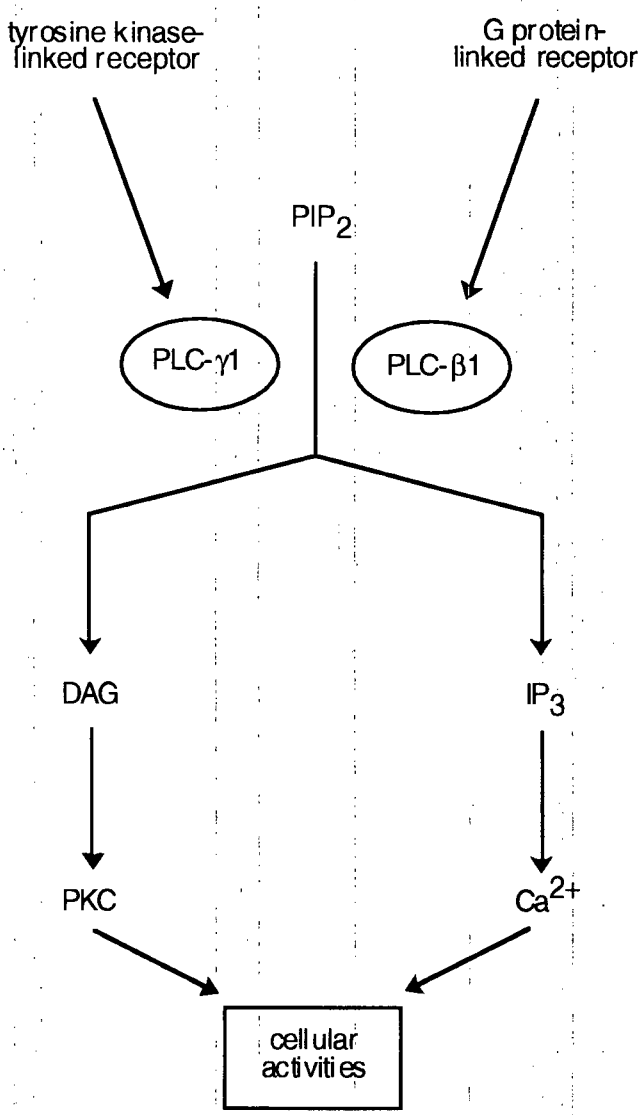
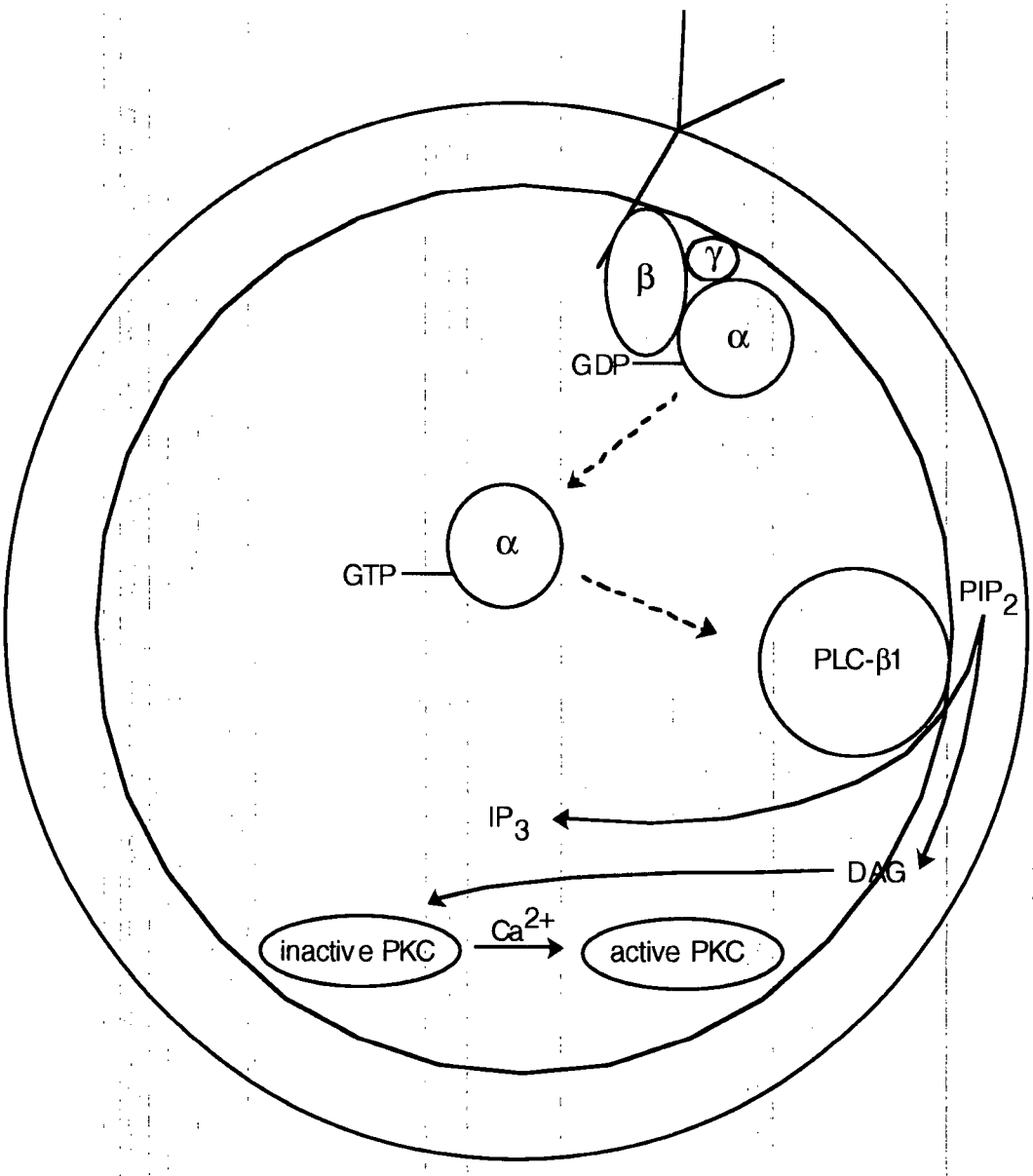


Figure 3: Phospholipase C-ß1 Pathway



When a ligand binds to a receptor, the resulting ligand-receptor complex activates the receptor-coupled G-protein (Figure 3). Once the G-protein is activated, the α-subunit-bound GDP is released, allowing GTP to bind in its place and the α-subunit dissociates from the ßγ-complex. The GTP-bound α-subunit in turn activates phospholipase C-ß1. The α-subunit exhibits intrinsic GTPase activity capable of hydrolysing GTP to GDP. Once inactivated (i.e. GDP-bound), the α-subunit re-associates with the ßγ-complex [Berridge, 1993; Hanley and Steiner, 1989]. Phospholipase C-ß1 hydrolysis membrane-bound PIP_2 to produce IP_3 and DAG which act as second messengers for the mobilisation of calcium and activation of protein kinase C, respectively. IP_3 is released into the cytoplasm where it binds IP_3 receptors and mobilises internal calcium stores. DAG remains membrane-bound and activates protein kinase C. DAG can also be hydrolysed to arachidonic acid. Protein kinase C and the increased intracellular calcium levels then promote cellular activities [Berridge, 1993; Hanley and Steiner, 1989].

The tyrosine kinase-linked receptor directly activates phospholipase C-γ1 (Figure 2). The tyrosine kinase-linked receptor consists of a single transmembrane protein containing a cytoplasmic tyrosine kinase. When a ligand binds to the receptor, it induces receptor dimerisation, allowing two kinase domains to phosphorylate each other at specific tyrosine residues; this action provides a docking site for the SH2 domain of phospholipase C-γ1. Once phospholipase C-γ1 is phosphorylated, it can then hydrolyse PIP_2 to yield IP_3 and DAG.

At least nine distinct protein kinase C isoenzymes have been identified, and differ in their tissue expression as well as in their mode of activation and

their substrate specificities. The individual enzymes will probably prove to have distinct functions in signal transduction and in the control of metabolism, secretion, differentiation, and proliferation [Hug and Sarre, 1993]. The nine isoenzymes can be subdivided into the conventional calcium-dependent isoforms (α, ßI, ßII, and γ) and the calcium-independent isoforms (δ, ε, η, θ, and ζ). The former are single polypeptide chains with catalytic domains containing the ATP and substrate binding sites located in the carboxy-terminal half of the molecule, and regulatory domains containing the calcium, phospholipid, and DAG/phorbol ester binding sites in the amino-terminal half. The regulatory domains are similar among the calcium-dependent α, ß, and γ enzymes, but the calcium-independent δ-ζ enzymes lack the calcium binding domain, and ζ is not activated by DAG or phorbol ester [Nishizuka, 1988].

The major lipid activator of protein kinase C is DAG, acting in conjunction with PS as a cofactor. Following ligand-activation, the calcium released from $InsP_3$-sensitive stores binds to the conventional protein kinase C isoenzymes and promotes their translocation to the plasma membrane, where they are activated by the PS present in the lipid bilayer and the DAG produced from phosphoinositide hydrolysis. Phorbol esters act by mimicking the action of DAG, and lowering the calcium requirement for enzyme activation. In the case of calcium-independent protein kinase Cs, phosphoserine and DAG or other lipid derivatives are required for activation. Several of the protein kinase C isoenzymes are activated by other phospholipid metabolites including *cis*-unsaturated fatty acids, arachidonic acid and its derivatives, and PIP_2. Differential activation can also result from DAG produced during phosphatidylcholine breakdown stimulated by certain hormones and cytokines, and from PIP_3 formed during activation of growth factor receptors.

In this manner, the several protein kinase C isoenzymes could be differentially activated by specific stimuli to phosphorylate their substrates at defined cellular locations.

Calcium and Cellular Regulation

Calcium is the fifth most abundant element in the human body and the most common of the mineral ions [Lehninger, 1982]. It is also the most important structural element, occurring not only in combination with phosphate in bone and teeth, but also with phospholipids and proteins in cell membranes where it plays a vital role in the maintenance of membrane integrity and in controlling the permeability of the membrane to many ions including calcium itself. It is involved in a myriad of physiological and biochemical processes [Lehninger, 1982]: blood coagulation, coupling of muscle excitation and contraction [Ebashi, et al., 1978], regulation of nerve excitability [Katz, 1966], sperm motility , fertilisation [Epel, 1982], cell reproduction [Hepler, 1994; Morrill and Kostellow, 1986], control of enzymatic reactions, and as the second messenger in the many hormone-induced pathways [Berridge, 1993; Rasmussen, 1989]. Because of its importance, many mechanisms have evolved to preserve body stores of the ion and to ensure a sufficient supply to the organism so that it can maintain relatively constant concentrations of both intra- and extracellular calcium. It is so vital to the body's normal functioning that if the plasma levels of ionised calcium falls below 0.6-0.7 mmol/L (normal range being 1.10 - 1.30 mmol/L) then the neuromuscular system ceases to function normally and bone fails to mineralise properly. On the other hand, abnormally high levels of ionised calcium (> 1.6 mmol/L) are toxic to many enzyme systems so that the level must also be kept

below this critical upper limit to ensure the continuance of normal cellular function. Thus a finely tuned mechanism for calcium homeostasis has evolved to maintain a constant extracellular fluid (ECF) concentration of the cation [Lehninger, 1982].

Extracellular fluid calcium homeostasis is achieved by the steady-state control of calcium fluxes into and out of the ECF by a number of hormones, namely parathyroid hormone, calcitonin, and the active metabolites of vitamin D. These act on the main target organs for calcium, namely kidney, intestine and bone, of which the kidney is by far the most important regulatory organ for calcium homeostasis. Deviations from the normal ECF level of calcium occur in certain disease states, particularly those involving alterations in the circulating concentrations of the aforementioned hormones.

In order to fulfill its various functions, calcium must often be transferred from one body compartment to another or from one cellular compartment to another. The cells involved in the translocation of calcium must be able to protect themselves against a surfeit of the cation, which, although necessary for some intracellular activities is toxic to many others. To achieve both objectives, highly specific transport and buffering mechanisms for calcium have had to be developed within these cells.

Modulation of Intracellular Free Calcium Concentrations

Being a critical mediator in a myriad of cellular responses, the concentration of free, ionised calcium in the cytosol is carefully regulated [Berridge, 1993]. Basal intracellular calcium levels are approximately 0.1 μM and can rise over 100-fold in response to influx of extracellular calcium (~1 mM)

or mobilisation of intracellular calcium stores in the endoplasmic reticulum. The elevation of cytosolic free calcium concentrations in a hormone-responsive tissue can be due to several mechanisms [Meldolesi and Pozzan, 1987]: (i) influx of extracellular calcium by the activation and opening of second messenger-activated channels, receptor-operated calcium channels and/or voltage-dependent calcium channels; (ii) release of calcium from intracellular membrane-bound stores; (iii) inhibition of calcium extrusion systems such as the calcium pump and the Na^+/Ca^{2+} antiport; and (iv) release of calcium from intracellular binding proteins. These mechanisms may also work in synergy, as in the process of calcium-induced calcium response. Each of these mechanisms has been implicated in the different tissues in response to various calcium-mobilising agents. The return of calcium concentrations to resting levels after stimulation is brought about in essence by the reversal of these events; i.e. by the release of the hormone from its receptor, the destruction of intracellular second messengers, the active extrusion of calcium from the cell and the sequestration of calcium by intracellular organelles and binding proteins. Much of the calcium that enters the cytoplasm during agonist stimulation is rapidly re-sequestrated into the endoplasmic reticulum via Ca^{2+}-ATPase pumps. In addition, agonist-induced elevations in cytosolic calcium often activate the Ca^{2+}-calmodulin-sensitive enzyme, Ca^{2+}-Mg^{2+}-ATPase, which extrudes calcium from the cell [Berridge, 1992].

Calcium as Intracellular Regulator

The importance of calcium as an intracellular messenger has long been recognised, but only in the last decade has the complexity of this signalling system been fully appreciated. Most of the signalling actions of calcium are

dependent upon its interaction with binding proteins such as calmodulin and regulatory enzymes such as protein kinase C. The calcium-calmodulin complex regulates the activities of numerous enzyme systems, including adenylate and guanylate cyclase, cyclic nucleotide phosphodiesterase, Ca^{2+}-Mg^{2+}-ATPase, and calcineurin. By influencing the cytoplasmic levels of cyclic nucleotides and calcium, calmodulin links the intracellular messenger systems as well as controlling enzymes involved in signalling, secretion, and contractility.

Calcium also binds directly to several calcium-dependent enzymes, the most important of which is protein kinase C, a calcium- and phospholipid-dependent phosphokinase. The phospholipase C pathway is the predominant mechanism of calcium-mobilising receptors (Figure 3). Both the G-protein-linked receptor and the tyrosine kinase-linked receptor stimulate release of IP_3 - the G-protein-linked receptor via phospholipase C-ß1, while the tyrosine kinase-linked receptor works through phospholipase C-γ1 [Jayaraman, et al., 1996]. Once PIP_2 is converted to IP_3 and DAG, the latter acts by activating protein kinase C, while IP_3 diffuses into the cytosol to release calcium from intracellular reservoirs. IP_3 acts as the intracellular second messenger by binding to the specialised tetrameric IP_3 receptor that spans the endoplasmic reticular membrane and triggers the release of calcium from the ER [Li, et al., 1995].

Inositol 1,4,5-trisphosphate and ryanodine receptors

IP_3 receptors are located on the nuclear membrane and on certain parts of the ER. IP_3 appear to release only a portion (usually 30-50%) of the calcium from the non-mitochondrial stores [Berridge and Irvine, 1984]. Calcium that is

sequestered in IP_3-insensitive stores may not necessarily be inert, but may be released by the processes of calcium-induced calcium release [Endo, et al., 1970].

IP_3 and ryanodine receptors are the two principal intracellular calcium channels involved in mobilisation of stored calcium [Coronado, et al., 1994; Li, et al., 1995]. Both receptors are tetramers composed of large subunits (300 and 550 kDa, respectively), and share considerable structural and functional similarities [Tsien and Tsien, 1990]. A significant degree of homology exists in the domain located toward the carboxy-terminal, that spans the membrane and participates in the assembly of the calcium channel. The remainder of the molecule, where no homology is evident, protrudes into the cytosol [Pozzan, et al., 1994].

IP_3 receptor channel activity is influenced by a number of cellular factors: cAMP and guanosine 3',5'-cyclic monophosphate (cGMP) protein kinases [Danoff, et al., 1991; Komalavilas and Lincoln, 1994], protein kinase C [Ferris, et al., 1991], calcium/calmodulin-dependent protein kinase II [Hanson, et al., 1994], ATP [Bezprozvanny and Ehrlich, 1993], pH, etc. Upon binding to the ligand, the IP_3 receptor undergoes a conformational change that is thought to be related to the coupling process leading to channel opening. Gating of the IP_3 receptor channel by IP_3 and intracellular calcium concentrations are key factors in calcium signalling. The probability of channel opening increases with the concentration of IP_3, and saturates at very high levels of IP_3. Enhancement of IP_3-induced channel opening is associated with higher oscillation frequency in cell types exhibiting agonist-induced calcium oscillations [Berridge, 1990].

Ligands known to open the ryanodine receptor channel and stimulate calcium release include: micromolar calcium concentrations, millimolar ATP, and caffeine [Berridge, 1993; Coronado, et al., 1994]. These receptors contribute to calcium signalling in many different cell types: skeletal muscle, cardiac muscle, neurons, chromaffin cells, smooth muscle, pituitary cells, and sea urchin eggs [Berridge, 1993].

Calcium induced calcium release is one of the most interesting aspect of the ryanodine receptor. This positive feedback process allows calcium to trigger its own release. A small influx of calcium through voltage-operated calcium channels can trigger an larger release of stored intracellular calcium. This process allows for the amplification of the calcium signal, and possibility results in the generation of repetitive calcium spikes [Berridge, 1993]. This calcium-induced calcium release property of ryanodine receptors is also exhibited by IP_3 receptors.

Apart from the calcium-sensitive regenerative ability of IP_3 receptors, the other intriguing aspect of IP_3-induced calcium mobilisation is its all-or-none effect. This property is manifested as a sudden or near-maximal release of calcium if the level of IP_3 is gradually increased. Low concentrations IP_3 will elicit small intermittent bursts of calcium release; these calcium bursts continue until a threshold concentration of IP_3 is attained, after which an explosive release of stored calcium occurs [Berridge, 1993].

Cytosolic Calcium Oscillations

Given the multiplicity of receptors which stimulate $InsP_3$ turnover, it

remains unclear how specific signal information is transmitted to different cells, or how single cells distinguish between different receptor inputs.

Cytosolic calcium oscillations are widespread, occurring in both undifferentiated (e.g. mouse oocytes and hamster eggs) and specialised cells (e.g. gonadotrophs and GLCs). The oscillations are based upon fluctuations in cytosolic free calcium, and are classified by their source of calcium influx: (i) membrane oscillators originate from the influx of extracellular calcium, and (ii) cytosolic oscillators arise from the mobilisation of intracellular calcium stores [Berridge and Galione, 1988]. Membrane oscillators depend upon the opening and closing of voltage-dependent calcium channels in the plasma membrane. Examples of such oscillators are sinoatrial node cells and various pacemaker neurones in the brain where oscillations are set. Cytosolic oscillators depend upon the periodic release of calcium from intracellular reservoirs. Such cytosolic calcium oscillators are frequently associated with stimuli that act through the phosphoinositide signalling pathway, and they probably reflect the complex feedback interactions responsible for regulating intracellular calcium. Although considerable progress has been made in understanding the mechanism of membrane oscillators, less is known about the cellular basis of the cytosolic oscillators.

Intracellular calcium oscillations can be triggered by a variety of stimuli. Of the natural stimuli (neurotransmitters, hormones, and growth factors), many are calcium-mobilising agents that hydrolyse phosphoinositides to generate both diacylglycerol and IP_3 [Berridge, 1987]. The significance of receptor activation is supported by observations that GTPγS can trigger oscillatory activity when injected into hamster eggs or HeLA cells [Berridge and Galione, 1988]. In both cases, the GTPγS-induced oscillations were different from those

produced by the natural stimuli of fertilisation or histamine. These experiments, nevertheless, indicate that the activation of a G-protein can initiate oscillatory activity, most likely by stimulating the hydrolysis of phosphoinositides.

Characteristics of Calcium Oscillations

Calcium oscillations appear in various forms. Although they may be specific for any given cell type, they can vary depending upon the agonist. The two major oscillation patterns are: transient and sinusoidal oscillations. Transient calcium oscillations are characterised by a series of discrete spikes separated by quiescent phases when the level of calcium remains close to basal concentrations. Sinusoidal oscillations are calcium fluctuations whereby the oscillatory cycles are continuous with each other and are usually found riding on the elevated plateau level of calcium. Sinusoidal oscillations also display a high frequency, that is independent of agonist concentration [Berridge, 1992].

Calcium transient profiles remain relatively constant, in spite of agonist-induced changes in frequency. Most calcium profiles may be divided into three separate phases: the initial slow pacemaker rise, which then leads into the rapid upstroke of the spike, followed by the recovery phase. Although the pattern of the calcium spikes in response to different agents may vary considerably, the calcium transient profile should remain constant for any given cell [Berridge, 1992].

The rapid upstroke of calcium spikes suggests that there is a mechanism for synchronising the individual calcium stores distributed throughout the cytosol. Calcium imaging studies have revealed that each calcium spike has a

precise spatial organisation. A calcium response is often initiated at one point and then spreads throughout the cell in the form of a wave or tide [Berridge, 1990; Miyazaki, et al., 1986]. Curiously, there appears to be a loss of synchronisation shortly after the initial response. This is manifested by the rapid dampening of calcium spikes, accompanied by a broadening of the spikes.

Adenosine Triphosphate and Purinergic Agonists

Adenosine triphosphate is a ubiquitous nucleotide and serves as the principal immediate donor of free energy in biological systems. Intracellular ATP is present in millimolar concentrations, while the micromolar-nanomolar concentrations of extracellular ATP are maintained by ectonucleotidases and ectophosphatases [Dubyak, 1991]. The source of extracellular ATP is thought to be mainly neuronal in origin; either from purinergic terminals or co-released with traditional neurotransmitters such as acetylcholine and noradrenaline [Gordon, 1986; Morel and Meunier, 1981; Morley, et al., 1994]. Extracellular ATP and its metabolites have been implicated in a myriad of biological systems: cardiovascular function [Olsson and Pearson, 1990], neurotransmission [Edwards, et al., 1992], muscle contraction [Satchell, 1990], and insulin secretion [Squires, et al., 1994].

It has long been established that the ovaries are well innervated. The nerves of the ovaries are derivatives of the ovarian plexus and uterine nerves. All vessels and nerves enter the ovary through the hilum. Most of the nerves are non-myelinated and sympathetic and supply the muscular coats of arterioles. Some non-myelinated fibres form plexuses around multilaminar

follicles. Whether nerves are associated also with generalised smooth muscles cells in the ovary is unknown. A few sensory nerve endings have been described in the ovarian stroma.

The purinergic receptors can be divided into two main categories: P_1 purinoreceptors (adenosine receptors), and P_2 purinoreceptors (ATP receptors) [Burnstock, 1978]. P_1 purinoreceptors are more responsive to adenosine and AMP than to ADP and ATP. P_2 purinoreceptors, conversely, are more responsive to ATP and ADP than to AMP and adenosine [Burnstock, 1978; Burnstock and Buckley, 1985; Dalziel and Westfall, 1994]. P_2 purinoreceptors are heterogeneous [Burnstock, 1978; Dalziel and Westfall, 1994; Kennedy and Burnstock, 1985; Kennedy, et al., 1985; White, et al., 1985] subtypes of P_2 purinoreceptors characterised thus far include: P_{2T}, P_{2U}, P_{2X}, P_{2Y}, and P_{2Z} [Dalziel and Westfall, 1994]. The P_{2T}, P_{2U}, and P_{2Y} purinoreceptors are coupled to G-proteins [Dalziel and Westfall, 1994; Lustig, et al., 1993; Webb, et al., 1993]. The P_{2X} purinoreceptor is an intrinsic ion channel [Bean, 1992]; while the P_{2Z} purinoreceptor remains to be fully elucidated [Cockcroft and Gomperts, 1979; Cockcroft and Gomperts, 1980; Dalziel and Westfall, 1994].

Stimulation of the G protein-coupled P_2 purinoreceptor activates phospholipase C and phosphatidylinositide hydrolysis, generating diacylglycerols and IP_3, which activate protein kinase C and mobilisation of intracellular calcium [Berridge, 1984]. Stimulation of the cation channel-coupled P_2 purinoreceptors also activate calcium mobilisation. The role of ATP in the human ovary remains equivocal.

Objectives

The primary objective of this thesis was to examine the role of calcium as messenger in human ovarian cells. Over the last two decades, it has become evident that the concentration of intracellular calcium is critical to the regulation of normal cellular activities. Calcium plays a pivotal role in mediating the contraction of muscles, the secretion of exocrine, endocrine, and neurocrine products, the metabolic processes of glycogenolysis and gluconeogenesis, the transport and secretion of fluids and electrolytes, and the growth of cells [Rasmussen, 1986].

Various events occurring over the course of the menstrual cycle are mediated by the two female sex hormones. The LH/CG and FSH receptors belong to the large gene family known as the seven transmembrane-guanine nucleotide regulatory (G) protein-coupled receptors [Berridge and Galione, 1988; Loosfelt, et al., 1989; McFarland, et al., 1989; Minegishi, et al., 1993; Minegishi, et al., 1990; Segaloff, et al., 1990; Tsai-Morris, et al., 1990]. It has been established that both the LH/CG and FSH receptors are coupled to the adenylate cyclase/cAMP pathway [Dufau and Catt, 1978; Hunzicker-Dunn and Bimbaumer, 1985; Leung and Steele, 1992]. That the hormones mediate various events, suggests that they may also act via other signal transduction pathways.

It has long been established that the ovaries are well innervated. Adenosine triphosphate is a ubiquitous nucleotide and serves as the principal immediate donor of free energy in biological systems. Intracellular ATP is present in millimolar concentrations, while the micromolar-nanomolar concentrations of extracellular ATP are maintained by ectonucleotidases and

ectophosphatases [Dubyak, 1991]. The source of extracellular ATP is thought to be mainly neuronal in origin; either from purinergic terminals or co-released with traditional neurotransmitters such as acetylcholine and noradrenaline [Gordon, 1986; Morel and Meunier, 1981; Morley, et al., 1994]. Extracellular ATP and its metabolites have been implicated in a myriad of biological systems: cardiovascular function [Olsson and Pearson, 1990], neurotransmission [Edwards, et al., 1992], muscle contraction [Satchell, 1990], and insulin secretion [Squires, et al., 1994]. The role of ATP in the human ovary remains equivocal.

The role of calcium was investigated in human granulosa-lutein cells (GLCs) acquired from the University of British Columbia *In Vitro* Fertilisation Programme. The cells were obtained from women with fertility, including endocrine, problems, and who recently have received sufficient amounts of hCG to simulate the natural LH surge. As some of the studies involved the monitoring of intracellular calcium concentrations in response to the activation of gonadotrophic receptors, human embryonic kidney 293 (HEK293) cells transfected with wild-type and chimeric gonadotrophic receptors were used in lieu of the human GLCs. The following were studies were conducted:

1. To investigate the possibility that the phospholipase pathway is also coupled to the human LH/CG receptor in human granulosa-lutein cells (GLCs) and in HEK293 cells expressing the human LH/CG receptor.

2. To investigate the possibility that the phospholipase C pathway is also coupled to the human FSH receptor, in HEK293 cells expressing the human FSH receptor.

3. To investigate the segments of the hLH receptor involved in signal transduction, in HEK293 cells expressing the wild-type and chimeric human gonadotrophin receptor.

4. To investigate the segments of the hFSH receptor involved in signal transduction, in HEK293 cells expressing the wild-type and chimeric human gonadotrophin receptor.

5. To investigate the effects of ATP and other purinergic agonists on intracellular calcium signalling in single human GLCs.

6. To investigate the effects of ATP and other purinergic agonists on steroid production in single human GLCs.

Materials and Methods

I. Reagents and Materials

Adenosine diphosphate (ADP), adenosine monophosphate (AMP), adenosine 5′-o-(3-thiotriphosphate) (ATPγS), adenosine triphosphate (ATP), 4-androstene-3,17-dione, 8-bromoadenosine 3′:5′-cyclic monophosphate (8-Br-cAMP), caffeine, dantrolene, ethylene glycol-bis(ß-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA), human chorionic gonadotrophin (hCG), N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid (BES), N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), nifidepine, 17ß-oestradiol, Percoll, potassium chloride (KCl), progesterone, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), thapsigargin (TPG), verapamil (VP) were obtained from Sigma (St. Louis, MO, U.S.A.). 5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-gal), Dulbecco's Modified Eagle's Medium (DMEM), Hanks' balanced salt solution Ca^{2+}-, Mg^{2+}-free (HBSS), penicillin-streptomycin, trypsin were obtained from Gibco-BRL (Burlington, ON, Canada). Tritiated oestradiol-17ß and progesterone were obtained from Amersham (Oakville, ON, Canada). Heat-inactivated foetal bovine serum (FBS) was obtained from Professional Diagonistics (xxx, xxx, U.S.A.). Fura-2-AM was obtained from Molecular Probes (Eugene, OR, U.S.A.). Scintran Cocktail EX was obtained from Fisher Scientific (Vancouver, BC, Canada).

Rabbit anti-oestradiol-17β and anti-progesterone antisera were obtained from Dr. D. T. Armstrong. Human luteinising hormone (hLH) and follicle-stimulating hormone (hFSH) were obtained from NIH (Maryland, U.S.A.).

Falcon culture plates (48-wells), 25 mm circular coverglasses, and 12 x 75 mm borosilicate glass tubes were obtained from Fisher Scientific (Edmonton, AB, Canada). Simport Plastics polyethylene scintallation vials with snap-on caps from VWR-Canlab (Edmonton, AB, Canada)

II. Human Granulosa-Lutein Cells

The use of human GLCs was approved by the UBC Clinical Screening Committee for Research and Other Studies Involving Human Subjects.

Follicular development was stimulated by using one of several protocols. One of the more commonly used protocol involved administering a GnRH analogue to down-regulate pituitary function. Once pituitary down-regulation is achieved, human menopausal gonadotrophin was administered to stimulate follicular growth. Serum oestradiol levels and ultrasound measurements of follicular size and number were used as indicators of oocyte maturity. Once at least three follicles exceed 17mm in diameter, 10,000 IU of hCG was administered and oocyte retrieval was performed 34 to 36 hours later.

Human GLCs were harvested from the follicular aspirate collected during oocyte retrieval. Harvested human GLCs were centrifuged (1000 g; 5 min) and re-suspended in DMEM containing 2% penicillin-streptomycin (v/v). The cell suspension was then layered onto a Percoll:HBSS (40:60, v/v) column, and centrifuged (1000 g; 5 min). After centrifugation, cells on the surface of the Percoll:HBSS column were collected and suspended in DMEM. This suspension was centrifuged (1000 g; 5 min) and re-suspended in DMEM containing 5% FBS and 2% penicillin-streptomycin (DMEM/FBS). Cell viability was determined to be ~ 95% by trypan blue exclusion.

III. Culture and Drug Treatments

Human GLCs were seeded onto 48-well plates at a density of 50,000 cells/well, and cultured in DMEM/FBS at 37°C in humidified air with 5% CO_2. Medium was replaced after the initial 24 hrs, and then every 48 hrs thereafter. The cells were incubated with serum-free DMEM at least 6 hrs prior to drug treatments. Cells were cultured for 7 days prior to drug treatment. Treatment periods ranged from 22-26 hrs. Treatments were made up in serum-free DMEM containing androstenedione (0.5 μM).

IV. Radioimmunoassays for Oestradiol and Progesterone

Oestradiol content was determined using a classical competitive binding radioimmunoassay. The rabbit anti-oestradiol antisera was raised against 1,3,5 (10)-estratiene-3,17ß-diol-6-one-6-carboxy-methyl-oxime:BSA conjugate (Steraloids, Wilton, NH). This antisera was used at a final dilution of 1:200,000 (v/v), with approximately 60% binding of label.

Progesterone content was determined using a classical competitive binding radioimmunoassay. The rabbit anti-progesterone antisera were raised 4-pregnen-6ß-ol-3,20-dione hemisuccinate:bovine serum albumin. This antisera was used at a final dilution of 1:10,000 (v/v), with approximately 50% binding of label.

A. Reagents

The assay buffer used was a 0.1 M phosphate buffered saline (PBS; 4.3

mM $NaH_2PO_4 \cdot H_2O$, 11.7 mM $Na_2HPO_4 \cdot 7H_2O$, 13 mM NaCl, 0.01% (w/v) thimerasol), supplemented with 0.1% gelatin (PBS-G; pH 6.9).

The tritiated oestradiol (1 µl), with an initial activity of 1 µCi/µl, was dissolved in 1 ml of pure ethyl alcohol. The ethanol was evaporated, and the label was then reconstituted in 15 ml PBS-G, yielding ~17,000 cpm/100 µl. The tritiated progesterone (1 µl), with an initial activity of 1 µCi/µl, was dissolved in 1 ml of pure ethyl alcohol. The ethanol was evaporated, and the label was then reconstituted in 15 ml PBS-G, yielding ~17,000 cpm/100 µl.

The steroid standards were serially diluted with PBS from an initial 0.32 mM stock solution which was reconstituted in distilled absolute ethanol. A standard curve was set up with 8 reference concentrations ranging from 1 to 128 ng/ml.

The separation reagent comprised charcoal (0.25%, w/v) and dextran (0.025%, w/v) in PBS-G. This reagent is prepared 24 hrs prior to the assay, and was continuously stirred at 4°C.

Scintran Cocktail EX was the scintillation cocktail used.

B. Protocol:

Standards were assayed in triplicate, while the samples were in duplicate. All assays were performed in 12 x 75 mm borosilicate glass tubes. The assays were counted in polyethylene scintillation vials with snap-on caps.

1. PBS-G was added to all tubes: 300 µl buffer into each of the total counts (TC) and non-specific binding (NSB) tubes; 200 µl into each maximum binding

(B_{max}) tubes; and 100 µl into each of the sample and remaining reference tubes.

2. Diluted antibody solution (100 µl) was added to all tubes except the TC and NSB tubes.

3. Tritiated oestradiol (100 µl) was added to every tube in the assay.

4. All tubes were vortex gently, and incubate at 4°C for 16-24 hrs.

5. Oestradiol assay: following the overnight incubation at 4°C, 1 ml charcoal-dextran separating reagent was added to all but the TC tubes. The tubes were gently vortexed and incubated at 4°C for 15 min.

   Progesterone assay: following the overnight incubation at 4°C, 0.5 ml charcoal-dextran separating reagent was added to all but the TC tubes. The tubes were gently vortexed and incubated at 4°C for 15 min.

6. All tubes, except the TC, were centrifuged at 16,000 g for 15 min, at 4°C. All tubes were decanted into scintillation vials immediately after centrifugation.

7. Scintillation cocktail (3 ml) was added to all tubes, mixed, and then allowed to equilibrate in the counter (LKB Wallace) for 1 hr prior to counting.

V. Microspectrofluorimetry

Cells were seeded onto 25 mm circular coverglasses and incubated in DMEM/FBS at 37°C in humidified air with 5% CO_2 before microfluorimetric measurements.

Intracellular calcium concentrations were measured using established fluorimetric techniques [Buchan and Meloche, 1994]. All fura-2 ratio measurements were performed using the Attofluor™ Digital Fluorescence Microscopy System (Atto Instruments, Rockville, MD, U.S.A.). The temperature-controlled perifusion chamber was connected to a six channel perifusion system with a flow rate of 1-2 ml/min. All experiments were completed using the Zeiss 40x Fluar™ oil immersion objective lens. The cells were illuminated alternately with light at 340 and 380 nm. Measurements of intracellular free calcium levels were collected at 1-2 sec intervals. All data presented have been corrected for background fluorescence, as determined from cell-free regions of the coverglass. Changes in the fluorescence ratio recorded at 340 and 380 nm correspond to changes in cytosolic free calcium.

The cells were incubated with fura-2-AM loading buffer (5 μM) for 15 min at 37°C in humidified air with 5% CO_2. The coverglass was mounted onto the temperature-controlled perifusion chamber and equilibrated for 10 min prior to the start of the experiment. Fura-2-loaded cells were perifused with a balanced salt solution (BSS; 137 mM NaCl, 5.36 mM KCl, 1.26 mM $CaCl_2$, 0.81 mM $MgSO_4 \cdot 7H_2O$, 0.34 mM $Na_2HPO_4 \cdot 7H_2O$, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 10 mM HEPES, 2.02 mM glucose; pH 7.4). The treatment intervals ranged from 2-10 min, whereas the wash intervals varied from 2-15 min, depending upon the magnitude of the preceding calcium response.

VI. Transfection of Human Embryonic Kidney 293 Cells

A. Transient Transfection of Human Embryonic Kidney 293 Cells

Human gonadotrophin receptor cDNA was subcloned into the pcDNA3

vector [Hirsch, et al., 1996; Kudo, et al., 1996] and transiently transfected into 293 cells derived from human embryonic kidney fibroblasts (HEK293) by the calcium phosphate method [Raymond, et al., 1996]. The HEK293 cells were cultured until 80% confluency, then trypsinised (0.0625% in calcium- and magnesium-free HBSS) and re-seeded at a density of 1×10^6 cells per 100 mm culture dish. The HEK293 cells were incubated with DMEM/FBS at 37°C in humidified air with 5% CO_2 for 24 hr prior to transfection. Thirty minutes prior to transfection, the HEK293 cells were incubated at 37°C in humidified air with 3% CO_2. Ten to twenty micro-grams of cDNA per 100 mm culture dish were used.

The 10-20 μg of cDNA was precipitated with 3 M sodium acetate (1% v/v) and 100% ethanol (1 ml). The cDNA solution was centrifuged at 4°C at 14,000 rpm for 15 min. The supernatant was discarded and the cells were washed with 1 ml of 100% ethanol. The cDNA was re-suspended in 0.1x TE solution (450 μl), and 2.5 mM $CaCl_2$ (50 μl) and 2X BES (500 μl). Following a 20 min incubation at room temperature, the cDNA solution was introduced into the HEK293 cell culture.

Following a 14 hr incubation at 37°C in humidified air with 3% CO_2, the HEK293 cells were washed twice with DMEM and then trypsinised (0.0625% trypsin), as aforementioned. The cells were centrifuged, re-suspended in DMEM/FBS, and seeded onto 25 mm circular coverglasses. The transiently transfected cells were assayed 45-80 hr post-transfection.

B. Transfection Efficiency

To monitor transfection efficiency, the RSV-ß-gal plasmid was routinely included in the transfection mixture, and ß-galactosidase activity was determined by X-gal staining. Transfected HEK293 cells were washed with phos-

phate buffer solution (PBS), incubated at room temperature with fixative for 15 min, and washed again (see Appendix B for formulation for PBS and fixative). The fixed cells were then incubated at 37°C with the X-gal stain for approximately 12 hrs.

Results

Figure 4 shows that hCG does evoke calcium oscillations in human GLCs cells. As aforementioned, human GLCs were obtained from the UBC *In Vitro* Fertilisation Programme. The cells were obtained from women with fertility, including endocrine, problems, and who have recently received pharmacological doses of hCG to simulate the natural LH surge. To facilitate the study of LH-induced intracellular calcium mobilisation, human wild-type and chimeric receptors where transfected into HEK293 cells.

I. Gonadotrophin-Induced Calcium Oscillations in HEK293 Cells Express-ing the Human Luteinising Hormone/Chorionic Gonadotrophin Receptor

A. Specificity of the Human LH/CG Receptor

We have examined the effects of gonadotrophins in transfected HEK293 cells using single-cell dual-excitation microfluorimetry. The control groups were untransfected HEK293 cells, and HEK293 cells transfected with lac-Z cDNA and/or pcDNA3 plasmid (Table 1). Gonadotrophin treatment failed to elicit cal-cium signals in all four control groups. Figure 5 shows the specificity of the human LH/CG receptor. Both human FSH and LH were administered at a dose of 4 μg/ml for a duration of 180 sec. Human FSH failed to elicit a calcium response from the transfected cells (n=42, #=2). Under the same conditions, human LH consistently evoked oscillatory calcium signals (n=42, #=2). The on-set of the $[Ca^{2+}]_i$ oscillations was rapid, well within 15 sec of the LH treatment.

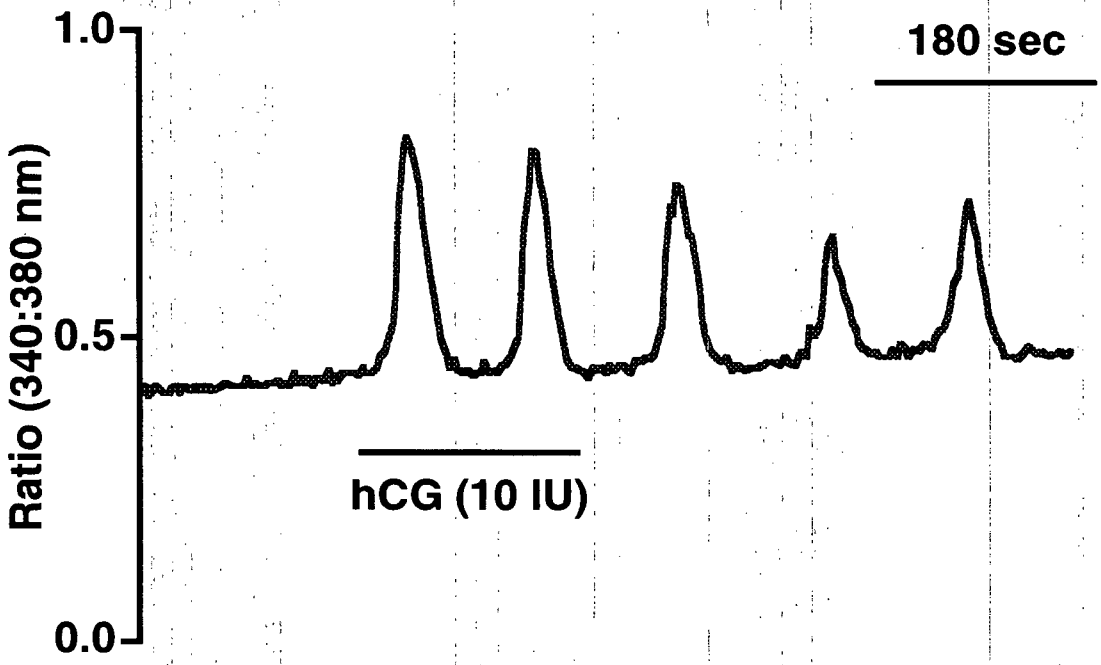


Figure 4: Effects of hCG treatment on human GLCs. Single-cell microfluorimetric studies demonstrated that hCG successfully evoked mobilisation of intracellular calcium in human GLCs. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber. The agonist was administered at a concentration of 10 IU/ml, for a duration of 180 sec.

control groups	cells imaged (n)	number of transfections (#)
HEK293	57	2
HEK293/ß-gal	45	2
HEK293/pcDNA3	72	2
HEK293/ß-gal/pcDNA3	42	1

Table 1: Mobilisation of intracellular calcium in response to gonadotrophin treatment was investigated in HEK293 cells transfected with gonadotrophic receptors. Several control groups were established to demonstrate that the intracellular calcium response was due to activation of the transfected gonadotrophic receptors. The control groups were all treated with human FSH and LH (2-4 µg/ ml) for a duration of 180 sec.

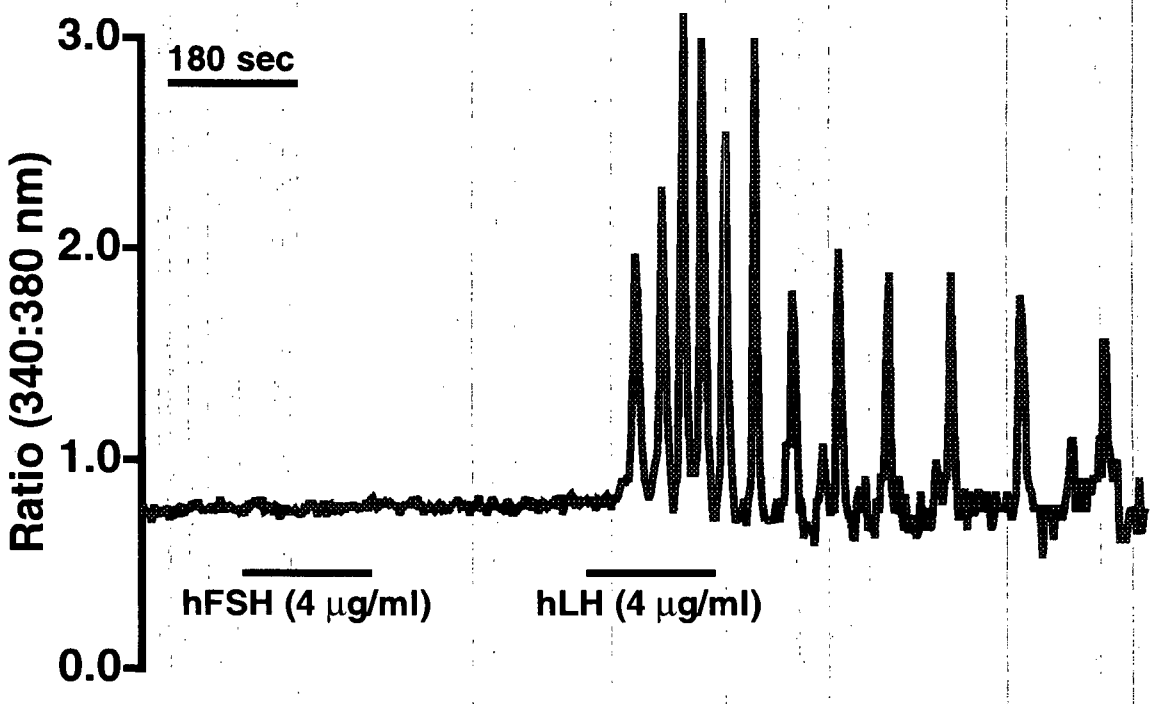


Figure 5: Effects of gonadotrophin treatment on human LH receptors expressed in HEK293 cells. Only hLH was capable of eliciting an intracellular calcium response. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber. Transfected cells were treated with both human FSH (4 μg/ml) and LH (4 μg/ml) for a duration of 180 sec.

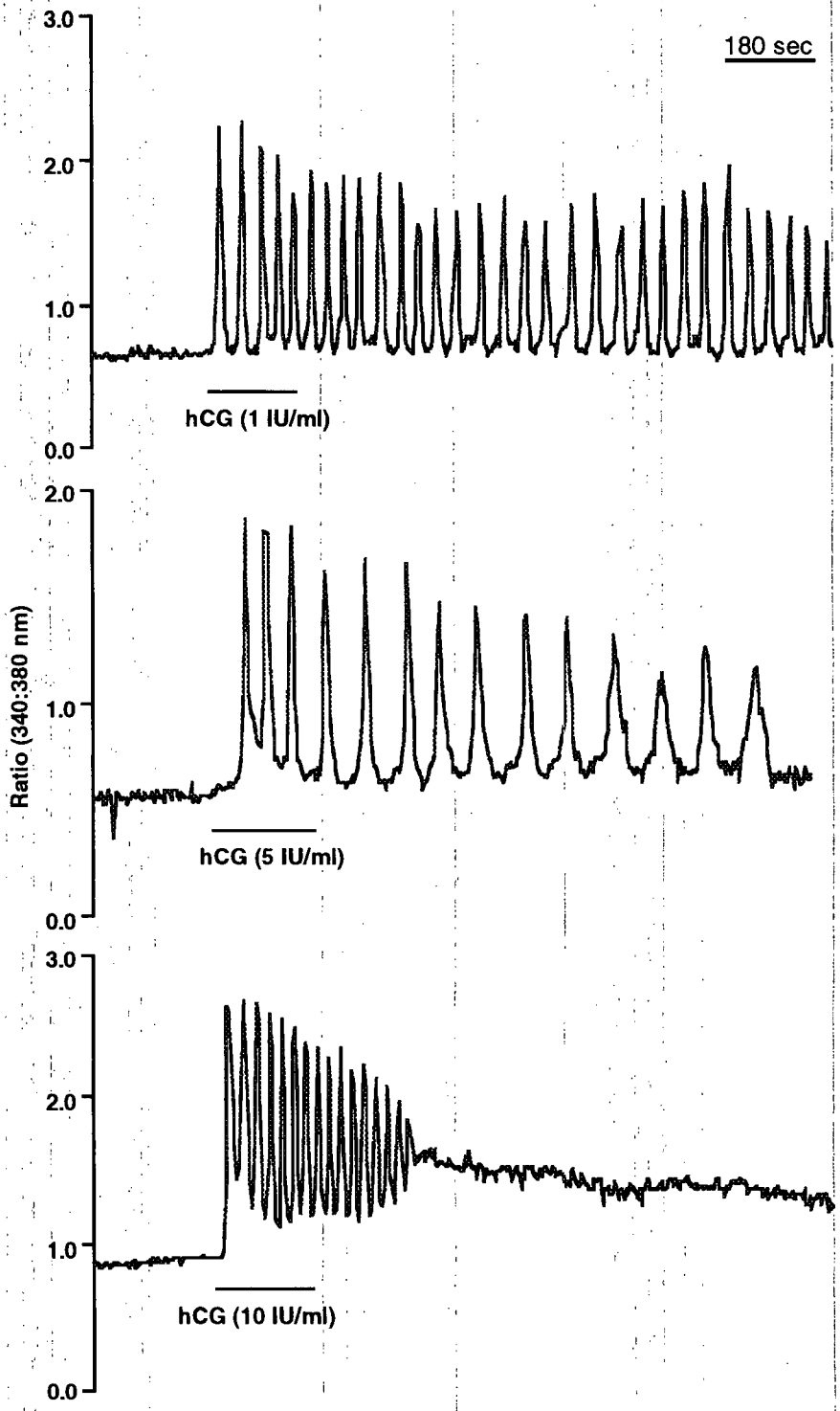


Figure 6: Human CG concentration-response relationship. Human CG was administered at the various concentrations for a duration of 180 sec.

The calcium oscillations lasted throughout the entire treatment period, and persisted for at least 25 min after the cessation of LH treatment.

B. Effect of Human Chorionic Gonadotrophin on $[Ca^{2+}]_i$

Figure 6 shows the concentration-response relationship between hCG and $[Ca^{2+}]_i$. Human CG was administered at 1, 5 and 10 IU/ml, for a duration of 180 sec. At 1 and 5 IU/ml (n=54 and 10, respectively; #=2 and 1, respectively) hCG elicited baseline calcium oscillations which were sustained even after treatment withdrawal. At 10 IU/ml, hCG evoked a rise in $[Ca^{2+}]_i$, with oscillations superimposed on the quasi-sustained plateau phase (n=81, #=3). The cessation of the oscillations is likely due to the depletion of internal calcium stores.

C. Calcium Influx vs. Calcium Mobilisation

To determine the relative contribution of calcium influx vs. calcium mobilisation of cytosolic stores in the initiation and maintenance of the gonadotrophic response, hCG was administered in the absence of extracellular calcium. Under calcium-containing conditions, hCG (1 IU/ml) reproducibly evoked calcium oscillations, sustained even after treatment withdrawal (Figure 6). Under calcium-free conditions, in the presence of 1 mM EGTA, hCG still evoked calcium oscillations, but the response now was transient (n=64, #=2; Figure 7). The second calcium elevation in Figure 7 is due to the influx of extracellular calcium into the cell following a return to calcium-containing conditions.

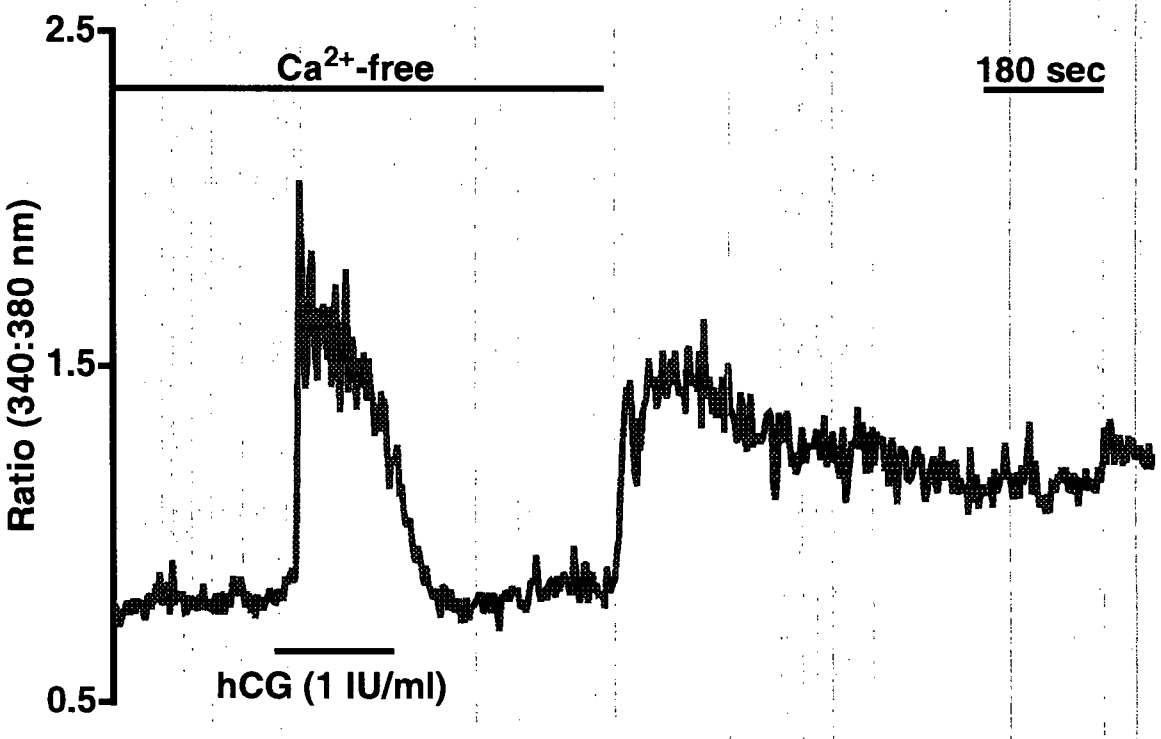


Figure 7: The involvement of extracellular calcium on hCG-evoked calcium mobilisation. In the absence of extracellular calcium, the hCG-induced calcium response was not sustained beyond the treatment period. The calcium-free buffer contained 1 mM EGTA. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

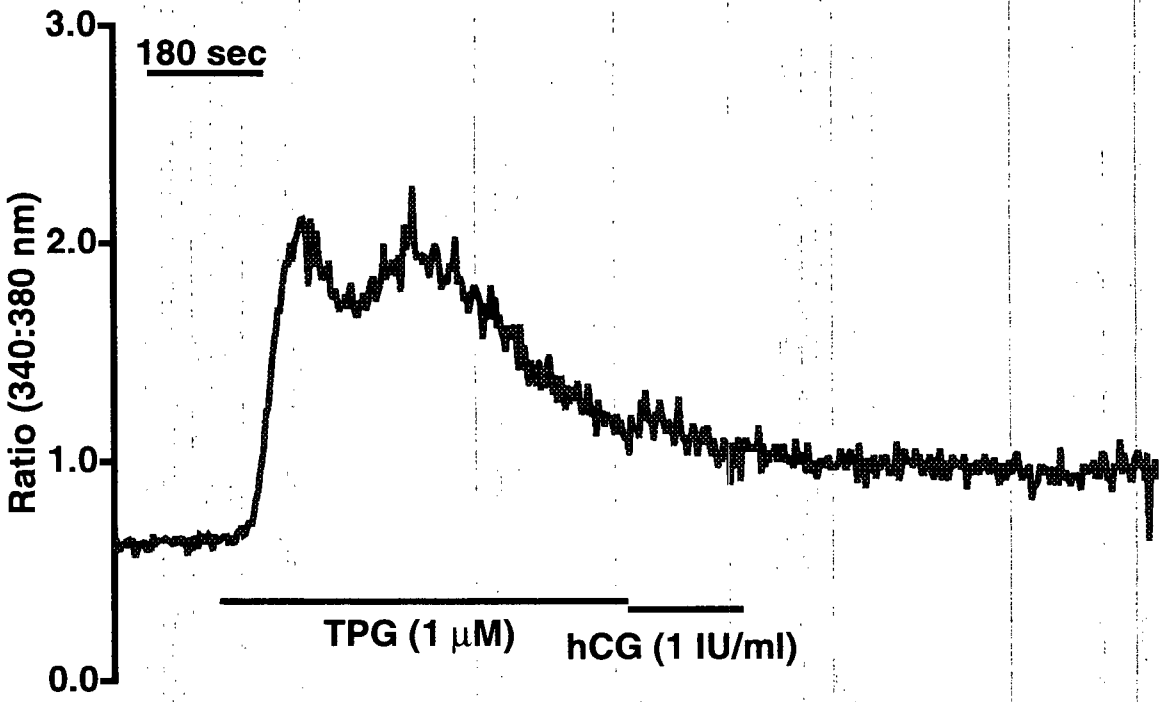


Figure 8: Effect of thapsigargin (TPG) pre-treatment on transfected HEK293 cells. Human CG fails to elicit intracellular calcium mobilisation when cells are depleted of their endoplasmic reticular calcium stores. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

To identify the internal calcium stores mobilized in the LH-evoked calcium response, transfected HEK293 cells were pre-treated with thapsigargin (n=83, #=2). Thapsigargin is a plant-derived lactone, whose mode of action appears to result from the emptying of intracellular calcium stores by inhibiting sequestration pathways [Thastrup, et al., 1989]. Thapsigargin specifically inhibits all members of the endoplasmic and sarcoplasmic reticulum calcium pump family [Lytton, et al., 1991]. Following thapsigargin pre-treatment (1 μM), hCG failed to elicit a calcium response (Figure 8). The cells in this, and all experiments, were co-transfected with ß-gal cDNA; ergo, the presence of the human LH/CG receptor was indirectly determined by X-gal staining.

To determine the involvement of intracellular IP_3-sensitive calcium stores in the hCG-evoked calcium signals, caffeine was used. High concentrations of caffeine have been shown to inhibit the mobilisation of non-mitochondrial, IP_3-sensitive calcium stores [Toescu, et al., 1992]. In Figure 9, hCG treatment produces the usual oscillatory calcium signals; the introduction of 20 mM caffeine eradicates the calcium oscillations to almost baseline levels. The withdrawal of caffeine resulted in an elevation in $[Ca^{2+}]_i$, but the oscillations are not restored (n=33, #=2).

To determine whether the human LH/CG receptor is coupled to calcium signalling through the G_i-protein, transfected HEK293 cells were pre-treated with pertussis toxin (PTX). Following a 16 hr pre-treatment with PTX (100 ng/ml), hCG failed to elicit a calcium response (n=163, #=4). Again, the cells were co-transfected with ß-gal cDNA; ergo, the presence of the human LH/CG receptor was indirectly determined by X-gal staining.

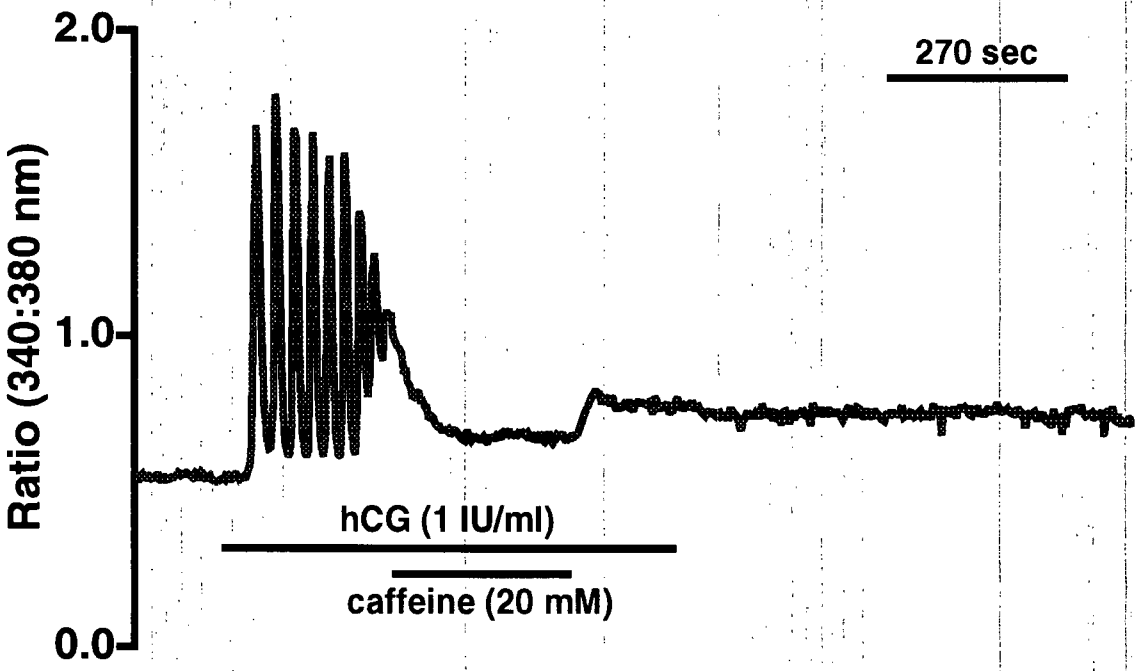


Figure 9: Effect of caffeine on hCG-evoked calcium signals in transfected HEK 293 cells. High concentrations of caffeine (20 mM) inhibits the mobilisation of non-mitochondrial, IP_3-sensitive calcium stores. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

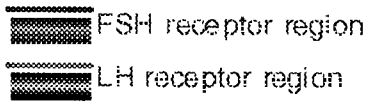
II. Calcium Signalling in HEK293 Cells Transfected with the Wild-Type or Chimeric Human Gonadotrophin Receptors

The twelve chimeric and the two wild-type gonadotrophin receptors were individually transfected into HEK293 cells. Gonadotrophin treatment failed to elicit calcium mobilisation in five of the fourteen receptor types (Table 2): FFR (n=212, #=5); LFR (n=189, #=4); LF(5-C)R (n=177, #=3); FL(1-4)FR (n=202; #=4); FL(i3-VI)FR (n=143, #=4). Various agents used in the experiments were dissolved in DMSO (20%, v/v). Figure 10 shows that the vehicle, DMSO, did not elicit a calcium response in the transfected HEK293 cells (Figure 10; n=87, #=2).

A. Phospholipase C Involvement in Gonadotrophin-Induced Calcium Responses

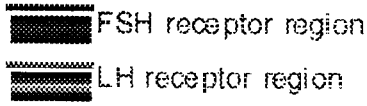
To determine whether adenylate cyclase plays a role in gonadotrophin-stimulated intracellular calcium mobilisation, HEK293 cells transfected with either the wild-type human LH receptor or the chimeric human gonadotrophin receptor FLR were treated with 50 μM forskolin, an adenylate cyclase stimulator. Figures 11 and 12 show that forskolin failed to elicit intracellular calcium signals in HEK293 cells transfected with either the wild-type human LH receptor (Figure 11; n=98, #=2) or the chimeric human gonadotrophin receptor FLR (Figure 12; n=93, #=3). Conversely, U-73122 (10 μM), a phospholipase C activator, was clearly shown to degrade hCG-induced intracellular calcium mobilisation (Figure 13; n=107, #=3).

Table 2A: Wild-type and chimeric human gonadotrophin receptor schematics and detectability of intracellular calcium mobilisation. Calcium response results are from the experiments documented in this section.



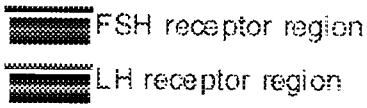
Receptor	Schematic	Calcium Response
FFR		**no**
F(1-4)LR		**yes**
FL(1-4)FR		**no**
FL(7-C)R		**yes**

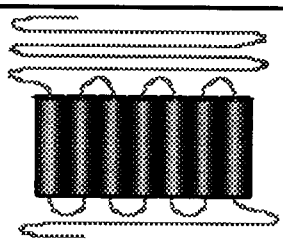
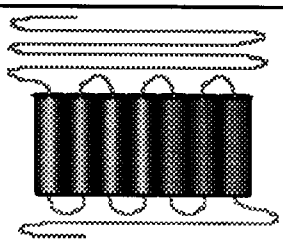
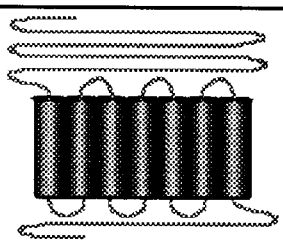
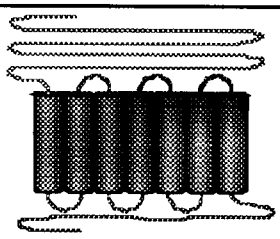
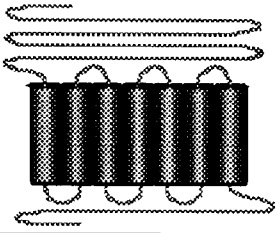
Table 2B: Wild-type and chimeric human gonadotrophin receptor schematics and detectability of intracellular calcium mobilisation. Calcium response results are from the experiments documented in this section.



Receptor	Schematic	Calcium Response
FL(C)R		yes
FL(i3-VI)FR		no
FL(V-i3)FR		yes
FL(V-VI)R		yes
FL(V/VI)R		yes

Table 2C: Wild-type and chimeric human gonadotrophin receptor schematics and detectability of intracellular calcium mobilisation. Calcium response results are from the experiments documented in this section.



Receptor	Schematic	Calcium Response
FLR		**yes**
LF(5-C)R		**no**
LF(C)R		**yes**
LFR		**no**
LLR		**yes**

B. Effect of Gonadotrophin on Chimeric Human Gonadotrophin Receptors

LF(C)R (Figure 14; n=131, #=3): the intracellular carboxy-terminal of the human LH receptor has been replaced with that of the human FSH receptor. This alterations results in an altered hCG-induced calcium profile. The calcium oscillations are lost and the signal is only sustained for the duration of the gonadotrophin treatment. Porcine FSH (40 μg/ml) fails to elicit a calcium response from this chimeric receptor.

FLR (Figure 15 and 16; n=292, #=5): the transmembrane and intracellular portions of the human FSH receptor has been replaced with that of the human LH receptor. The FSH-induced calcium profile is less consistent than that observed in the wild-type receptor. There is a marked hysteresis in the various calcium profiles of these altered human FSH receptors. Human CG (10 IU/ml) failed to elicit a calcium response.

FL(C)R (Figure 17; n=156, #=3): the intracellular carboxy-terminal of the human FSH receptor has been replaced with that of the human LH receptor. Porcine FSH (40 μg/ml) elicits a single calcium spike. The calcium oscillations and sustained calcium mobilisation is not evident in the FSH-induced calcium responses for this receptor.

F(1-4)LR (Figure 18; n=143, #=3): the latter segment (from part of extracellular loop two to the end of the carboxy-terminal) of the human FSH receptor has been replaced with that of the human LH receptor. Porcine FSH (40 μg/ml) elicits a similar calcium profile to that normally observed in the human LH/CG activation of the wild-type human LH receptor.

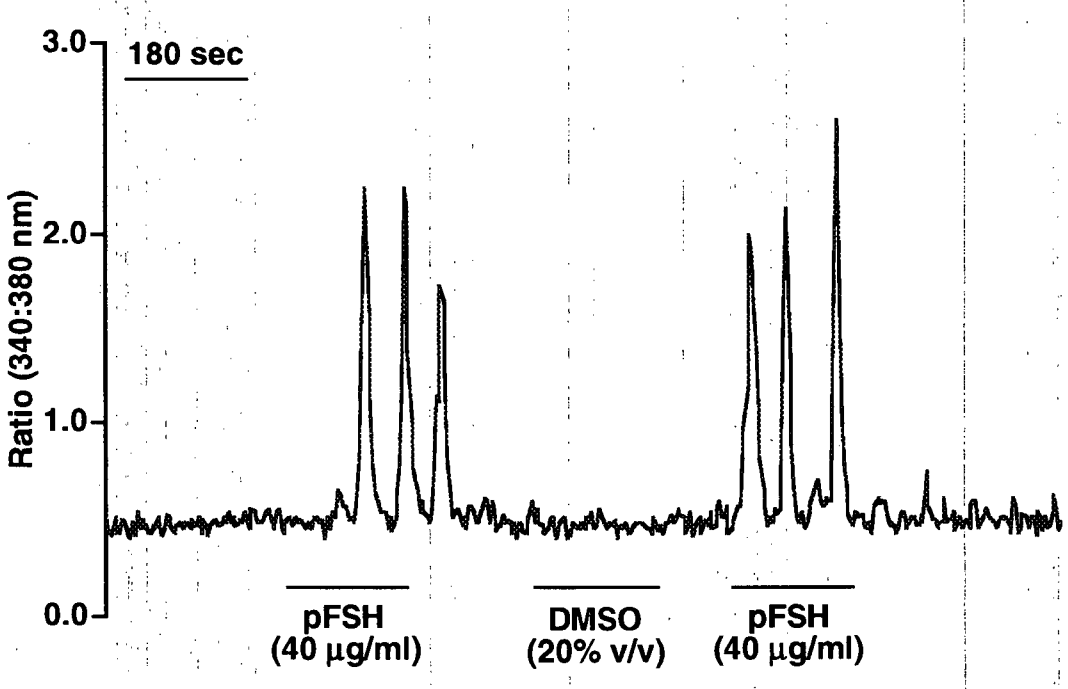


Figure 10: Effects of pFSH and DMSO on HEK293 cells transfected with the chimeric human gonadotrophin receptor FLR. Porcine FSH (40 µg/ml) and DMSO (20%, v/v) were both administered for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

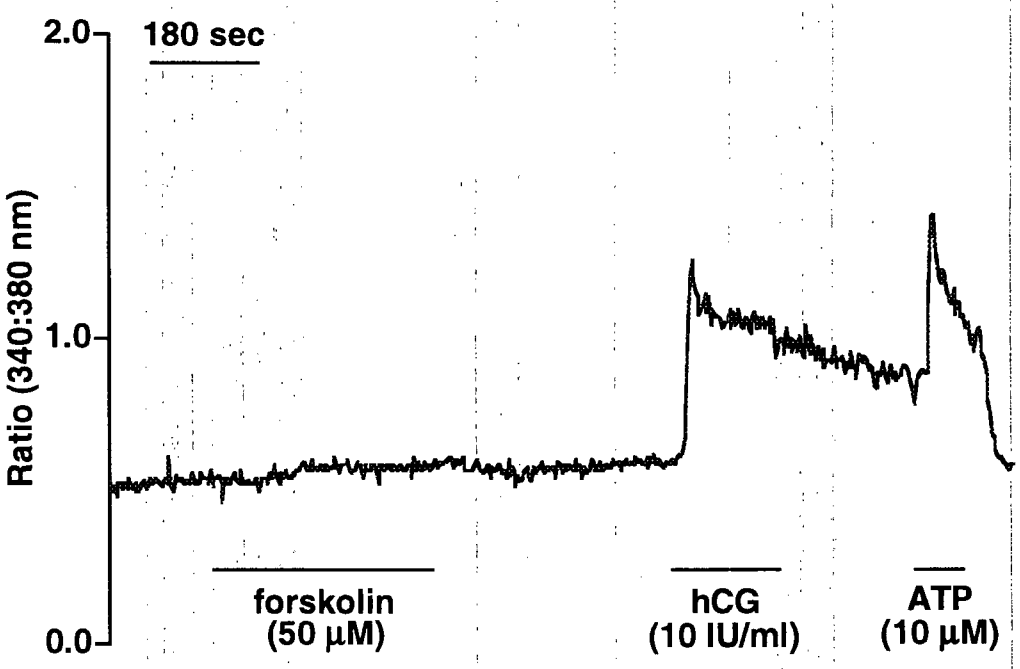


Figure 11: Effecta of forskolin treatment on HEK293 cells transfected with the wild-type human LH receptor. Forskolin, an adenylate cyclase stimulator, was administered at a concentration of 50 µM for a duration 360 sec, while hCG was administered at 10 IU/ml for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All micro-fluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

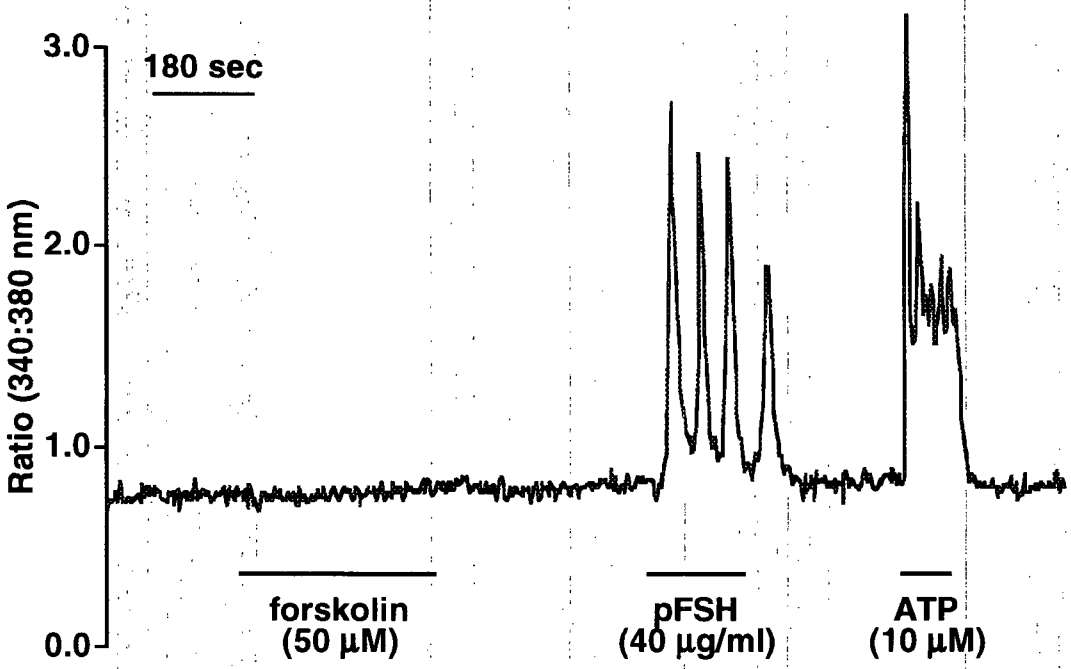


Figure 12: Effects of forskolin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor FLR. Forskolin, an adenylate cyclase stimulator, was administered at a concentration of 50 µM for a duration 360 sec, while hCG was administered at 10 IU/ml for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

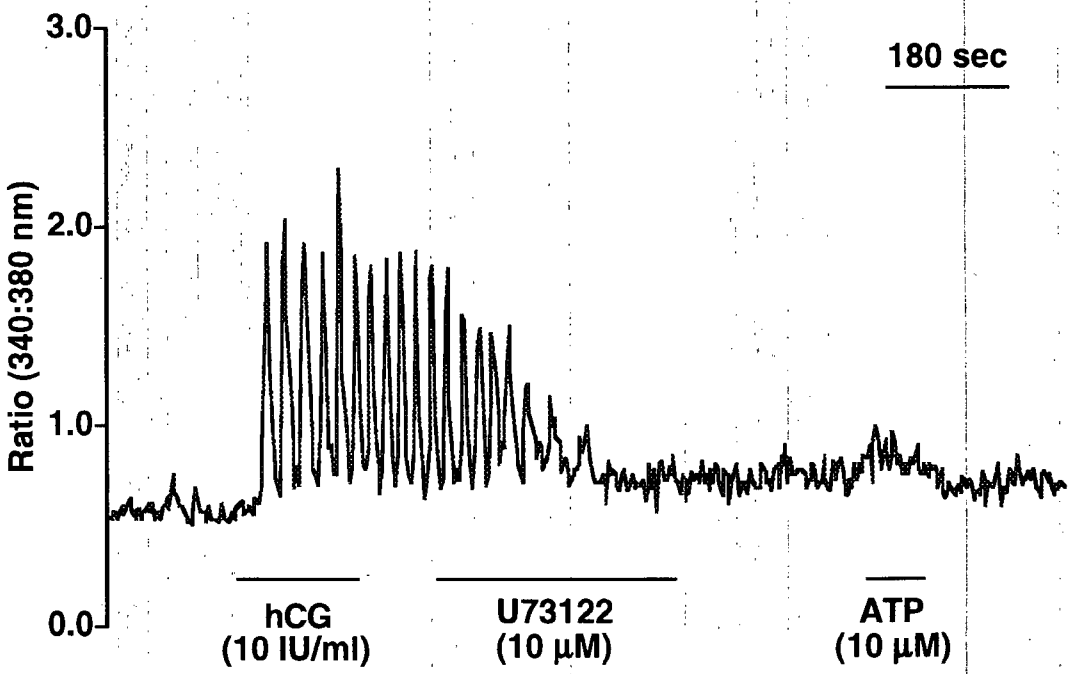


Figure 13: Effects of U-73122 treatment on HEK293 cells transfected with the wild-type human LH receptor. U-73122, a PLC stimulator, was administered at a concentration of 10 µM for a duration 360 sec, while hCG was administered at 10 IU/ml for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

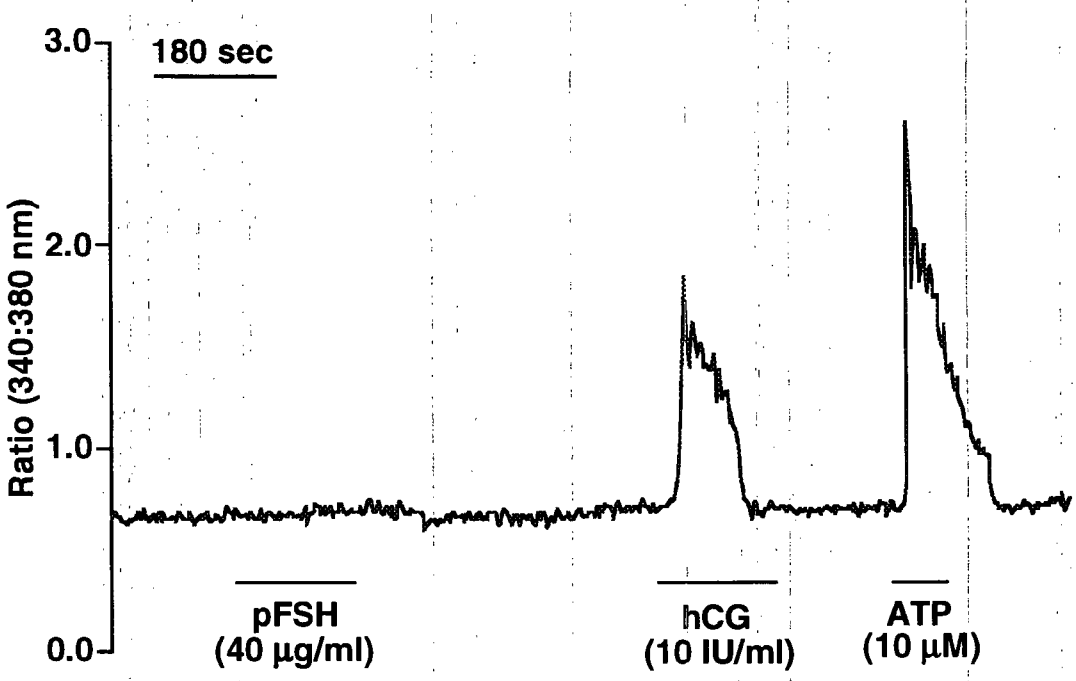


Figure 14: Effects of gonadotrophin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor LF(C)R. Porcine FSH (40 µg/ml) and hCG (10 IU/ml) were both administered for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

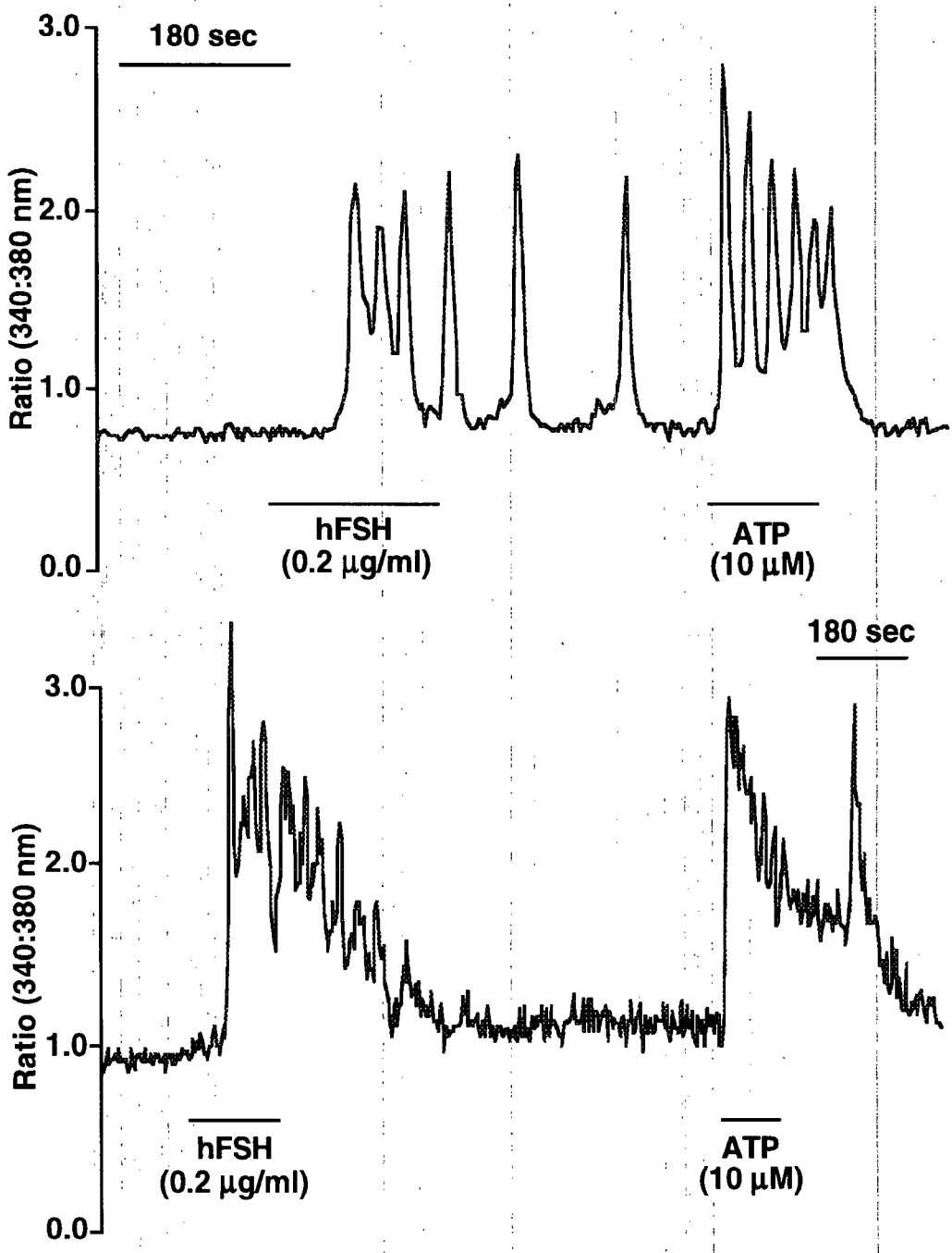


Figure 15: Effects of gonadotrophin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor FLR. Human FSH was administered at a concentration of 0.2 μg/ml for a duration of 180 sec. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

FL(7-C)R (Figure 19; n=97, #=2): the third extracellular loop, the seventh transmembrane segment, and the carboxy-terminal of the human FSH receptor have been replaced with those of the human LH receptor. FSH is still capable of eliciting a calcium response, but the sustained oscillatory pattern in essentially lost, although a few oscillations may occasionally be observed. There is also a marked hysteresis in the calcium responses in these receptors.

FL(V-i3)FR (Figure 20; n=174, #=3): part of the second extracellular loop, the fifth transmembrane segment, and the third intracellular loop of the human FSH receptor have been replaced with those of the human LH receptor. FSH is still able to elicit calcium transients in HEK293 cells transfected with this chimeric receptor; however, the calcium transients are severely attenuated, and no oscillations were observed.

FL(V/VI)R (Figure 21, n=173, #=3): the fifth and sixth transmembrane segments of the human FSH receptor have been replaced by those of the human LH receptor. FSH is again able to elicit calcium transients in HEK293 cells transfected with this chimeric receptor; however, the calcium response is attenuated.

FL(V-VI)R (Figure 22; n=167, #=3): the fifth and sixth transmembrane segments and the third intracellular loop of the human FSH receptor have been replaced by those of the human LH receptor. The ligand-induced calcium mobilisation profile is similar that normally expected for hCG-induced calcium mobilisation; however, the calcium oscillations have

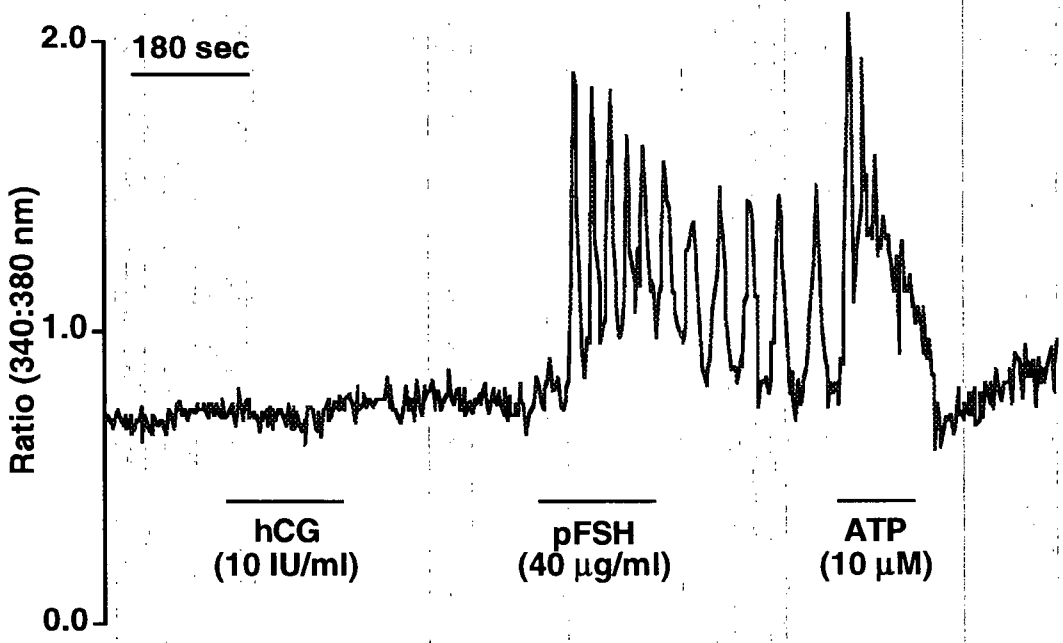


Figure 16: Effects of gonadotrophin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor FLR. Porcine FSH (40 µg/ml) and hCG (10 IU/ml) were administered for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

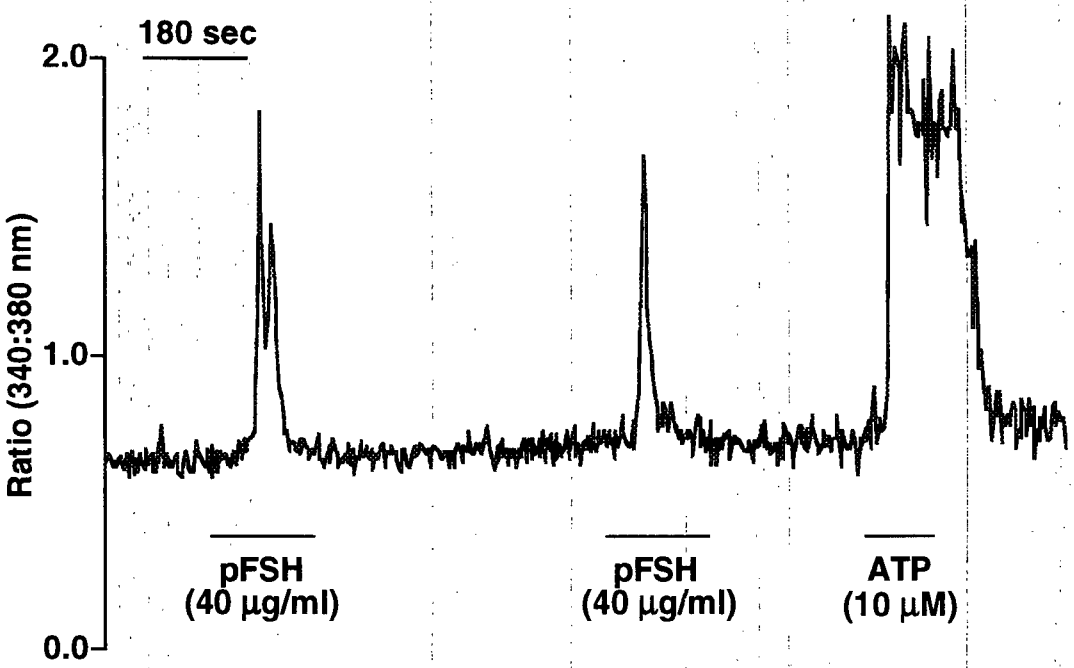


Figure 17: Effects of gonadotrophin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor FL(C)R. Porcine FSH was administered at a concentration of 40 µg/ml for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

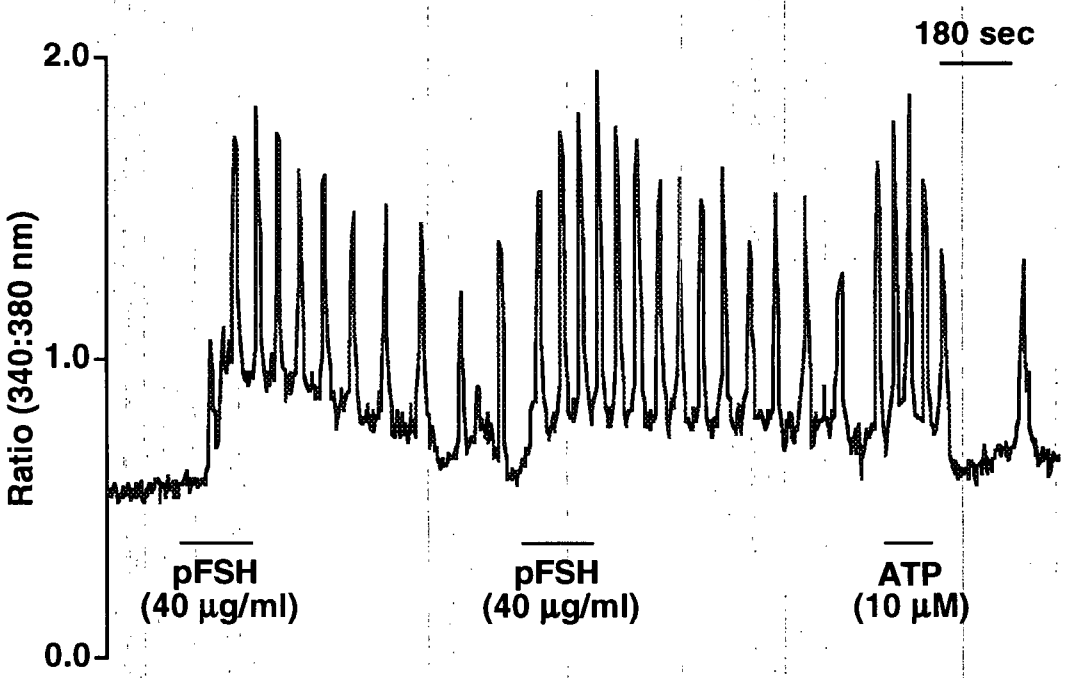


Figure 18: Effects of gonadotrophin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor F(1-4)LR. Porcine FSH was administered at a concentration of 40 µg/ml for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

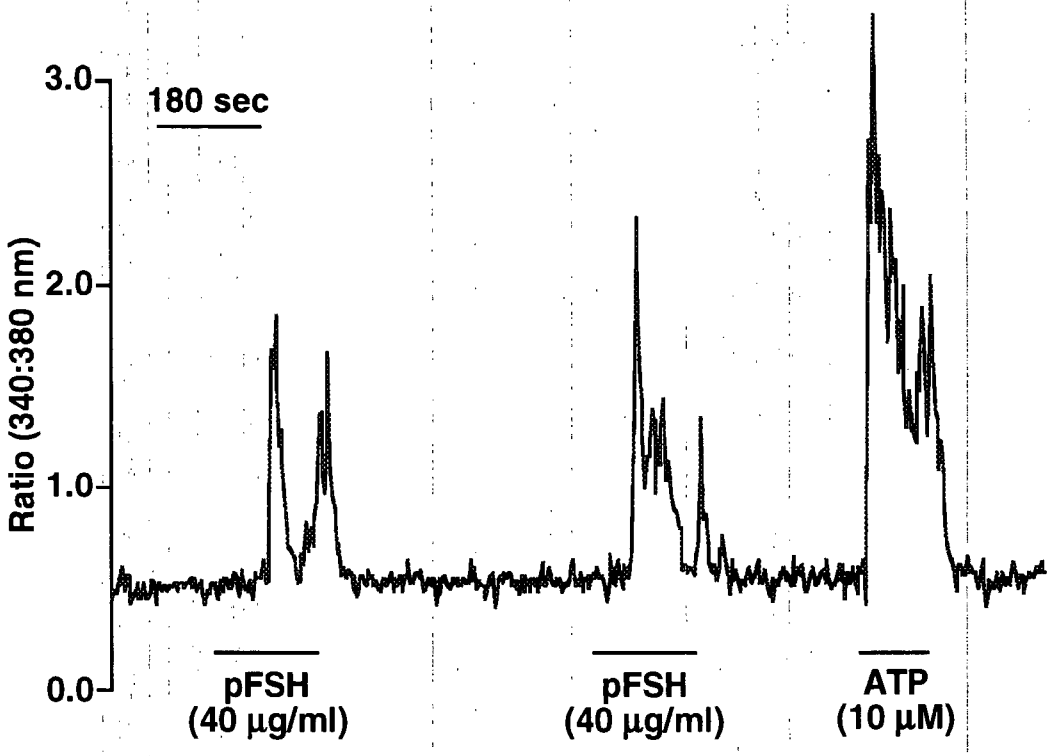


Figure 19: Effects of gonadotrophin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor FL(7-C)R. Porcine FSH (40 µg/ml) and hCG (10 IU/ml) were administered for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

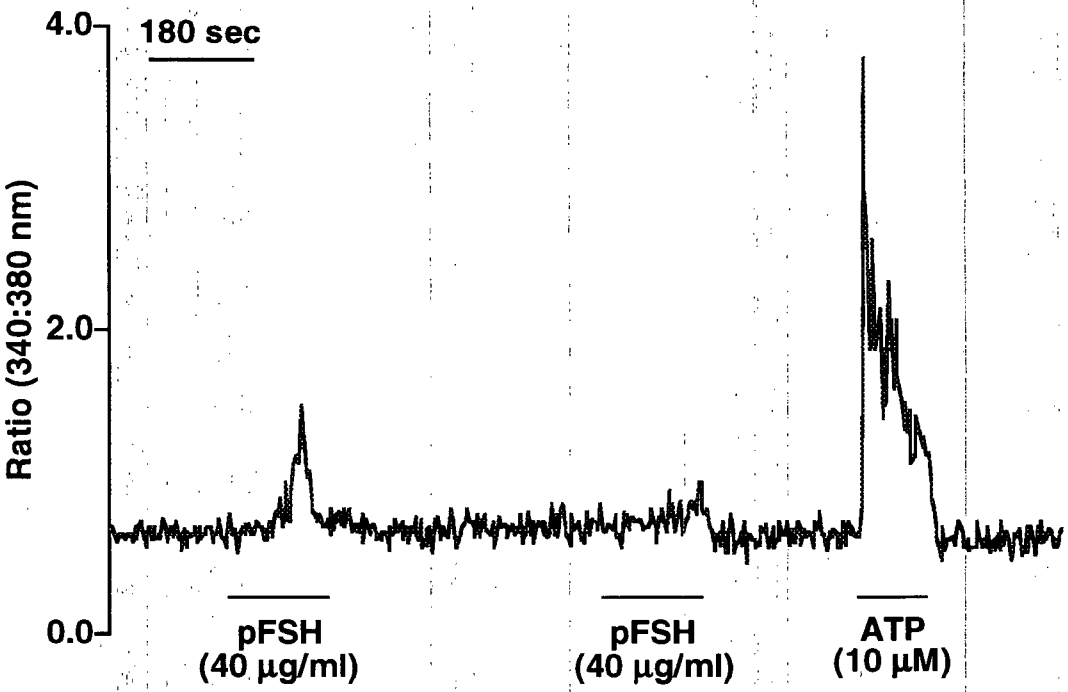


Figure 20: Effects of gonadotrophin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor FL(V-i3)FR. Porcine FSH was administered at a concentration of 40 µg/ml for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

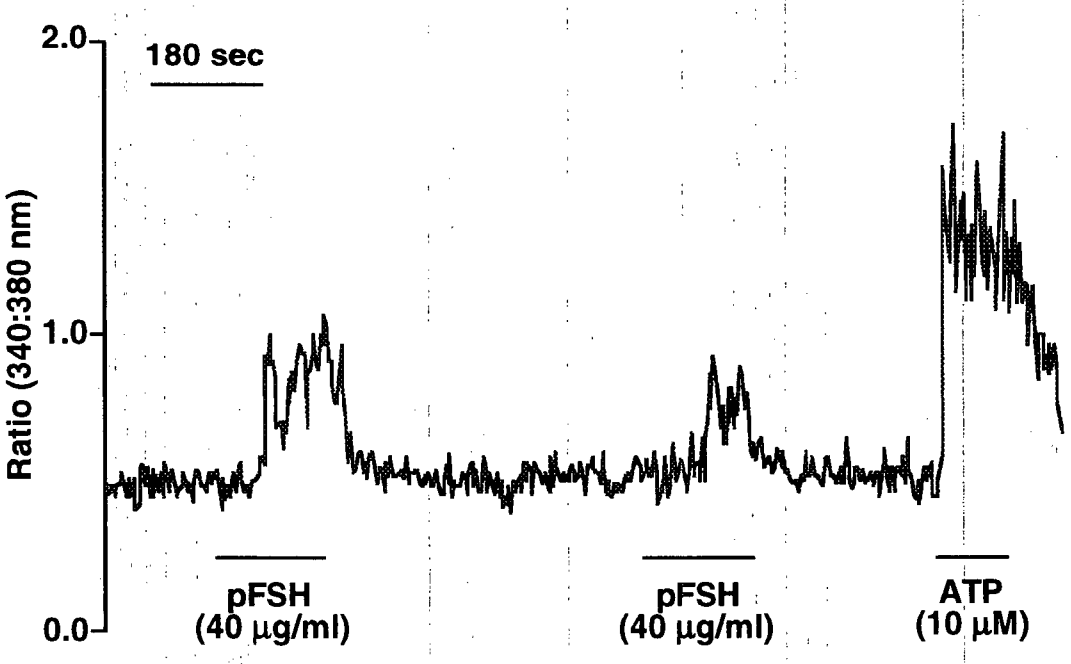


Figure 21: Effects of gonadotrophin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor FL(V/VI)R. Porcine FSH was administered at a concentration of 40 µg/ml for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

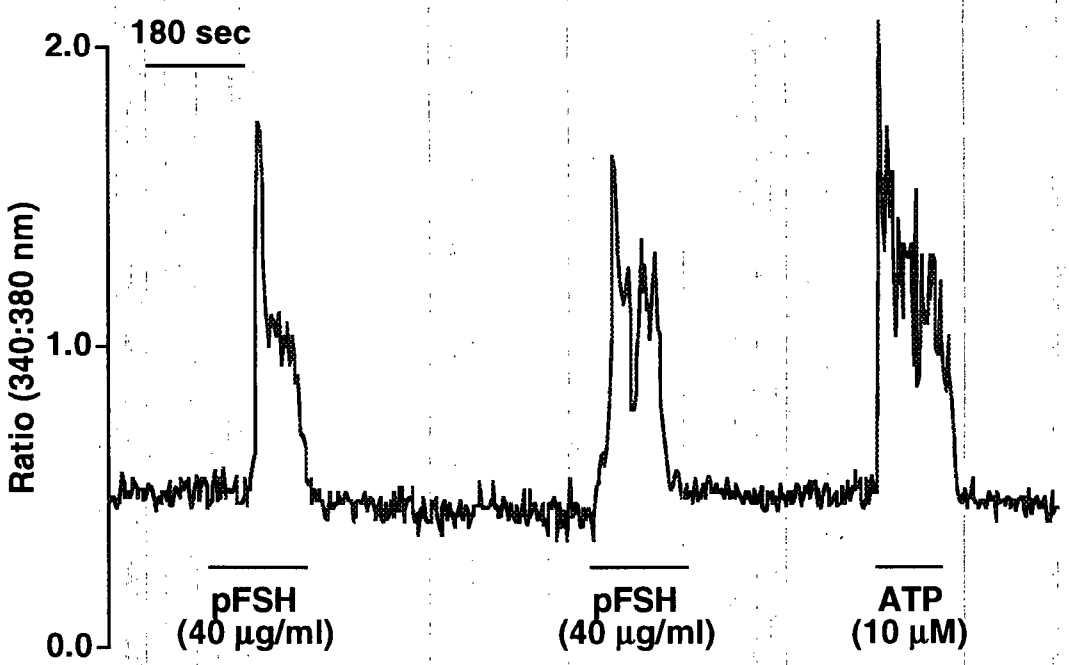


Figure 22: Effects of gonadotrophin treatment on HEK293 cells transfected with the chimeric human gonadotrophin receptor FL(V-VI)R. Porcine FSH was administered at a concentration of 40 µg/ml for a duration of 180 sec. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

III. P_2-Purinoreceptor Agonist-Evoked Calcium Oscillations in Single Human Granulosa-Lutein Cells

We have examined the effects of purinergic receptor agonists ATP, ADP, AMP, adenosine, UTP, and the non-hydrolysable analogue ATPγS on intracellular calcium concentration over a range of concentrations (1-100 μM) in isolated human GLCs, using the techniques of single-cell dual-excitation microfluorimetry. The data presented are representative of the changes in intracellular calcium, and are reported as the total number of cells imaged (TC) and number of patients (n) for each protocol.

A. Effects of purinergic receptor agonists on intracellular calcium concentrations

ATP consistently evoked a marked increase in cytosolic calcium (TC=750, n=11). As shown in Figure 23, above micromolar levels, the response to ATP was concentration dependent. No change in intracellular calcium concentration was observed at submicromolar concentrations, whilst the plateau phase produced by 100 μM ATP exhibited partial run-down; a phenomenon consistent with desensitisation at the level of the receptor. The desensitisation effect was independent of the order of administration. The patterns of intracellular calcium rises were generally characterised as either non-oscillatory (25%; TC=187, n=11), or oscillatory calcium transients (75%; TC=563, n=11) originating from a plateau of elevated intracellular calcium (Figures 23 and 24). In a efficacy profile experiment of UTP, ATP, ADP, AMP, and adenosine (Figure 24), cells were exposed to 10 μM concentrations (TC=75, n=4). The data in Fig-

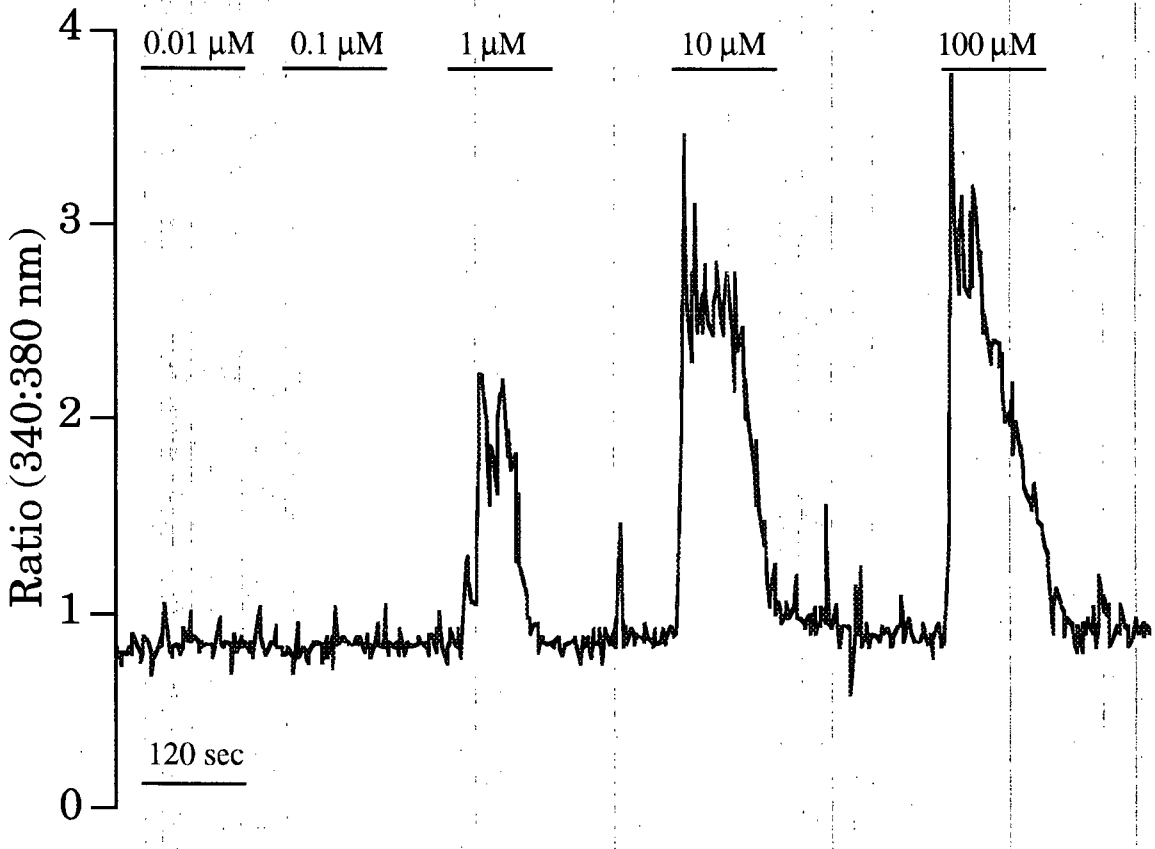


Figure 23: ATP concentration-response relationship. Submicromolar concentrations of ATP were incapable of calcium mobilisation. Note the oscillatory pattern at 10 μM ATP. The cells were loaded with Fura-2-AM, and perifused with a balanced salt solution. All microfluorimetric studies were conducted in a temperature-controlled (37°C) chamber.

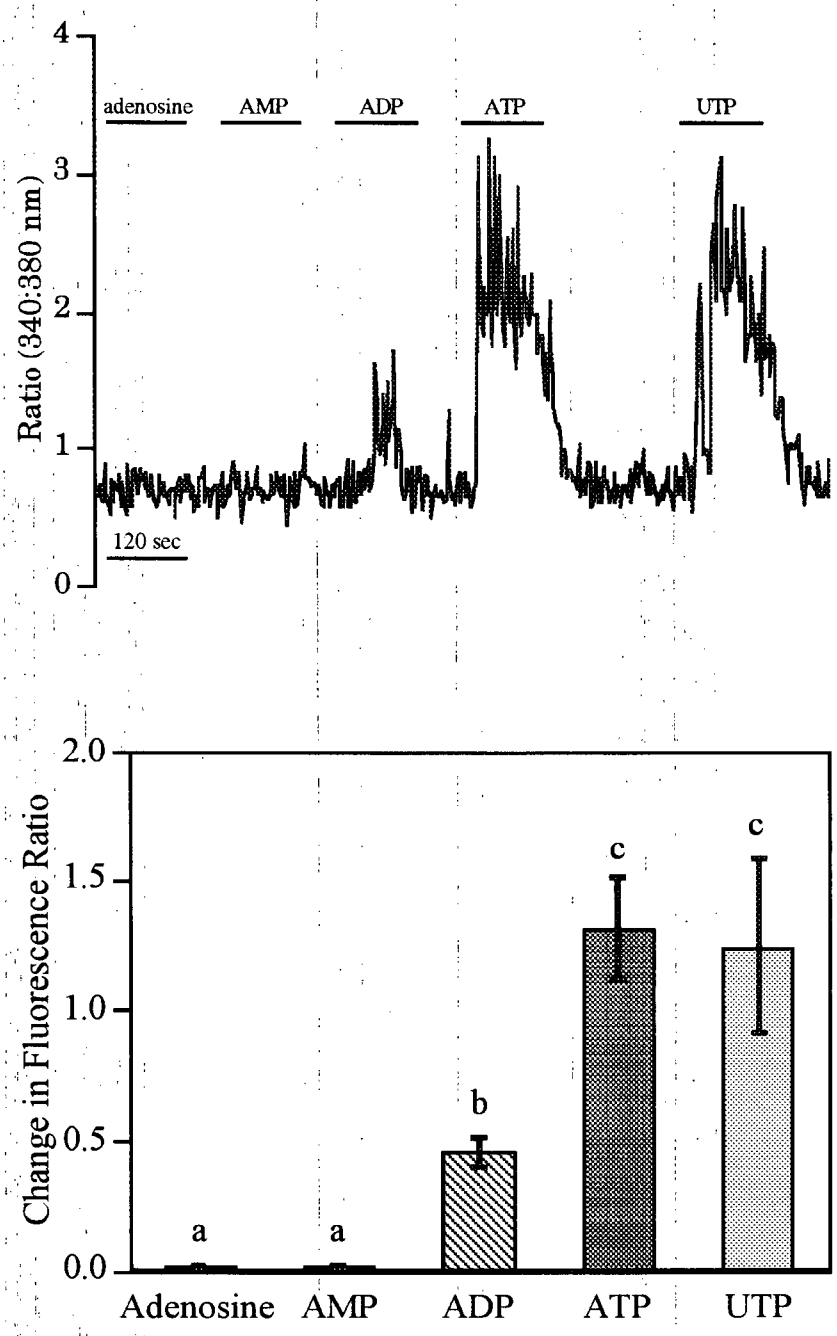


Figure 24: Upper panel: Efficacy profile of various purinergic agonists. All agonists were used at a concentration of 10 μM.

Lower panel: Comparison of the relative potencies of the various P_{2U} agonists. ($a \neq b \neq c$, $p < 0.05$).

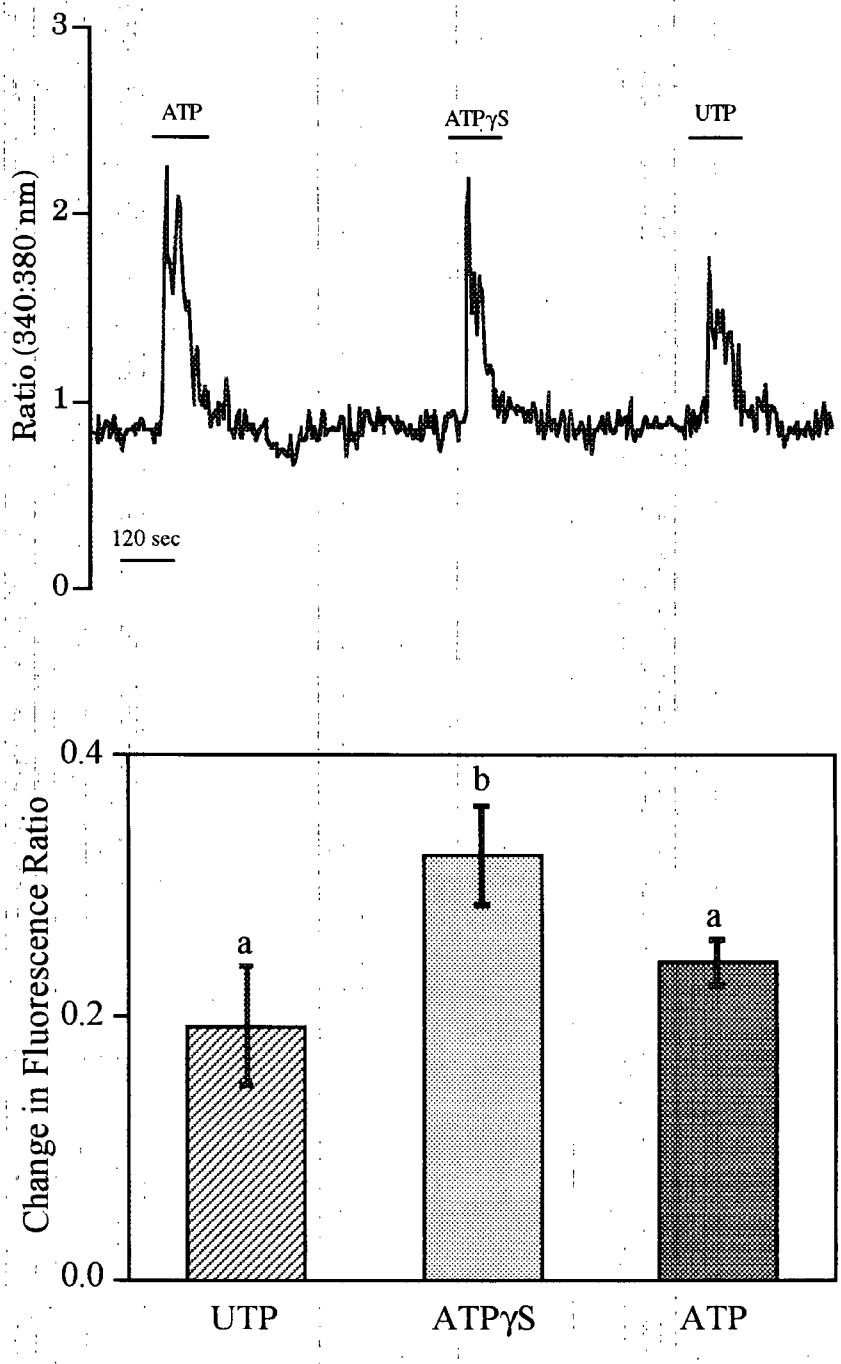


Figure 25: Upper panel: Effects of ATPγS, a non-hydrolysable ATP analogue, on hGLCs. Agonists were used at a concentration of 10 μM.

Lower panel: Comparison of the relative potencies of the various purinergic agonists. ($a \neq b$, $p < 0.05$).

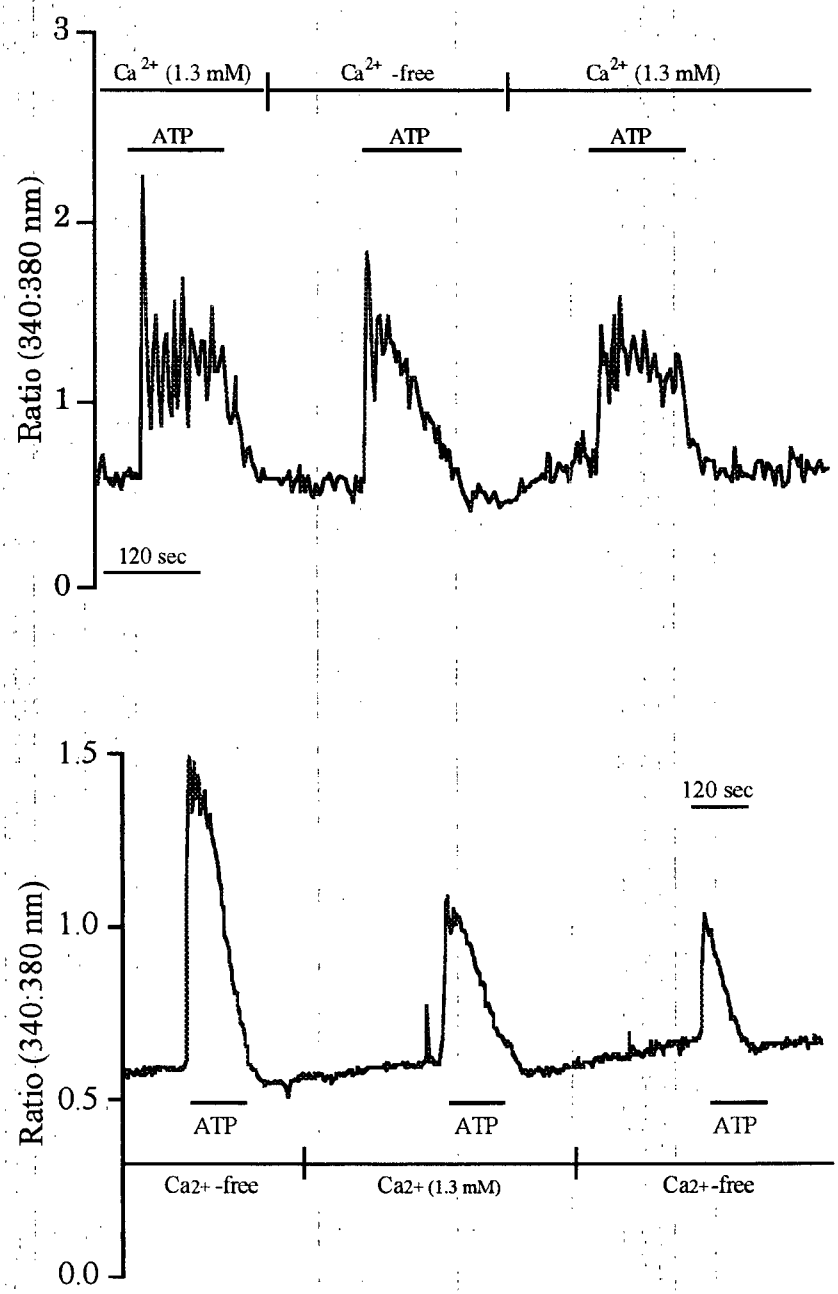


Figure 26: Upper panel: The involvement of extracellular calcium on ATP -induced calcium mobilisation. ATP was used at a concentration of 10 μM. The calcium-free buffer contained 1 mM EGTA.

Lower panel: The involvement of extracellular calcium on ATP-induced calcium mobilisation. ATP was used at a concentration of 10 μM. The calcium-free buffer contained 1 mM EGTA.

ure 24 shows that while 10 μM ATP evokes a substantial signal, there is no effect of either AMP or adenosine. Under the same experimental conditions, ADP (10 μM) consistently evoked smaller changes in intracellular calcium concentration than ATP (TC=50, n=4), whilst UTP was equipotent (TC=75, n=4).

B. Effects of ATPγS on intracellular calcium concentrations

Figure 25 is a representative profile of the effects of the non-hydrolysable analogue ATPγS on intracellular calcium levels in human GLCs (TC=75, n=4). The response to 10 μM ATPγS mirrors that evoked by ATP both in the time course of the onset of the response and the oscillatory nature of the sustained plateau phase. The amplitude of the change in calcium is comparable for both ATP and ATPγS. Figure 25 Lower panel shows that the effects of ATPγS are greater than those of ATP and UTP.

C. Calcium-influx vs. calcium-mobilisation

In order to determine the relative contribution of calcium-influx vs. calcium-mobilisation from cytosolic stores in the initiation and maintenance of the purinergic response, ATP was added in either the presence or absence of extracellular calcium. Under calcium-containing conditions, ATP (10 μM) reproducibly evokes a sharp rise in cytosolic calcium, which is maintained as either an oscillatory (Figure 26 Upper panel) or smooth (Figure 26 Lower panel) plateau in the continued presence of the agonist. In calcium-free experiments, in the presence of the selective calcium chelator EGTA (1 mM), ATP evokes an initial rise in intracellular calcium levels, but the response is now transient, returning to basal levels in the continued presence of ATP.

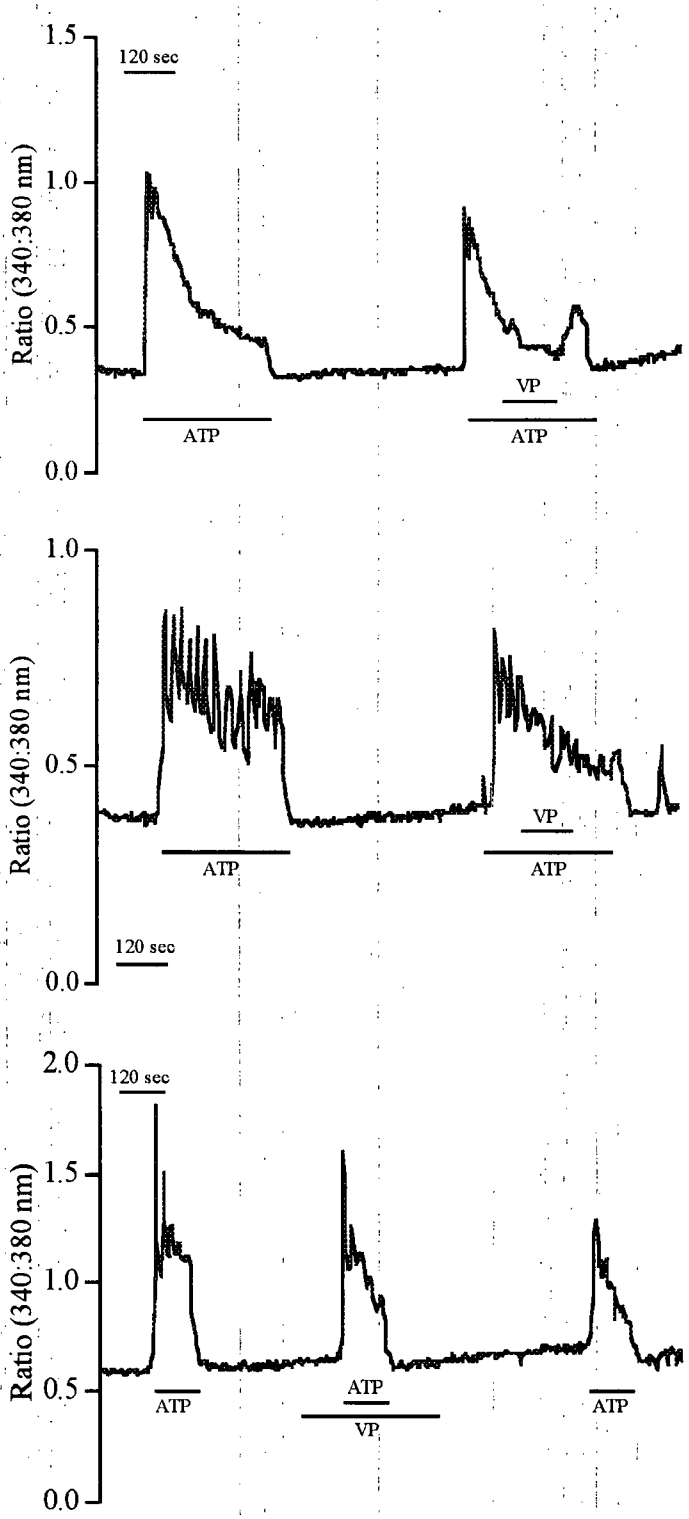


Figure 27: Upper, Middle, and Lower panels: Effects of verapamil (VP) on ATP-stimulated calcium mobilisation. Reagents were used at a concentration of 10 μM.

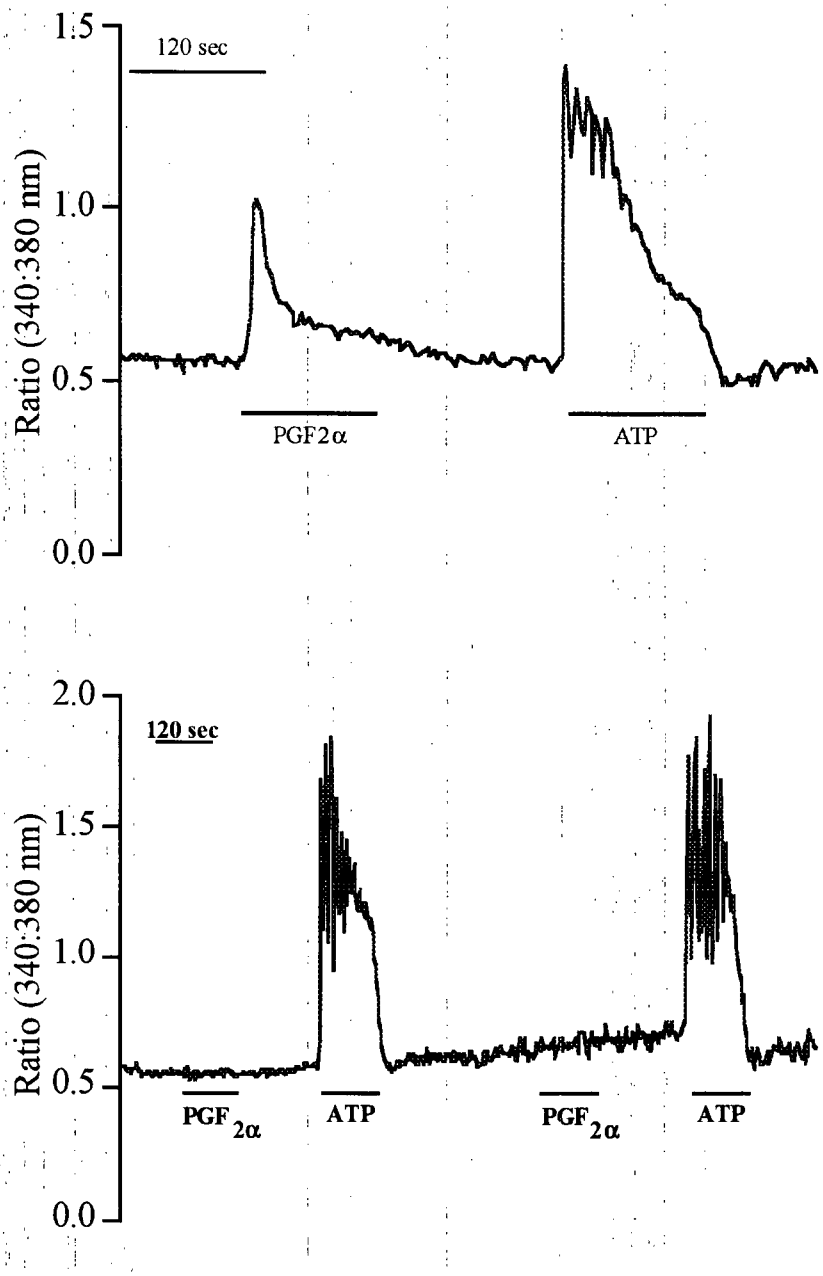


Figure 28: Upper panel: Effects of $PGF_{2\alpha}$ and ATP on intracellular calcium mobilisation. Reagents were used at a concentration of 10 µM.

Lower panel: Effects of $PGF_{2\alpha}$ and ATP on intracellular calcium mobilisation in human GLCs pretreated with PTX (100 ng/ml) for 18 h prior to experiment. $PGF_{2\alpha}$ and ATP were used at a concentration of 10 µM.

However, note how the response still exhibits oscillations (TC=60, n=3; Figure 26 Upper panel).

To determine whether VDCC were involved in the influx component of the ATP evoked change, we used the broad acting blocker, verapamil (10 μM), in an attempt to inhibit the plateau phase of the response. As shown in Figure 27 Upper panel (TC=18, n=4) and 27 Middle panel (TC=36, n=4), verapamil was able to block the maintained phase of the response in 33% of the cells. In addition, verapamil did not prevent an ATP-evoked rise in cytosolic calcium when added prior to the P_2-purinergic receptor agonist (TC=75 and 75, n=4 and 3; Figure 27 Lower panel).

D. Pertussis Toxin Pre-treatment

To determine whether the ATP evoked calcium response was coupled to a PTX sensitive G protein, we pretreated the cells with PTX (100 ng/ml) for 18 h. PTX failed to alter the profile of the ATP evoked calcium response (TC=75, n=3; Figure 28 Lower panel). $PGF_{2\alpha}$ was used as the control in determining the effectiveness of PTX.

E. Effects of purinergic receptor agonists on steroid secretion

Human GLCs were treated after a 7 day incubation. The data presented are representative of the steroidal responses elicited by the various reagents; the data are presented in this manner because the basal steroidal concentrations varied, at time considerably, amongst the patients.

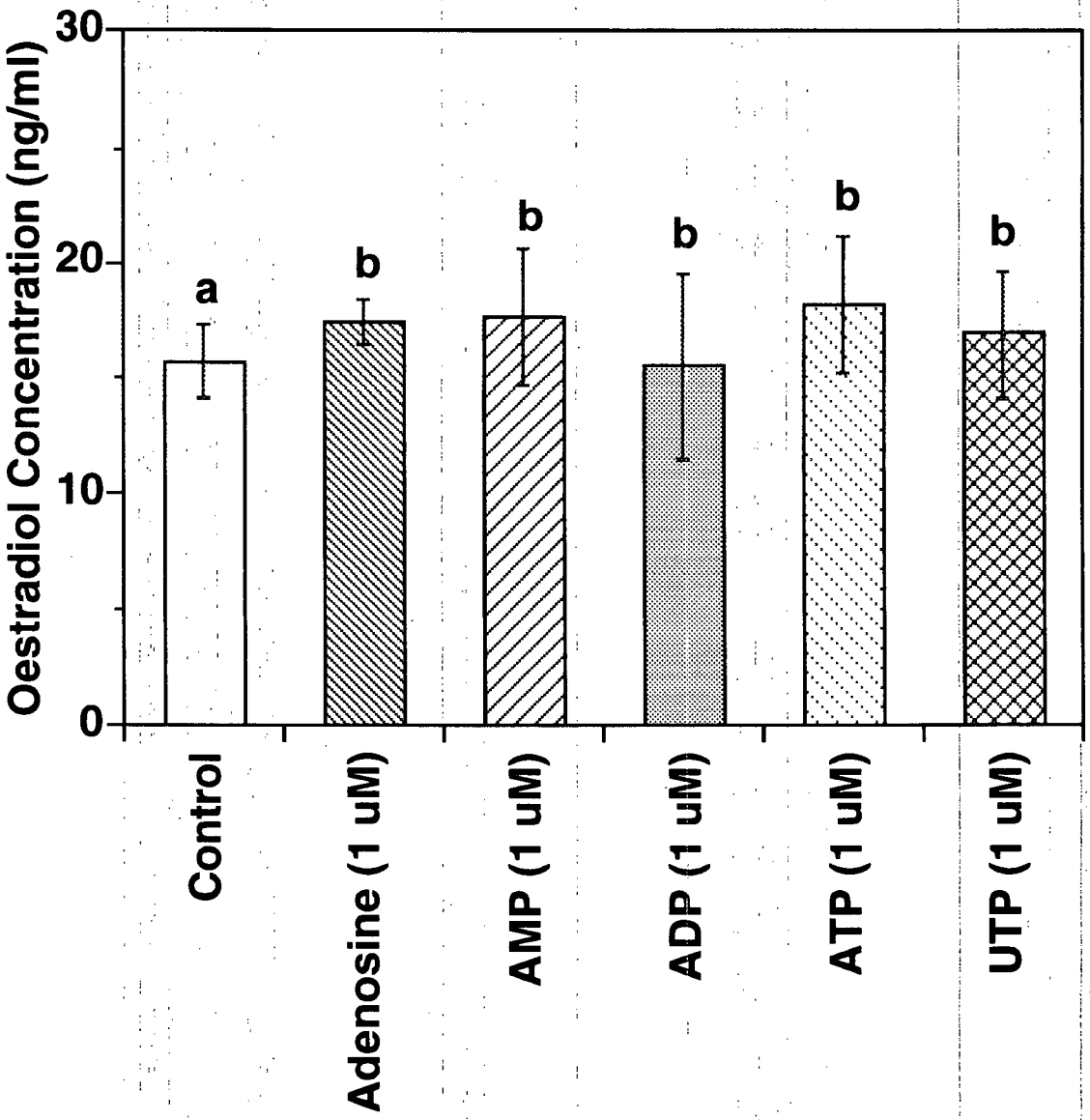


Figure 29: Effects of purinergic agonists on basal oestradiol production in human GLCs. The cells were incubated with serum-free DMEM at least 6 hrs prior to drug treatments. Cells were cultured for 7 days prior to drug treatment. Treatment periods ranged from 22-26 hrs. Treatments were made up in serum-free DMEM containing androstenedione. (a=b, $p > 0.05$)

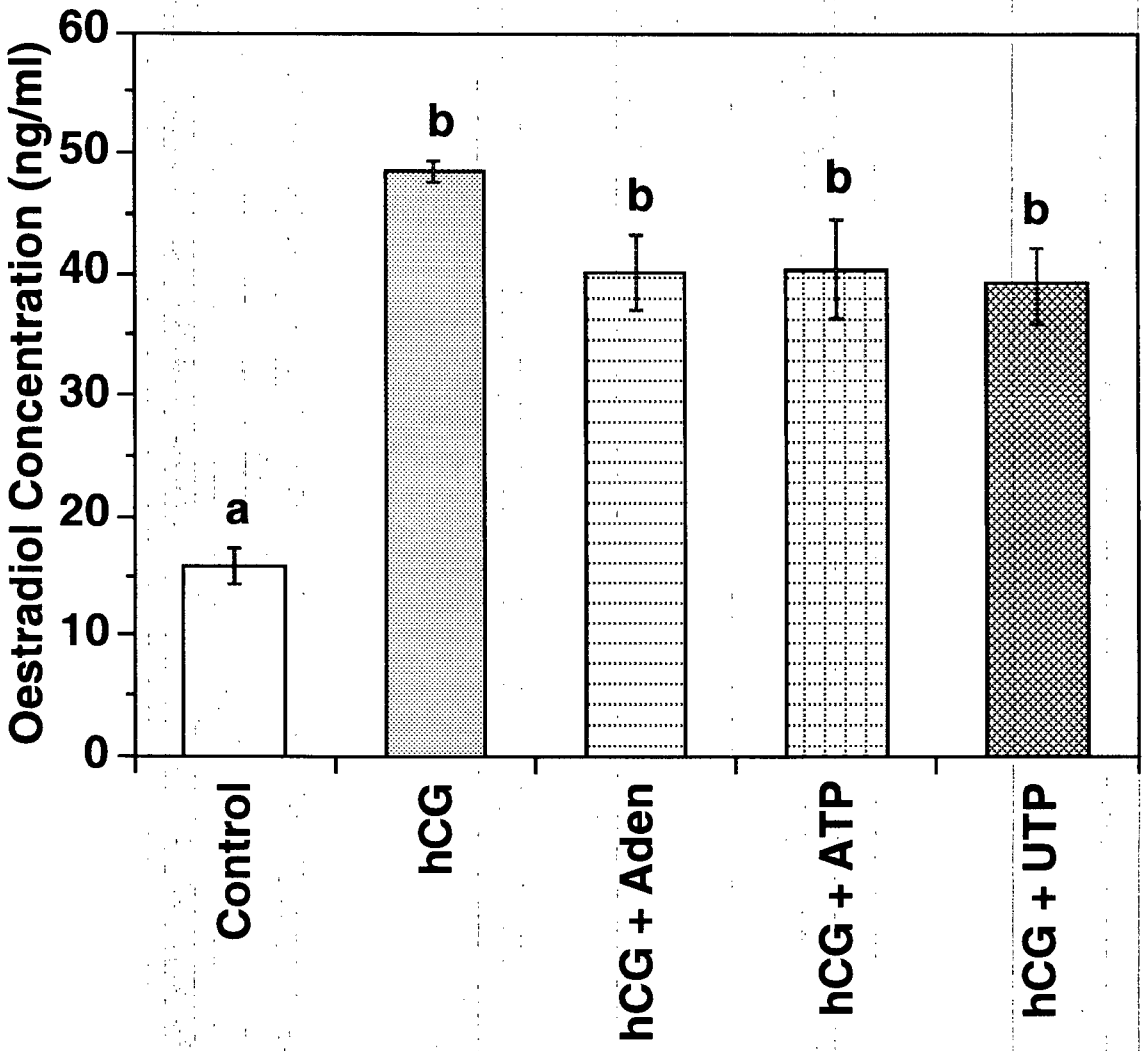


Figure 30: Effects of purinergic agonists on hCG-stimulated oestradiol production in human GLCs. The cells were incubated with serum-free DMEM at least 6 hrs prior to drug treatments. Cells were cultured for 7 days prior to drug treatment. Treatment periods ranged from 22-26 hrs. Treatments were made up in serum-free DMEM containing androstenedione. ($a \neq b$, $p < 0.05$)

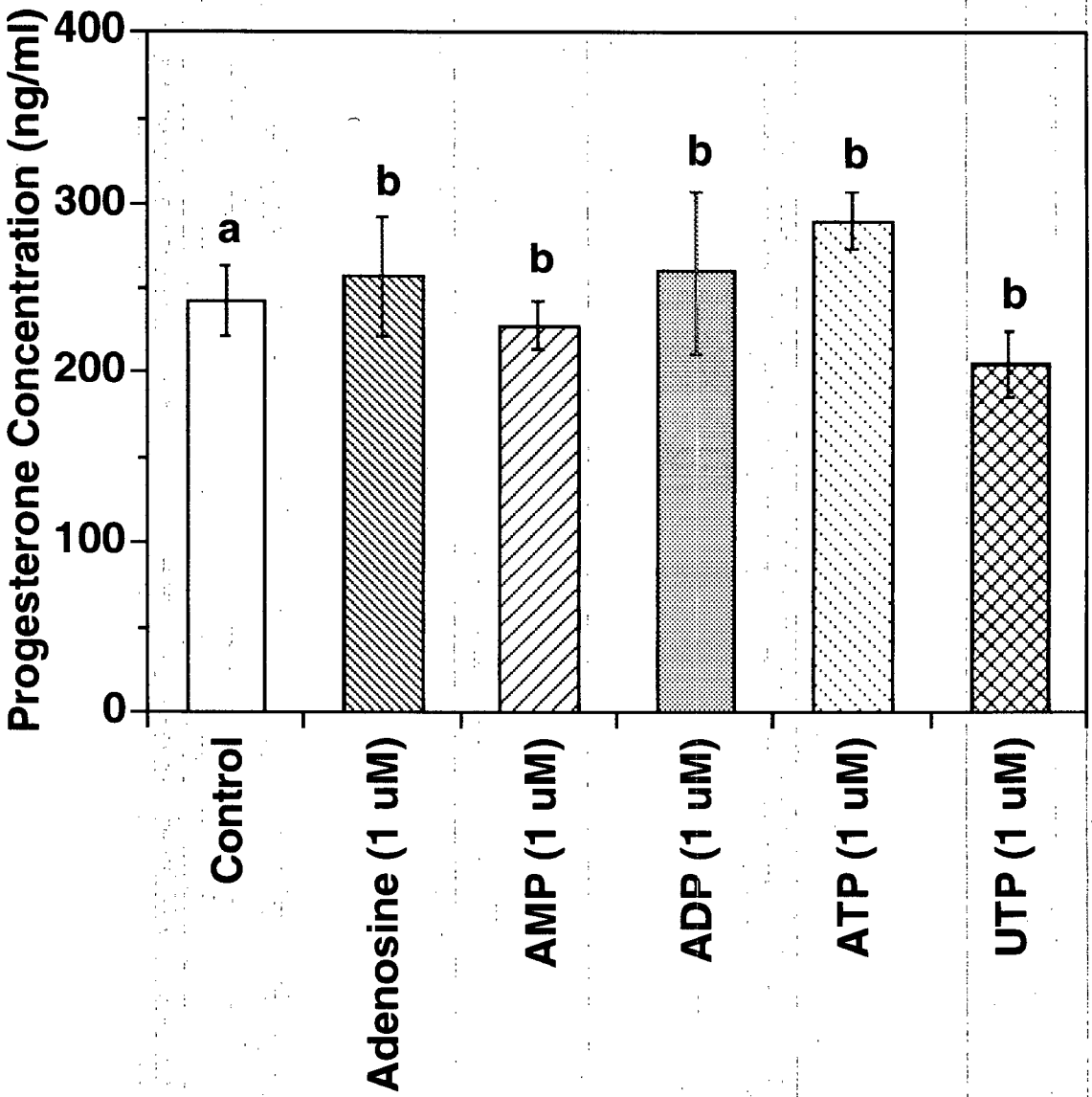


Figure 31: Effects of purinergic agonists on basal progesterone production in human GLCs. The cells were incubated with serum-free DMEM at least 6 hrs prior to drug treatments. Cells were cultured for 7 days prior to drug treatment. Treatment periods ranged from 22-26 hrs. Treatments were made up in serum-free DMEM containing androstenedione. (a=b, $p > 0.05$)

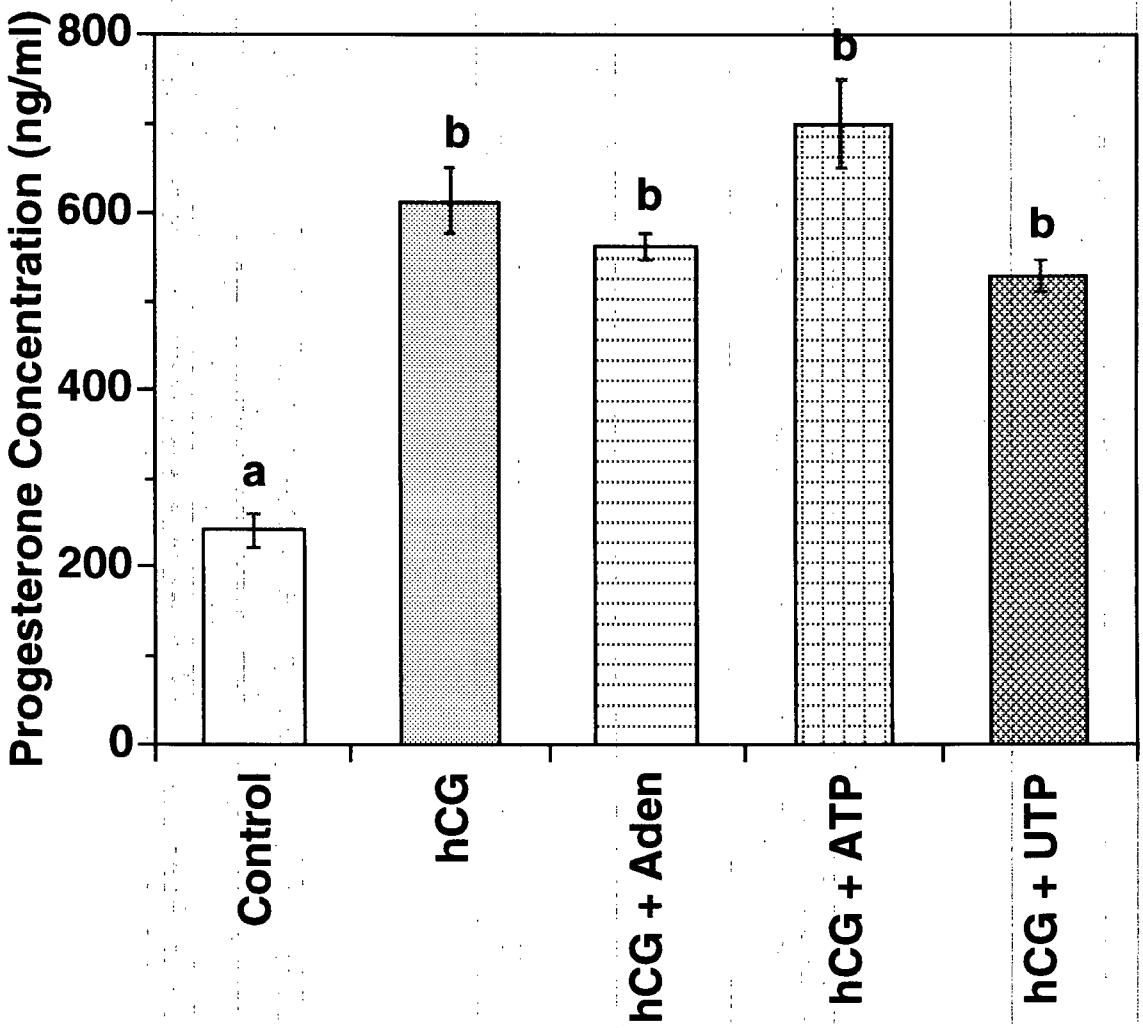


Figure 32: Effects of purinergic agonists on hCG-induced progesterone production in human GLCs. The cells were incubated with serum-free DMEM at least 6 hrs prior to drug treatments. Cells were cultured for 7 days prior to drug treatment. Treatment periods ranged from 22-26 hrs. Treatments were made up in serum-free DMEM containing androstenedione. ($a \neq b$, $p < 0.05$)

To determine whether purinergic agonists have any effect on basal steroid secretion, human GLC cultures were treated with 1 μM concentrations of adenosine, AMP, ADP, ATP, and UTP. The cells were incubated with serum-free DMEM at least 6 hrs prior to drug treatments. Cells were cultured for 7 days prior to drug treatment. Treatment periods ranged from 22-26 hrs. Treatments were made up in serum-free DMEM containing androstenedione. The purinergic agonists did not appear to have any effects on basal steroid production, nor on hCG-stimulated steroid production (Figures 29-32).

Discussion

I. Gonadotrophin-Induced Calcium Oscillations in HEK293 Cells Express-ing the Human Luteinising Hormone/Chorionic Gonadotrophin Receptor

To our knowledge, this is the first report of sustained calcium oscillations in response to the activation of human LH/CG receptors. Previous studies have reported LH/CG-induced calcium elevations in *Xenopus* oocytes [Gudermann, et al., 1992b] and in HEK293 cells transfected with the rat LH/CG receptor [Lakkakorpi and Rajaniemi, 1994]. The type of calcium response elicited by the activation of the rat LH receptor was dependent upon hormone concentration and the presence of extracellular calcium. Lakkakorpi *et al.* [Lakkakorpi and Rajaniemi, 1994] observed calcium oscillations in 72% of the cells in the presence of extracellular calcium, and only 33% of cells in the absence of extracellular calcium. We observed human CG-induced calcium oscillations in all HEK293 cells expressing the human LH receptor, the pattern and frequency of oscillation altered with increasing concentrations of the hormone. In the absence of extracellular calcium oscillations were still observed although the frequency was reduced. Thus is appears that the human LH receptor affects intracellular calcium levels in a manner significantly different to the rodent receptor. Hirsch *et al.* [Hirsch, et al., 1996] have previously reported that HEK293 cells transiently transfected with the human LH/CG receptor do exhibit human LH-induced elevations in intracellular IP_3 and cAMP.

The induction of intracellular oscillations by transfection of the human LH receptors into HEK293 cells was similar to the effect of human CG on

human granulosa cells maintained in short term culture. These results indicate that the coupling of the human LH receptor to both adenylate cyclase- and IP_3-mediated oscillations is a response of the normal target cells as well as transfected cell lines. Thyrotrophin, structurally related to LH. FSH and hCG, appears to share the ability to activate multiple signaling pathways with LH/hCG. Treatment of human thyroid cells with thryotrophin results in the generation of calcium oscillations [D'Arcangelo, et al., 1995]. The thyrotrophin receptor is well known to couple to the adenylate cyclase/cAMP cascade in a manner similar to LH and FSH, however, thyrotrophin has been reported to stimulate the inositol phosphate/calcium signaling pathway in primary cultures of thyroid cells, thyroidal cell lines, and transfected cell lines [Corda and Kohn, 1986; D'Arcangelo, et al., 1995; Hidaka, et al., 1994]. D'Arcangelo *et al.* [D'Arcangelo, et al., 1995] These results suggest that the inositol phosphate/ intracellular calcium cascade plays an integral role in the complex signal transduction pathways in both gonadal and thyroidal cells.

Intracellular calcium oscillations were first described by Prince and Berridge [Prince and Berridge, 1972], but not appreciated until Woods *et al.* [Woods, et al., 1986] described the linkage between surface membrane receptors and cellular function. Calcium oscillations are involved in the potentiation of a ligand response [Alkon and Rasmussen, 1988; Berridge and Galione, 1988]. Calcium oscillations have been reported following the activation of several receptors in the reproductive system, notably P_2-purinoreceptor in human ovarian cells [Lee, et al., 1996; Squires, et al., 1997] and GnRH receptor in gonadotrophs [Stojilkovic and Catt, 1995]. Gonadotrophin releasing hormone and endothelin-1 induce biphasic intracellular calcium transients in gonadotroph cell suspensions (measured using the cuvette technique), but

oscillatory intracellular calcium responses in single gonadotrophs [Stojilkovic and Catt, 1995]. The pattern of oscillations reported in the latter study resemble the LH-induced intracellular calcium oscillations observed in the present studies.

Although much remains to be explained with regard to their significance, it has been suggested that the calcium oscillations may code for multiple signals in their amplitudes and frequencies. It is believed that the various calcium profiles differentially activate signaling pathways such as gene transcription by activating the calmodulin pathway and intracellular protein phosphorylation by activating the calcium sensitive protein kinase C isozymes.

The data presented in this thesis clearly indicates that the binding of LH/CG to the human LH/CG receptor activates the phospholipase C pathway. In order to determine the source of the calcium driving the oscillatory behaviour a number of pharmacological manipulations of the transfected cells were undertaken. High concentrations of caffeine have been shown to inhibit the mobilisation of non-mitochondrial, IP_3-sensitive calcium stores [Toescu, et al., 1992]. The data obtained using both the primary granulosa cells and the transfected HEK cells show that the calcium oscillations initiated by stimulation of the LH receptor were inhibited by caffeine. These results would be consistent with activation of the phospholipase C pathway and generation of IP_3 and diacylglycerol.

Inositol 1,4,5-trisphosphate stimulates the release of calcium from intracellular stores by binding to and opening the IP_3 receptor, a calcium release channel in the endoplasmic reticulum. Inositol trisphosphate receptor activity is not only sensitive to intracellular IP_3 concentrations, but also to

intracellular calcium concentrations [Iino, 1987]. The IP_3-induced rate of calcium release depends on a feed forward mechanism whereby the initial calcium released through the channel stimulates opening of additional IP_3 receptors and a further increase in calcium levels [Iino, 1990; Iino and Endo, 1992]. Studies have shown that IP_3 is only a partial agonist of the IP_3 receptor; for the full activation of the receptor, both calcium and IP_3 are required [Bezprozvanny, et al., 1991; Finch, et al., 1991; Iino, 1990; Iino and Endo, 1992]. The LH/CG-induced calcium oscillations observed were most probably generated by the process of calcium-induced calcium release. The IP_3 receptor functions as a calcium-induced calcium release channel in the continued presence of IP_3 [Iino, 1999].

Additional confirmation of the stimulation of phospholipase C by the LH receptor was obtained using thapsigargin to deplete ER calcium stores. After thapsigargin pre-treatment neither LH nor human CG were capable of initiating calcium oscillations. In addition if thapsigargin was given after stimulation of the LH receptors additional calcium release was observed indicating that human CG did not completely empty the IP_3-sensitive endoplasmic reticular calcium stores. When thapsigargin was applied first, twin peaks of calcium release were observed suggesting that more than one isoform of the IP_3 receptor is expressed on the endoplasmic reticulum. The expression of multiple IP_3 is not unusual and many cells types have been reported to contain at least two of the three known IP_3 receptors [Cardy, et al., 1997; Fissore, et al., 1999]. The data obtained from the HEK cells would be consistent with the initial activation by thapsigargin of the high affinity IP_3 receptor (most probably IP_3 type II) . The presence of thapsigargin in the perfusion medium prevents the re-sequestration of released calcium into the

endoplasmic reticulum, and in the continued presence of IP_3 in the cytosol would result in activation of the second lower affinity IP_3 receptor.

Multiple subtypes of IP_3 receptors (types I, II, and III) are expressed in a tissue- and development-specific manner [Rosemblit, et al., 1999] Calcium signalling patterns are dependent on the IP_3 receptor subtypes, which differ significantly in their responses to agonists (i.e. IP_3, calcium, and ATP). Type I IP_3 receptors are highly sensitive to ATP, and mediate irregular calcium oscillations [Miyakawa, et al., 1999]. The type II IP_3 receptors form channels with permeation properties similar to type I. However, IP_3 and calcium are more effective at activating type II IP_3 receptors, therefore, these channels mobilise substantially more calcium than type I channels [Ramos-Franco, et al., 1998]. Type II IP_3 receptor are the most sensitive to IP_3 and are required for the long-lasting, regular calcium oscillations that occur upon surface receptor activation [Miyakawa, et al., 1999]. High cytoplasmic calcium concentrations inactivate type I, but not type II, IP_3 receptors, indicating that calcium is not inherently self-limiting thus calcium passing through an active type II channel cannot feed back and turn the channel off [Ramos-Franco, et al., 1998]. Type III IP_3 receptors are the least sensitive to IP_3 and calcium, and tend to generate monophasic calcium transients [Miyakawa, et al., 1999]. It forms calcium channels similar to those of type I receptors; however, the open probability increases monotonically with increased intracellular calcium concentration, whereas the type I isoform has a bell-shaped dependence on cytoplasmic calcium. Type III IP_3 receptors provide positive feedback as calcium is released; the lack of negative feedback allows complete calcium release from intracellular stores [Hagar, et al., 1998] Differential expression of IP_3 receptor subtypes helps to encode IP_3-mediated calcium signalling; thus the complement

of IP_3 receptors in the cell defines the spatial and temporal nature of calcium signalling in response to stimulation of phospholipase C.

The significance of intracellular calcium oscillations remains to be elucidated. The unravelling of the mechanisms that give rise of these oscillations will provide insights into the mechanisms that regulate and set cytosolic calcium levels within physiological limits. Baseline calcium oscillations are defined as rapidly rising transient increases in intracellular concentrations from close to baseline levels. These oscillations are characterised by increased oscillation frequencies, without concomitant increases in spike amplitude, in response to increasing agonist concentrations. Sinusoidal oscillations are intracellular calcium oscillations superimposed on a sustained plateau of intracellular calcium. Increased agonist concentrations increases the overall amplitude of the sinusoidal oscillations, but not the frequency of the oscillations. Baseline oscillations may continue throughout prolonged periods of stimulation, while sinusoidal oscillations tend to diminish with time, generally lasting for only a few minutes [Putney, 1992]. Clearly the response to stimulation of the LH receptor generates baseline rather than sinusoidal oscillatory responses in the cells examined in the present studies.

Several investigators have suggested that calcium oscillations encode hormone signals. Because oscillation frequencies can vary with agonist concentrations, calcium transients might be part of a frequency-encoded signalling system [Berridge and Galione, 1988; Putney, 1992]. Calcium oscillations might encode information to be detected over a broader range than with just sustained, tonic increases. This may be of especial importance for hormones of very low concentrations. Prolonged exposure to extremely low

concentrations of some phospholipase C-linked hormones, can induce biological responses such as changes in gene expression [Stachowiak, et al., 1990; Suh, et al., 1992] Low agonist concentrations could evoke agonist concentration-sensitive calcium oscillations; these low frequency oscillations could, over a prolonged interval, could be integrated into a biological response. Meyer *et al.* [Meyer, et al., 1992] suggested that the kinetic behaviour of the calmodulin/calcium-calmodulin-dependent protein kinase interaction can detect and respond to calcium oscillations. If calcium oscillations are capable of activating calmodulin/calcium-calmodulin-dependent protein kinase, then a sustained series of oscillations would be sufficient to alter the expression of proteins encoded by genes regulated by CAM activity.

II. Role of calcium oscillations in gonadal physiology

It has long been established that LH action is mediated primarily via the adenylate cyclase signalling pathway [Dufau and Catt, 1978; Hunzicker-Dunn and Bimbaumer, 1985; Leung and Steele, 1992]. However, recent reports have raised the possibility that the phospholipase C signal transduction pathway is also involved in LH action [Davis, 1994; Gudermann, et al., 1992a; Herrlich, et al., 1996; Hipkin, et al., 1993]. Although the mechanism underlying the bifurcating signal transduction remains unknown, it has been shown that in bovine corpora lutea and L cells stably expressing the murine LH receptor, LH stimulation can couple to both G_s and G_i, and the ßγ-subunits released from either G protein contribute to the stimulation of phospholipase Cß isoforms [Herrlich, et al., 1996] Hipkin *et al.* [Hipkin, et al., 1993] have shown that the LH/CG receptor phosphorylation is induced by a phorbol ester, but not with a

calcium ionophore. Although the phorbol ester-induced phosphorylation of the LH/CG receptor can be correlated with uncoupling, other experiments indicate that human CG-induced uncoupling of the LH/CG receptor can occur under conditions where the cAMP-mediated receptor phosphorylation is greatly reduced or abolished [Hipkin, et al., 1993]. Gudermann *et al.* have shown that L cells stably expressing murine LH receptors respond to human CG with an increase in their rate of phosphoinositide hydrolysis, and an increase in intracellular calcium concentrations [Gudermann, et al., 1992a].

It has been suggested that a single LH/CG receptor can couple to both adenylate cyclase and phospholipase C, and the ability of LH/CG to activate phospholipase C is independent of cAMP accumulation [Davis, 1994]. The concentrations of LH and human CG used in the present study were in the same range as those employed to induce IP_3 accumulation associated with the human LH receptor [Hirsch, et al., 1996]. Interestingly, it has been reported that higher concentrations of LH are required to activate the phospholipase C pathway than that which is required for the adenylate cyclase pathway, at least in rodents. In light of the data from Zhu *et al.* [Zhu, et al., 1994], the activation of the phospholipase C pathway appears to be associated with events surrounding ovulation and pregnancy, when circulating levels of LH and human CG are high. This notion is corroborated by the increase in the number of LH receptors during follicular maturation [Kammerman and Ross, 1975]. Moreover, recent studies have shown that the ability of LH to induce ovulation is impaired by protein kinase C inhibitors [Shimamoto, 1993 #70; Kaufman, 1992 #311], further supporting a role for the phospholipase C pathway in LH action during the periovulatory period.

Calcium signals generated by the LH-activated phospholipase C pathway during ovulatory process may also act via protein kinase C. Cutler *et al.* [Cutler, et al., 1993] reported that a calcium-independent protein kinase C was involved in ovulation in the rat; however, this does not negate the possibility that a calcium-dependent protein kinase C is involved in the ovulatory process in other species. Rodents do not produce a dominant follicle during the follicular phase and give birth to between 12 - 15 pups, thus, the endocrinological dynamics regulating ovulation will differ significantly from animals producing only a single offspring by ovulating one follicle (such as humans).

Our study shows that the human LH/CG receptor is specific for LH and human CG. While purified human FSH failed to elicit calcium signals, human LH and CG consistently evoked calcium oscillations that were sustained even after treatment withdrawal. The initial phase of the human CG-evoked increase in intracellular calcium concentrations results from the mobilisation of cytosolic calcium stores, and is then sustained by an influx of extracellular calcium. The intracellular calcium stores in question were the intracellular IP_3-sensitive calcium stores in the endoplasmic reticulum. The human LH/CG receptor also appears to be coupled to calcium signalling through the G_i-protein, as is the case in the murine LH receptor [Herrlich, et al., 1996] Taken together with recent report of increased IP_3 accumulation following the activation of the human LH/CG receptor [Hirsch, et al., 1996], the present results support the concept that in addition to adenylate cyclase, activation of phospholipase C is a parallel signalling pathway coupled to the human LH/CG receptor.

III. Calcium Signalling in HEK293 Cells Transfected with the Wild-Type or Chimeric Human Gonadotrophin Receptors

Follicle stimulating hormone, LH/CG, and TSH receptors belong to the large gene family known as the seven transmembrane spanning, G-protein-coupled receptors [Lefkowitz and Caron, 1988; McFarland, et al., 1989]. Molecular cloning of cDNAs and genes for proteins of this family has revealed that they share a common structure, consisting of seven α-helical hydrophobic putative transmembrane regions, joined by three extra- and intracellular loops that display significant homology within the family. The glycoprotein hormone receptors for LH, FSH, and TSH represent a small subclass of this superfamily that with a large extracellular amino-terminal region responsible for high affinity binding of their large (28-38 kDa) ligands [Segaloff and Ascoli, 1993; Segaloff, et al., 1990; Xie, et al., 1990]. The extracellular region of these receptors is encoded by multiple exons and comprises approximately one-half of the full-length protein. The transmembrane carboxy-terminal half of these receptors is encoded by a single exon and represents the signal-transducing component of the glycoprotein [Segaloff and Ascoli, 1993]].

To investigate the segments of the human LH receptor involved in signal transduction, we studied the response of intracellular calcium concentrations to gonadotrophin treatment, in HEK293 cells expressing the wild-type and chimeric human gonadotrophin receptor.

The chimeric receptor approach was originally used to investigate the functional domains of adrenergic receptors [Kobilka, et al., 1988]. The advantage of studying gonadotrophic receptors is that binding and activation

are inter-related but separate phenomena [Fernandez and Puett, 1996; Ji and Ji, 1991b]. Ryu *et al.* [Ryu, et al., 1996] have reported that human CG binding at the high affinity site in the amino-terminal half of the receptor induces conformational adjustments. This leads to low affinity secondary contacts of the complex of the human CG-amino terminal end of the receptor with the carboxy-terminal end of the receptor. This low affinity secondary contact is responsible for activating the receptor. This property allows the generation of chimeric receptors with alterations in the signal-transducing transmembrane domains without perturbation of ligand binding.

It has been confirmed that all the chimeric human gonadotrophic receptors are efficiently synthesized, recognise the appropriate ligands, and respond to ligand activation with increases in cAMP production. There were no major differences in cell surface expression and k_d values of various wild-type and chimeric receptors, ruling out promiscuous coupling due to changes in receptor number [Hirsch, et al., 1996].

Our findings support the work of Kudo, *et al.* [Kudo, et al., 1996]. Replacing the extracellular domain of the human LH receptor with that of the human FSH receptor did not alter receptor activation. Apart from the delay in calcium response onset, the intracellular calcium profile elicited by the binding of FSH to the human chimeric gonadotrophin FLR receptor is very similar to that evoked by LH stimulation of the human LH receptor. This would suggest that the extracellular domain of the receptor is important for ligand binding, but is not involved in the activation of the intracellular signalling pathways. It has previously been reported that receptor activation results in a conformational change to the LH receptor that promotes the binding of the cytoplasmic tail to the main body of the receptor. The data obtained using the

FLR chimeric receptors indicate that switching the external binding site to that of the FSH receptor resulted in FSH binding initiating the conformational change required for C-terminal attachment.

Alterations in the transmembrane regions of the human LH receptor results in perturbations of the agonist-induced intracellular calcium profile. In all cases this resulted in the ablation of the basal calcium oscillations. Our findings suggest that the transmembrane regions V through VII are crucial in retaining a normal intracellular calcium profile for gonadotrophin-induced activation. Any alteration to these regions resulted in a significantly perturbed calcium profile. These data would be consistent with the conclusion that the carboxy-terminal third of the human LH receptor mediates the activation of phospholipase C. Although the chimeric receptors containing transmembrane regions V and VI were capable of initiating a transient increase in intracellular calcium levels these were not equivalent to the sustained basal oscillations produced by stimulation of the native receptor. These results indicate that the intact carboxy-terminal third of the receptor is required to achieve the normal intracellular calcium oscillation profile.

IV. P_2-Purinoreceptor Agonist-Evoked Calcium Oscillations in Single Human Granulosa-Lutein Cells

Adenosine trisphosphate and other agonists of purinergic receptors are known to be potent stimulators of hormone secretion. Several reports in the literature have demonstrated that ATP will cause a marked increase in cytosolic calcium concentration from endocrine tissues [Bertrand, et al., 1990; Filippini, et al., 1994; Kamada, et al., 1994; Squires, et al., 1994]. It is known that ATP is co-

localised with neurotransmitters at concentrations in excess of 100 mM and is co-released with both adrenaline and acetylcholine [Dubyak and el-Moatassim, 1993; el-Moatassim, et al., 1992; Gordon, 1986]. The sympathetic innervation of the ovaries is extensive [Dissen, et al., 1993] which would provide the source of extracellular ATP within the follicles.

The work presented in this thesis clearly demonstrates that human GLCs possess functional purinoreceptors. Oscillatory changes in intracellular calcium concentrations can be triggered equipotently by either ATP or UTP and to a lesser extent by ADP. The P_1-purinergic receptor agonists, adenosine and AMP, failed to alter basal levels of intracellular calcium, whilst the non-hydrolysable analogue ATPγS evoked a rise in calcium with a similar potency to ATP. These data suggest the existence of the P_{2U} class of receptor where ATP=ATPγS=UTP>ADP>>AMP=adenosine. Although the existence of P_{2U} receptors has been previously reported in single chicken granulosa cells [Morley, et al., 1994] and in dissociated human granulosa cells using the cuvette measuring technique [Kamada, et al., 1994], this is the first study to address the activity of P_2-purinergic receptor agonists in single isolated human GLCs.

In chicken granulosa cells, ATP has been shown to evoke calcium oscillations, which return to basal values between successive spikes, whilst in the one study in human cells to date, the use of populations of cells precludes the identification of oscillatory behaviour. Single human GLCs respond to ATP and other P_{2U}-purinoreceptor agonists with calcium oscillations and that these transients differ from those previously reported in the chicken. In human, the calcium transients originate from an elevated plateau of intracellular calcium. Moreover, it appears that the mechanism driving these transients differs amongst species. Unlike the changes observed in chicken where only an initial

spike of reduced amplitude was observed under calcium-free conditions, the calcium oscillations seen in the present study still occurred in the absence of extracellular calcium. However, there is a gradual decline in the oscillatory pattern as the cytosolic calcium stores are depleted. It therefore appears that the calcium transients in human GLCs originate from the release of intracellular stores of calcium and are maintained by the influx of calcium from the extracellular media. However, in the chicken, oscillations are only seen when extracellular calcium is present, although the initial response involves mobilisation of intracellular stores.

The effect of verapamil on the sustained phase of the calcium response was variable with a small proportion of the cells showing a decrease which returned to plateau levels after removal of the drug. In the majority of the cells, verapamil had no effect on the alteration in intracellular calcium levels suggesting that voltage-dependent calcium channels are not involved.

The P_{2U} purinoreceptors may be coupled to pertussis toxin-sensitive and/or -insensitive G proteins [Dubyak and el-Moatassim, 1993; Rhee, et al., 1989; Sternweis and Smrcka, 1992]; both pertussis toxin-sensitive and -insensitive pathways are capable of activating PLC. The failure of pertussis toxin to alter the ATP evoked calcium changes in human GLCs would suggest that these cells possess P_{2U} purinoreceptors which are coupled to pertussis toxin-insensitive G proteins. This observation differs from that of the P_{2U} purinoreceptor of rat Sertoli cells which is pertussis toxin-sensitive as reported by Filippini *et al.* [Filippini, et al., 1994].

The P_2-evoked oscillatory changes in intracellular calcium are distinct from the calcium transients previously reported for $PGF_{2\alpha}$ in these cells

[Currie, et al., 1992]. The effect of $PGF_{2\alpha}$ on human and rat ovarian cells also involved inositol phosphate metabolism, but was pertussis toxin sensitive [Davis, et al., 1989; Leung, et al., 1986; Rodway, et al., 1991]. Recent cloning and expressions studies of the $PGF_{2\alpha}$ receptor confirmed that, like ATP, it acts via the phospholipase C-mediated phosphoinositide hydrolysis/calcium signalling pathway [Abramovitz, et al., 1994; Kitanaka, et al., 1994; Lake, et al., 1994] Prostaglandin $F_{2\alpha}$-stimulated changes in intracellular calcium are transient, returning to baseline levels despite the continued presence of the agonist. In addition, the concentration related effects of ATP differ from the all-or-none effects $PGF_{2\alpha}$ on human GLCs [Currie, et al., 1992].

The pattern of change in intracellular calcium levels and the ability of human GLCs to instigate and maintain sophisticated calcium oscillations clearly have important implications to ovarian physiology; however, the precise role of these changes have yet to be elucidated. The present study has confirmed and extended a previous report suggesting the existence of P_{2U}-purinoreceptors on human GLCs. Intracellular calcium signalling was achieved via both influx and mobilisation and, for the first time, the cytosolic release of calcium has been identified as the source of calcium oscillations in single human GLCs.

Based on the cell culture experiments, P_2 agonists are unlikely to be directly involved in steroidogenesis (Figure 29-32). However, they may be involved in the mobilisation of steroidogenic precursors, e.g. via the steroidogenic acute regulatory (StAR) protein. The StAR protein is deemed essential for the transfer of cholesterol from the outer to the inner mitochondrial membrane, where the cytochrome P_{450} cholesterol side chain cleavage enzyme is located [Ferguson, 1963; Garren, et al., 1965]. Clark *et al.*

have established a temporal relationship between levels of StAR expression and steroidogenesis [Clark, et al., 1995b] and have shown that agonists which increase intracellular calcium also increase the level of the StAR protein [Clark, et al., 1995a].

V. Summary and Conclusion

Activation of the human LH receptor by LH or human CG results in the stimulation of at least 2 signal transduction pathways. The data presented concerning intracellular calcium dynamics in stimulated cells were consistent with the stimulation of the phospholipase C pathway in addition to the established linkage with adenylate cyclase. The human receptors were more responsive to low levels of agonist than was previously reported in rat models suggesting that low LH levels early in the follicular period may play an important role in the function of granulosa cells.

The response of both human granulosa and transfected HEK cells to low agonist levels was characterised by the presence of long lasting trains of basal calcium oscillations. This pattern of calcium mobilization could be linked to modulation of gene transcription in both cell types.

The studies with the chimeric receptors showed that the sequence of the long extracellular portion of the receptor was not critical for stimulation of phospholipase C activity but maintained the specificity of agonist binding. The C-terminal sequence of the receptor is clearly important for the generation of the basal oscillations but the precise extent of the critical sequence has yet to be identified.

Stimulation of both the purinergic and LH receptors in human granulosa cells resulted in calcium oscillations, although, with clearly different dynamics. These results strongly suggest that the precise spatial and temporal regulation of intracellular calcium in these cells will be important in the regulation of cellular function. Clearly further studies into the physiological significance of these oscillations will be required.

VI. Future Directions

Women differ from the majority of experimental animal species in that late in the follicular phase a single follicle becomes dominant and the remaining follicles undergo atrophy. The precise factors regulating dominance have yet to be identified, however, there is strong support for the follicle that develops LH receptors first becoming the Graffian follicle. If this is true then the ability of human Granulosa cells to respond to low LH levels by initiating calcium transients could play a critical role in the development of the dominant follicle.

The dominant follicle is characterised by an increased output of oestradiol resulting from the ability to produce the steroid de-novo without the requirement of androstenedione secreted from the adjoining Theca cells. The ability to synthesize oestradiol is dependent on the Granulosa cells expressing significant levels of P450 side chain cleavage and StAR. Clearly an important extension of the present studies will be to determine the effect of LH with and without concomitant ATP on expression of the two proteins in Granulosa cells.

From the two-cell theory of ovarian follicular steroidogenesis, we know that: FSH receptors are present on granulosa cells, and increased levels are induced by FSH itself; LH/CG receptors are present on theca cells and initially absent on granulosa cells; as the follicle matures, FSH induces the expression of LH/CG receptors on the granulosa cells; and FSH induces aromatase activity in granulosa cells.

Theca cells are characterised by LH-induced androgen production. During the middle of the follicular phase prior to selection of the dominant follicle while LH concentrations are low, it is possible that a sufficient number of LH/CG receptors are activated to evoke intracellular calcium transients. The calcium spikes would form part of a frequency-encoded signalling system, and over a period of several days, these signals may integrate into a biological response such as the expression of the StAR protein. Activation of the calcium-sensitive StAR gene in the theca cell results in the transfer of cholesterol from the outer to the inner mitochondrial membrane, where cytochrome P_{450} side chain cleavage enzyme is located.

The cytochrome P_{450} side chain cleavage enzyme converts cholesterol to pregnenolone, and is the key steroidogenic intermediate common to all classes of steroid hormones. Both LH and cAMP regulate transcription of the cytochrome P_{450} gene but cAMP is the more potent of the two. Interestingly, FSH receptors activate adenylate cyclase resulting in an increase in cAMP levels, however, in the early follicular stage in the absence of LH receptors, FSH is incapable of stimulating the expression of P450 side chain cleavage. This suggests that a transcriptional repressor must be present in the granulosa cells and that the actions of this repressor protein are reversed once the cells express LH receptors.

There is at present no evidence that ATP has a direct effect on steroidogenesis in human GLCs, but it may be another candidate for regulation of the StAR protein. The possible effect of P_2 agonists in this regard warrants further investigation.

Finally, the phospholipase C pathway, and protein kinase C in particular, has been implicated in the ovulatory processes in several animals, therefore, protein kinase C could play a role in ovulation in women. Protein kinase Cs are subdivided into calcium-dependent and calcium independent isoforms. There is no information available concerning which of the 12 known isoforms of protein kinase C are expressed in human GLCs. If the cells possess a calcium-dependent protein kinase C, LH-induced calcium transients would function as a co-activator of this enzyme along with the diacylglycerol generated by phospholipid hydrolysis. The involvement of protein kinase C in the ovulatory process suggests that the enzyme may be involved in the increased expression and secretion of proteolytic enzymes required for rupture of the follicle. In order to test the hypothesis that the LH-induced oscillations activate a calcium-sensitive protein kinase C, which in turn increases proteolytic enzyme production, the effect of LH on tissue type plasminogen activator activity must be investigated in the presence and absence of inhibitors protein kinase C.

An extension of these studies would be to identify the subtypes of protein kinase Cs present in human GLCs; and if a calcium-dependent isoform is present, to inhibit it and to examine the effects of LH stimulation.

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