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Function of Protein Kinase A in Xenopus oocyte Maturation

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Function of protein kinase A in *Xenopus* oocyte maturation

by

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Abstract

Like most other vertebrate oocytes, oocytes from *Xenopus laevis* are physiologically arrested at the first meiotic prophase. Re-initiation of meiosis, or oocyte maturation, is triggered by progesterone. Progesterone-induced oocyte maturation is thought to involve inhibition of cAMP-dependent protein kinase (protein kinase A or PKA). However, changes in PKA activity in live oocytes have never been demonstrated. I have developed a novel approach to monitor endogenous PKA activity in live oocytes, by expressing a PKA-specific substrate in oocytes followed by monitoring its PKA phosphorylation status during progesterone-induced oocyte maturation. I demonstrate that PKA is fully activated in prophase oocytes and that progesterone causes a rapid and permanent inhibition of PKA (Chapter 3). Utilizing this PKA activity indicator in live oocytes, I further demonstrate that progesterone-induced oocyte maturation requires persistent inhibition of PKA such that critical maturation-specific proteins can be synthesized and accumulated to threshold levels necessary for activation of maturation promoting factor (MPF) (chapter 4). In addition to analyzing PKA activity dynamics during oocyte maturation, I have also provided the first biochemical evidence supporting the notion that in prophase oocytes, a constitutively activated G protein coupled receptor system is responsible for elevated cAMP and the high levels of PKA activity (chapter 2).

Dedication

The thesis is dedicated to the memory of my Dad. Without his love and support, I could not accomplish all of these. His spirit was and will be inspiring me to pursue my ultimate academic goals.

Acknowledgements

I express great thanks to my supervisor Dr. Johné Liu for his patience, support and guidance throughout my Ph.D. project. The experience in his laboratory opened my mind to new avenues of research and built a solid base for my future research career. His motivation, energy and demand for excellence have been and always will be an inspiration to me. Also, the guidance that I received from my advisory committee (Dr. Robert Haché and Dr. Paul Albert) was much appreciated.

I would also like to thank all the members in the lab, including Dr. Yinglun Sheng, Dr. Chunqi Ma, Dr. Ronald Booth and Ling Wang for their help and numerous discussions that aided in the completion of this thesis.

I would especially like to thank my family, Dad, Mom and my older sister, for their love and encouragement.

Finally, I thank my husband for his daily support, patience and encouragement. Without him none of these could have been possible.

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List of Abbreviations

M	molar
nM	nanomolar
μM	micromolar
mL	millilitre
μL	microlitre
mg	milligram
U	international units
μCi	microcurrie
s.e.m.	standard error of the mean

2-D	two-dimensional
AA	amino acid
AC	adenylyl cyclase
ADP	adenosine diphosphoate
Ala	alanine
Asn	asparagine
AGS	activator of G protein signaling
AKAP	protein kinase A anchor protein
β_2AR	β_2- adrenergic receptor
cAMP	adenosine 3',5'-cyclic monophosphate
Cdks	cyclin dependent kinases
Chk	check point kinase
Con A	concanavalin A
CPE	cytoplasmic polyadenylation
CPEB	cytoplasmic polyadenylation binding protein
CPSF	cleavage and polyadenylation specific factor
CTX	cholera toxin
DTT	dithiothreitol
eIF4E	eukaryotic translation initiation factor 4E
eIF4G	eukaryotic translation initiation factor 4G
Erk	extracellular signal-regulated kinase
Fsk	forskolin
Gαi	inhibitory G alpha subunit
Gαs	stimulatory G alpha subunit
GAP	GTPase activating protein
GDP	guanine nucleotide diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
Gly	glycine
GpCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
GTP	guanine nucleotide triphosphate

GVBD	Germinal Vesicle Break Down
H1	histone 1
IBMX	isobutylmethylxanthine
Lys	lysine
MII phase	metaphase II
MAPK	mitogen-activated protein kinase
MβCD	methyl-β-cyclodextrin
MBP	myelin basic protein
MDC	monodansylcadaverine
MPF	maturation Promoting Factor
mRNA	messenger RNA
OA	okadaic acid
PBS	phosphate-buffered saline solution
PCR	polymerize chain reaction
PDE	phosphodiesterase
PH	pleckstrin homology
PKA	cyclic adenosine monophosphate-dependent protein kinase
PKAc	protein kinase A catalytic subunit
PKAr	protein kinase A regulatory subunit
PKC	Ca^{2+}-dependent protein kinase, or protein kinase C
Plx1	polo-like kinase 1
PKI	protein kinase A inhibitor
PMSF	phenylmethylsulfonyl fluoride
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PR	progesterone receptor
PRKX	chromosome – encoded protein kinase X
p-S	phosphorylated serine
PTX	pertussis toxin
PVDF	polyvinylidene difluoride
R	arginine
RI (II)	PKA regulatory subunit I or II
RGS	regulator of G protein signaling
S phase	synthesis phase
Ser	serine
S	serine
SDS-PAGE	SDS polyacrylamide gels electrophoresis
Thr	threonine
Tyr	tyrosine
UTR	untranslated region
Xcdc25C	Xenopus cdc25C
XPR	Xenopus classical progesterone receptor

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Chapter One

General Introduction

General Introduction

Sexual reproduction involves a cyclic alternation of diploid and haploid states: germ cells with diploid genomes divide by meiosis to form gametes (sperm or egg); haploid genomes from gametes (sperm or egg) fuse in pair to form a new diploid genome state. Thus, meiosis is described as two consecutive cell divisions following a single phase of DNA replication. Although recent findings indicate that proliferative germ cells may exist and sustain oocyte production in postnatal mouse ovary (Johnson et al., 2004; Johnson et al., 2005), a long-held view in reproductive biology states that most mammalian females are born with a finite, non-renewing pool of germ cells, all of which are enclosed by follicles (Borum, 1961; McLaren, 1984). Hence, oocyte meiosis starts in embryonic stage and continues to the diplotene stage of the first meiotic prophase (prophase I), at which point the oocytes are arrested until puberty. During the prolonged prophase I, the primary oocytes increase in size and accumulate components necessary for early embryonic development, accompanied by the development of the surrounding follicle cells. Upon sexual maturation of the females, the fully-grown oocytes resume meiosis under the influence of pituitary gonadotropins (follicle-stimulating hormone and luteinizing hormone). These oocytes then complete meiosis I with the emission of the first polar body, progress to meiosis II without an intervening S phase (DNA replication) and are arrested at metaphase II (MII phase). The transition from prophase I to MII phase is termed oocyte maturation, as ovulated mature eggs are arrested in metaphase II. At fertilization, sperm binding triggers completion of meiosis II with the emission of the second polar body (Gilbert, 2000; Masui and Clarke, 1979).

The african clawed frog, *Xenopus laevis*, represents a highly advantageous system for studying oocyte maturation. Isolated frog oocytes remain stably arrested at prophase I and respond *in vitro* to the steroid hormone progesterone to undergo oocyte maturation (Figure. 1.1). In contrast, denuded mammalian oocytes undergo "spontaneous" oocyte maturation, making it difficult to synchronize the oocytes or manipulate gene expression. In addition, the giant size of frog oocytes (~1.2 mm in diameter) and the large number (several thousands) available from each female are features that are particularly suitable for biochemical characterization of signaling pathways controlling oocyte maturation.

Although there is ongoing controversy whether other steroids also play a role in gonadotropin-mediated oocyte maturation (Gill et al., 2004; Hammes, 2004; Lutz et al., 2001; Lutz et al., 2003), it is generally agreed that progesterone is the natural steroid hormone to trigger *Xenopus* oocyte maturation. A unique feature of progesterone-induced oocyte maturation is its independence of transcription regulation in the oocytes. This so called "nongenomic" action of progesterone is in contrast to its classical role in binding the nuclear progesterone receptor and regulating gene expression (Iwashita et al., 1998; Masui and Markert, 1971; Smith and Tenney, 1980; Smith and Ecker, 1969). Therefore progesterone activates a cytoplasmic receptor, which in turn causes a reduction of intracellular adenosine 3',5'-cyclic monophosphate (cAMP) and consequently inhibition of cAMP-dependent protein kinase (PKA). It is thought that inhibition of PKA stimulates *de novo* synthesis of certain maturation-specific proteins. Ultimately, maturation promoting factor (MPF) is activated (Figure. 1.2). MPF is the key cell cycle kinase thought to be responsible for nuclear envelop breakdown (also known as germinal

vesicle breakdown or GVBD), which signifies the entry to metaphase I (Masui and Markert, 1971).

The focus of my thesis work was to determine endogenous PKA activity and its function during oocyte maturation as well as to study molecular mechanisms regulating intracellular cAMP/PKA signaling.

Figure 1.1

Schematic representation of oocyte maturation and fertilization in *Xenopus laevis*. Modified from **Developmental Biology** (Gilbert, 2000). During oogenesis, the oocytes are arrested in prophase while they accumulate the components necessary for early embryogenesis. Once stage VI is reached the oocytes are competent to undergo maturation in response to follicular progesterone and arrest for a second time at metaphase II. The mature oocytes, now referred to as eggs, are able to be fertilized. The red and green lines refer to the activities of MAPK and MPF, respectively, during oocyte maturation.

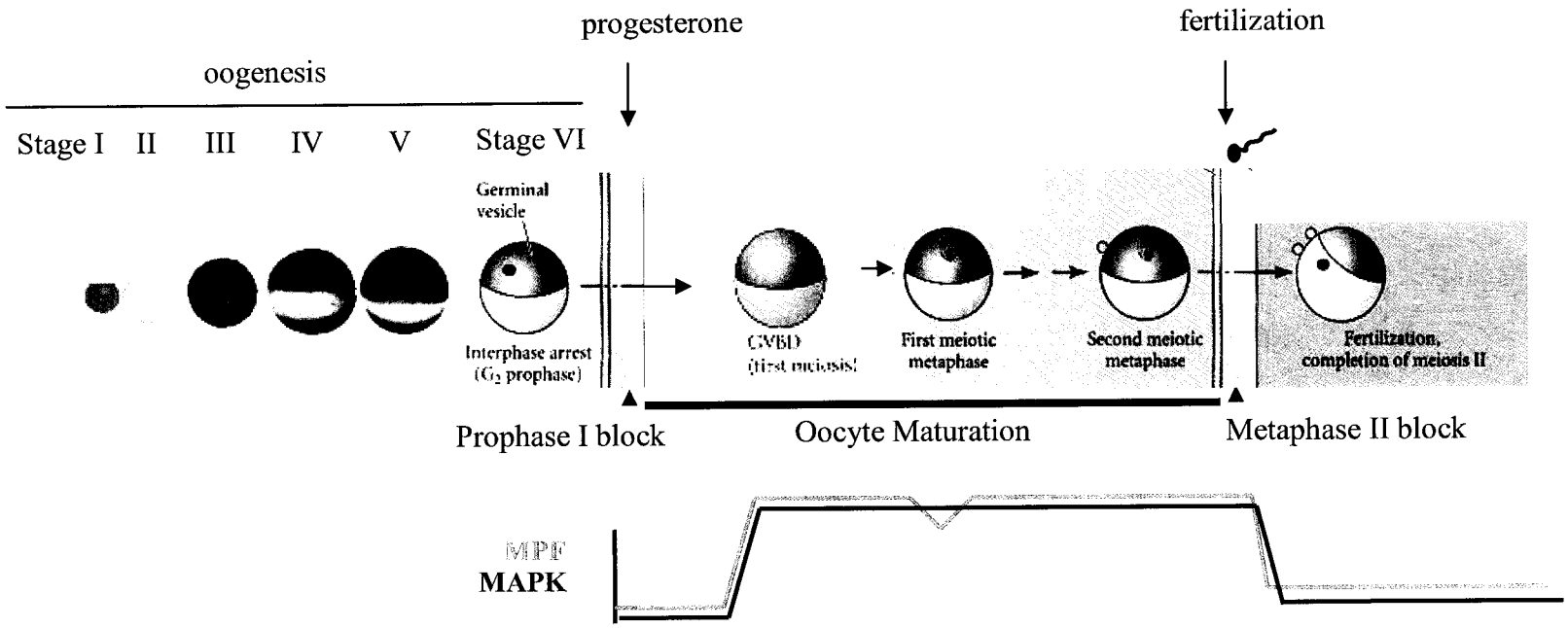
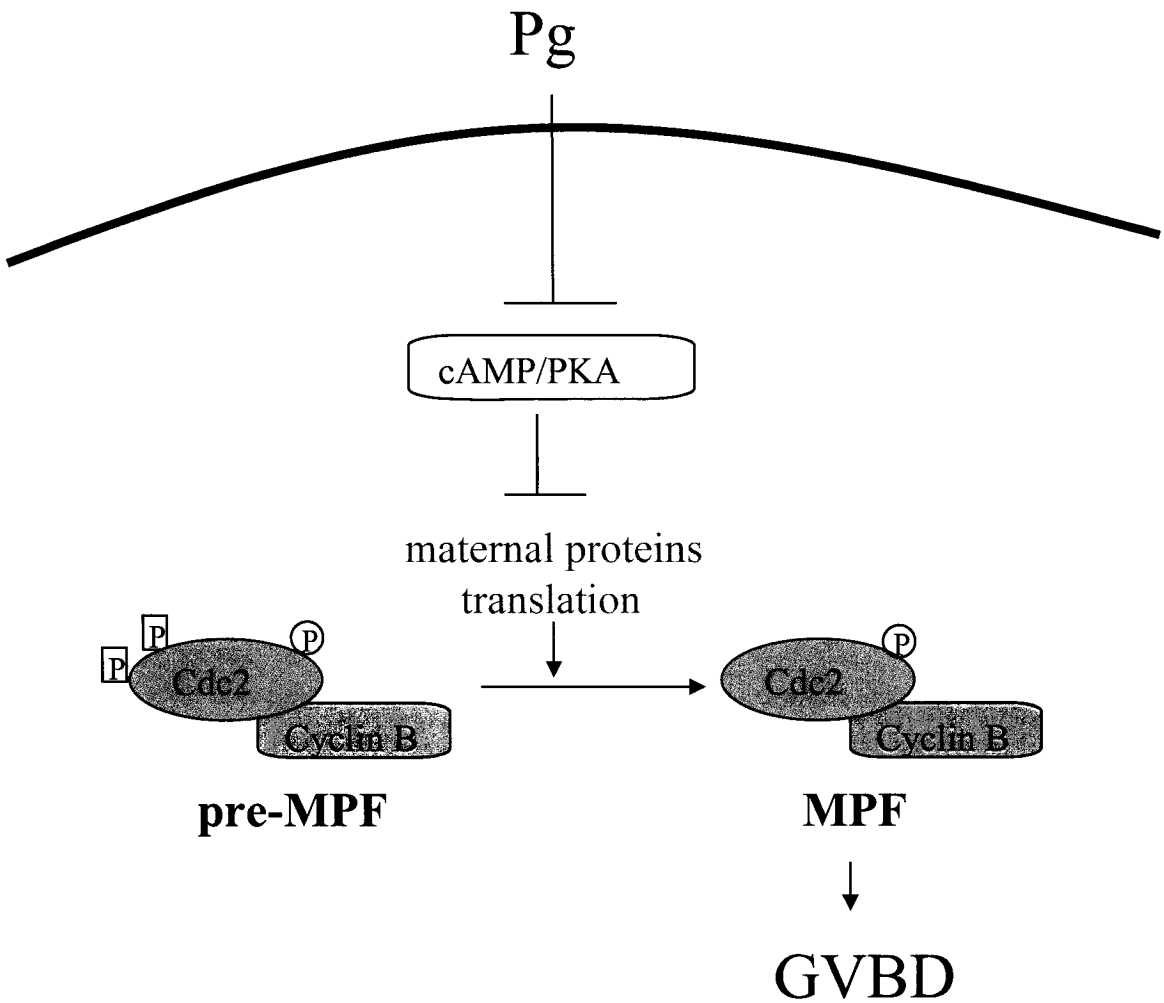

progesterone
fertilization
oogenesis
Stage I
II
III
IV
V
Stage VI
Germinal vesicle
Interphase arrest (G2 prophase)
GVBD (first meiosis)
First meiotic metaphase
Second meiotic metaphase
Fertilization, completion of meiosis II
Prophase I block
Oocyte Maturation
Metaphase II block
MPF
MAPK

Figure 1.2

A simplified model representing progesterone-induced non-genomic action during oocyte maturation. Upon progesterone stimulation of stage VI oocytes, cAMP/PKA signaling is inhibited. PKA inhibition causes increase of protein synthesis and ultimately activates MPF and GVBD.


Pg
cAMP/PKA
maternal proteins
translation
P
P
P
Cdc2
Cyclin B
P
Cdc2
Cyclin B
pre-MPF
MPF
GVBD

I. Nongenomic Progesterone Receptor in frog oocytes

It was demonstrated more than 30 years ago that progesterone can activate MPF in enucleated frog oocytes, or induce GVBD in the presence of the general transcription inhibitor, actinomycin D (Iwashita et al., 1998; Masui and Markert, 1971; Smith and Tenney, 1980; Smith and Ecker, 1969). To account for this "nongenomic" (or transcription independent) action of progesterone in frog oocytes, two possible models are envisaged. The first is that the classical progesterone receptor (PR) has a novel, nongenomic function in the oocytes. The second is that progesterone action is mediated by a novel receptor distinct from PR. Although current evidence favors the involvement of PR, this issue is not completely resolved.

I.1 A nongenomic role for the classical progesterone receptor

Progesterone is synthesized in somatic follicle cells in the ovaries and secreted to the blood. Progesterone exists as two forms in plasma circulation, the free form and in a complex with steroid-binding proteins. Traditionally, it is thought that only free steroids are biologically relevant, whereas the complex forms are inactive because they are unable to enter target cells (Petra, 1991). However, a recent study by Hammes *et al* demonstrates that megalin, an endocytic receptor in reproductive tissues, functions in cellular uptake of steroids that are bound to steroid-binding proteins (Hammes et al., 2005). Either way, after progesterone is transported into the target cell, the biological activites of progesterone are mediated by two isoforms of PR, PR-A and PR-B. The two PRs arise from the same gene with two alternatively spliced mRNA. PR belongs to the nuclear receptor superfamily of transcription factors (Conneely and Lydon, 2000;

Conneely et al., 2001; Lydon et al., 1995). PR consists of a large amino-terminal transcriptional activation domain followed by the DNA binding domain and the carboxyl-terminal hormone-binding domain. Upon progesterone binding, PR dimerizes and then binds to specific progesterone response DNA elements located in the promoter regions of target genes (Gronemeyer, 1991; Tsai and O'Malley, 1994). PR activates transcription by interacting with specific transcriptional coactivators and general transciption factors to form a productive transcription initiation complex on specific target gene promoters (Kamei et al., 1996; Onate et al., 1995).

Recently, work in our lab and Dr. Ruderman's lab strongly suggests that in *Xenopus* oocytes, the classical PR (XPR) is responsible for the nongenomic action of progesterone in the induction of oocyte maturation (Bayaa et al., 2000; Tian et al., 2000). Contrary to previous belief, XPR protein is present in frog oocyte (Tian et al., 2000) and, in response to progesterone stimulation, undergoes hyperphosphorylation (Liu et al., 2005). Over-expression of XPR enhances progesterone-induced oocyte maturation (Bayaa et al., 2000; Tian et al., 2000). Antisence oligos specific for XPR inhibit progesterone-induced oocyte maturation and the inhibition is rescued by subsequent injection of mRNA encoding the cloned XPR (Tian et al., 2000). How XPR activation in the cytoplasm leads to reduction of cAMP remains unknown.

I.2 A membrane bound progesterone receptor in the oocytes?

Zhu *et al* have recently identified a putative membrane-bound progesterone receptor (mPR) in sea trout (Zhu et al., 2003b). mPR appears to be a seven-transmembrane G-protein coupled receptor (GpCR) and regulates pertussis toxin-

sensitive G (Gi, inhibitory G) proteins (Thomas et al., 2002; Zhu et al., 2003a; Zhu et al., 2003b). However, whether mPR is a physiologically relevant progesterone receptor and whether mPR plays any role in progesterone-induced frog oocyte maturation remains unknown. First, it is not known whether mPR is necessary for oocyte maturation in sea trout, or other species. Second, mPR is thought to couple to a pertussis toxin-sensitive Gi and yet it is well established that progesterone-induced frog oocyte maturation is insensitive to pertussis toxin (Sadler et al., 1984; Sheng et al., 2001). Therefore until proven otherwise, XPR remains the only plausible receptor responsible for the nongenomic action of progesterone in the induction of frog oocyte maturation (Bayaa et al., 2000; Hammes, 2004; Tian et al., 2000).

II. cAMP/PKA signaling

The adenosine 3', 5'-cyclic monophosphate (cyclic AMP; cAMP) is perhaps the most important second messenger, functioning to transmit signals from a large number of cell surface receptors (GpCR) to generate diverse cellular responses. Intracellular cAMP levels are controlled by its synthesis from ATP, catalyzed by various adenylyl cyclases (AC), and degradation by cAMP-specific phosphodiesterases (PDE). Although new effectors of cAMP, such as cyclic nucleotide-gated cation channels, have been identified recently (Tasken and Aandahl, 2004), cAMP-dependent protein kinase (PKA) is generally recognized as the primary effector of cAMP.

II.1 G Protein Coupled Receptor (GpCR)

II.1.a Structure, classification and activation of GpCR

GpCRs are an enormous family including over 800 genes (Pierce et al., 2002). The large superfamily mediates a wide variety of biological processes ranging from neurotransmission, hormone control and perception of taste, smell, light and pain. The only structural feature common to all GpCRs is the presence of seven transmembrane-spanning α-helical segments connected by alternating intracellular and extracellular loops, with the amino terminus located on the extracellular side and the carboxyl terminus on the intracellular side. The GpCR can be grouped into three distinct families, A, B and C, based on the sequence similarity among the proteins. Family A is by far the largest group, including the receptors for light (rhodopsin) and adrenaline (adrenergic receptors). Family B contains only about 25 members, including the receptors for gastrointestinal peptide hormone family. Family C is also relatively small and contains the metabotropic glutamate receptor family, the $GABA_B$ receptor and the calcium-sensing receptor (Gether, 2000; Pierce et al., 2002).

The most widely accepted model for GpCR activation is the two-state model (Leff, 1995). The receptor is thought to exist in equilibrium between an inactive conformation (R) and an active conformation (R*) (Samama et al., 1993). In the absence of agonist, the inactive R state is prevailing in most GpCRs; however, a certain fraction of the receptors spontaneously assume the active R* state (Chidiac et al., 1994; Costa and Herz, 1989) due to the low energy barrier between the R and R* state. On the other hand, some GpCRs exhibit 100% active R* state in the absence of ligand, and therefore referred to as constitutively activated (Leurs et al., 1998). An agonist is predicted to bind to the R* conformation with highest affinity and stabilize the R*, hence shifting the

equilibrium to increase the proportion of the receptor in R*. Conversely, inverse agonist (also called negative antagonist), is predicted to stabilize the inactive R state, shifting the equilibrium away from R* and thus inhibiting agonist-independent receptor activity. In this theory, the "R*" form of the receptor interacts with heterotrimeric G proteins and serves as a GEF (guanine nucleotide exchange factor) to promote GDP dissociation from the α subunit and the subsequent GTP binding. Once activated, Gα-GTP dissociates from its Gβγ subunits and from the GpCR. Both Gα-GTP and the free Gβγ can activate, often different, downstream effectors (Samama et al., 1993; Wickman and Clapham, 1995). Hydrolysis of GTP to GDP, a process that is regulated by RGS (regulators of G protein signalling) proteins and the intrinsic GTPase activity of Gα, leads to re-association of the heterotrimer and termination of the activation cycle (De Vries et al., 2000; Ross and Wilkie, 2000).

II.1.b Regulation of GpCR signaling by desensitization, internalization, re-sensitization and degradation pathways

Several mechanisms have evolved for fine-tuning and regulating GpCR signaling. In many cases a time-dependent decrease of the cellular response to the external signal occurs in the continuing presence of the GpCR ligand. This attenuation of signaling is termed desensitization. In principle, desensitization is the consequence of a combination of different mechanisms. These mechanisms include the uncoupling of GpCRs from heterotrimeric G proteins following receptor phosphorylation (Bouvier et al., 1988; Hausdorff et al., 1989; Lohse et al., 1990a); the internalization of the membrane-bound receptors to intracellular compartments (Hermans et al., 1997; Oakley et al., 1999; Trejo and Coughlin, 1999); and the downregulation of the total cellular complement of the

receptors due to receptor degradation and reduced *de novo* synthesis of the receptors protein (Tsao and von Zastrow, 2000; Collins et al., 1991). The time frames, over which these processes occur, range from seconds (phosphorylation) to minutes (endocytosis) and hours (down-regulation).

Classification of desensitization

Rapid dampening of GpCRs function is usually controlled by receptor phosphorylation, which is mediated either by second-messenger-activated kinases (for example, PKA and protein kinase C (PKC)), or by G protein coupled receptor kinases (GRK) (Pitcher et al., 1998a). PKA- and PKC-regulated receptor phosphorylation mediate heterologous desensitization, in which the activated kinase (PKA or PKC) phosphorylates and desensitizes other GpCR (hence heterologous), in addition to the GpCR that activates the kinase in the first place. PKA-mediated receptor phosphorylation has also been shown, in the case of the β_2-adrenergic receptor (β_2AR), to 'switch' receptor coupling from Gs to subsequent coupling to Gi (Daaka et al., 1997; Zamah et al., 2002).

In contrast to the heterologous desensitization, GRK/β-arrestin-mediated GpCR desensitization is termed "homologous" desensitization, as GRKs, whose activation is regulated by GpCR, only phosphorylate the activated (or the R* form) GpCR. GRK-phosphorylation of the receptors is not sufficient to elicit for desensitization, but rather serves to create high affinity binding sites for arrestin proteins, which sterically inhibits further interaction between the receptor and the G protein (Gurevich et al., 1995; Lohse et al., 1990b).

GRKs-regulated desensitization pathway

Seven distinct mammalian GRKs are classified into three subgroups based on their tissue distribution and regulatory properties. 1. Rhodopsin kinase (GRK1) and GRK7 are specifically expressed in retina. 2. β-adrenergic receptor kinases (GRK2 and GRK3) are translocated to the membrane through binding of free Gβγ. 3. GRK4, GRK5 and GRK6 are intrinsic membrane-bound kinases.

Structurally, GRKs contain a central catalytic domain (about 260 AAs) flanked by amino- and carboxyl-terminal regulatory domains. The amino-terminal domain of GRKs, about 185 AAs, is thought to be important for substrate recognition. The amino-terminal region of GRKs also contains a RGS domain. The RGS domain of GRK2 and GRK3 sequesters Gαq protein and functions as a GAP (GTPase activating protein) to inhibit Gαq signaling (Carman et al., 1999; Day et al., 2004; Pitcher et al., 1998a; Sterne-Marr et al., 2004). The carboxyl-terminal domain of GRKs is highly variable in both length and structure. The most prominent function of the carboxyl-termini is to mediate membrane association of the GRKs. Several distinct mechanisms are employed to regulate plasma membrane association of different GRKs through the carboxyl-terminus. GRK1 and GRK7 are targeted to the membrane by farnesylation at CAAX motifs in their carboxyl terminus upon light activation (Chen et al., 2001; Hisatomi et al., 1998; Inglese et al., 1992; Weiss et al., 1998). GRK2 and GRK3 bind to the membrane through a PH domain (pleckstrin homology), which binds phospholipids and membrane-bound free Gβγ subunits (Pitcher et al., 1995; Pitcher et al., 1992). The last 46 residues of GRK5 are predominantly positively charged amino acids and may play a role in GRK5 plasma localization (Kunapuli et al., 1994; Premont et al., 1994). GRK4 and GRK6 are

palmitoylated at a carboxyl terminal cysteine residue to accomplish plasma membrane association (Stoffel et al., 1998; Stoffel et al., 1994).

GRKs phosphorylate both serine and threonine residues localized within either the third intracellular loop or carboxyl-terminal tail of GpCRs (Bouvier et al., 1988; Fredericks et al., 1996; Nakata et al., 1994). Given the large numbers of GpCR (in the hundreds) and a relatively small numbers of GRKs, it is not surprising that each GRK appears to regulate large and overlapping groups of the GpCRs.

The activity of GRKs towards GpCRs is controlled by several factors. Most important among those is the activated conformation of GpCRs, which allosterically activates the enzyme (Chen et al., 1993). Plasma membrane association of GRKs is another important factor in GRK activation. Membrane association dramatically enhances GRK2-mediated phosphorylation of the activated receptor (Pitcher et al., 1995). Plasma membrane localization of GRK1-3 and 7 is dependent on agonist-activation of their membrane-bound receptor targets. On the other hand, GRK4-6 exhibit substantial membrane localization in the absence of GpCR activation. The GRKs activity is also regulated by direct phosphorylation (Kohout and Lefkowitz, 2003) and protein interaction (Ferguson, 2001; Pierce et al., 2002; Pitcher et al., 1998a).

β-arrestin-mediated internalization

To date, four arrestin family members have been identified and can be divided into two groups based on sequence homology, function, and tissue distribution: 1. visual arrestin (Shinohara et al., 1987) and cone arrestin (Craft and Whitmore, 1995) are expressed exclusively in the retina. 2. β-arrestins (β-arrestin 1 and β-arrestin 2) (Attramadal et al., 1992; Lohse et al., 1990b) are ubiquitously expressed outside the

retina, but are most abundantly expressed in neuronal tissues and in the spleen. Arrestins exhibit two distinct domains known as a primary receptor activation recognition domain and a secondary receptor binding site. The two domains are held intact by intramolecular interactions of the amino-terminal and carboxyl-terminal regions. Upon receptor activation, GRK-phosphorylated receptor initially interacts with arrestins, causing the carboxyl-terminal tail of arrestins released from amino-terminal region and eliciting its high affinity binding to the activated receptor (Gurevich and Benovic, 1995; Lefkowitz and Shenoy, 2005). The carboxyl terminus of β-arrestins, but not visual arrestins, contains the clathrin and β2-adaptin-binding region (Krupnick et al., 1997; Laporte et al., 1999).

β-arrestins play a key role in coordinating the various processes that are involved in receptor desensitization and internalization. In response to GpCR activation, plasma membrane-associated β-arrestins directly interact with the agonist-occupied, GRK-phosphorylated GpCR, preventing further G protein binding (initiation of desensitization) (Lohse et al., 1990b). In the same time, the β-arrestins also bind to two major components of the clathrin-coated-pit, the heavy chain of clathrin itself (Goodman et al., 1997; Goodman et al., 1996) and the β2-adaptin subunit of clathrin adaptor protein AP-2 (Laporte et al., 1999). The coordinated interaction of β-arrestins with clathrin, AP-2 as well as phosphoinositides recruits the GpCRs/β-arrestins complex to clathrin-coated pits at the cell surface (Ferguson, 2001; Pierce et al., 2002). These pits are then pinched off from the cell surface by the actions of the large GTPase, dynamin, which is a clathrin-associated monomeric G protein functioning in vesicle scission (Marsh and McMahon, 1999).

In addition to their role in GpCR desensitizastion, β-arrestins have new-found roles as adaptor/scaffolding proteins to recruite additional components to GpCR signaling. For example, β-arrestins interact with c-Src tyrosine kinase to promote tyrosine phosphorylation of dynamin and enhance receptor endocytosis (Ahn et al., 2002; Ahn et al., 1999; Luttrell et al., 1999). β-arrestins also directly interact with MAP kinase (MAPK) cascades components including MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK and bring the activity and subcellular localization of the active MAPK under the control of the receptor (DeFea et al., 2000; Luttrell et al., 2001; McDonald et al., 2000).

Other GpCR internalization pathways

GpCR internalization is also mediated by caveolae, flask-shaped membrane invaginations that are rich in caveolin protein as well as cholesterol (Pierce et al., 2002). Caveolae-mediated GpCR internalization may require dynamin for fission (Henley et al., 1998; Oh et al., 1998). In addition, the caveolae might also function as lipid rafts that compartmentalize different components of GpCR signalings (Smart et al., 1999). Other, dynamin-independent GpCR internalization has been found in angiotensin II1A receptor, M_2-muscarinic receptor and D_2 dopamine receptors (Hosey et al., 1999).

Resensitization and downregulation of GpCR

Endocytosis via clathrin-coated vesicle is not only a general mechanism for GpCR internalization, but also crucial for some GpCRs resensitization, such as β_2AR (Barak et al., 1997). When the receptors proceed from clathrin-coated vesicle to early endosomes, they are dephosphorylated and recycled back to the cell surface, or "re-sensitized". The resensitization starts with a conformational change of the receptor

caused by acidification in the endosomal compartment (Krueger et al., 1997). The conformational change leads to dephosphorylation of their GRK-phosphorylation sites by a membrane-associated GpCR phosphatase (Krueger et al., 1997). In addition, the stability of receptor-β-arrestin complex also regulates the rate of receptor resensitization (Oakley et al., 1999). Some receptors (family A, such as β_2AR), which dissociate from β-arrestin at or near the plasma membrane, are rapidly dephosphorylated and recycled (Morrison et al., 1996; Pippig et al., 1995; Zhang et al., 1997), whereas other receptors (family B, such as the vasopressin type 2 receptor), which remain associated with β-arrestin for longer time period, are slowly resensitized (Fonseca et al., 1995; Innamorati et al., 1998; Oakley et al., 1999).

Prolonged agonist stimulation (hours) results in GpCR down-regulation, defined by an overall decrease in receptor amounts. Down-regulation of the GpCR arises from both degradation of internalized receptors in lysosomes (Tsao and von Zastrow, 2000) and reduction of new receptor synthesis through inhibition of transcription and translation (Collins et al., 1991).

II.2 Heterotrimeric G proteins and their regulation

The α subunit of heterotrimeric G proteins (Gα, Gβ, Gγ) is responsible for binding guanine nucleotides (GDP or GTP) through its Ras-like domain (Hamm, 1998). They are membrane-associated proteins through an isoprenyl group covalently attached to the Gγ subunit and lipid groups attached to the Gα subunit (Forse, 2000; Neer, 1995). The Gβ and Gγ subunits form a dimer that only dissociates when it is denatured (under nonphysiological conditions). So far, 20 Gα, 6 Gβ, and 12 Gγ have been identified. The

heterotrimeric G proteins are historically classified based on sequence homology of their α subunits and the effector systems they regulate. Four distinct G protein subfamilies are recognized: Gs proteins (containing Gαs subfamily) couple to stimulation of adenylyl cyclases; Gi proteins (containing Gαi subfamily) couple to inhibition of adenylyl cyclases; Gq proteins (containing Gαq subfamily) couple to activation of phospholipase Cβ; and G12 proteins (containing Gα12 subfamily) couple to activation of ion exchange, phospholipase-D, c-Src and PKC (Hamm, 1998; Huang et al., 2003; Neves et al., 2002; Simon et al., 1991).

The heterotrimeric G protein acts as a molecular switch. When Gα is bound to GDP, the α subunit associates with the βγ subunits, and remains inactive. When GDP is exchanged for GTP, however, the α subunit dissociates from the βγ subunits and becomes activated to trigger downstream effector functions (Neves et al., 2002). In addition to Gα subunit, the free Gβγ dimmer also initiates intracellular signal transduction. These include the muscarinic K^+ channel, adenylyl cyclases, phospholipase Cβ, phospholipase A_2, phosphoinositide 3-kinase (PI3K) and GRKs. The βγ subunit may also act through Ras to activate MAPK (Schwindinger and Robishaw, 2001; Vanderbeld and Kelly, 2000).

As their name implies, GpCRs are the primary regulators of G proteins. Upon ligand binding, the GpCR acts as a guanine-nucleotide exchange factor (GEF) that promotes the release of GDP from Gα and the subsequent binding of GTP. The active GTP-Gα is released from both the receptor and from its βγ subunits. It is translocated to the cytoplasm (Can et al., 2000; Svoboda and Novotny, 2002; Wedegaertner et al., 1996; Yu and Rasenick, 2002). Following eliciting its cellular function through an effector

molecule, the α subunit, which has intrinsic GTPase activity, hydrolyzes its bound GTP to GDP, often aided by action of a GTPase activating protein (GAP) (see below). The G protein returns to its inactive state, in which the Gα subunit binds to Gβγ and is translocated back to plasma membrane.

In addition to GpCRs, other accessory proteins also regulate heterotrimeric G proteins through influencing guanine nucleotide binding, guanine nucleotide hydrolysis or subunit interaction. Regulators of G-protein signaling (RGS) proteins accelerate the intrinsic GTPase-activity of Gα subunit and hence are referred to as GAPs, Gα inhibitors. These proteins share the conserved structure designated as "RGS domain", which is responsible for accelerating GTPase activity (Ishii and Kurachi, 2003; Ross and Wilkie, 2000; Wieland and Chen, 1999). Whether each α subunit has a strict RGS specificity remains to be established.

Activators of G protein signaling (AGS) refer to a functionally defined group of proteins that activate heterotrimeric G protein signaling independently of GpCR. AGS1 activates heterotrimeric G proteins by increasing GDP/GTP exchange on the Gα subunit, therefore mimicking an activated GpCR. AGS2 interacts with Gβγ subunit to actively promote subunits dissociation independently of nucleotide exchange, while AGS3 actively promote subunit dissociation by "stabilizing" GDP-Gα, preventing the association of GDP-Gα with Gβγ and increase the duration of Gβγ signaling (Blumer and Lanier, 1905; Cismowski et al., 2001). In the last few years, it also has become apparent that a large number of receptors with a single transmembrane domain, such as some receptor tyrosine kinases, can associate with and/or activate heterotrimeric G proteins

(Patel, 2004). Therefore, seven-transmembrane receptors are no longer the only receptors that "couple" to heterotrimeric G proteins.

II.3 Adenylyl cyclases and phosphodiesterases

Molecular cloning has identified nine membrane-bound mammalian ACs (Cooper, 2003), which are responsive to heterotrimeric G protein activation. A typical adenylyl cyclase contains two tandom repeats of catalytic domains, each functioning in catalyzing conversion from ATP to cAMP. All the AC isoforms exhibit a basal activity that is enhanced upon binding of the Gαs-GTP and reduced upon binding of the Gαi-GTP (Sunahara et al., 1996). Gβγ subunits may also modulate the activity of some ACs (Avidor-Reiss et al., 1997; Choi et al., 1993; Clapham and Neer, 1997; Sunahara et al., 1996; Tang and Gilman, 1991; Taussig et al., 1993). For example, in *Xenopus* oocytes, AC7 is thought to be stimulated by both Gαs-GTP and Gβγ subunits cooperately (Guzman et al., 2005; Sheng et al., 2005a).

PDEs are enzymes responsible for the hydrolysis of cyclic nucleotides. They play an important and highly regulated role in controlling the resting state levels of cAMP or cGMP, intracellularly. Two families of PDEs, PDE3 and PDE4, are known as the major PDE to downregulate cAMP level in the cells. cAMP hydrolysis in mouse and rat oocytes is primarily accomplished by PDE3A (Richard et al., 2001; Shakur et al., 1905; Shitsukawa et al., 2001; Tsafriri et al., 1996). Although endogenous PDE activity does not appear to change during *Xenopus* oocyte maturation, inhibitors of PDE3 block progesterone-induced oocyte maturation (Sadler and Maller, 1987). These results suggest that PDE3 function is necessary in oocyte cAMP metabolism. On the other hand,

endogenous membrane-bound AC activity is reduced during the maturation process (Finidori-Lepicard et al., 1981; Sadler and Maller, 1981), suggesting that in *Xenopus* oocytes, progesterone reduces cAMP level probably through downregulation of AC but not upregulation of PDE.

II.4 Protein kinase A (PKA)

II.4.a Structure and functional regulation of PKA

The PKA holoenzyme consists of two catalytic subunits (PKAc) bound noncovalently to a regulatory subunit (PKAr) dimer (Taylor et al., 1990). There are two subfamilies of PKAr subunits, PKArI (PKArIα, PKArIβ), PKArII (PKArIIα, PKArIIβ) as well as four PKAc subunits, PKAcα, PKAcβ, PKAcγ and PRKX (the human X chromosome-encoded protein kinase X) (Tasken and Aandahl, 2004). Two major functionally distinct isozymes, designated type I and II PKA, are recognized. This classification is based on the regulatory subunits, PKArI and PKArII, as PKAr is the cAMP-binding subunit and is therefore functionally important (see below). In *Xenopus* oocytes, two subtypes of PKAc, which are homologues to mammalian PKAcα and PKAcβ, have been cloned (Schmitt and Nebreda, 2002). Also, type II PKA appears to be the major PKA in frog oocytes (Schmitt and Nebreda, 2002).

In the absence of cAMP binding, the PKAr subunits associate with and inactivate PKAc subunits. The PKAr subunit contains two tandem, gene-duplicated cAMP-binding sequences at their carboxyl terminus (Takio et al., 1984; Titani et al., 1984), and protein interaction regions at the amino-terminus (Taylor et al., 1990). Kinetic evidence indicates that cAMP binding to one site stimulates binding at the other site, in a manner

of positive cooperativity (Francis and Corbin, 1994; Taylor et al., 1990). In addition, the PKAr subunits exhibit different cAMP binding affinities, giving rise to PKA holoenzymes with different thresholds of cAMP for activation. Activation constant (Kact) of type II PKA is 200-400nM of cAMP, while type I PKA has higher affinity with Kact of 50-100nM of cAMP (Tasken and Aandahl, 2004). Once PKAr binds cAMP, the dimer dissociates from PKAc. Thus, monomeric PKAc becomes active.

The first 40 residues at the amino terminus of PKAr are responsible for PKAr dimer formation (Leon et al., 1997; Potter et al., 1978; Rannels and Corbin, 1980). The proteolytically sensitive "hinge region", generally lying between residues 90 and 100 in the PKAr, is essential for making contact with the PKAc subunit. The hinge region contains amino acid sequences that are similar to the PKAc subunit substrate motif (RRXS) for PKArII or pseudosubstrate motif (RRXA(G)) for PKArI. Hereby, the hinge region of PKAr occupies the substrate-binding site on PKAc, making it inaccessible to other potential PKAc substrates (Taylor et al., 1990).

The eukaryotic PKAc isoforms contain the conserved catalytic core shared by all protein kinases. The conserved triad of glycine, Gly50, 52 and 55, together with Lys72, form the major part of the ATP-binding site. The anionic sites in the carboxyl-terminus of the catalytic core are designated for recognizing the arginine residues in the RRXS substrate motif (Francis and Corbin, 1994; Taylor et al., 1990). The heat stable protein inhibitor of PKA (PKI) contains a PKA pseudosubstrate motif (RRXA) and is a highly selective and potent competitive inhibitor of PKAcα and PKAcβ function, but not of PKAcγ (Francis and Corbin, 1994). Another important regulator of PKA is a family of proteins called A kinase anchoring proteins (AKAPs). All the AKAPs contain PKA-

binding tether domain, which interacts with the dimerized region of PKAr dimer, and a unique targeting domain directing the PKA-AKAP complex into defined subcellular structures, membrane and organelle (Tasken and Aandahl, 2004). In addition to the two domains, some AKAPs are also able to form multivalent signal transduction complex by interaction with phosphatases as well as other kinases and proteins involved in signal introduction (Tasken and Aandahl, 2004; Wong and Scott, 2004).

II.4.b cAMP/PKA signalling in oocyte maturation

It has long been established that cAMP and PKA play important roles in maintaining vertebrate oocyte prophase arrest. Injection of PKAr or PKI is sufficient to cause hormone-independent oocyte maturation in *Xenopus*, presumably by inhibiting endogenous PKAc. Conversely, artificially maintaining high levels of cAMP or injection of PKAc inhibits progesterone-induced oocyte maturation in *Xenopus* (Huchon et al., 1981; Maller and Krebs, 1977; Sadler and Maller, 1987). Although it is clear that cAMP and PKA play dominant roles in prophase arrest, the mechanisms through which PKA maintains prophase arrest are still poorly understood. One issue is the timing of progesterone-induced cAMP reduction in frog oocytes. While some investigators have concluded that progesterone induces a rapid and transient reduction of cAMP (Maller et al., 1979), others have reported more persistent inhibition of the cAMP pathway (Bravo et al., 1978). In addition, the reported 20% reduction of cAMP cannot account for inhibition of PKA required for oocyte maturation, as the basal cAMP in prophase oocytes is about 1 μM (Maller et al., 1979; Masui and Markert, 1971). It therefore seems likely that physiological relevant reduction of cAMP in frog oocytes is perhaps spatially restricted, as has been demonstrated in muscle cells (Zaccolo and Pozzan, 2002).

The other important and unresolved issue is the physiological substrate profile of PKA in frog oocytes. Recently, Duckworth *et al* have identified the dual specificity phosphatase Xcdc25C as a direct substrate for PKA in frog ooytes (Duckworth et al., 2002). Xcdc25C dephosphorylates and activates cdc2, the catalytic subunit of MPF. Therefore it is thought that PKA phosphorylates Xcdc25C at Ser287 and inhibits its phosphatase activity toward cdc2 (Duckworth et al., 2002). Interestingly, a recent study has added mouse wee1B as a physiological substrate of PKA. Wee1B is a protein kinase that phosphorylates and inhibits cdc2. The PKA phosphorylation at Ser15 in the mouse wee1B enhances the activity of mouse wee1B to inhibit cdc2. These findings therefore suggest that PKA maintain meiotic prophase arrest via dual regulation of the kinase (wee1B) that phosphorylates cdc2 and the phosphatase (cdc25C) that dephosphorylates the same sites (Han et al., 2005). Although both Xcdc25C and wee1B appear to be good candidate substrates for PKA in the oocytes, other substrates must exist to explain the tempo differences between cAMP reduction (early) and activation of MPF (late), as well as the involvement of *de novo* protein synthesis during oocyte maturation (see below).

III. Protein translation

III.1 Translational requirement for GVBD induction

Although fully-grown frog oocytes are transcriptionally inactive during oocyte maturation, these oocytes are very active in protein translation. It's been known for a long time that a protein synthesis inhibitor, cycloheximide, blocks progesterone-induced maturation in *Xenopus* oocyte (Brachet et al., 1975), indicating that *de novo* protein synthesis is necessary for the initiation of meiotic maturation. It is also known that the

addition of cycloheximide at different time points during oocyte maturation causes different phenotypic changes, suggesting that multiple translational events at multiple time points are required for proper oocyte maturation (Furuno et al., 1994; Huchon et al., 1993).

III.1.a Translational regulation mechanism leading to GVBD induction

Cytoplasmic polyadenylation appears to be a major translational control mechanism in the oocytes. Inhibition of the cytoplasmic polyadenylation blocks not only *de novo* protein synthesis but also progesterone-induced oocyte maturation (Kuge and Inoue, 1992; McGrew et al., 1989). Generally, the maternally inherited messenger RNAs (mRNAs) contain short poly (A) tail (20-40 nucleotides long) and are translationally dormant in prophase-arrested oocytes. Selected mRNAs are polyadenylated (to 150 nucleotides) in the cytoplasm and occur at various stages of oocyte maturation (Barkoff et al., 1998; Hake and Richter, 1997; McGrew et al., 1989; Mendez and Richter, 2001; Sheets et al., 1994). In *Xenopus* oocytes, two cis elements in the 3'-untranslated region (UTR) of these mRNAs direct the cytoplasmic polyadenylation. They are termed the cytoplasmic polyadenylation element (CPE) and the polyadenylation hexanucleotide AAUAAA (Fox et al., 1989; McGrew et al., 1989), respectively. The CPE is bound by cytoplasmic polyadenylation element binding protein (CPEB), a highly conserved ZINC FINGER- and RNA recognition motif type RNA-binding protein (Hake and Richter, 1994; Hake et al., 1998; Paris et al., 1991), while the hexanucleotide is bound by a cleavage and polyadenylation specific factor (CPSF) (Bilger et al., 1994; Dickson et al., 1999). The instigation of cytoplasmic polyadenylation by the 3'-UTR requires the serine/threonine protein kinase Eg2, an enzyme activated by progesterone in the *Xenopus*

oocytes (Andresson and Ruderman, 1998; Frank-Vaillant et al., 2000). Eg2, a member of the Aurora family of serine/threonine protein kinase, phosphorylates CPEB at serine 174 (Mendez et al., 2000a) to enhance CPSF binding to the hexanucleotide. The stabilization of CPSF on the AAUAAA sequence (Mendez et al., 2000b), in turn, attracts poly (A) polymerase to the end of the mRNA, resulting in the elongation of poly(A)-tail to initiate translation.

In addition, CPEB is involved in translation repression (masking) in prophase-arrested oocytes. In immature oocytes, CPE-bound CPEB interacts with an additional factor, maskin. Maskin, in turn, binds eukaryotic translation initiation factor 4E(eIF4E) at the same region for eukaryotic translation initiation factor 4G (eIF4G) binding, thereby preventing formation of initial translation complex (eIF4E-eIF4G-eIF4A) on CPE-containing mRNA. Following progesterone-induced oocyte maturation, the CPEB-dependent polyadenylation help eIF4G to displace maskin from eIF4E, thereby stimulating translation (Stebbins-Boaz et al., 1999).

III.1.b Mos/MEK/MAPK signaling in GVBD induction

Mos is a germ cell specific serine/threonine protein kinase. Mos functions as MAP kinase kinase kinase, phosphorylating and activating MEK1 (MAP kinase kinase). MEK1 is a dual-specificity kinase that activates Erk1/2 MAPK by tyrosine and threonine phosphorylation within their activation loop (Karin, 1998). Indeed, MEK1 phosphorylates the only Erk MAPK in frog ocoytes, p42 MAPK, at its Thr183 and Tyr185 and activates it. Mos protein is undetectable in prophase-arrested oocytes and *de novo* synthesis of mos is one of the biochemical hallmarks of oocyte maturation (Sagata et al., 1988; Sagata et al., 1989; Sheets et al., 1994). *De novo* synthesis of mos is an early

event stimulated by progesterone. However, *de novo* synthesis of mos is also subjected to a positive feedback mechanism that depends on the activation of MAPK and MPF (Matten et al., 1996). This unique feature of the Mos-MEK1-MAPK pathway renders MAPK activation essentially an all-or-none biochemical event during *Xenopus* oocyte maturation (Ferrell and Machleder, 1998).

De novo synthesis of mos is required for activation of MAPK in the ocoytes (Dupre et al., 2002a; Verlhac et al., 2000; Verlhac et al., 1996). However, the early assertion that *de novo* synthesis of mos is absolutely required for MPF activation and GVBD (Sagata et al., 1988) is a subject of recent debate. Inhibition of mos synthesis by morpholino antisense oligonucleotides effectively inhibits *de novo* synthesis of mos but only delays, not inhibits, MPF activation and GVBD induction (Dupre et al., 2002a). Consistent with this, oocytes also undergo MPF activation and maturation upon progesterone stimulation in the absence of MEK1 or MAPK activation (Bodart et al., 2002; Fisher et al., 1999; Gross et al., 2000). It appears that the phosphorothioate antisense oligos used in previous studies (Sagata et al., 1988) might have undesired toxic effect, in addition to the inhibition of mos protein translation.

III.1.c Regulation of MPF activity in GVBD induction

MPF was first found (Masui and Markert, 1971) as an activity present in the cytoplasm from mature frog oocytes. Microinjection of MPF into immature oocytes induces precocious maturation in the absence of progesterone and protein synthesis. MPF activity also appears in cleaving embryos concomitantly with metaphase initiation (Gerhart et al., 1984; Wasserman and Smith, 1978), suggesting that MPF is a universal regulator of the G2/M transition and might have evolutionarily conserved components.

Xenopus MPF was biochemically purified and shown to be a dimer containing the vertebrate $p34^{Cdc2}$ serine/threonine protein kinase and a regulatory 45 KD protein, cyclin B (Lohka et al., 1988). Cdc kinases, also known as cyclin dependent kinases (Cdks), regulate the progression of cell division. Cdks interact with cyclins, whose protein levels oscillate during cell cycle, to regulate the cyclic entry and exit from different phases in the cell cycle (Morgan, 1995).

Immature *Xenopus* oocytes contain inactive MPF (also called pre-MPF). The pre-MPF is a complex of cyclin B and cdc2, with the latter triphosphorylated on Thr14, Tyr15 and Thr161 (De Smedt et al., 2002). Thr161 phosphorylation is required for MPF activity, and appears to be constitutively phosphorylated in immature oocytes (Yamashita, 1998). Phosphorylation of Thr14/Tyr15 by the wee1 and myt1 kinases inhibits MPF (Morgan, 1995), while dephosphorylation of Thr14/Tyr15 by the dual specificity phosphatase cdc25C (Gautier et al., 1991; Yamashita, 1998) promotes MPF activity.

The activity of cdc25C is regulated by multiple phosphorylation and dephosphorylation events and through the binding of other proteins. Cdc25C has a low level of phosphatase activity during interphase of the cell cycle (Izumi et al., 1992; Kumagai and Dunphy, 1992) and this form is phosphorylated at Ser216 in human cdc25C (Ser287 in *Xenopus* cdc25C (Xcdc25C)) (Kumagai et al., 1998; Ogg et al., 1994; Peng et al., 1997). Ser287 lies in a consensus 14-3-3 recognition motif. The binding of 14-3-3 to the phospo-Ser287 residue of Xcdc25C in prophase oocytes protects Xcdc25C from premature dephosphorylation by protein phosphatase 1 (PP1) (Margolis et al., 2003).

In addition to the negative regulation by PKA-catalyzed Ser287 phosphorylation, cdc25C is also regulated by positive phosphorylation. Accompanying Ser287 dephosphorylation and activation, Xcdc25C also undergoes a significant shift in electrophoretic mobility due to extensive phosphorylation on other Ser/Thr residues (Perdiguero and Nebreda, 2004). Several kinases have been reported to phosphorylate and activate cdc25C. Polo-like kinase (Plx1) is identified as an initial triggering kinase to phosphorylate and activate Xcdc25C at multiple sites prior to MPF activation (Kumagai and Dunphy, 1996; Qian et al., 1998; Qian et al., 2001). Plx1 is activated by an upstream kinase, Plkk1, suggesting a kinase cascade may be involved in the activation of cdc25C (Karaiskou et al., 1999). A member of the *Xenopus* MAPK family, Xp38γ/SAPK3, has been recently reported to phosphorylate Ser205 of Xcdc25C, which might create a Plx1 recognition motif (Perdiguero and Nebreda, 2004). Once Xcdc25C is phosphorylated by Xp38γ and Plx1, it dephosphorylates and actives pre-MPF in the oocyte. Interestingly, activated MPF also contributes to Xcdc25C activation by phosphorylating at least 3 residues: Thr48, Thr67, and Ser285 in Xcdc25C, thus triggering a MPF auto-amplification (Izumi and Maller, 1993; Perdiguero and Nebreda, 2004). All these phosphorylated residues on Xcdc25C (by Plx1 and by MPF) can be dephosphorylated by protein phosphatase 2A (PP2A) (Karaiskou et al., 1999). Hence, okadaic acids (OA), when used in concentrations that specifically inhibit PP2A activity, triggers oocyte maturation in the absence of progesterone stimulation (Perdiguero and Nebreda, 2004).

The prototype of the cdc2 inhibitory kinases is wee1, the negative regulator of mitosis in *Schizosaccharomyces pombe.* A wee1 homologue kinase in *Xenopus*, myt1, has been cloned and reported to be present in immature oocyte (Mueller et al., 1995b;

Murakami and Vande, 1998). Thus, myt1 is recognized as the inhibitory kinase to phosphorylate cdc2 at Thr14 and Tyr15 and keep pre-MPF inactive in prophase-arrested oocytes (Mueller et al., 1995b). Just prior to pre-MPF activation, myt1 becomes extensively phosphorylated and inactivated by $p90^{rsk}$, a protein kinase that can be phosphorylated and activated by p42 MAPK (Mueller et al., 1995a; Mueller et al., 1995b; Palmer et al., 1998). This demonstrates a link between the activation of p42 MAPK and the subsequent activation of MPF, participating in the initial activation and maintenance of MPF activity.

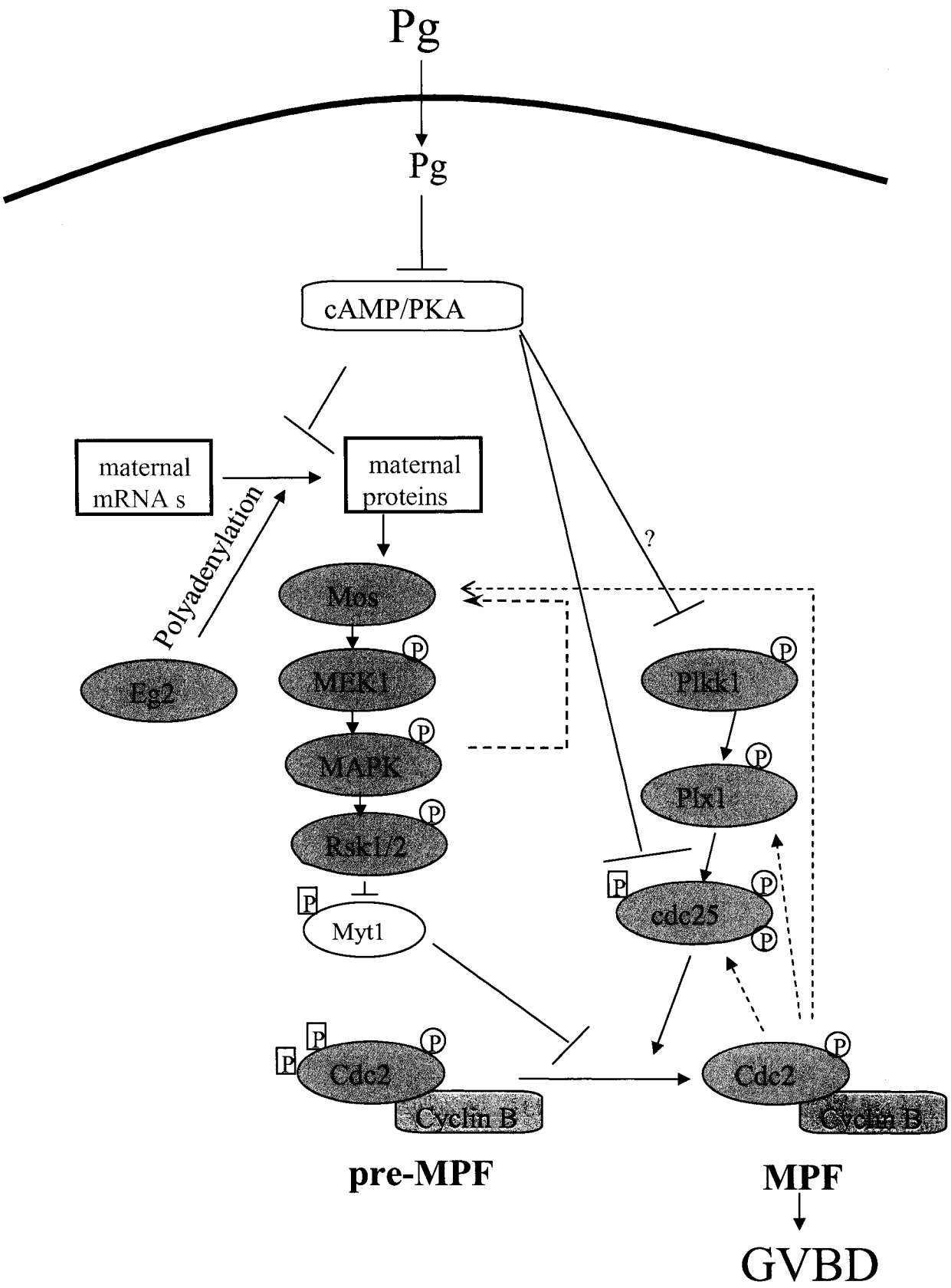
Like MAPK, MPF activation also exhibits an all-or-none feature. This is the result of extensive positive feedback mechanism (Figure. 1.3) (Ferrell, 1999). In addition to the activity of MPF to stimulate mos synthesis, MPF also has the ability to directly phosphorylate and activate cdc25C (Izumi and Maller, 1993) as well as to activate Plx1 (Abrieu et al., 1998). These different levels of positive feedback loops contribute to the abrupt transition of MPF activity during oocyte maturation.

III.2 Translational Requirement for proper completion of meiosis I and entry into meiosis II

The presence of cycloheximide before GVBD prevents progesterone-induced GVBD, indicating that *de novo* protein synthesis is required for GVBD. *De novo* protein synthesis is also necessary for the proper completion of oocyte maturation following GVBD, as cycloheximide, when given at or shortly after GVBD, causes metaphase I spindle disorganized and the oocytes rapidly form "nuclear-like structures" containing decondensed chromosome (Hochegger et al., 2001; Huchon et al., 1993; Wasserman and

Figure 1.3

The complex signaling pathways leading to MPF activation. Upon progesterone stimulation of stage VI oocytes, reduction of cAMP/PKA signaling activates translation of maternal proteins including mos protein. The translation of the maternal proteins is also regulated by Eg2, which facilitates mRNA cytoplasmic polyadenylation process. The translated mos protein activates the p42 MAPK pathway, which through Rsk and Myt1, activates MPF. In the absence of mos synthesis, other proteins proposed to be involved in activation of MPF include Plkk1, Plx1 and cdc25c. Cdc25c also has been suggested to be directly and negatively regulated by cAMP/PKA signaling. The dot lines in the figure indicate multi-layer positive feedback pathways involved in abrupt activation of MAPK and MPF.


Pg
Pg
cAMP/PKA
maternal mRNA s
maternal proteins
Polyadenylation
Eg2
Mos
MEK1
MAPK
Rsk1/2
Myt1
?
Plkk1
Plx1
cdc25
Cdc2
Cyclin B
Cdc2
Cyclin B
pre-MPF
MPF
GVBD

Masui, 1975). The newly synthesized proteins include mos protein, B type cyclins and other not yet identified proteins.

Although *de novo* synthesis of mos protein is dispensable for MPF activation and GVBD, it is required to prevent DNA replication after meiosis I and progress through meiosis II until metaphase II-arrest. Therefore, inhibition of mos synthesis by morpholino antisense oligonucleotides following GVBD causes the oocytes entering a series of embryonic-like cell cycle accompanied by oscillation of cdc2 activity and DNA synthesis (Dupre et al., 2002a). In this regard, mos serve as a cytostatic factor to prevent mitotic cell cycle of female gamete until fertilization (Colledge et al., 1994; Dupre et al., 2002a; Hashimoto et al., 1994; Sadler and Ruderman, 1998). Consistent with this, inhibition of other MAPK cascade components, MEK1/MAPK, causes a similar phenomenon (Gross et al., 2000).

Following GVBD, cyclin B is partially degraded (Gross et al., 2000), causing a partial inhibition of MPF. Afterward, re-synthesis of cyclin B and reactivation of MPF is necessary for the oocyte to progress into meiosis II until metaphase II-arrest stage (Hochegger et al., 2001; Taieb et al., 2001). However, whether partial degradation of cyclin B is involved in meiosis I exit and progress into meiosis II remains controversial (Hampl and Eppig, 1995; Herbert et al., 2003; Iwabuchi et al., 2000; Taieb et al., 2001).

Chapter Two

Probing G Protein Coupled Receptor Signaling in Frog Oocytes

INTRODUCTION

One important signaling pathway regulated by progesterone is the reduction of intracellular cAMP during *Xenopus* oocyte maturation. It is generally agreed that progesterone induces a modest (20%) reduction of cAMP (Smith, 1989), likely by inhibiting membrane-bound adenylyl cyclases (Finidori-Lepicard et al., 1981; Sadler and Maller, 1981). The importance of cAMP reduction is underlined by the demonstration that both forskolin (Schorderet-Slatkine and Baulieu, 1982) and isobutylmethylxanthine (IBMX) (Sadler and Maller, 1987) block progesterone-induced oocyte maturation. However, the classical Gi inhibitor *Bordetella pertussis toxin* does not prevent progesterone from inhibiting adenylyl cyclase or inducing oocyte maturation (Sadler et al., 1984; Sheng et al., 2001), suggesting that progesterone action is not mediated by the activation of classical Gi proteins. This puzzle appears to be resolved by the finding that inhibition of endogenous adenylyl cyclase-activating G proteins causes spontaneous oocyte maturation. Jaffe and colleagues demonstrated, first in the frog (Gallo et al., 1995; Kalinowski et al., 2004) and later in mice (Mehlmann et al., 2002), that injection of neutralizing antibodies against mammalian Gsα causes oocyte maturation, suggesting that endogenous *Xenopus* Gsα plays a dominant role in maintaining prophase arrest. On the other hand, work in our lab demonstrated that inhibition of endogenous G protein βγ subunits lowers oocyte cAMP and induces oocyte maturation (Sheng et al., 2001). Furthermore, overexpression of Gβγ subunits increases oocyte cAMP (Sheng et al., 2001) and inhibits progesterone-induced oocyte maturation (Lutz et al., 2000; Sheng et al., 2001). Together these studies suggest the existence of an activated G protein(s) in prophase oocytes that maintains high levels of cAMP and prophase arrest. We postulated

that this G protein(s) is activated by an endogenous G protein-coupled receptor (GpCR) that is activated in prophase oocytes (Sheng et al., 2001).

In this chapter, I wished to explore a well-characterized aspect of GpCR signaling, receptor desensitization, to validate the existence and function of the putative GpCR in maintaining prophase arrest in frog oocytes. A general mechanism governing GpCR desensitization is regulated through the GRK/β-arrestin pathway (Claing et al., 2002). I reasoned that overexpression of GRKs and β-arrestin in frog oocytes may disrupt the tonic GpCR signaling in prophase oocytes by causing GpCR desensitization and endocytosis. Such interventions may therefore cause spontaneous oocyte maturation. Indeed, injection of mRNA for rat GRK3 caused hormone-independent GVBD. The kinase activity of GRK3 was essential for GVBD induction as its kinase-dead mutant (GRK3-K220N) was completely ineffective. Another GRK3 mutant (GRK3-ΔC), which lacked the carboxyl-terminal Gβγ-binding domain and which was not associated with oocyte membranes, also failed to induce GVBD. Furthermore, injection of rat β-arrestin-2 mRNA also induced hormone-independent GVBD. Finally, I showed that blocking clathrin-mediated endocytosis prevented GRK/β-arrestin from inducing GVBD.

MATERIALS and METHODS:

Materials

Monodansylcadaverine (MDC), Concanavalin A (Con A) and Methyl-β-cyclodextrin (MβCD) were purchased from Sigma. β-casein, myelin basic protein

(MBP), histone 1 (H1) and forskolin (Fsk) were also purchased from Sigma. Radioactive compounds were from Amersham. Antibody against mos was purchased from Santa-Cruz. Antibody against p-MAPK was from Upstate Biotechnology. Antibody against p42 *Xenopus* MAPK was provided by Dr. Jonathan Cooper.

Oocyte isolation and mRNA injection

Sexually mature, oocyte positive *Xenopus laevis* were purchased from NASCO and maintained according to local animal care guidelines. Ovaries were surgically removed immediately after euthanasia of the female *Xenopus* laevis 3-7days after gonadotropin priming (pregnant mare serum gonadotropin, 50IU per frog). Stage VI oocytes were selected and manually isolated. The fully-grown stage VI oocytes were incubated and injected in OR2 medium minus Ca^{++} (83mM NaCI, 2.5mM KCI, 1mM $CaCI_2$, 1mM $MgCl_2$, 1mM Na_2HPO_4, 5mM HEPES [pH 7.8]).

mRNAs were generated through *in vitro* transcription using linearized plasmid DNAs as templates and the MessageMachine kit with Sp6 polymerase (Ambion, Texas). Typically, oocytes were microinjected with 20-30nl of mRNA (10ng per oocyte, unless otherwise indicated) and incubated overnight at 18°C in OR2 containing (100μg/ml) gentamycin and (1mM) Ca^{2+} to allow protein expression.

cDNA constructs

The nucleotide sequence encoding full-length rat GRK3 (Arriza et al., 1992) (a gift from Dr. Robert Lefkowitz) was PCR-amplified using the following primer: forward primer, 5'-TAT AGG CCT GCC ATG GCG GAC CTG GAG G- 3'; reverse primer, 5'-

TAT AGG CCT CAG AGG CCG CTG CTA TTT CTG- 3'. The amplified DNA was digested with StuI, and ligated into StuI-digested pCS2+ (Turner and Weintraub, 1994) or pCS2+HA (Booth et al., 2002) vectors. The resultant plasmid encoded, respectively, wildtype untagged or HA-tagged GRK3.

The kinase deficient mutants GRK3-K220N was constructed as the following steps: 1. Two segments of PCR products from GRK3 were amplified using the same forward and reverse primers (above), in combination with the following internal primers changing the catalytically essential Lys(K)-220 to Asn (N); reverse primer, 5'-AA GGA ATT CAT GGC ATA CAT TTT CCC GGT- 3'; forward primer, 5'- C ATG AAT TCC TTA GAC AAG AAG AGA GTG AAG- 3', 2. Both PCR products were then digested with EcoRI and StuI and ligated together with StuI-predigested vectors, either pCS2+ or pCS2+HA, forming constructs GRK3-K220N or HA-GRK3-K220N, respectively.

A C-terminally truncated GRK, GRK3-ΔC (containing residues 1-545 AAs (Arriza et al., 1992)) were PCR-amplified using the following primers: forward primer, 5'-TAT AGG CCT GCC ATG GCG GAC CTG GAG G- 3'; reverse primer, 5'-AGG CCT TCA TTT ATT TTT AGC CTT CTT CCT GGC- 3'. The amplified DNA was digested with stuI and then ligated into the pCS2+HA that had been previously digested with StuI. These manipulations resulted in HA-GRK3-ΔC.

Rat β-arrestin-2 (Attramadal et al., 1992) (a gift of Dr. Robert Lefkowitz) was PCR-amplified using the following primers: forward primer, 5'-TAT CCA TGG GTG AAA AAC CCG GGA CC- 3'; reverse primer, 5'-TAT CCA TGG CAG AAC TGG TCA TCA CAG TC- 3'. The amplified DNA was digested with NcoI (limited digestion was necessary due to the presence of an NcoI within the coding sequence of β-arrestin-2),

and ligated into pCS2+HA that had previously been digested with NcoI. The carboxyl-terminal clathrin-binding domain of β-arrestin-2 (residues 319-410 AAs) (Krupnick et al., 1997) was PCR-amplified using the same reverse primer (above) and the following forward primer, 5'-TAT CCA TGG GAA TCC TAG TAT CCT AC- 3'. The amplified cDNA was digested and ligated into pCS2+HA, as described above for full-length β-arrestin-2. The resultant plasmid encoded HA-β-arrestin2-C.

Schematic representation of the above plasmid cDNA constructs without HA tag were shown in Figure 2.1.

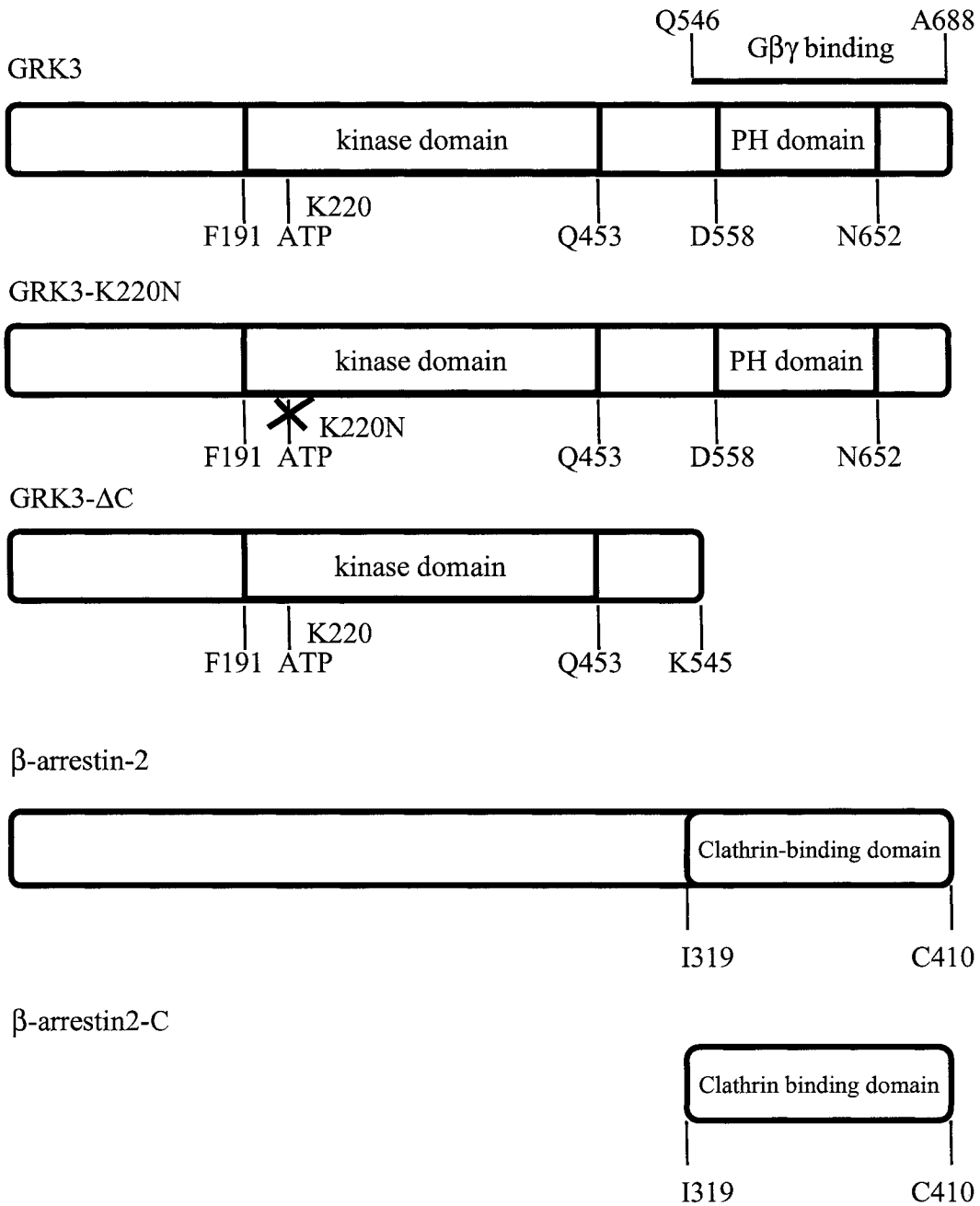
Western blotting

Oocytes were crushed through pipette tip in ice-cold MPF assay buffer (20mM Hepes, 80mM glycerophosphate, 20mM EGTA, 15mM $MgCl_2$, 1mM dithiothreitol (DTT), 10μM ATP, 0.15mM NaF, 10μg/mL leupeptin, 200μM phenylmethylsulfonyl fluoride (PMSF), 25μg/mL benzamadine; 10μL/oocyte). Following centrifugation at 13,500rpm×5min, the supernatants were mixed with 2×Laemmli sample buffer (250mM Tris-HCl, pH 6.8; 20% glycerol; 10% β-mercaptoethanol; 6% SDS; 0.04% bromophenol blue) for electrophoresis. Proteins were separated using SDS polyacrylamide gels (15%) electrophoresis (SDS-PAGE), transferred to polyvinylidene difluoride (PVDF) membrane (Schleicher & Schuell, Germany), and immunoblotted with the indicated primary antibodies. The immunoblots were then developed using chemiluminescence system (Amersham Bioscience, UK).

Preparation of total oocytes extracts, total membrane and cytosol fractions

Figure 2.1

Schematic representation of plasmid constructs used in this study. Details are presented under "Materials and Methods." The N-terminal HA tag is not represented here. The boundaries of the identified domains are marked by single-letter codes of amino acids and their positions. PH, pleckstrin homology.


GRK3
Q546
A688
Gβγ binding
kinase domain
PH domain
F191
ATP
K220
Q453
D558
N652
GRK3-K220N
kinase domain
PH domain
F191
ATP
K220N
Q453
D558
N652
GRK3-ΔC
kinase domain
F191
ATP
K220
Q453
K545
β-arrestin-2
Clathrin-binding domain
I319
C410
β-arrestin2-C
Clathrin binding domain
I319
C410

Oocytes were lysed in detergent-free homogenization buffer (10mM Hepes, 83mM NaCI, pH 7.9) (Sheng et al., 2001) (250μL for 15 oocyte). Following low-speed (900rcf×15min at 4°C) centrifugation, the clarified supernatants were subjected to high-speed centrifugation (100,000rcf×60min at 4°C). The pellets (total membrane fractions) following high-speed (100,000rcf×60 min at 4°C) centrifugation were directly lysed in 2×Laemmli sample buffer. The aliquots of supernatants from both low-speed and high-speed centrifugation, representing un-fractionated extracts (total extracts) and cytosol fractions respectively, were mixed with equal volumes of 2×Laemmli sample buffer. All the samples were subjected to SDS-PAGE and immunoblotting with anti-HA antibodies.

In vitro kinase assay

GRK3 kinase assay--Oocytes were injected with mRNA encoding wildtype HA-GRK3, HA-GRK3-K220N or HA-GRK3ΔC. Following an overnight incubation, twenty oocytes per group were lysed in ice-cold phosphate-buffered saline (PBS) lysis buffer (10mM sodium phosphate buffer pH7.5, 150mM NaCl, 1% Triton X-100, 1μg/ml leupeptin, 200μM PMSF; 10μl/oocyte). The lysates were clarified by centrifugation and then were mixed with 50μl agarose-conjugated HA antibody containing 10μg protein G and 4μg anti-HA. The immune complexes were incubated for 2h at 4°C on a rotator. The beads were washed twice with PBS and one time with kinase buffer (50mM Hepes pH7.3; 10mM $MgCl_2$; 2mM $MnCl_2$; 1mM DTT; 0.05% Triton X-100). Aliquots of immune complexes (1/3) were subjected to direct protein separation using SDS-PAGE. The remaining immune complexes were resuspended in kinase buffer (20μL per sample).

Kinase reaction was initiated with the addition of 5μCi of [γ-^{32}P] ATP, 10μM ATP, 10μg each of MBP and β-casein (Benovic et al., 1989; Kim et al., 1993). The kinase reaction was carried out at room temperature for 30min and was stopped with the addition of equal volume of 2×Laemmli sample buffer. Proteins were separated on a 15% SDS-PAGE, dried and visualized by autoradiography.

MPF kinase assay- MPF extracts were prepared and assayed according to Nebreda *et al* (Nebreda and Hunt, 1993). Briefly, oocytes were crushed in MPF-assay buffer (20mM Hepes pH7.3, 80mM glycerophosphate, 20mM EGTA, 15mM $MgCl_2$, 1mM DTT, 10μM ATP, 0.15mM NaF, 10μg/mL leupeptin, 200μM PMSF, 25μg/mL benzamadine; 20μL/oocyte). Following centrifugation, 8μL of lysates were used in a kinase reaction (total volume of 12μL) with the addition of 2μg of H1, 5μCi (Oppermann et al., 1996) γ-ATP (10mCi/mL > 3000Ci/mmol; Amersham, USA) and 33μM ATP. Kinase reactions were carried out at room temperature for 20min before the addition of 12μL of 2×Laemmli sample buffer. Proteins were separated on a 15% SDS-PAGE, dried and visualized by autoradiography.

RESULTS

Overexpression of GRK3 induces oocyte maturation

To test whether mammalian GRKs can induce frog oocyte maturation, I subcloned rat GRK3 into pCS2+ (Turner and Weintraub, 1994) for *in vitro* mRNA synthesis. Injection of rat GRK3 mRNA indeed caused efficient GVBD, with maturation

spots indistinguishable from that induced by progesterone (Figure. 2.2). Typically, GRK3-induced GVBD lagged several hours behind progesterone-induced GVBD (Figure. 2.2), presumbly due to the time required for translation and posttranslational modification of GRK3 protein.

Others in our lab have previously shown that the non-catalytic carboxyl-terminus of bovine GRK2 (71% identical to its counterpart in rat GRK3), when engineered with a geranylgeranylation site for membrane attachment, caused GVBD by scavenging endogenous G protein βγ complexes (Sheng et al., 2001). The geranylgeranylation site was necessary since the wild type carboxyl-terminus was ineffective (Sheng et al., 2001). As GRK3 (or GRK2) does not contain a geranylgeranylation site (or any other sites for posttranslational modifications for membrane attachment), it was unlikely that GRK3 functioned as efficient Gβγ scavengers to induce GVBD. Nevertheless, to rule out this possibility, I substituted the catalytically essential lysine residue (Lys220) (Arriza et al., 1992) with asparagine (K220N). In contrast to wildtype GRK3, GRK3-K220N was completely ineffective in GVBD induction (Figure. 2.3A). To facilitate biochemical analyses of GRK3 and GRK3-K220N, I engineered an amino-terminal hemagglutinin (HA) tag (Booth et al., 2002) to each of them, generating HA-GRK3 and HA-GRK3-K220N respectively. HA-GRK3 was slightly less efficient than untagged GRK3 in GVBD induction whereas HA-GRK3-K220N was, as expected, incapable of inducing GVBD (Figure. 2.3A). To confirm that GRK3-induced oocyte maturation is accompanied by activation of the various maturation-specific protein kinases (mos, MAPK and MPF), I analysed them in extracts derived from mRNA-injected oocytes. Clearly, HA-GRK3 or GRK3, but not their kinase-deficient counterparts, induced mos

Figure 2.2

GRK3-induced GVBD lags several hours behind progesterone-induced GVBD. Fifty or more oocytes were either injected with water (control) or GRK3 mRNA, or incubated with progesterone (Pg, 1μM). At the indicated time following the injection (or the addition of progesterone), GVBD were scored and expressed as % of total treated oocytes. Although different batches of oocytes varied in their GVBD response time, GRK3-induced GVBD always lagged several hours behind Pg-induced GVBD. Typical images of oocytes in each group were shown.

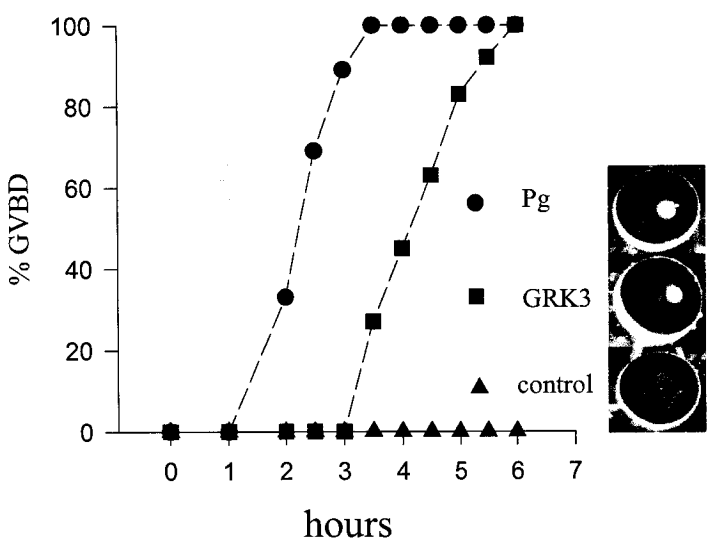
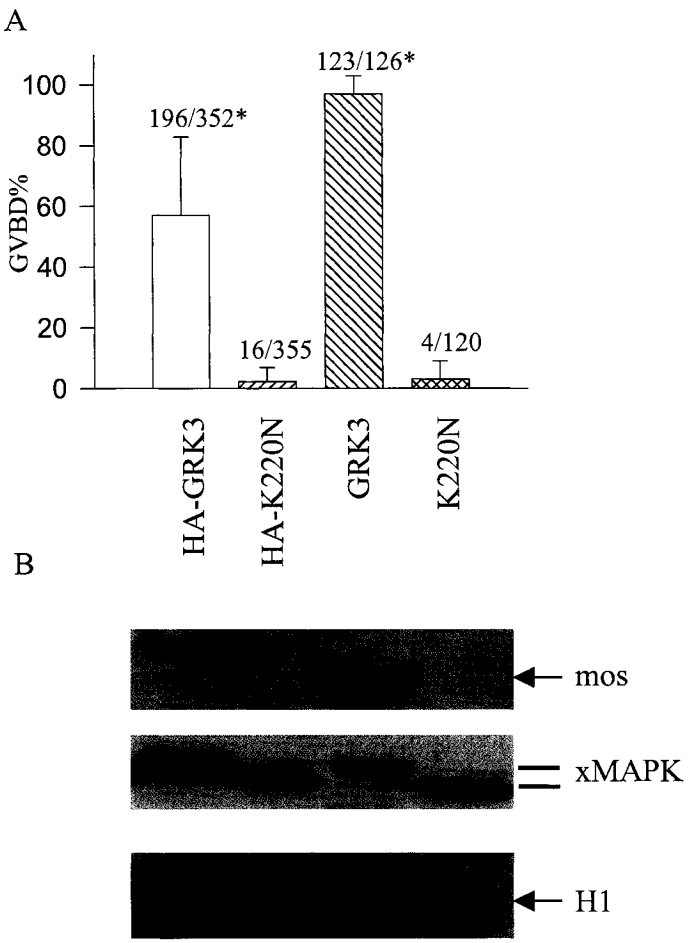

100
80
60
40
20
0
% GVBD
0
1
2
3
4
5
6
7
hours
Pg
GRK3
control

Figure 2.3

Kinase activity of GRK3 is essential for GVBD induction. (A) Oocytes injected with the indicated mRNAs were incubated overnight in OR2. GVBD were scored and expressed as % of total injected oocytes. Shown are means (with s.e.m) of four to six independent experiments. Shown above the bars are actual numbers of GVBD-positive oocytes over those of treated oocytes. The sign * denotes $p<0.001$ in pair-wise student t test. (B) Following GVBD scoring (as in A), oocytes were lysed and the resultant extracts were analyzed for mos accumulation, xMAPK phosphorylation and MPF assays (using histone H1 as an in vitro substrate) (Nebreda and Hunt, 1993).


A
GVBD%
100
80
60
40
20
0
196/352*
16/355
123/126*
4/120
HA-GRK3
HA-K220N
GRK3
K220N
B
mos
xMAPK
H1

synthesis and activated both MAPK and MPF (Figure. 2.3B). Finally, I carried out immune kinase assays to determine the kinase activites of HA-GRK3 and HA-GRK3-K220N. Anti-HA antibodies pulled down similar amounts of the two proteins in mRNA-injected oocytes (Figure. 2.4, lower panel). However, only HA-GRK3 immunoprecipitates contained significantly greater kinase activities, when compared to control immunoprecipitation (derived from extracts of water-injected oocytes) (Figure. 2.4, upper panel). These results clearly indicate that the kinase activity of GRK3 is essential for GVBD induction and suggest that GRK3 induced GVBD via a mechanism different from scavenging oocyte Gβγ (Sheng et al., 2001).

In intact cells, the abilities of GRKs to phosphorylate their substrates, agonist-occupied GpCRs, are dependent on their membrane association (Pitcher et al., 1998a). Whereas some GRKs (GRK4 and GRK6) are intrinsically membrane-bound because they contain posttranslational lipid attachments (Pitcher et al., 1998a), GRK3 and GRK2 need to bind membrane-bound Gβγ complex for membrane association (Pitcher et al., 1998a). To determine whether Gβγ binding is required for GRK3 to induce GVBD, I constructed GRK3-ΔC in which the carboxyl-terminal Gβγ binding domain (residues 546-670 AAs) (Inglese et al., 1994) had been deleted. Figure 2.5 shows that both HA-GRK3 (lane 1) and HA-GRK3-ΔC (lane 2) were expressed efficiently in mRNA-injected oocytes. However, only wildtype HA-GRK3 was partially associated with oocyte membranes (Figure. 2.5, lane 5) whereas HA-GRK3-ΔC was completely "cytosolic". Following immunoprecipitation, both HA-GRK3 and HA-GRK3-ΔC exhibited *in vitro* kinase activities, although HA-GRK3-ΔC was slightly less robust in phosphorylating MBP and β-casein (Figure. 2.6). Despite retaining considerable *in vitro* kinase activities, HA-

Figure 2.4

HA-GRK3-K220N is deficient in kinase activity. Oocytes injected with water or mRNA for HA-GRK3 or HA-GRK3-K220N were incubated overnight in OR2. Extracts were prepared and subjected to immunoprecipitation with anti-HA monoclonal antibodies. The immune complexes were subjected to in vitro kinase assays using both β-casein and myelin basic protein (MBP) as substrates (Benovic et al., 1989) (upper panel). An aliquot of the immune complexes were also subjected to immune blotting with anti-HA antibodies (lower panel). Shown are representatives of four independent experiments.

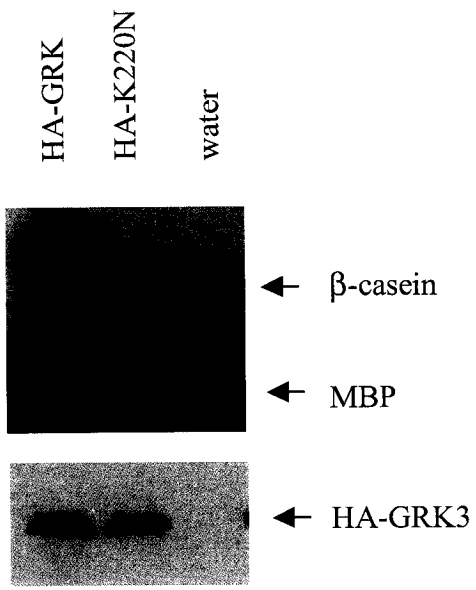

HA-GRK
HA-K220N
water
β-casein
MBP
HA-GRK3

Figure 2.5

GRK3-ΔC is defective in membrane association. Oocytes injected with mRNA for HA-GRK3 (lanes 1, 3 and 5) or HA-GRK3-ΔC (lanes 2, 4 and 6) were incubated overnight in OR2. Oocytes were then subjected to isolation of total membrane fractions. Un-fractionated extracts (total, representing one oocyte), the supernatant ("cytosol", representing one oocyte) or pellets ("membranes", representing one and half oocytes) were analyzed by immunoblotting with anti-HA antibodies. Shown is a representative of three independent experiments.

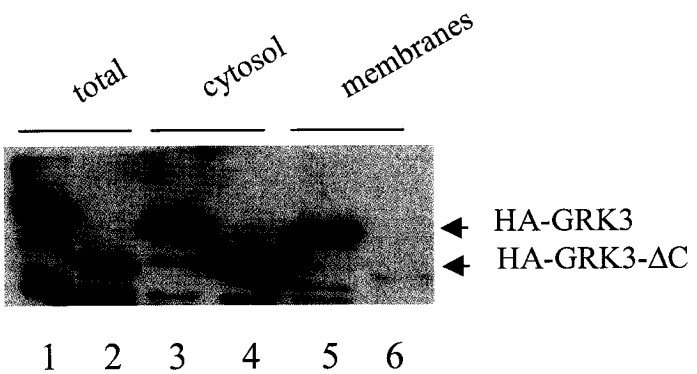
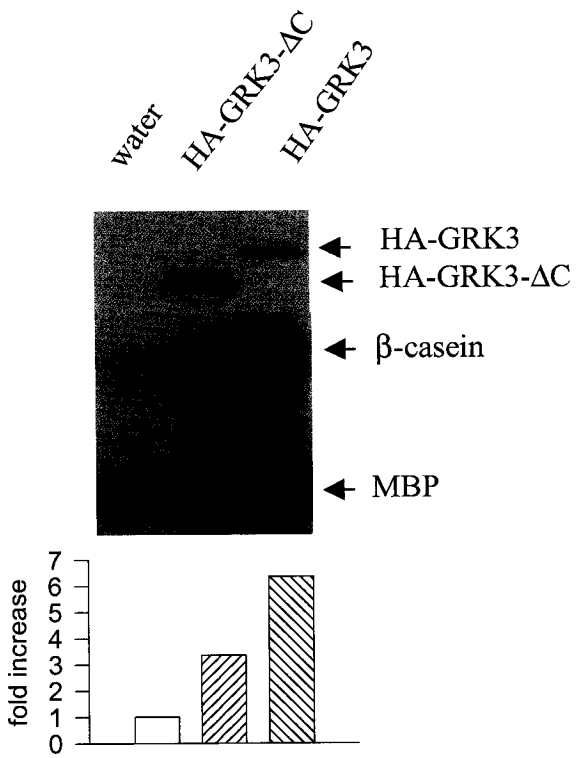

total
cytosol
membranes
HA-GRK3
HA-GRK3-ΔC
1 2 3 4 5 6

Figure 2.6

Kinase assays of GRK3 and GRK3-ΔC. Extracts from the variously injected oocytes were subjected to immune kinase assays as described in Figure. 2.4. The upper panel shows a representative immunoprecipitation, as probed by anti-HA immunoblotting. The middle panel shows the corresponding in vitro kinase assays, using β-casein and MBP as substrates. The lower panel shows the relative kinase activities of each immunoprecipitation, as determined by phosphoimaging scanning (total phosphorylation of both substrates)


water
HA-GRK3-ΔC
HA-GRK3
HA-GRK3
HA-GRK3-ΔC
β-casein
MBP
fold increase
0
1
2
3
4
5
6
7

GRK3-ΔC was not able to induce any GVBD, nor was it capable of activating any of the maturation-specific protein kinases in frog oocytes (Figure. 2.7). These results indicate the essential role of the carboxyl-terminal Gβγ-binding domain of GRK in membrane association and in GVBD induction and, therefore, lend further support that GRK3 induced GVBD via phosphorylating a membrane-bound GpCR.

Synergistic effect of co-overexpression of GRK3 and β-arrestin 2 on GVBD induction

If GRK3-induced GVBD was mediated via phosphorylation and desensitization of an endogenous GpCR*, it must work together with another regulatory proteins, β-arrestins (Claing et al., 2002). β-arrestins are well known as adaptor proteins to desensitize and internalize activated GpCRs by binding to GRK-phosphorylated GpCRs. Therefore, I wished to test whether over-expression of mammalian β-arrestin would also induce GVBD. Injection of rat HA-β-arrestin-2 mRNA caused robust GVBD (Figure. 2.8). In contrast, the carboxyl-terminal clathrin-binding domain of β-arrestin-2, HA-β-arrestin-2-C, was unable to induce GVBD. Like GRK3-induced GVBD, HA-β-arrestin-2-induced GVBD also lagged behind progesterone-induced GVBD (Figure. 2.9). To determine if GRK3 and HA-β-arrestin-2 co-operate in GVBD induction, I injected low concentrations (1ng per oocyte) of GRK3 or HA-β-arrestin-2 mRNA alone, or in combination. As shown in Figure. 2.10, injection of either mRNA alone caused little GVBD whereas co-injection of both caused significant percentages of GVBD. These results clearly indicate synergism between β-arrestin-2 and GRK3 in GVBD induction, consistent with the involvement of GpCR desensitization (Pitcher et al., 1998a).

Figure 2.7

GRK3-ΔC is defective for GVBD induction. Oocytes injected with water or mRNA for GRK3 or GRK3-ΔC were incubated overnight in OR2. GVBD were scored and expressed as % of total injected oocytes (means with s.e.m. of four independent experiments). The lower panels show representative analyses for mos accumulation and MPF assays. The sign* denotes $p<0.001$ in pair-wise student t test.

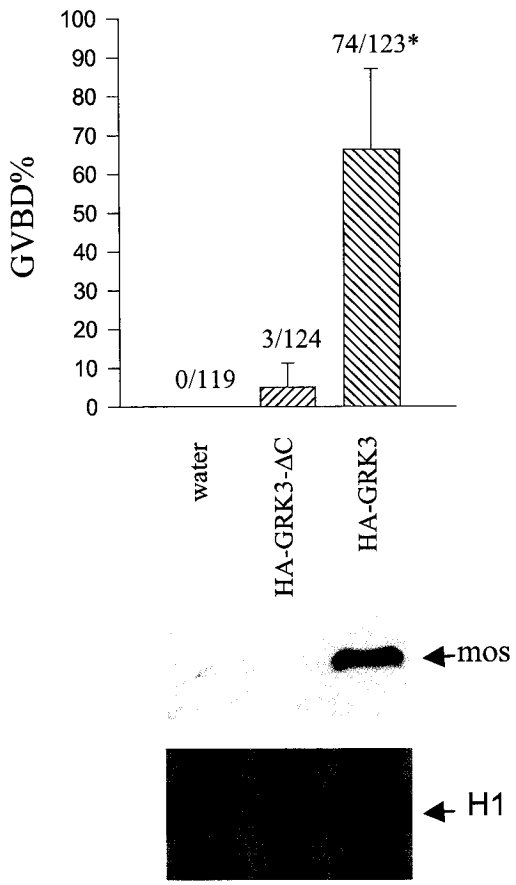

100
90
80
70
60
50
40
30
20
10
0
GVBD%
74/123*
3/124
0/119
water
HA-GRK3-ΔC
HA-GRK3
mos
H1

Figure 2.8

β-arrestin-2 induces GVBD. Groups of twenty or more oocytes were injected with water or mRNA for β-arrestin-2 or β-arrestin-2-C. Following overnight incubation in OR2, GVBD were scored and expressed as % of total injected oocytes. Shown are means with s.e.m. of three to four independent experiments.

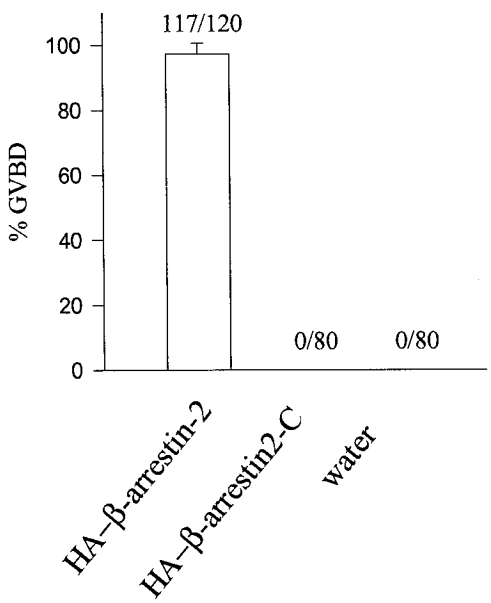

117/120
100
80
60
40
20
0
% GVBD
0/80
0/80
HA-β-arrestin-2
HA-β-arrestin2-C
water

Figure 2.9

β-arrestin-2-induced GVBD lags several hours behind progesterone-induced GVBD. Groups of fifty or more oocytes were incubated in OR2 (control) or OR2 containing progesterone (Pg, 1μM), or injected with mRNA for GRK3 or HA-β-arrestin-2. GVBD were scored at the indicated time following the addition of progesterone or the injection of mRNAs. Shown is a representative of three independent experiments.

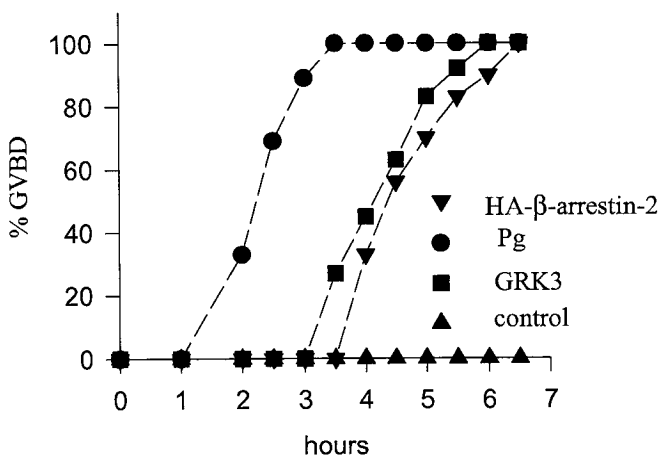
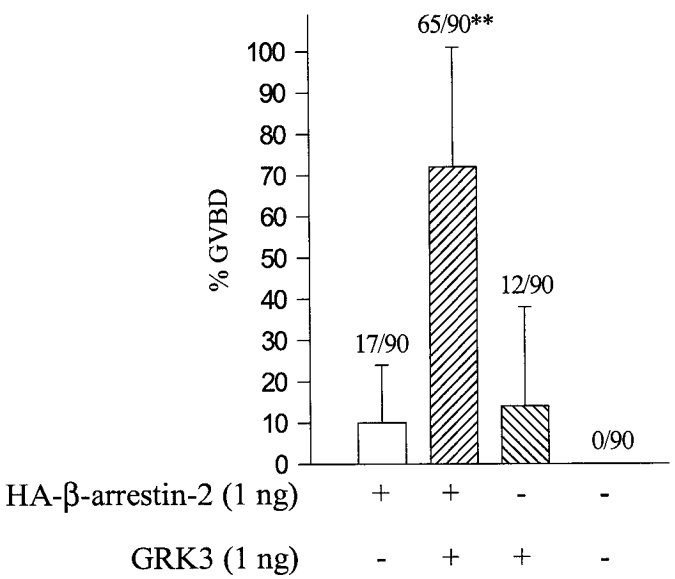

100
80
60
40
20
0
% GVBD
0
1
2
3
4
5
6
7
hours
HA-β-arrestin-2
Pg
GRK3
control

Figure 2.10

Co-overexpression of GRK3 and β-arrestin 2 synergistically induces GVBD. Groups of thirty or more oocytes were injected with water or the indicated mRNA (1 ng per oocyte). Following overnight incubation in OR2, oocytes were scored for GVBD. Shown are means with s.e.m. of three independent experiments. The sign** denotes $p<0.05$ in pair-wise student t test.


65/90**
100
90
80
70
60
50
40
30
20
10
0
% GVBD
12/90
17/90
0/90
HA-β-arrestin-2 (1 ng) + + - -
GRK3 (1 ng) - + + -

If GRK3/β-arrestin-2 induced GVBD via the desensitization of a cAMP-raising GpCR, I expected that the GVBD induction would be blocked by forskolin, an activator of AC. Indeed, incubation of GRK3- or β-arrestin-2-injected oocytes with forskolin completely blocked GVBD induction (Figure. 2.11). Upon removal of forskolin (by simply transferring oocyte to fresh OR2) and further incubation, significant percentages of the mRNA-injected oocytes eventually underwent GVBD (Figure. 2.11).

Inhibitors of clathrin-mediated endocytosis partially block GRK3/β-arrestin-2-induced GVBD

In addition to GpCR desensitization, GRKs/arrestins also promote GpCR endocytosis (Claing et al., 2002). In particular, β-arrestins (non-visual arrestins) serve to target GpCR to clathrin-coated pits (Claing et al., 2002). Krupnick *et al* (Krupnick et al., 1997) have previously demonstrated that the overexpression of the clathrin-binding domain of β-arrestin blocks GpCR endocytosis. If clathrin-mediated GpCR endocytosis played any role in GRK3/β-arrestin-2-induced GVBD, HA-β-arrestin2-C should interfere with the GVBD induction. Indeed, injection of β-arrestin2-C mRNA significantly reduced, but did not eliminate, the ability of GRK3 or β-arrestin-2 to induce GVBD (Figure. 2.12). As a control, I injected equal amounts of an mRNA (pCS2+MT (Turner and Weintraub, 1994)) that encoded a similarly sized polypeptide.

To further examine the involvement of clathrin-mediated endocytic pathway in GRK3-induced GVBD, I tested two widely used chemical inhibitors. The plant lectin Con A binds cell-surface glycoproteins and interferes with the formation of clathrin-coated pits (Salisbury et al., 1982). Treatment of cells with Con A blocks β_2AR

Figure 2.11

Forskolin blocks GRK3/β-arrestin-2-induced GVBD. Oocytes were injected with water or the indicated mRNA. mRNA-injected oocytes were immediately split into two groups. One group was placed in OR2 and the other in OR2 containing forskolin (50μM). Following overnight incubation, oocytes were scored for GVBD. Shown are means (with s.e.m.) of at least three independent experiments. In two experiments, after GVBD scoring, forskolin-treated oocytes were transferred to fresh OR2 without forskolin and were further incubated for 7-8 hours before being scored again for GVBD (indicated by+/- forskolin).

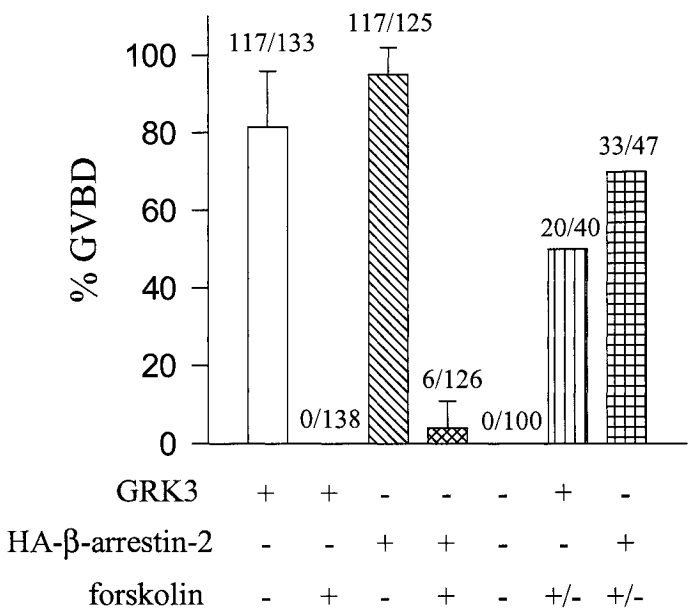
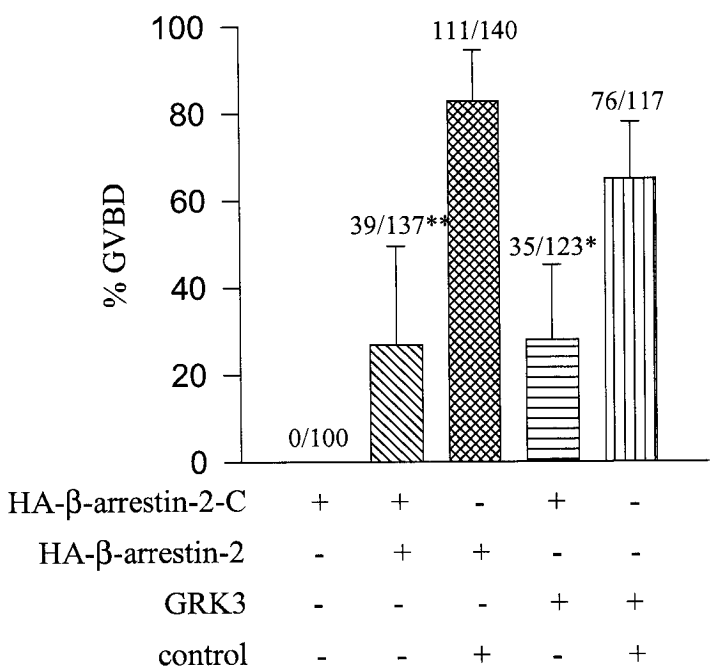

% GVBD
100
80
60
40
20
0
117/133
0/138
117/125
6/126
0/100
20/40
33/47
GRK3 + + - - - + -
HA-β-arrestin-2 - - + + - - +
forskolin - + - + - +/- +/-

Figure 2.12

HA-β-arrestin-2-C, an inhibitor of clathrin-pits, reduces GRK3 (or β-arrestin-2)-induced GVBD. Groups of ninety or more oocytes were injected with a control mRNA (pCS2+MT, coding for six copies of the 13-amino acid Myc tag (Turner and Weintraub, 1994)) or mRNA for HA-β-arrestin-2-C. Each of the two groups of oocytes was split and immediately injected with mRNA for either GRK3 or HA-β-arrestin-2. Oocytes were incubated in OR2 for 6–8 h (when either GRK3- or HA-β-arrestin-2-injected oocytes had reached maximum GVBD response). All groups were scored for GVBD at the same time. Shown are means with s.e.m. of three independent experiments.


100
80
60
40
20
0
% GVBD
0/100
39/137**
111/140
35/123*
76/117
HA-β-arrestin-2-C + + - + -
HA-β-arrestin-2 - + + - -
GRK3 - - - + +
control - - + - +

endocytosis without affecting G protein activation and activation of the second messenger cAMP (Pippig et al., 1995; Wang et al., 1989). Indeed, treatment of oocytes with Con A significantly reduced, but did not eliminate, the ability of GRK3 to induce GVBD (Figure. 2.13, lane 2). Another inhibitor for clathrin-pits is the polyamine MDC. Unlike Con A, which acts extracellularly, MDC acts intracellularly to block clathrin polymerization (Nandi et al., 1981). As shown in Figure. 2.14, MDC similarly reduced GRK3-induced GVBD. To rule out general toxicity or other nonspecific effects of the inhibitors in oocytes, I tested the effects of these inhibitors on v-Ras-induced GVBD. Although the mechanism by which v-Ras induces GVBD remains controversial (Dupre et al., 2002b), it is unlikely that it would require receptor endocytosis. Indeed, neither Con A (Figure. 2.13) nor MDC (Figure. 2.14) had any inhibitory effect on v-Ras-induced GVBD. To further strengthen the argument that Con A and MDC inhibited GRK-induced GVBD through blockade of clathrin-mediated GpCR endocytosis, I tested the cholesterol-binding agent MβCD (Neufeld et al., 1996), which inhibits caveolae-mediated endocytosis (Orlandi and Fishman, 1998). MβCD had no effect on GRK3- or v-Ras-induced GVBD (Figure. 2.14), suggesting that caveoli are not involved in GRK3-induced GpCR endocytosis.

DISCUSSION

Recent studies have clearly indicated that G proteins play critical roles in maintaining meiosis arrest in both amphibian (Gallo et al., 1995; Lutz et al., 2000; Sheng et al., 2001) and mouse (Mehlmann et al., 2004) oocytes. However, the identity of the G

Figure 2.13

Concanavalin A, an inhibitor of clathrin-pits, reduces GRK3-induced GVBD. Oocytes were injected with mRNA for GRK3 or v-Ras and were immediately transferred to OR2 with (+) or without concanavalin A (250 μg/ml). Oocytes were incubated for 6–8 h (when GRK3- or v-Ras injected oocytes had reached maximum GVBD responses). Therefore, the GRK3 groups (lanes 1 and 2) were always scored for GVBD and lysed at the same time. So were the v-Ras groups (lanes 3 and 4). Shown are means with s.e.m of three to four independent experiments. The lower panels show representative immunoblots with anti-xMAPK or anti-phosphor MAPK.

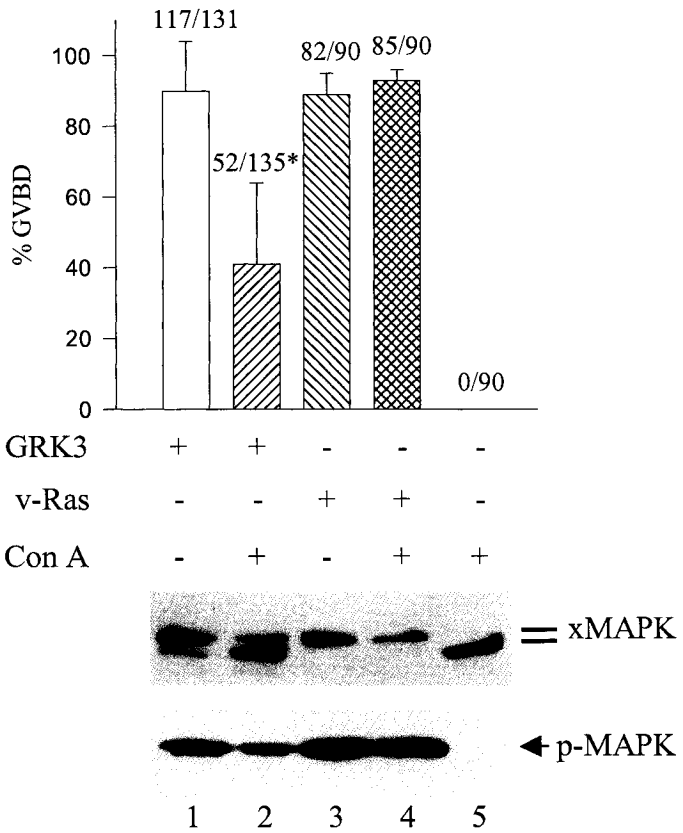
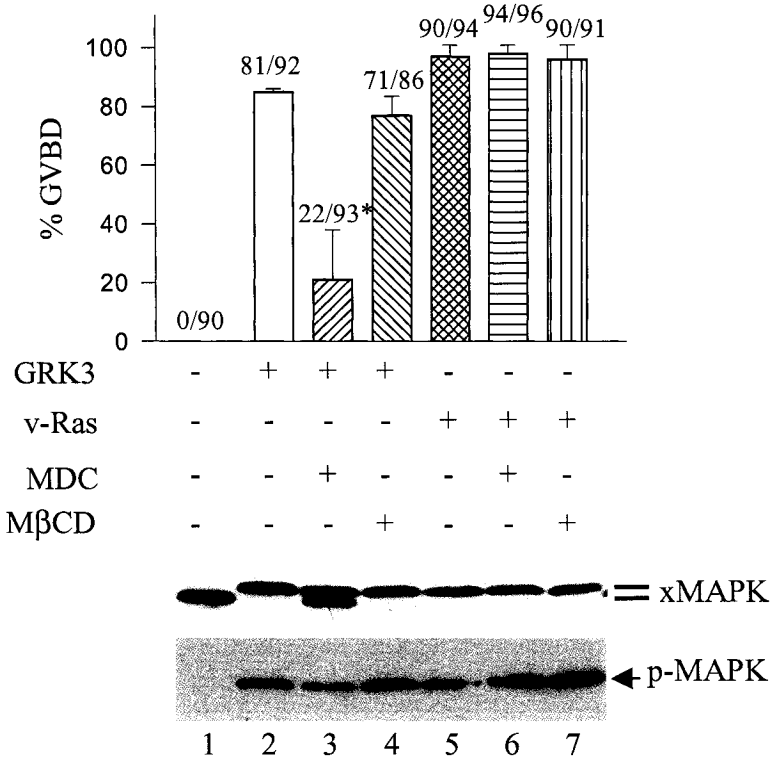

117/131
82/90
85/90
52/135*
0/90
100
80
60
40
20
0
% GVBD
GRK3 + + - - -
v-Ras - - + + -
Con A - + - + +
xMAPK
p-MAPK
1 2 3 4 5

Figure 2.14

Inhibition of clathrin-pits, but not inhibition of caveoli, reduces GRK3-induced GVBD. Oocytes were injected with mRNA for GRK3 or v-Ras and were immediately transferred to OR2 or OR2 containing the indicated inhibitors monodansyl cadaverine (MDC, 300 μM) or methyl-β-cyclodextrin (MβCD, 1%). GVBD scoring and oocyte lysis were performed as described in Figure. 2.13. Shown are means with s.e.m of three independent experiments. The lower panels show representative immunoblots with anti-xMAPK or anti-phosphor-MAPK. The sighs * and ** denote $p<0.001$ and $p<0.05$, respectively, in paire-wise student t test.


% GVBD
100
80
60
40
20
0
0/90
81/92
22/93*
71/86
90/94
94/96
90/91
GRK3 - + + + - - -
v-Ras - - - - + + +
MDC - - + - - + -
MβCD - - - + - - +
xMAPK
p-MAPK
1 2 3 4 5 6 7

proteins or whether these G proteins are regulated by classical GpCRs remains unclear. For example, receptors with intrinsic protein tyrosine kinase activities have been implicated in regulating G proteins (Booth et al., 2002; Kuemmerle and Murthy, 2001; Lou et al., 2001; Luttrell et al., 1995), with direct binding of the receptors to the α subunits of the G proteins (Dalle et al., 2001; Hallak et al., 2000). GRKs are highly specialized protein kinases that preferentially phosphorylate activated (agonist-occupied), as opposed to inactive or antagonist-occupied, GpCRs (Pitcher et al., 1998a). That GRK3 was able to induce GVBD therefore represents the first and compelling experimental support for the notion that prophase oocytes contain an active GpCR and that this GpCR is responsible for maintaining meiosis arrest (Mehlmann et al., 2004; Sheng et al., 2001).

Recent studies have indicated that GRKs are able to phosphorylate non-GpCR proteins. Tubulin (Pitcher et al., 1998b) and synuleins (a class of low molecular weight proteins of unknown biological functions) (Pronin et al., 2000) can be phosphorylated by GRK2 and GRK3. However, physiological significance of these phosphorylation events remains unknown. Another example of a non-GpCR substrate is the Na(+)/H(+) exchanger regulatory factor (NHERF) which is specifically phosphorylated by a splice variant of GRK6 (GRK6A), but not by other GRK6 variants or other GRKs (Hall et al., 1999).

I believe that GRK3-induced GVBD involves phosphorylation of a GpCR, rather than any of these non-GpCR substrates. First, GRK3-induced GVBD required membrane association of the kinase mediated by the carboxyl-terminal Gβγ-binding domain, consistent with the involvement of a membrane-bound GpCR. Second, GRK3-induced GVBD was blocked by forskolin, indicating the involvement of inhibition of cAMP

signaling pathway. Third, both GRK3 and β-arrestin-2 were potent GVBD inducers and acted synergistically, consistent with the involvement of GpCR desensitization. Furthermore, several inhibitors of clathrin-mediated endocytosis significantly reduced the abilities of GRK3/β-arrestin-2 to induce GVBD, supporting a role of GpCR endocytosis in GVBD induction. However, as only partial inhibition was observed with these inhibitors (Figures. 2.12, 2.13 and 2.14), it appears that clathrin-mediated endocytosis may not be absolutely required for GVBD induction. In other words, GpCR desensitization caused by GRK3/β-arrestin-2 might be sufficient to cause GVBD by uncoupling heterotrimeric G proteins from GpCR. In any event, these data strongly support the presence of an activated GpCR* that is responsible for maintaining prophase I arrest.

Although the identity of the GpCR remains unknown, there are several candidates. Recent work by others in our lab have suggested that the GpCR* is related to a serotonin receptor, based on the fact that a well-known serotonin receptor antagonist, ritanserine, causes hormone-independent oocyte maturation (Sheng et al., 2005b). Clearly, another candidate is the *Xenopus* homolog of GPR3 (a constitutively activated orphan GpCR*) as mouse oocytes lacking GPR3 undergo spontaneous maturation in follicle-enclosed ovary (Ledent et al., 2005; Mehlmann, 2005; Mehlmann et al., 2004). It remains to be determined whether GPR3 plays similar role in maintaining prophase arrest in *Xenopus* oocyte.

Chapter Three

Progesterone inhibits protein kinase A (PKA) in *Xenopus* oocytes: demonstration of endogenous PKA activities using an expressed substrate

INTRODUCTION

Maller *et al* first found that injection of PKAr, or PKI, caused spontaneous oocyte maturation. In contrast, injection of PKAc prevented progesterone-induced oocyte maturation (Maller and Krebs, 1977). These results clearly suggest that endogenous PKA plays a critical role in maintaining prophase arrest. Consistent with this notion, many previous studies show that progesterone causes a modest reduction (20%) of intracellular cAMP (Bravo et al., 1978; Maller et al., 1979). In contrast, the mechanism by which PKA helps to maintain prophase arrest is not well understood. Over the years, many investigators have searched for physiological substrates for PKA in frog oocytes. Earlier studies suggested that PKAc can phosphorylate and inhibit the germ cell specific protein kinase mos (Lazar et al., 2002; Matten et al., 1994; Yang et al., 1996). However, as mos protein is absent in prophase-arrested oocytes, phosphorylation of mos cannot be a primary mechanism by which PKA maintains prophase arrest. The best characterized PKA substrate in frog oocytes appears to be the dual specificity phosphatase cdc25C (Duckworth et al., 2002; Matten et al., 1994). PKA catalyzed phosphorylation of cdc25C inhibits the ability of the latter to dephosphorylate and activate cdc2 (the catalytic subunit of MPF). On the other hand, it is unlikely that Xcdc25C is the only physiological substrate of PKA in frog oocytes, as PKAc clearly inhibits mos translation, which precedes Xcdc25C dephosphorylation and activation (Daar et al., 1993; Faure et al., 1998; Matten et al., 1994).

Despite the importance of PKA in prophase arrest and its proposed regulation by progesterone during the initiation of frog oocyte maturation, activities of endogenous PKA have never been directly demonstrated. As PKA is regulated by intracellular

cAMP, measuring PKA activities after cell lysis may not accurately determine the kinase activities in live cells. Therefore I sought to develop a novel approach to determine PKA activities in live oocytes. The idea was to express a specific PKA substrate whose phosphorylation status can be analyzed following cell lysis. I constructed a tripartite fusion protein, myr-HA-β_2AR-C, containing an amino-terminal myristylation (myr) sequence derived from the c-Src protein (Aronheim et al., 1994), followed by an antigenic epitope tag (hemagglutinin, HA) and a PKA substrate motif (the carboxyl-terminus of β_2AR) (Kemp et al., 1977). In prophase oocytes, the expressed protein, myr-HA-β_2AR-C, was fully phosphorylated on a single PKA site (Ser346 of human β_2AR), indicating that endogenous PKA is activated in prophase oocytes. Within 30 minutes of the addition of progesterone, the PKA site became mostly dephosphorylated, indicating that PKA activity is inhibited by progesterone. These results represent the first biochemical demonstration of regulation of endogenous *Xenopus* oocyte PKA by progesterone.

MATERIALS AND METHODS

Materials

H89, was purchased from Calbiochem. Recombinant PKAc was from Promega and stored in aliquots at -80°C (350 mM potassium phosphate buffer, pH6.8, 0.1 mM DTT). Antibodies against phospho-MAPK were purchased from Upstate Biotechnology. Antibodies against phospho-β_2AR (S345S346) were from Santa Cruz. Antibodies against

cyclin B2 was a gift from James Maller (Taieb et al., 2001). Radioactive compounds were from Amersham. Other chemicals were from Sigma, unless otherwise indicated. Polyacrylamide gel electrophoresis materials (for both first dimension mini-tube gels and second dimension slab gels) were purchased from BioRad.

Animal and oocyte manipulation

Refer to: Materials and Methods in chapter 2

Plasmid constructs

Full-length cDNA constructs containing wildtype human β_2AR and its mutants lacking PKA phosphorylation sites or GRK phosphorylation sites (or both) were provided by Dr. Robert J. Lefkowitz. The nucleotide sequence encoding human β_2AR carboxyl-terminal region (residues 328-413 AAs) was PCR-amplified using the following primer: forward primer, 5'-TAT GGA TCC ATG GGG TAC CCA TAC GAT GTT CCA GAT TAC GCT CGG AGC CCA GAT TTC AGG AT- 3'; reverse primer, 5'-TAT GAA TTC ACA GCA GTG AGT CAT TTG TAC TA- 3'. The amplified segment was digested with BamHI and EcoRI, and then ligated into pCS2+ vector, which had been similarly digested. The resultant plasmid encoded HA-tagged β_2AR carboxyl-terminus (HA-β_2AR-C). The plasmid was further linearized with NcoI. Double stranded oligos encoding the myristylation sequence (forward 5' -C ATG GGG AGT AGC AAG AGA AAG CCT AAG GAC CCC AGC CAG CGC CGG CC- 3'; reverse 5' -CAT GGG CCG GCG CTG GCT GGG GTC CTT AGG CTT GCT CTT GCT ACT CCC- 3') and containing cohesive NcoI was then inserted into the linearized HA-β_2AR-C, resulting in

myr-HA-β_2AR-C. The myr-HA-β_2AR-C mutants lacking PKA-phosphorylated sites or GRK-phosphorylated sites (or both) were produced in the same way as wildtype myr-HA-β_2AR-C, with the exception that the corresponding full-length β_2AR mutants were used as PCR template (Figure. 3.1). To generate the two single Ser to Ala (S345A and S346A) mutants of myr-HA-β_2AR-C, I used the two-step PCR mutagenesis protocol (Vallette et al., 1989), using myr-HA-β_2AR-C as a template. PCR products containing SP6 sequence at the 5′ end and T3 sequence at the 3′ end, including the SV40 poly(A) tail, were used directly for in vitro transcription. Both PCR products were also analyzed by DNA sequencing to confirm the respective single mutation (S345A and S346A).

Preparation of oocyte extracts and two-dimentinal (2-D) immunoblotting

Oocytes were crushed through pipette tip in ice-cold extraction buffer (20mM Hepes pH 7.2, 50mM glycerophosphate, 10mM EDTA, 2.5mM $MgCl_2$, 0.25M sucrose, 0.1M NaCl, 1% triton X-100, 200μM PMSF, 10μg/ml leupeptin, 1μM sodium orthovanadate, 10μM H89 and 1μM OA; 2 μl/oocyte). The extracts were centrifuged for 15min at 13,500rpm (in a refrigerated eppendorf centrifuge). The clarified supernatant was mixed with equal volume of first dimension sample buffer (8M urea, 2% triton X-100, 5% β-mercaptoethanol, 1.6% Bio-lyte 5/7 ampholyte, 0.4% Bio-lyte 3/10 ampholyte). Samples (20μl) were loaded onto mini-tube gels for first dimension electrophoresis. Electrophoresis was carried out at 750V for 3.5h. Subsequently, the tube gels were extruded and loaded onto the SDS polyacrylamide slab gels. I typically loaded two tube gels side-by-side to reduce the number of slab gels. In addition, an extra sample in Laemmli sample buffer was loaded directly onto the slab gel as a marker (see

Figure. 3.3F for example). Transfer of proteins onto PVDF membrane and the subsequent immunoblotting with HA antibodies and ECL detection were all standard procedures.

SDS-PAGE western blotting and MPF assay

Refer to: Materials and Methods in chapter two

Protein phosphatases assay

Groups of four oocytes were lysed in phosphatase assay buffer (20mM Tris PH 7.5, 4mM EDTA, 15mM β-mercaptoethanol, 200μM PMSF, 10μg/ml leupeptin, benzamadine 25μg/ml and 1% triton X100; 20μl per group). Extracts were clarified by centrifugation (13,500rpm×5min). The supernatants were further diluted fifty times with the same buffer. Phosphatase reaction was started by mixing of 8.5μl of assay buffer and 4μl of the diluted lysates with 12.5μl synthetic phosphopeptide (Ser/Thr phosphatase Assay kit, Upstate Biotechnology) in wells of microtiter plate. The reaction was carried out at room temperature for 30min and stopped by addition of 100μl of Malachite Green phosphate detection solution (Ser/Thr phosphatase Assay kit, Upstate biotechnology). Absorbance was measured at 650nm and readings were converted to amounts of free phosphate released from reaction using standard curve. Then, the relative ratios of free phosphates released were normalized by defining a group without progesterone stimulation as 100%.

RESULTS

Characterization of myr-HA-β_2AR-C as a PKA activity indicator: 2-D gel approach

The carboxyl-terminal intracellular domain of the β_2AR contains one of the two well-characterized PKA phosphorylation sites (in the context of RRSS^{346}; the other is in the third intracellular loop) and all eleven identified and possible phosphorylation sites by GRKs (Fredericks et al., 1996; Kemp et al., 1977; Zamah et al., 2002). Phosphorylation of β_2AR by PKA and GRKs represents an important regulatory mechanism that controls β_2AR desensitization (Claing et al., 2002). To generate a potential expressed substrate for PKA, I added two features to the carboxyl-terminus of β_2AR. The first was to add a myristylation sequence to target the truncated protein to the membranes, where β_2AR normally resides. I also included an antigenic epitope (HA) to facilitate detection in immunoblotting. In addition to myr-HA-β_2AR-C, I also constructed myr-HA-β_2AR-C/PKA- (substituting both Ser-345 and Ser 346 with alanine(Ala)), myr-HA-β_2AR-C/GRK- (substituting all eleven GRK sites with Ala or glycine(Gly)) and myr-HA-β_2AR-C/PKA-&GRK- (substituting PKA site and GRK sites with Ala or Gly) (Figure. 3.1).

mRNAs for myr-HA-β_2AR-C and the three mutant forms were individually injected into prophase oocytes. Following overnight incubation, extracts were prepared and analyzed by SDS-PAGE followed by immunoblotting using antibodies against HA. These experiments showed that myr-HA-β_2AR-C (Figure. 3.2) and its phosphorylation mutants (not shown, but see Figure.3.12) were properly expressed. These experiments also indicated that myr-HA-β_2AR-C exhibited identical migration patterns whether the oocytes were treated with progesterone or not, suggesting that changes in the

Figure 3.1

Schematic representation of cDNA constructs used in this study. "See text for details".

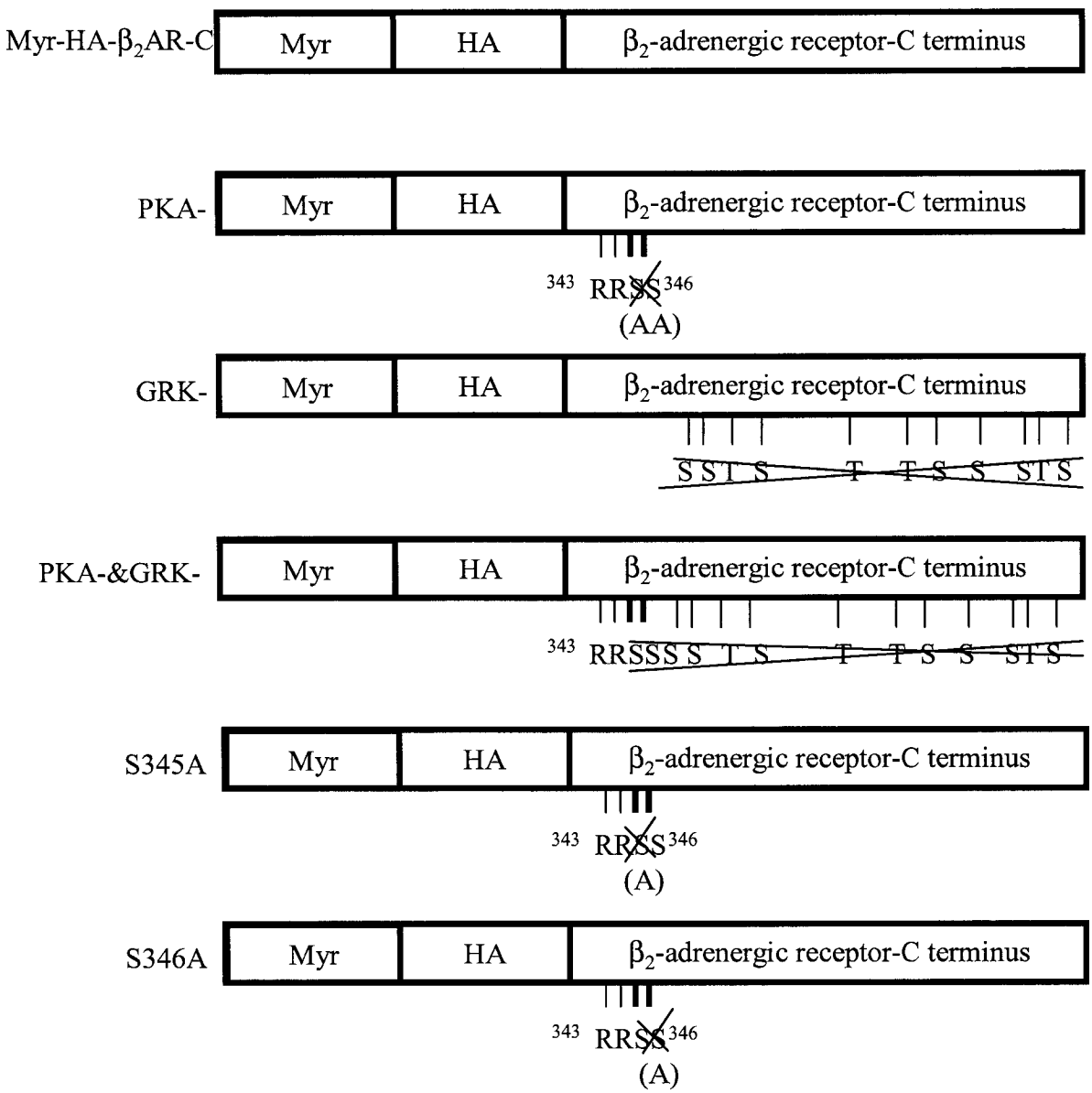
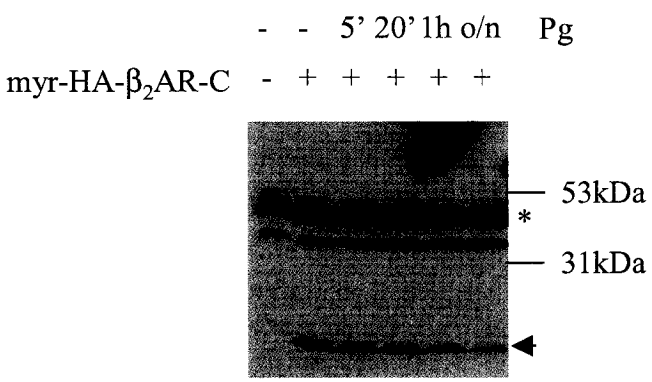

Myr-HA-β2AR-C
Myr
HA
β2-adrenergic receptor-C terminus
PKA-
343 RRSS 346
(AA)
GRK-
SSTS T TSS STS
PKA-&GRK-
343 RRSSSSTS T TSS STS
S345A
343 RRSS 346
(A)
S346A
343 RRSS 346
(A)

Figure 3.2

Expression of myr-HA-β_2AR-C in *Xenopus* oocytes. Oocytes were injected with water (–) or mRNA for myr-HA-β_2AR-C (+). Oocytes injected with myr-HA-β_2AR-C mRNA were incubated in OR2 (–) or with progesterone for the indicated periods. Oocytes were lysed and analyzed by immunoblotting with antibodies against HA. Oocytes treated overnight (o/n) with progesterone (Pg) exhibited 100% germinal vesicle breakdown (GVBD). The arrow indicates the position of myr-HA-β_2AR-C and the asterisk indicates a nonspecific protein existing in frog oocytes.

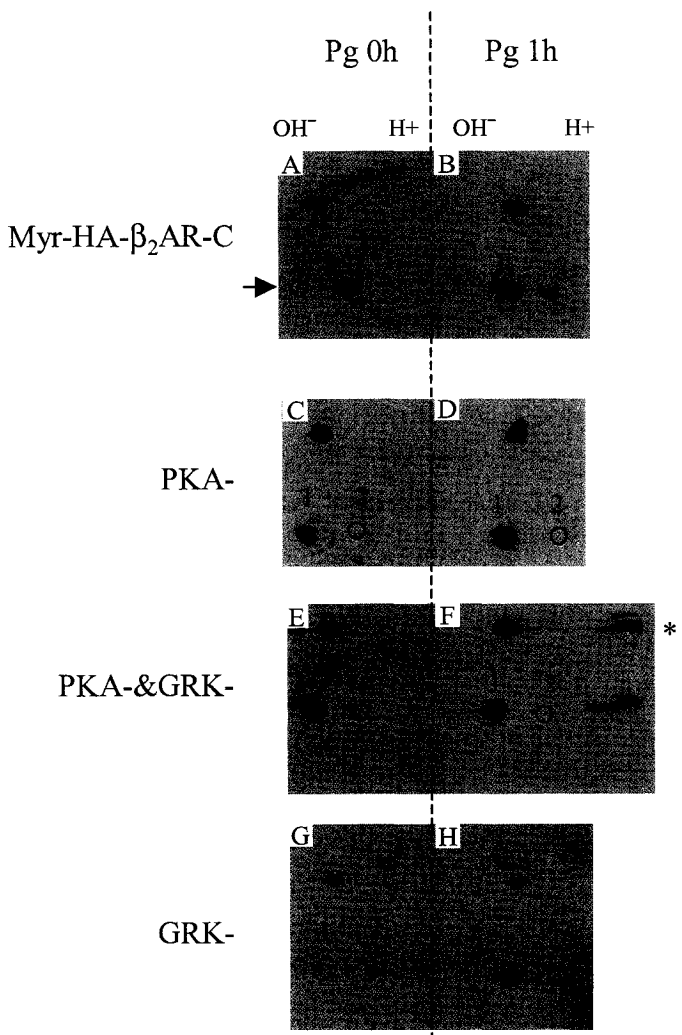

- - 5' 20' 1h o/n Pg
myr-HA-β2AR-C - + + + + +
53kDa
*
31kDa

phosphorylation status of myr-HA-β_2AR-C, if they occurred, did not result in any shift on one-dimensional gels (Figure. 3.2). I therefore carried out two-dimensional gel electrophoresis followed by HA immunoblotting to identify specific PKA-phosphorylated form of myr-HA-β_2AR-C. As shown in Figure. 3.3, myr-HA-β_2AR-C (panel A) and myr-HA-β_2AR-C/GRK- (panel G) migrated as a single and identical spot (designated as 2; identity confirmed by 2-D analyses of mixed sample, data not shown). Myr-HA-β_2AR-C/PKA- (panel C) and myr-HA-β_2AR-C/PKA-&GRK- (panel E) also appeared as a single and identical spot (designated as 1; identity similarly confirmed), but distinct from spot 2 in the first dimension. Clearly, spot 1 and spot 2 differed only in their isoelectrical points, with spot 2 being more acidic as a result of higher levels of phosphorylation. The correlation of spot 2 (phosphorylated form) with the presence of the PKA phosphorylation site suggested that myr-HA-β_2AR-C (or myr-HA-β_2AR-C/GRK-) was fully phosphorylated by PKA in prophase oocytes. An unknown endogenous protein (indicated by *in Figure. 3.3) that was recognized by HA antibodies and that did not undergo progesterone-induced migration pattern change (compared to first dimensional protein markers with various pHi values, not shown) served as a convenient reference point in the 2-D analyses.

Following one hour incubation with progesterone, the more phosphorylated form (spot 2) diminished for both myr-HA-β_2AR-C (Figure. 3.3, panel B) and myr-HA-β_2AR-C/GRK- (Figure. 3.3, panel H). Correspondingly, spot 1 (dephosphorylated form) was the predominant form for both. In contrast, the migration pattern of myr-HA-β_2AR-C/PKA- (Figure. 3.3, panel D) or myr-HA-β_2AR-C/PKA-&GRK- (Figure. 3.3, panel F) was not changed and remained as a single spot (spot 1). In the presence of forskolin

Figure 3.3

myr-HA-β_2AR-C is a specific PKA substrate in frog oocyte. *Xenopus* oocytes injected with the indicated mRNAs were incubated in OR2 for overnight. Groups of at least twenty oocytes were incubated in OR2 or OR2 containing 1μM progesterone (Pg). Shown are representative examples of three to ten independent experiments. The long dashed line separates two first dimension mini-tube gels. Shown in the PKA-&GRK- panel (on the right side of panel F) is one example of an extra SDS sample directly loaded on the slab gels. OH^- and H+ denote the anion and cation ends, respectively, of the tube gels. Circles represent positions of missing spots (1 or 2). Arrow, myr-HA-β_2AR-C; asterisk *, nonspecific protein.


Pg 0h
Pg 1h
OH⁻
H+
OH⁻
H+
A
B
Myr-HA-β2AR-C
C
D
PKA-
E
F
*
PKA-&GRK-
G
H
GRK-

(Figure. 3.4, panel C) or recombinant PKAc (Figure. 3.4, panel D), progesterone was unable to cause dephosphorylation of myr-HA-β_2AR-C and therefore it remained predominantly as spot 2. Forskolin and recombinant PKAc completely blocked progesterone-induced oocyte maturation (not shown). These data clearly demonstrated that progesterone caused dephosphorylation of the PKA site in intact oocytes and implicated that the spot 2 reflect PKA-phosphorylated status and the spot 1 is a corresponding dephosphorylated form.

Characterization of myr-HA-β_2AR-C as a PKA activity indicator: H89 experiments

To further ensure that spot 2 represented a PKA-phosphorylated form of myr-HA-β_2AR-C, I employed a highly specific cell permeable PKA inhibitor, H89 (Chijiwa et al., 1990). Indeed, treating oocytes with H89 for one hour resulted in a significant decrease in concentration of spot 2 and a corresponding increase of spot 1 (Figure. 3.5, panel A and B). Not surprisingly, oocytes incubated with H89 eventually underwent hormone-independent GVBD (Figure. 3.6).

Progesterone induces dephosphorylation of myr-HA-β_2AR-C: 2-D gel analysis

Based on the results from Figure 3.3, 3.4 and 3.5, I was confident that spot 2 is the PKA-phosphorylated form of myr-HA-β_2AR-C. Therefore, I used myr-HA-β_2AR-C to determine endogenous PKA activities during progesterone-induced oocyte maturation. In prophase oocytes, myr-HA-β_2AR-C was always fully phosphorylated on the PKA site (Figure. 3.7A), indicating that PKA is activated in prophase oocytes. When these oocytes were examined one hour (Figure. 3.7B) or two hours (Figure. 3.7C) following the

Figure 3.4

Activation of PKA prevents progesterone-induced dephosphorylation of myr-HA-β_2AR-C. *Xenopus* oocytes injected with myr-HA-β_2AR-C mRNAs were incubated in OR2 for overnight. Groups of at least twenty oocytes were incubated in OR2 (A) or OR2 containing 1μM progesterone (Pg) (B, C, D). Group C and D received incubation of 100μM forskolin (Fsk) and injection of recombinant PKAc (0.8unit/oocytes), respectively, 20 minutes prior to the addition of progesterone. One hour later, oocytes were lysed and analyzed by 2-D gel electrophoresis followed by HA immunoblotting. Shown are representative examples of three independent experiments. OH^- and H+ denote the anion and cation ends, respectively, of the tube gels. Circles represent positions of missing spots 1.

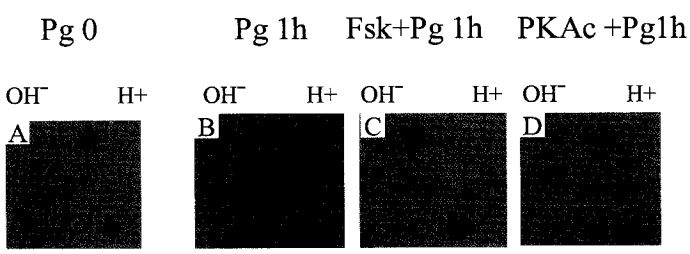

Pg 0
Pg 1h
Fsk+Pg 1h
PKAc +Pg1h
OH-
H+
A
B
C
D

Figure 3.5

H89, a PKA inhibitor, causes dephosphorylation of myr-HA-β_2AR-C in *Xenopus* oocytes. Oocytes injected with mRNA for myr-HA-β_2AR-C were incubated in OR2 for overnight. Oocytes were further incubated in OR2 or OR2 containing H89 (100μM). One hour later, twenty oocytes were retrieved from each group for lysis and 2-D gel electrophoresis followed by immunoblotting with HA antibodies. Shown are representative of three independent experiments.

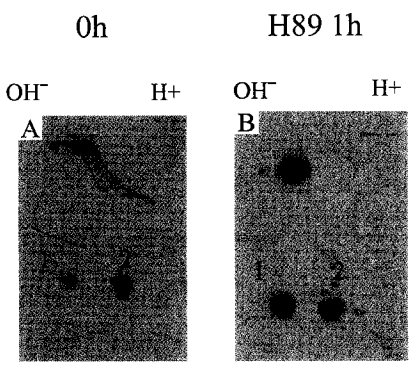

0h
H89 1h
OH⁻
H+
A
OH⁻
H+
B
1
2

Figure 3.6

H89 induces oocyte maturation. Oocytes were injected with mRNA for myr-HA-β_2AR-C and incubated overnight. Following the addition of H89 (100μM), oocytes were monitored for GVBD. Shown are representative of three independent experiments.

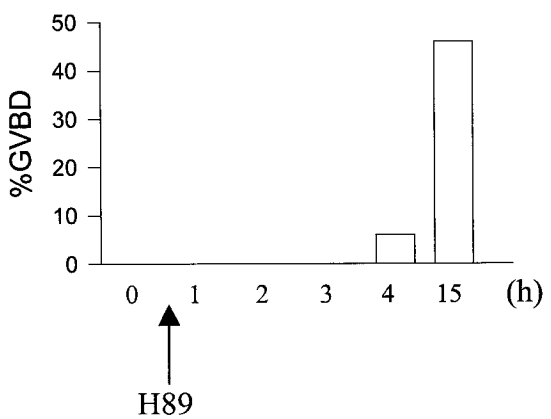
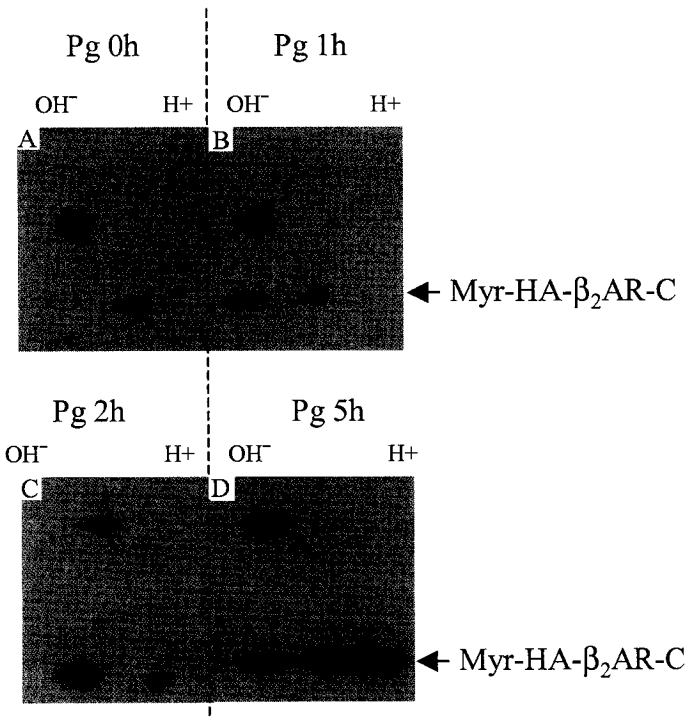

50
40
30
20
10
0
%GVBD
0
1
2
3
4
15
(h)
H89

Figure 3.7

Multiple phosphorylation events of myr-HA-β_2AR-C in frog oocytes. *Xenopus* oocytes injected with mRNA for myr-HA-β_2AR-C were incubated for overnight. Twenty oocytes were set aside as control (Pg0h) and the rest were incubated with 1μM progesterone (Pg). At each of the indicated times, twenty more oocytes were removed and were then subjected to 2-D gel electrophoresis and HA immunoblotting. (D) Multiple phosphorylated forms of myr-HA-β_2AR-C were seen in oocytes that had undergone GVBD (represented by X). Shown are representative of three independent experiments.


Pg 0h
Pg 1h
OH-
H+
OH-
H+
A
B
Myr-HA-β2AR-C
Pg 2h
Pg 5h
OH-
H+
OH-
H+
C
D
Myr-HA-β2AR-C

addition of progesterone, myr-HA-β_2AR-C appeared predominantly as dephosphorylated spot 1, suggesting that progesterone caused PKA inhibition. The time course of progesterone-induced dephosphorylation of PKA site, and hence inhibition of endogenous PKA, was clearly ahead of the activation of maturation-specific protein kinases (eg. MAPK and MPF) and GVBD (Figure. 3.8). In oocytes that had undergone GVBD (5 hours after the addition of progesterone in this particular experiment), myr-HA-β_2AR-C exhibited multiple phosphorylated forms (collectively designated as X) (Figure. 3.7D). Similar analyses of the various mutant forms of myr-HA-β_2AR-C in oocytes that had undergone GVBD indicated that all four constructs exhibited more or less the same pattern of multiple phosphorylated forms (X) (Figure. 3.9). These results argued strongly that a kinase(s) other than PKA or GRK was responsible for myr-HA-β_2AR-C phosphorylation in oocytes that had undergone GVBD.

Progesterone does not change serine/threonine phosphatases activities in frog oocytes

As prophase oocytes contained myr-HA-β_2AR-C that was fully phosphorylated at its PKA site, the decrease in PKA phosphorylation following progesterone stimulation could also be the result of activation of protein phosphatases such as PP1 and/or PP2A, two enzymes thought to be mainly responsible for dephosphorylating PKA sites (Cormier et al., 1990; Wadzinski et al., 1993; Zhang and Lee, 1997). I carried out *in vitro* phosphatase assays to determine whether progesterone caused any significant changes in oocyte phosphatase activities. As shown in Figure. 3.10, prophase oocytes contained significant levels of phosphatase activity capable of dephosphorylating a synthetic phosphopeptide (K-R-pT-I-RR) (Harder et al., 1994). The phosphatase activities in

Figure 3.8

Activation of MAPK and MPF lags behind PKA inhibition. Progesterone-treated oocytes from Figure. 3.7 were scored for GVBD. The same groups of oocytes were also analyzed for MAPK phosphorylation (p-MAPK), and for histone H1 kinase (MPF) activities (H1). Representative examples from three independent experiments are shown.

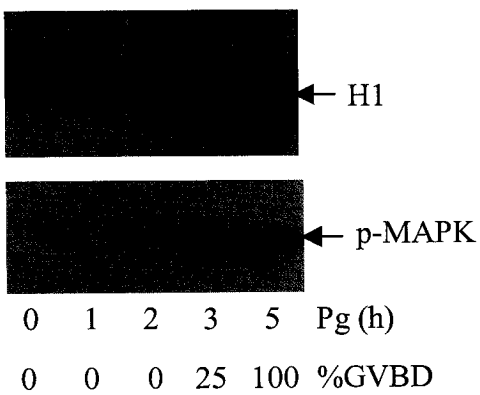

H1
p-MAPK
0 1 2 3 5 Pg (h)
0 0 0 25 100 %GVBD

Figure 3.9

Complex phosphorylation of myr-HA-β_2AR-C in oocytes following GVBD. *Xenopus* oocytes injected with the indicated mRNAs were incubated overnight before the addition of progesterone. Following progesterone-induced GVBD, oocytes were lysed and analyzed by 2-D gel electrophoresis and immunoblotting with anti-HA antibodies. X, multiple phosphorylated forms of myr-HA-β_2AR-C.

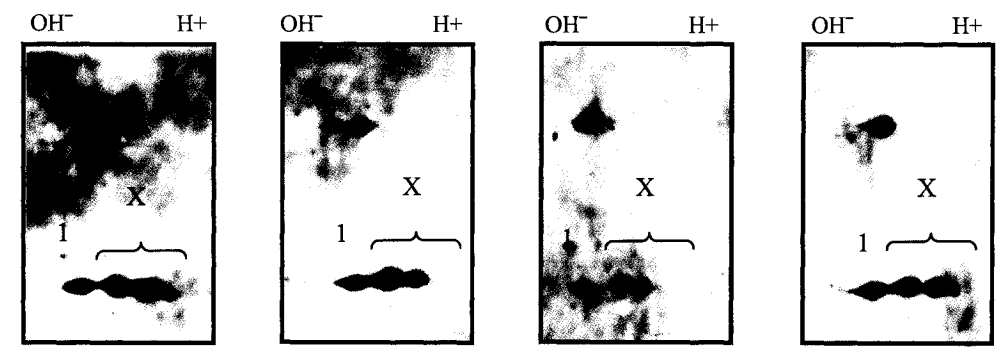
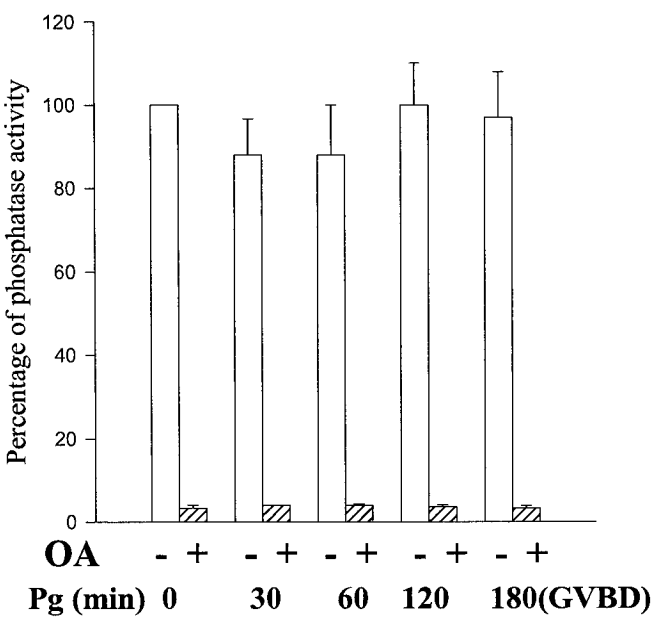

OH⁻
H+
X
1
myr-HA-β2AR-C
PKA-
PKA-&GRK-
GRK-

Figure 3.10

Progesterone does not alter *Xenopus* oocyte serine/threonine phosphatase activities. Phosphatase activities were determined in the absence (-) or presence (+) of 100nM OA. Means±s.e.m. of three (with OA) and five (without OA) independent experiments are shown. In this experiment, more than 50% of the ocoytes had undergone GVBD at 180 minutes after the addition of progesterone. The level of phosphatase activities in prophase oocytes (100%) was shown as 76±6.5 nmol phosphate released per oocyte in thirty minutes.


Percentage of phosphatase activity
120
100
80
60
40
20
0
OA - + - + - + - + - +
Pg (min) 0 30 60 120 180(GVBD)

prophase oocytes were eliminated by okadaic acid (100 nM), a highly specific inhibitor of PP2A (IC50=1 nM) and, to a lesser extent, PP1 (IC50=100-500 nM) (Bialojan and Takai, 1988). However, no significant changes were observed in the levels of phosphatase activities throughout progesterone-induced GVBD (Figure. 3.10).

To employ antibodies to detect PKA-phosphorylated myr-HA-β_2AR-C

To overcome the limitation of 2-D electrophoresis in distinguishing phosphorylation events caused by PKA compared to other protein kinases (e.g. Figure. 3.9), I sought to develop antibodies against PKA-phosphorylated myr-HA-β_2AR-C. I first wished to determine whether myr-HA-β_2AR-C was phosphorylated at Ser-345, Ser-346 or both. I generated two more myr-HA-β_2AR-C constructs, each bearing single serine to alanine mutation (S345A or S346A). As shown in Figure. 3.11, in prophase oocyte myr-HA-β_2AR-C/S345A appeared exclusively as spot 2 (PKA-phosphorylated form) whereas myr-HA-β_2AR-C/S346A only as spot 1 (PKA-dephosphorylated form). Clearly, myr-HA-β_2AR-C is phosphorylated by PKA on single serine (Ser-346) in frog oocytes.

During the course of my studies, Santa Cruz developed commercial phospho-antibodies against RRp-S^{345}p-S^{346} sites of β_2AR. To test whether these antibodies recognize PKA-phosphorylated myr-HA-β_2AR-C in prophase oocytes, I injected mRNA for myr-HA-β_2AR-C (Figure. 3.12 lane 5 and 8) and the various phosphorylation site mutants (Figure. 3.12, Lane1-4). Clearly, despite the fact that the antibodies were raised against doubly (artificially) phosphorylated (RRp-S^{345}p-S^{346}) peptide, they recognize myr-HA-β_2AR-C singly phosphorylated at Ser-346 (Figure. 3.12, lane 1, 4, 5 and 8).

Figure 3.11

PKA phosphorylates myr-HA-β_2AR-C at Ser346 in prophase oocytes. Oocytes were injected with mRNAs for myr-HA-β_2AR/S345A (A) or myr-HA-β_2AR-C/S346A (B). Following overnight incubation, PKA phosphorylation of the two mutants was analyzed by 2-D gel electrophoresis followed by anti-HA immunoblotting.

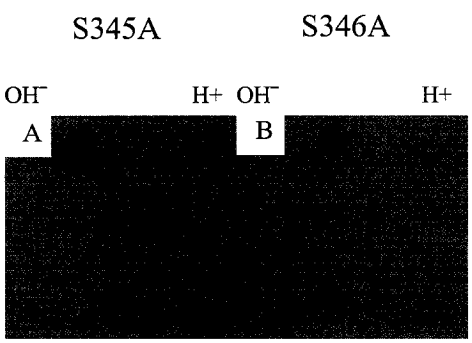
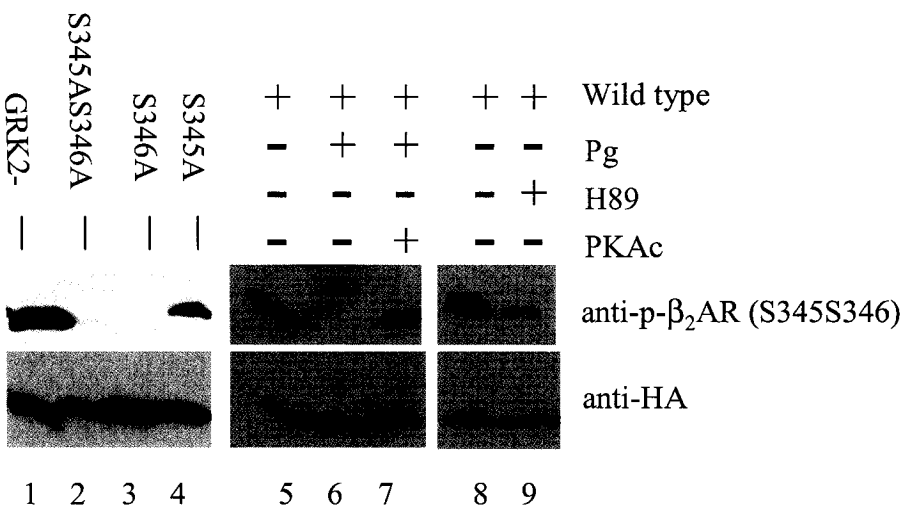

S345A
S346A
OH⁻
H+
OH⁻
H+
A
B

Figure 3.12

Ser-346-phosphorylated-myr-HA-β_2AR-C is recognized by anti-p-β_2AR(S345S346). Oocytes were injected with myr-HA-β_2AR-C/GRK2- (lane 1), myr-HA-β_2AR-C/PKA- (lane2), myr-HA-β_2AR-C/S346A (lane 3), myr-HA-β_2AR-C/S345A (lane 4) or myr-HA-β_2AR-C (lanes 5-9). After overnight incubation, oocytes in lanes 1-5 and 8 were directly lysed and analyzed by SDS-PAGE followed by immunoblotting with anti-p-β_2AR(S345S346) (upper panel) or anti-HA (lower panel). Oocytes in lane 6 were treated with progesterone for 1 hour. Oocytes in lane 7 were injected with PKAc (0.8 units/oocytes) 1 hour prior to the progesterone treatment. Oocytes in lanes 9 were treated with 100μM H89 for one hour.

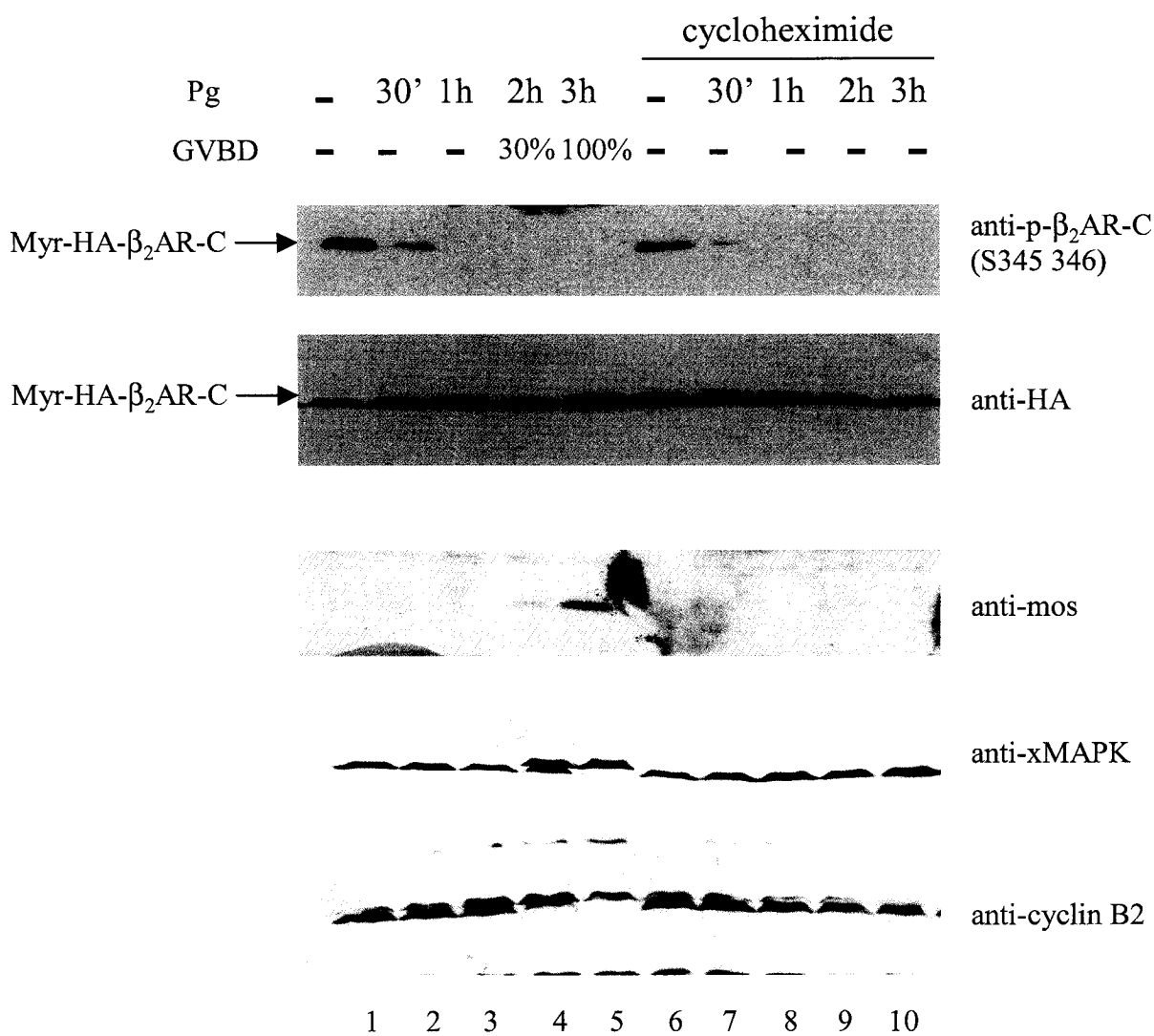

GRK2-
S345AS346A
S346A
S345A
+ + + + + Wild type
- + + - - Pg
- - - - + H89
- - + - - PKAc
anti-p-β2AR (S345S346)
anti-HA
1 2 3 4 5 6 7 8 9

The weaker recognition of Ser345 mutant (comparing lane 4 to lane 1) was probably caused by amino acids mismatch (Ala instead of Ser at position 345). As expected, treating oocytes with progesterone or H89 caused a complete loss of the antibody recognition (Figure. 3.12, lane 6 and 9). In contrast, injection of recombinant PKAc restored antibody recognition, even in the presence of progesterone (Figure. 3.12, lane 7). Expression and stability of the various form of the substrate were confirmed by anti-HA immunoblotting.

With these phosphorylation-specific antibodies, I reinvestigated the time course of myr-HA-β_2AR-C dephosphorylation during progesterone-induced oocyte maturation. Within 30 min of the addition of progesterone, myr-HA-β_2AR-C was significantly dephosphorylated (Figure. 3.13 comparing lane 2 to 1). As 2-D analysis suggested, myr-HA-β_2AR-C was never re-phosphorylated at Ser-346 during the maturation process (Figure. 3.13). Progesterone induced dephosphorylation of myr-HA-β_2AR-C, and therefore inactivation of PKA, occurred long before the almost simultaneous accumulation/activation of mos, MAPK (indicated by phosphorylation-specific shift) and MPF (indicated by phosphorylation-specific shift of cyclin B2) (Figure. 3.13). Interestingly, the protein synthesis inhibitor, cycloheximide, which blocked oocyte maturation and activation of any of the protein kinases, did not affect progesterone-induced dephosphorylation of myr-HA-β_2AR-C (Figure. 3.13, lanes 6-10). Thus, progesterone-induced PKA inactivation is an early event independent of *de novo* protein synthesis. Inhibition of PKA is also persistent during oocyte maturation.

Figure 3.13

Progesterone-induced PKA inhibition is independent of de novo protein synthesis. Oocytes were injected with mRNA for myr-HA-β_2AR-C. After overnight incubation, half of the oocytes were pre-incubated for two hours in OR2 containing 50μg/ml cycloheximide. Both groups were then incubated with progesterone. At the indicated times following the addition of progesterone, oocytes were scored for GVBD and ten oocytes were randomly withdrawn for lysis. The extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. Shown are representative examples of five independent experiments.


cycloheximide
Pg
- 30' 1h 2h 3h - 30' 1h 2h 3h
GVBD
- - - 30% 100% - - - - -
Myr-HA-β2AR-C
anti-p-β2AR-C (S345 346)
Myr-HA-β2AR-C
anti-HA
anti-mos
anti-xMAPK
anti-cyclin B2
1 2 3 4 5 6 7 8 9 10

DISCUSSION

I chose the carboxyl-terminus of β_2AR as the PKA substrate motif for the following reasons. First, phosphorylation of the carboxyl-terminal $RRS^{345}S^{346}$ of β_2AR by PKA has been demonstrated both *in vitro* and *in vivo* (Doronin et al., 2000; Kemp et al., 1977; Zamah et al., 2002). Second, PKA phosphorylation of β_2AR serves an important physiological function in promoting receptor desensitization (Hausdorff et al., 1989; Hausdorff et al., 1990). Third, the carboxyl-terminus of β_2AR, when expressed in isolation, retains its ability to be an efficient and specific substrate for PKA in vitro (Doronin et al., 2000). The decision to target β_2AR-C to the membrane was based on the belief that a membrane-bound β_2AR-C will more closely mimic its physiological precursor, β_2AR.

Despite the importance of cAMP and PKA in cellular signal transduction, direct measurement of cAMP and PKA activity in live cells had not been possible until very recently. The best known are the several fluorescence resonance energy transfer (FRET)-based assays to analyse intracellular cAMP (Zaccolo et al., 2000) and PKA activities (Nagai et al., 2000; Zhang et al., 2001) in live cells. These important advances clearly pave the way for analysing cAMP/PKA signaling in live cells/organism. However, the intrinsic background FRET (in the absence of PKA activation) and the overlapping fluorescence spectra of the donor and acceptor green fluorescence proteins (GFPs) result in rather modest FRET changes following pharmacological activation of PKA (with forskolin or db-cAMP) in live cells (Nagai et al., 2000). On the other hand, neither withdrawing db-cAMP from the culture medium nor the inclusion of PKA inhibitor (H-89) reverses the FRET change induced by db-cAMP (Zhang et al., 2001), raising the

possibility that the substrate motif within AKAR (A-kinase activity reporter) may be phosphorylated by other protein kinases in addition to PKA in live cells. Recently, another group has reported the development of a cell-based assay to monitor intracellular PKA activity by using capillary electrophoresis and a green fluorescence protein (GFP)-tagged substrate containing the PKA phosphorylation site consensus RRRp-SIN (Zarrine-Afsar and Krylov, 2003). However, it appears that in intact cells the peptide only exists in phosphorylated form, with no more than 1% of the substrate existing in unphosphorylated form under any conditions (judged from the data presented in the paper). Again, it seems possible that such a minimal consensus sequence, in the context of a carrier protein (GFP), may be subjected to more promiscuous phosphorylation by other protein kinases.

In contrast, myr-HA-β_2AR-C exhibited almost 100% convertibility between the phosphorylated and dephosphorylated forms when expressed in frog oocyte. Furthermore, the $RRSS^{346}$ motif appeared to be strictly a PKA phosphorylation site, as well as the only PKA phosphorylation site within myr-HA-β_2AR-C. Mutant proteins carrying $RRAA^{346}$ in its position (PKA- and PKA-&GRK-) were not phosphorylated in prophase oocytes (Figure. 3.3C,E and Figure. 3.12). In GVBD oocytes, on the other hand, myr-HA-β_2AR-C appeared to be phosphorylated by one or more protein kinases that become activated at this time. However, the fact that all four constructs exhibited the same pattern of phosphorylation argued strongly that neither the PKA site ($RRSS^{346}$) nor any of the GRK sites were phosphorylated in GVBD oocytes. Nonetheless, the presence of these multiple phosphorylation forms precluded the use of 2-D analyses of myr-HA-β_2AR-C to determine PKA activities in GVBD oocytes. This was confirmed using antibodies against the β_2AR peptides carrying doubly phosphorylated PKA site (RRp-Sp-

S346) (Figure. 3.13).

Earlier studies have clearly implicated cAMP/PKA as major players in maintaining prophase I arrest. Forskolin (Schorderet-Slatkine and Baulieu, 1982) and CTX (Maller et al., 1979), two highly specific cAMP-elevating agents, are both potent inhibitors of progesterone-induced oocyte maturation. Furthermore, whereas injection of PKAc inhibits progesterone-induced oocyte maturation, injection of PKAr or PKI induces hormone-independent oocyte maturation. On the other hand, considerable controversies still exist regarding the endogenous cAMP levels during progesterone-induced oocyte maturation. Overall, there appears to be a consensus that progesterone caused a rapid (within minutes) but modest (~20%) reduction of cAMP (Smith, 1989). However, some have also reported that this reduction is transient, with levels of cAMP recovered quickly (within 10-20 minutes) (Maller et al., 1979), whereas others have reported more persistently reduced levels of cAMP throughout the maturation process (Bravo et al., 1978). Hereby, the present study really clarified the controversies at the level of PKA activities and emphasized that progesterone-induced inhibition of cAMP/PKA signaling during oocyte maturation is not only rapid but also permanent.

For the first time, I have demonstrated that endogenous PKA is activated in prophase oocytes, by showing that a PKA substrate expressed in these oocytes is fully phosphorylated on the PKA site. Furthermore, I have demonstrated that progesterone caused an almost complete loss of PKA phosphorylation of the substrate within 30-60 minutes, long before MAPK activation or MPF activation, which usually occur simultaneously several hours after the addition of progesterone. The rapid loss of PKA phosphorylation is probably the result of dephosphorylation by the significant levels of

okadaic acid-sensitive phosphatase activities that were present in prophase oocyte and remained unchanged throughout the maturation process (Figure. 3.10). In prophase oocytes, PKA was dominant and therefore myr-HA-β_2AR-C was fully phosphorylated on its PKA site, despite these phosphatase activities. Progesterone caused a rapid reduction of oocyte cAMP and the corresponding inhibition of PKA. In the absence of PKA activities, myr-HA-β_2AR-C was rapidly dephosphorylated. However, not all PKA substrates were dephosphorylated equally in the absence of PKA activities. For example, dephosphorylation of the inhibitory PKA site on cdc25C occurs much later and coincides with activation of MAP kinase and MPF (Duckworth et al., 2002). Presumably, the PKA phosphorylation site of cdc25C is protected from phosphatase activities until this threshold point in the maturation process: the 'switch' point described by Ferrell and Machleder (Ferrell and Machleder, 1998).

Perhaps the most important contribution of my study is the demonstration that progesterone caused a rapid and permanent inhibition of PKA during oocyte maturation (Figure. 3.7, 3.12 and 3.13). This was in contrast to the prevailing view that progesterone causes a 'transient' reduction of cAMP. The notion of a transient reduction of cAMP (or inhibition of PKA) was clearly not compatible with several recent studies demonstrating that PKA phosphorylates cdc25C and inhibits its activities towards cdc2 (Duckworth et al., 2002; Schmitt and Nebreda, 2002). As cdc25C is an immediate activator of cdc2 and its activation coincides with activation of MAP kinase and MPF (Duckworth et al., 2002; Izumi and Maller, 1993), PKA must be inhibited at this time. My data clearly support such a notion.

Finally, the demonstration of PKA inhibition in the absence of *de novo* protein synthesis (Figure. 3.13) established that progesterone-induced PKA inhibition was truly an early event dissociable from all other biochemical (activation of numerous protein kinases) and cytological (germinal vesicle breakdown) events that are dependent on *de novo* protein synthesis. The inhibition of PKA and the maintenance of PKA inhibition did not require any feedback mechanism involving Mos-MAP kinase pathway or MPF activity. Together with earlier work that demonstrates inhibition of PKA alone is sufficient to induce oocyte maturation (Maller and Krebs, 1977), my data clearly establish that PKA plays a dominant role in maintaining oocyte prophase I arrest and that rapid and permanent suppression of PKA activity is the most prominent biochemical event in hormonal induction of re-initiation of meiosis (oocyte maturation).

Chapter Four

PKA Restrictive and PKA Permissive Phases of *Xenopus* Oocytes Maturation

INTRODUCTION

Although it is clear that protein kinase A plays a dominant role in maintaining prophase arrest in *Xenopus* ocytes, physiological PKA substrates are poorly characterized. Two recent studies (Duckworth et al., 2002; Schmitt and Nebreda, 2002), have implicated Xcdc25C, a dual specificity protein phosphatase and direct activator of cdc2, as a physiological substrate of PKA. Thus, PKA-catalyzed phosphorylation and inhibition of Xcdc25C is thought to be a major mechanism by which PKA activity helps to maintain prophase I arrest in *Xenopus* oocyte. This conclusion, however, is at odds with a long held view that progesterone causes a transient (within 20 minutes of the hormone addition) reduction of oocyte cAMP (and by extension a transient inhibition of PKA) (Smith, 1989), as Xcdc25 remains phosphorylated (on the PKA site) and inhibited until the time of GVBD, several hours following the addition of progesterone (Duckworth et al., 2002; Oe et al., 2001). To resolve this paradox, I have developed a novel approach to analyse endogenous PKA activity in live cells by expressing a PKA substrate whose phosphorylation status can be analyzed following cell lysis (Wang and Liu, 2004). Using this substrate as a PKA activity indicator in intact oocytes, I have demonstrated that PKA is inactivated within 30 minutes of the addition of progesterone and it remains inactivated at the time of GVBD (Wang and Liu, 2004).

However, the results to that do not address whether PKA is reactivated during the subsequent meiosis I to meiosis II transition, during which MPF undergoes a transient inactivation due to the partial degradation and subsequent re-synthesis of cyclin B (Gerhart et al., 1984). This is particularly important as previous studies have indicated

that the activities of PKA and MPF both oscillate, although opposite to each other, during mitosis (Grieco et al., 1994; Grieco et al., 1996).

In the current study, I demonstrated that during progesterone-induced oocyte maturation, PKA was inactivated within 30 min of the addition of progesterone and thereafter remained inactivated throughout the entire maturation process including meiosis I to meiosis II transition. However, artificial reactivation of endogenous PKA had differential consequences, depending on the timing of PKA reactivation. Reactivation of endogenous PKA at any time prior to germinal vesicle breakdown (GVBD) inhibited progesterone-induced GVBD. PKA reactivation at GVBD, or thereafter, did not interfere with meiosis I to meiosis II transition, nor did it interfere with metaphase II arrest. These results demonstrate for the first time a PKA-restrictive and a PKA-permissive phase in oocyte maturation.

MATERIALS AND METHODS

Materials

Forskolin, progesterone, and H1 were from Sigma. The following antibodies were purchased commercially: from Santa Cruz, phospho-β_2AR (S345S346) (anti-p-β_2AR), PKAc and PKAr; from Upstate Biotechnology, phospho-p42 MAPK (anti-p-MAPK). Antibodies against phosopho-Xcdc25C (Ser-287) (anti-p-cdc25C) were generously provided by Dr. Joan Ruderman. Antibodies against p42 *Xenopus* MAP kinase (anti-

MAPK) were provided by Dr. Jonathan Cooper. Antibodies against cyclin B2 were a gift from Dr. Maller. Sytox green was from Molecular Probe.

Animal and oocyte manipulation

Refer to: Chapter two Materials and Methods unless othervise indicated in the following description.

I isolated oocytes manually or by collagenase treatment (1.5mg/ml plus 1mg/ml trypsin inhibitor). For most experiments described here, it did not matter if oocytes were prepared one way or the other. Oocytes used for analyzing chromosome were isolated by collagenase treatment to ensure complete removal of follicle cells, which would otherwise interfere with visualization of the first polar body and oocyte chromosomes.

Synchronization of GVBD oocytes

In order to synchronize oocytes at GVBD, I typically started progesterone stimulation with a large number of oocytes (e.g. 100-200 per group). I monitored oocytes periodically until some oocytes in the group exhibited the maturation spot (GVBD). At this point, I began withdrawing GVBD oocytes every 5-10 min. Only GVBD oocytes withdrawn at the same time point (within 5-10 min) were further incubated for time course experiments. Therefore I typically selected 20-40 GVBD oocytes from each group and discarded the rest. One or two oocytes were then randomly withdrawn at the indicated time after GVBD and immediately lysed.

Expression plasmids

The PKA substrate construct, myr-HA-β_2AR-C, and its mutants have been described in the chapter three.

Preparation of oocyte extracts and western immunoblotting

Oocytes were crushed through pipette tip in ice-cold extraction buffer (20mM Hepes pH 7.2, 50mM glycerophosphate, 10mM EDTA, 2.5mM $MgCl_2$, 0.25M sucrose, 0.1M NaCl, 1% triton X-100, 200μM PMSF, 10μg/ml leupeptin, 1μM sodium orthovanadate, 10μM H89 and 1μM OA; 5μl/oocyte). Following centrifugation at 13,500rpm × 5min, the clarified supernatants were mixed with 2 × Laemmli sample buffer. Proteins were separated using SDS polyacrylamide gels electrophoresis. transferred to PVDF membrane (Schleicher & Schuell, Germany), and immunoblotted with the indicated primary antibodies. The immunoblots were then developed using a chemiluminescence system (Amersham Bioscience, UK).

MPF kinase assay

Refer to: Materials and Methods in chapter two

Chromosome arrays

Oocytes were fixed at room temperature in 100% methanol for at least 30 min. The fixed oocytes were rehydrated in OR2 followed by incubation in OR2 containing Sytox Green (Molecular Probes, diluted 3000 fold in OR2). Chromosomes were visualized by epifluorescence microscopy.

RESULTS

No PKA reactivation during the entire maturation process

In order to determine whether PKA undergoes a transient activation during transient inactivation of MPF in meiosis I to meiosis II transition, I synchronized oocytes by withdrawing them at the time of GVBD. As shown in chapter three (Figure. 3.13), Ser-346 of the PKA substrate was dephosphorylated within 30 min of the addition of progesterone (Figure. 4.1) and remained dephosphorylated before GVBD (Figure. 4.1) or during meiosis I to meiosis II transition (Figure. 4.2). The rapid and permanent dephosphorylation of the expressed PKA substrate is certainly in contrast to the late dephosphorylation of assumed physiological PKA substrate, cdc25C, which occurred only at GVBD (Figure. 4.1 and 4.2). As a control, MPF was inactive in prophase oocytes (lane 1 in Figure. 4.2), became fully active at GVBD (lane 2 in Figure. 4.2), and underwent partial and transient inactivation shortly after GVBD (lanes 6-12 in Figure. 4.2). MPF activity increased again and remained high in mature eggs (lane 15 in Figure. 4.2). *Xenopus* MAPK also exhibited its typical phosphorylation at GVBD (lane 5 in Figure. 4.1) and remained phosphorylated thereafter (data not shown). These data clearly suggest that no reactivation of PKA occurs during the entire maturation process including meiosis I to meiosis II transition once it is inactivated shortly following progesterone stimulation. These results also demonstrate a clear temporal separation of PKA inhibition and Xcdc25C dephosphorylation. No changes were detected in the protein levels of PKAc (Figure. 4.2,

Figure 4.1

Dephosphorylation of cdc25C lags behind PKA inactivation. myr-HA-β_2AR-C mRNA-injected oocytes were incubated overnight before the addition of progesterone. At each indicated time following the addition of progesterone, five to ten oocytes were withdrawn and lysed. In this particular experiment GVBD started at 2.5 h following the addition of progesterone. Only GVBD-positive oocytes were selected at 2.5 h. The extracts were immunoblotted with the indicated antibodies. Shown are representatives of four independent experiments. An arrow indicates the position of the expressed PKA substrate.

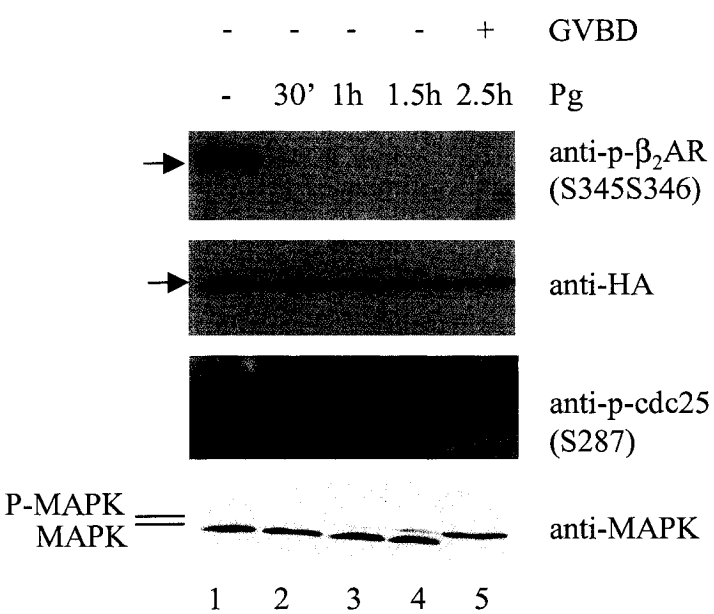
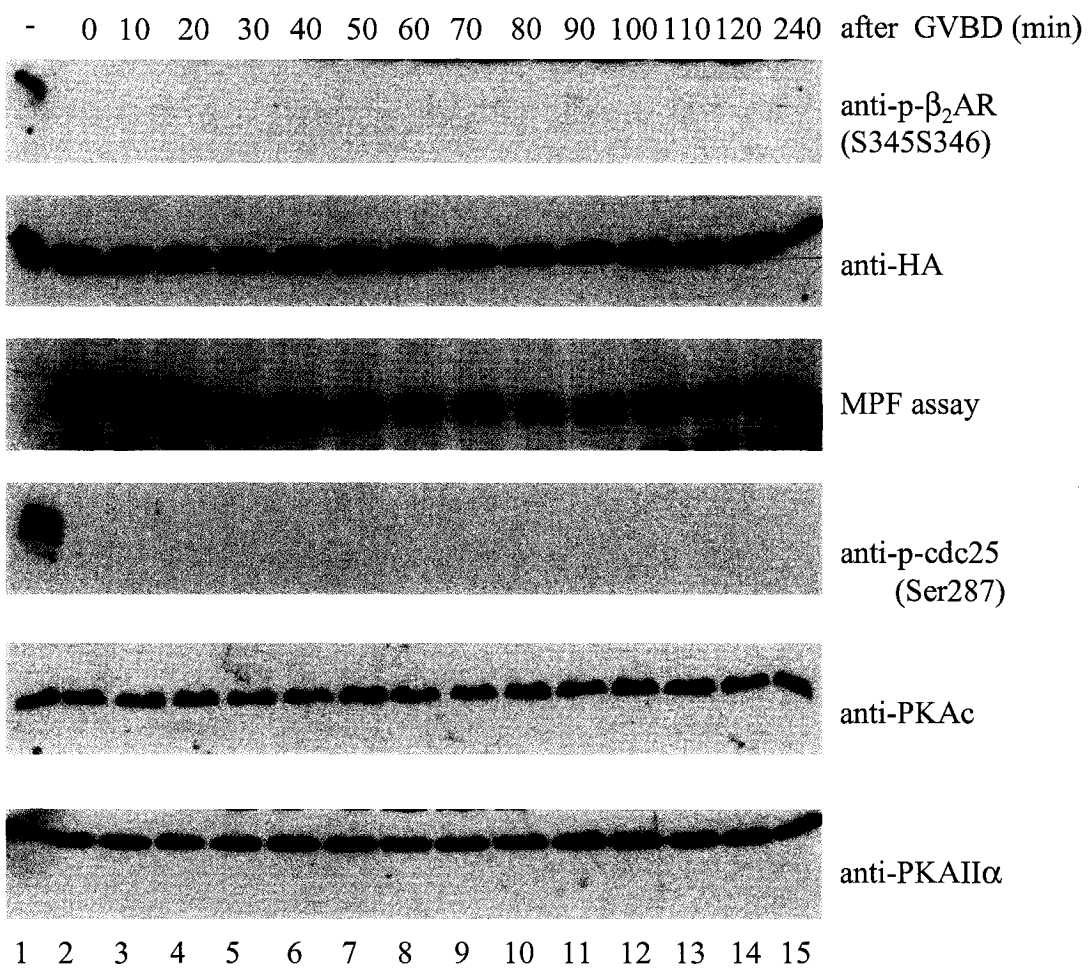

- - - - + GVBD
- 30' 1h 1.5h 2.5h Pg
anti-p-β2AR (S345S346)
anti-HA
anti-p-cdc25 (S287)
P-MAPK
MAPK
anti-MAPK
1 2 3 4 5

Figure 4.2

No reactivation of PKA following GVBD until MII arrest. Oocytes were injected with myr-HA-β_2AR-C mRNA and treated with progesterone following overnight incubation. Synchronized GVBD oocytes at interval time (5-10min) were withdrawn and further incubated for the indicated time before lysis. For each time point, a single oocyte was lysed for MPF assay, and two other oocytes were lysed for immunoblotting with the indicated antibodies. Lane 1 represents oocytes injected with myr-HA-β_2AR-C mRNA but not treated with progesterone. Shown are representatives of three independent experiments.


- 0 10 20 30 40 50 60 70 80 90 100 110 120 240 after GVBD (min)
anti-p-β2AR (S345S346)
anti-HA
MPF assay
anti-p-cdc25 (Ser287)
anti-PKAc
anti-PKAIIα
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

anti-PKAc) and PKAr (Figure 4.2, anti-PKAr) during oocyte maturation, indicating that the failure of PKA reactivation was not due to turn over in its subunits contents. Therefore once PKA was inhibited shortly after progesterone stimulation, it remained permanently inhibited throughout the maturation process and in the mature eggs.

Functional consequences of artificially reactivating PKA before and after GVBD

Schorderet-Slatkine *et al* (Schorderet-Slatkine and Baulieu, 1982) previously reported that the addition of forskolin at anytime prior to GVBD inhibits progesterone-induced GVBD. I have confirmed this finding (Figure. 4.3) but have extended their results by demonstrating that S-346 of the substrate is phosphorylated in the presence of forskolin, indicating that indeed PKA remains active in the presence of forskolin and progesterone. These results indicated that persistent inactivation of PKA is essential for the first phase of MPF activation and for GVBD.

As endogenous PKA remained inactivated following GVBD and during meiosis I to meiosis II transition (Figure. 4.2), I wished to determine the functional consequences of PKA reactivation following GVBD. Full PKA reactivation, as indicated by S-346 rephosphorylation, was achieved within 30 min of the addition of forskolin to GVBD oocytes (Figure. 4.5, top panel, lanes 3 and 4). Despite the rapid reactivation of PKA following GVBD, I observed no changes in the biphasic pattern of MPF activity in forskolin-treated oocytes (Figure. 4.5, MPF assay; compared to that in control oocytes (Figure. 4.4, MPF assay)). Surprisingly, I observed no rephosphorylation of XCdc25C Ser-287 in forskolin-treated oocytes (Figure. 4.5, bottom panel), given the notion that Xcdc25C is a direct substrate of PKA (Duckworth et al., 2002).

Figure 4.3

Forskolin inhibits progesterone-induced GVBD, accompanied by Ser-346 phosphorylation. The PKA substrate mRNA-injected oocytes were incubated overnight before being split into 7 groups (twenty or more oocytes each). Progesterone was added to all but group 1. At the indicated time after progesterone addition, forskolin (100μM) was added to individual groups, except for group 7 which received no forskolin as oocytes started to undergo GVBD 3h after the addition of progesterone in this particular experiment. Eight hours after the addition of progesterone, all groups were scored for GVBD and lysed. The extracts were immunoblotted with the indicated antibodies. p-MAPK antibodies recognized only active form of *Xenopus* MAPK. Shown are representatives of four independent experiments.

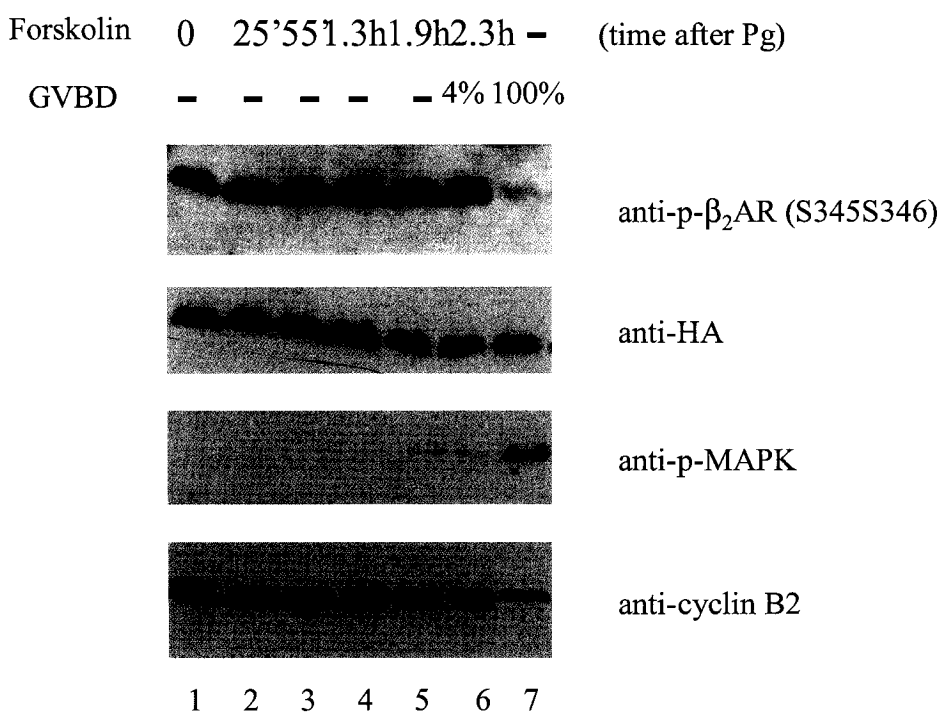

Forskolin 0 25'55'1.3h1.9h2.3h – (time after Pg)
GVBD – – – – – 4% 100%
anti-p-β2AR (S345S346)
anti-HA
anti-p-MAPK
anti-cyclin B2
1 2 3 4 5 6 7

Figure 4.4

Progesterone-induced oocyte maturation contains bi-phasic MPF activities. Oocytes injected with mRNA for myr-HA-β_2AR-C were incubated overnight and then were treated with progesterone. Synchonized GVBD oocytes at interval time 5-10min (20-25 oocytes/group) were withdrawn and immediately transferred to OR2. For each time points, a single oocyte was lysed for MPF assay, and another oocyte was lysed for immunoblotting with the indicated antibodies. Lane 1 represents oocytes injected with myr-HA-β_2AR-C but not treated with progesterone. Shown are representative of four independent experiments.

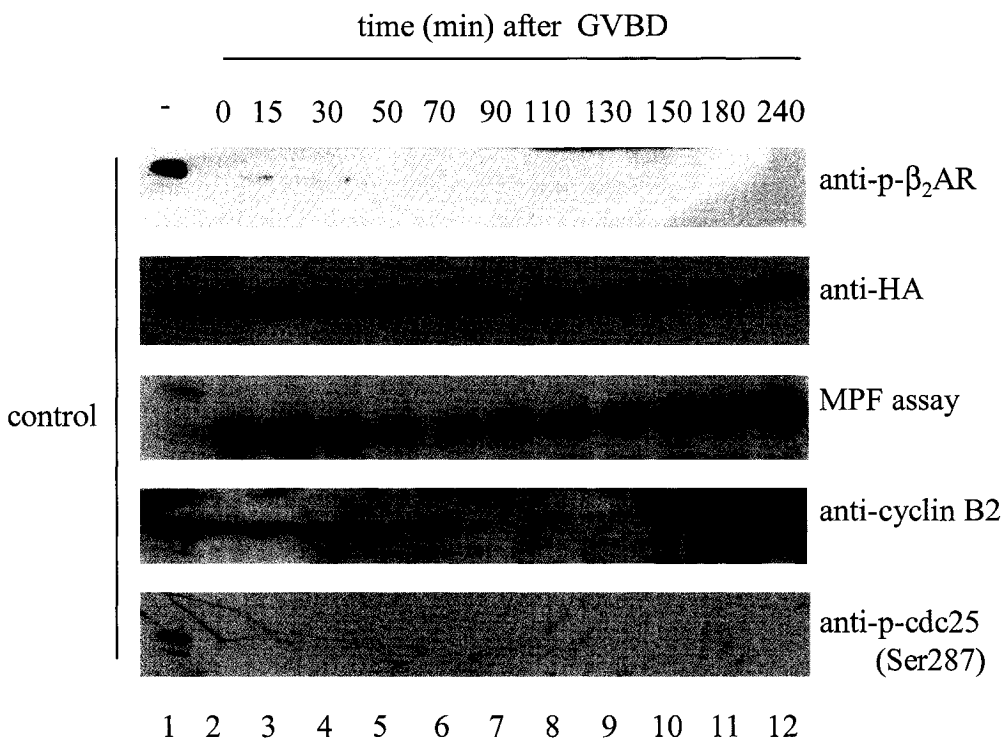
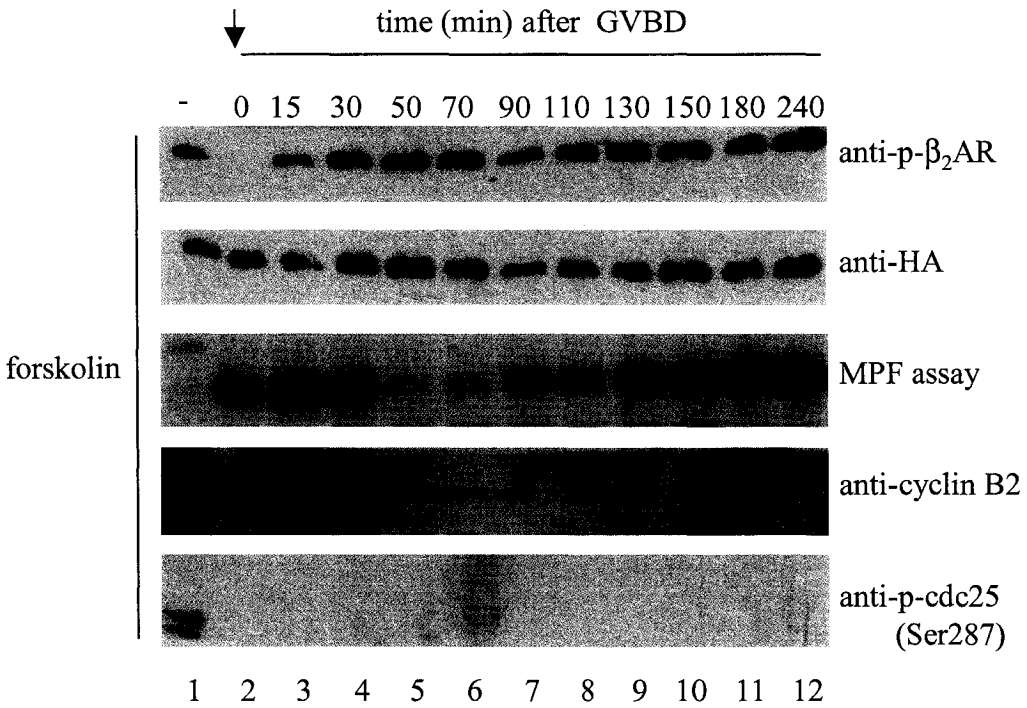

time (min) after GVBD
- 0 15 30 50 70 90 110 130 150 180 240
anti-p-β_2AR
anti-HA
control
MPF assay
anti-cyclin B2
anti-p-cdc25 (Ser287)
1 2 3 4 5 6 7 8 9 10 11 12

Figure 4.5

Reactivation of endogenous PKA at GVBD does not affect bi-phasic MPF activities. Oocytes injected with mRNA for myr-HA-β_2AR-C were incubated overnight and then were treated with progesterone. Synchonized GVBD oocytes at interval time 5-10min (20-25 oocytes/group) were withdrawn and immediately transferred to OR2 containing 100μM forskolin. For each time points, a single oocyte was lysed for MPF assay, and another oocyte was lysed for immunoblotting with the indicated antibodies. Lane 1 represents oocytes injected with myr-HA-β_2AR-C but not treated with progesterone. Shown are representative of four independent experiments. An arrow indicates time when oocytes were transferred to OR2 containing 100μM forskolin.


time (min) after GVBD
- 0 15 30 50 70 90 110 130 150 180 240
anti-p-β2AR
anti-HA
forskolin
MPF assay
anti-cyclin B2
anti-p-cdc25 (Ser287)
1 2 3 4 5 6 7 8 9 10 11 12

To determine whether reactivation of PKA at GVBD disrupted meiosis I to meiosis II transition, I incubated GVBD oocytes (within 5 min of the appearance of the maturation spot) in the presence of 100 μM forskolin. These oocytes were incubated overnight before being subjected to chromosome imaging. The majority of these oocytes (87/89, three experiments) completed maturation and were arrested in metaphase II (Figure. 4.6 and 4.7, forskolin). As a comparison, 92/97 control oocytes (no forskolin treatment, three experiments) were also properly matured and arrested in metaphase II (Figure. 4.6 and 4.7, water). Therefore reactivation of PKA at GVBD had no influence in the completion of oocyte maturation, as judged by the biphasic MPF activity (Figure. 4.5) and chromosome morphology (Figure. 4.6 and 4.7).

DISCUSSION

The most important discovery reported here is the demonstration that PKA activity is restrictive only during the early phase of oocyte maturation, i.e. up to GVBD. The later stages of oocyte maturation (meiosis I to meiosis II transition), including formation of metaphase I spindle, homolog separation (anaphase), emission of the first polar body and formation of metaphase II spindle, are not sensitive to PKA activation. Biochemically, this division of "early" and "late" stages of oocyte maturation coincides with before and after, respectively, the "switch-like" activation of MAPK, as first reported by Ferrell and Machleder (Ferrell and Machleder, 1998). When a single oocyte is analyzed, activation of MAPK is temporally indistinguishable from GVBD (the first appearance of the maturation spot) (Ferrell and Machleder, 1998; Ohan et al., 1999).

Figure 4.6

Reactivation of endogenous PKA at GVBD does not interfere with the completion of oocyte maturation. The PKA substrate mRNA-injected oocytes (100-150) were incubated overnight before the addition of progesterone. Individual oocytes were selected based on the first appearance of the maturation spot (GVBD) and immediately transferred to a well containing 100μM forskolin. The forskolin-treated oocytes were further incubated overnight and fixed and subjected to chromosome array analyses (four independent experiments).

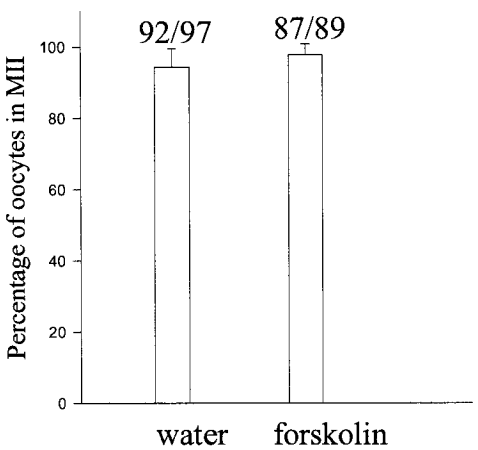
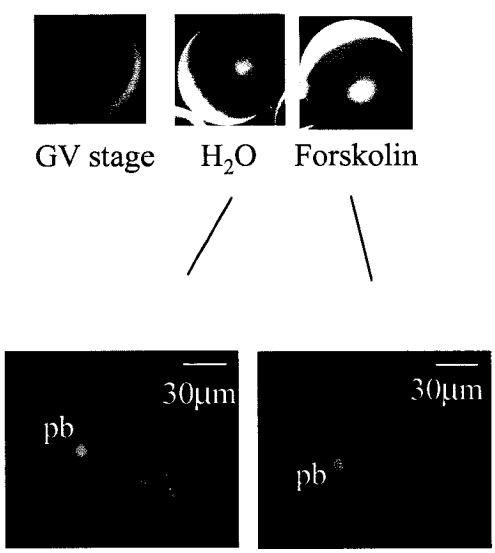

92/97
87/89
Percentage of oocytes in MII
100
80
60
40
20
0
water
forskolin

Figure 4.7

Typical images of prophase oocytes and mature eggs from experiments in Figure. 4.6. Upper level: oocyte images under light microscopy; Lower level: chromosome and pb (first polar body) images under epifluorescence microscopy.


GV stage
H_2O
Forskolin
30μm
pb
30μm
pb

Similarly, activation of Xcdc25C (indicated by Ser-287 dephosphorylation) and MPF (either by kinase assay or by dephosphorylation of Thr-14 and Tyr-15 of the $p34^{Cdc2}$ subunit) also coincides with MAP kinase activation and GVBD (Duckworth et al., 2002). In contrast to these coincidental biochemical events, inhibition of PKA is a temporally separable event, occurring well ahead of GVBD. In addition, progesterone-induced PKA inhibition occurs normally in the presence of cycloheximide, a potent inhibitor of protein synthesis (Figure. 3.13). In contrast, cycloheximide inhibits all the other biochemical events (Figure. 3.13). Therefore, following inhibition of PKA, at least one translational event must occur before the eventual Ser-287 dephosphorylation and activation of Xcdc25C. I suggest (Figure. 4.8) that the accumulation of this protein(s), whose synthesis is also likely regulated by a positive feedback mechanism involving MPF (Castro et al., 2001), to a threshold level dictates the timing of the switch-like MAPK and MPF activation (Ferrell and Machleder, 1998) and GVBD. The addition of forskolin at anytime before GVBD would reactivate PKA and therefore halt protein translation, hence inhibiting this switch.

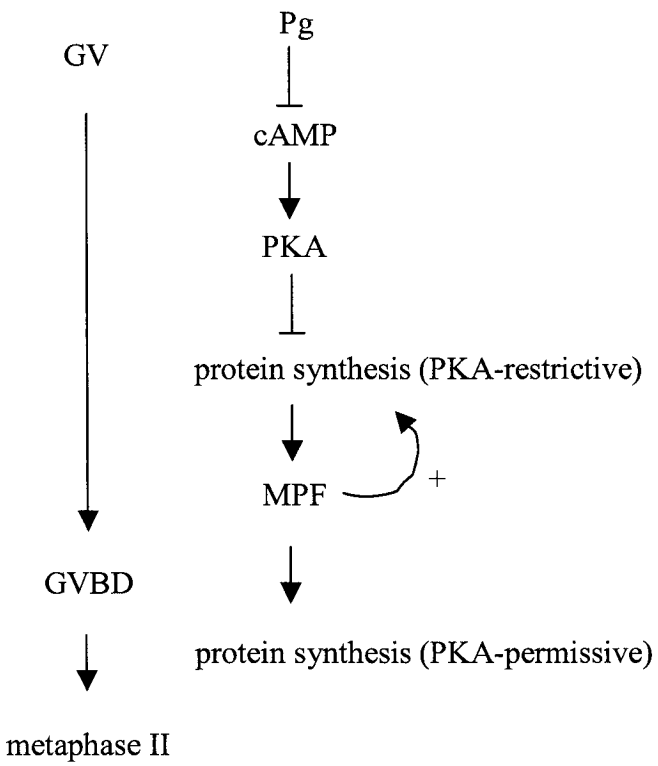
What is this translation event(s)? An earlier candidate, mos (Sagata et al., 1988), does not appear to fulfill this criterion. Mos is almost undetectable in prophase oocytes. Progesterone-induced mos translation becomes detectable shortly after the addition of progesterone (Sagata et al., 1989) and it is inhibited if forskolin is present (Sagata, 1997). However, a recent study reported that almost complete inhibition of mos translation by antisense morpholino oligos only slightly delays MPF activation (Dupre et al., 2002a). Therefore, defining the translational event(s) that is necessary for progesterone-induced MPF activation and GVBD remain an elusive goal.

In contrast to the early protein synthesis, which is inhibited by PKA (PKA-restrictive), protein synthesis following GVBD is not inhibited by PKA (PKA-permissive). This conclusion can be drawn from my observation that reactivation of PKA at GVBD does not interfere with de novo synthesis of cyclin B2 and the reactivation of MPF (Figure. 4.5). Furthermore, reactivation of PKA at GVBD does not interfere with the normal completion of meiosis I or the proper metaphase II arrest (Figure. 4.6 and 4.7). Inhibition of protein translation at GVBD, on the other hand, inhibits *de novo* synthesis of cyclin B or the second phase of MPF activation, and prevents completion of meiosis I and causes oocytes to enter an interphase-like state (Hochegger et al., 2001). Therefore, PKA-insensitive protein translation is required for the completion of oocyte maturation beyond GVBD (Figure. 4.8).

What are the physiological substrates for PKA in prophase I-arrested oocytes? It has recently been proposed that PKA phosphorylates the dual specificity phosphatase cdc25C at Ser-287 and, as a result, inhibits the latter's activity towards cdc2 kinase (Duckworth et al., 2002). However, as shown in Figure. 4.1, progesterone-induced PKA inhibition (indicated by Ser-346 dephosphorylation) occurs long before dephosphorylation and activation of Xcdc25C. Furthermore, while progesterone-induced PKA inhibition can occur in the presence of the protein synthesis inhibitor cycloheximide (Wang and Liu, 2004), dephosphorylation and activation of Xcdc25C requires *de novo* protein synthesis (Duckworth et al., 2002). These studies, though do not rule out PKA as the kinase responsible for Ser-287 phosphorylation, indicate that other events in addition to PKA inhibition are required for Ser-287 dephosphorylation (Kumagai et al., 1998; Margolis et al., 2003). Given these considerations, it is noteworthy that reactivation of

Figure 4.8

Schematic representation of PKA-restrictive and PKA-permissive protein synthesis during oocyte maturation. Reactivation of PKA at any time prior to GVBD blocks progesterone-induced oocyte maturation by inhibition of protein synthesis. Reactivation of PKA at GVBD, or thereafter, does not influence maternal protein degradation and re-synthesis (cyclin B2) during meiosis I to meiosis II transition.


Pg
GV
cAMP
PKA
protein synthesis (PKA-restrictive)
MPF
+
GVBD
protein synthesis (PKA-permissive)
metaphase II

PKA at GVBD does not cause rephosphorylation of Xcdc25C on Ser-287 (Figure. 4.5). These data raise the question whether Xcdc25C is a direct substrate of PKA in frog oocytes. In addition to PKA (Duckworth et al., 2002), several other protein kinases have been implicated in phosphorylating Ser-287 of Xcdc25C (Perdiguero and Nebreda, 2004). Of particular interest is *Xenopus* Chk1, a G2 checkpoint-activated protein kinase. Chk1 has been implicated as the inhibitory kinase responsible for phosphorylating Ser-216 of cdc25C (human sequence, equivalent to Ser-287 in Xcdc25C) in multiple organisms (Bulavin et al., 2002). *Xenopus* Chk1 potently inhibits progesterone-induced GVBD (Oe et al., 2001). It also potently counteracts the ability of Xcdc25C, but not Xcdc25C(S287A), in GVBD induction. Although in vitro kinase assays have not revealed appreciable changes in Chk1 activity during oocyte maturation (Oe et al., 2001), it remains to be determined whether XChk1 is inhibited by progesterone in vivo.

Regardless of whether PKA or Chk1 is the kinase responsible for phosphorylating Ser-287 of Xcdc25C, PKA must have other, temporally more proximal target(s) in maintaining prophase arrest. Progesterone-induced oocyte maturation is dependent on *de novo* protein synthesis (Wasserman and Masui, 1975). In the chapter three, I have shown that inhibition of protein synthesis does not affect progesterone-induced PKA inhibition. Inhibition of PKA therefore lies upstream of protein translation in progesterone induced oocyte maturation. The fact that inhibition of PKA by PKI leads to protein translation-dependent oocyte maturation (Duckworth et al., 2002) would also argue that other physiological substrate(s) of PKA must be dephosphorylated before the eventual, protein synthesis-dependent, dephosphorylation and activation of cdc25C.

In summary, by monitoring in vivo PKA activity, I have discovered a PKA-restrictive phase (prior to GVBD) and a PKA-permissive phase (following GVBD) during oocyte maturation. I propose the presence of one or more PKA-restrictive translational events, in addition to Mos, prior to GVBD. Identification of such translational events will lead to the mechanism by which PKA helps to maintain meiotic prophase arrest.

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Chapter Five

General Discussion

In vertebrates oogenesis starts in the embryonic stage and oogenic meiosis progresses to the diplotene stage of the first meiotic prophase (prophase I), at which point the oocytes are arrested until puberty. During the prolonged prophase I arrest, the primary oocytes increase in size, accompanied with growth of the surrounding follicle cells. The surrounding follicle cells secrete proteins, hormones and other molecules to allow the enclosed oocytes to grow, while in the meantime maintain the oocytes in prophase arrest. Prior to ovulation, the follicle-enclosed oocytes resume meiosis (oocyte maturation) under the influence of pituitary gonadotropins (follicle-stimulating hormone and luteinizing hormone). The gonadotropic hormones target to their cognated receptors localized in the plasma membranes of follicle cells. These receptors are Gs-coupled GpCR, hence resulting in elevation of cAMP in follicle cells. Signaling through cAMP in the follicle cells result in production of progesterone and androgen, which are thought to trigger oocyte maturation in amphibian species. However, definition of steroids in mammalian oocyte maturation remains controversial. In contrast to the cAMP elevation in follicle cells, cAMP level in the oocytes decreased during oocyte maturation.

Two alternative models of G protein signaling have been employed to explain the reduction of intracellular cAMP during oocyte maturation. First, G protein may be involved in releasing meiotic oocytes from prophase I arrest. Evidence from fish oocytes is in support of this model. Pertussis toxin (PTX), which ADP-ribosylates and inhibits the classical Gαi subunit, blocks starfish oocyte maturation in response to the meiosis inducing substance, 1-methyladenine (Chiba et al., 1992; Shilling et al., 1989). Furthermore, the treatment of 1-methyladenine redistributes Gαi from plasma membrane to intracellular structure (Lamash and Eliseikina, 2002). Therefore, resumption of

starfish oocyte maturation depends on activation of Gαi and the release of Gβγ subunits (Chiba and Hoshi, 1995; Jaffe et al., 1993; Thomas et al., 2002).

On the other hand, G protein signaling may be responsible for maintaining an oocyte prophase arrest in species like mouse and *Xenopus*. Microinjection of an antibody raised against the Gαs into mouse oocytes within the follicle (Mehlmann et al., 2002), or microinjection of a dominant negative form of Gαs into *Xenopus* oocyte (Kalinowski et al., 2004), causes oocyte maturation. In contrast, activation of Gs signaling pathway blocks oocyte maturation in both frogs and mice (Downs et al., 1992; Gallo et al., 1995; Romo et al., 2002; Vivarelli et al., 1983). Thus, prophase arrest in these vertebrate oocytes appears to be sustained by Gs signaling. A major part of my thesis work was designed to demonstrate the presence, and the proposed function, of a GpCR* as responsible for maintaining high level cAMP and oocyte prophase I arrest.

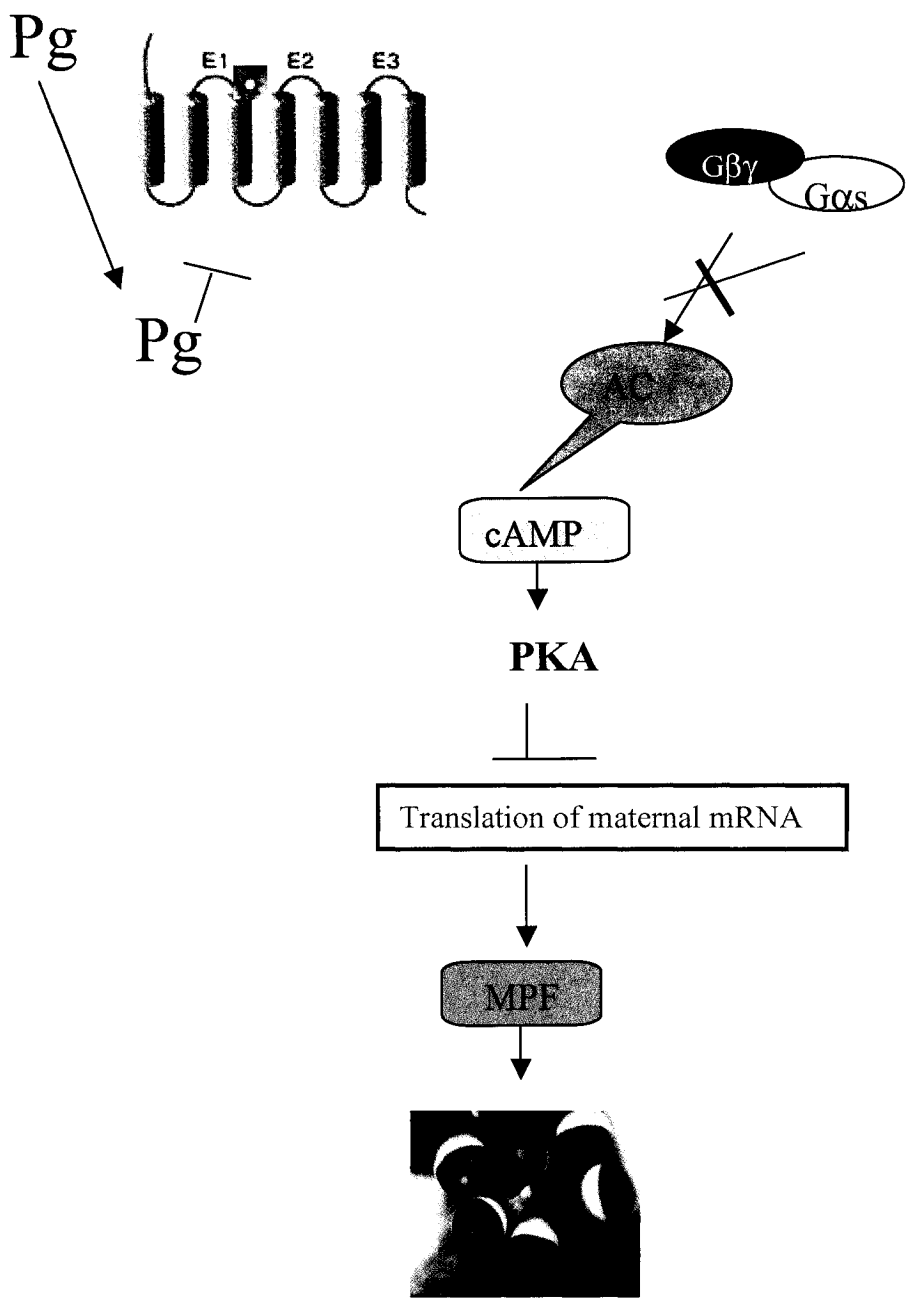
As recent studies have suggested that besides GpCRs, receptors with intrinsic protein tyrosine kinase activities can also regulate heterotrimeric G proteins (Booth et al., 2002; Kuemmerle and Murthy, 2001; Lou et al., 2001; Luttrell et al., 1995) with direct binding of the receptors to the α subunits of the G proteins (Dalle et al., 2001; Hallak et al., 2000; Patel, 2004). To demonstrate the presence of the activated GpCR* in maintaining prophase I arrest in frog oocyte, I employed GRK3 and β-arrestin-2, which cause GpCR* desensitization and internalization (Claing et al., 2002). The rationale of this project is that if indeed prophase I arrest is mediated by the GpCR*, possibly through an autocrine loop where its ligand is secreted by the oocyte or in a ligand-independent manner, overexpression GRK3 and β-arrestin-2 may desensitize the activity of the GpCR* and therefore should cause oocyte maturation. I demonstrated that injection of

mRNA for rat GRK3 or β-arrestin-2 caused hormone-independent GVBD. I further demonstrated that inhibition of clathrin-mediated receptor endocytosis significantly reduced the abilities of GRK3/ β-arrestin-2 to induce GVBD. As depicted in Figure. 5, my thesis work contributes significantly to the current scheme that progesterone induces oocyte maturation by inhibiting a constitutively activated GpCR.

Progesterone induces *Xenopus* oocyte maturation through a novel, cytoplasmic signaling pathway, ultimately leading to the activation of MPF (Figure. 5). The most prominent signaling pathway regulated by the "nongenomic" action of progesterone is the second messenger cAMP and its target, PKA. It has been known for three decades that hormonal induction of oocyte maturation in the frog, and in mammals, requires reduction of intracellular cAMP (Cho et al., 1974; Jagiello et al., 1981; Magnusson and Hillensjo, 1977; Sadler and Maller, 1987; Schorderet-Slatkine and Baulieu, 1982). Using radioimmunoassay to measure intracellular cAMP level during *Xenopus* oocyte maturation, some investigators observed that progesterone caused an early and transient reduction of intracellular cAMP (Maller et al., 1979), while others showed that the reduction of cAMP by progesterone is more persistent (Bravo et al., 1978). Regardless of the timing difference, it is also not clear whether the modest (20%) reduction (Bravo et al., 1978; Maller et al., 1979; Masui and Markert, 1971) of intracellular cAMP, estimated to be 1μM, is sufficient to cause inhibition of PKA. Possibly, physiological relevant reduction of cAMP in frog oocytes is spatially restricted, as has been demonstrated in muscle cells (Zaccolo and Pozzan, 2002). Therefore, measurement of total intracellular cAMP level might not be a good parameter for detecting the regulation of the second messenger and its target (PKA) by progesterone during oocyte maturation.

Figure 5

A constitutively activated GpCR* maintain prophase arrest in frog oocytes. See text for detailed information.


Pg
E1
E2
E3
Pg
Gβγ
Gαs
AC
cAMP
PKA
Translation of maternal mRNA
MPF

Despite of uncertainty of cAMP dynamic during oocyte maturation, PKA clearly plays a dominant role in maintaining prophase arrest and that inhibition of PKA is required for oocyte maturation (Maller and Krebs, 1977; Sadler and Maller, 1983; Sadler and Maller, 1981; Speaker and Butcher, 1977). However, direct measurement of endogenous PKA activity dynamics in live frog oocytes has never been reported. This is because PKA is regulated by intracellular cAMP, traditional in vitro protein kinase assay following cell lysis is thus not suitable for analyzing PKA activity. Determining the physiological change of endogenous PKA during oocyte maturation would lead us to explore mechanism by which PKA helps to maintain prophase arrest. My thesis work provides the first biochemical demonstration that endogenous PKA activity is high in prophase-arrest oocytes and progesterone causes a rapid and persistent reduction of PKA activity during oocyte maturation. I accomplished this by using an expressed PKA substrate as the in vivo PKA indicator (Wang and Liu, 2004). The novel assay, analyzing phosphorylation status of the specific PKA substrate after lysing cells, allows me to detect endogenous PKA activity in live oocytes. I also demonstrated the potential use of this strategy in a mammalian cell system. The quantitative conversion of the PKA substrate from phosphorylated form to dephosphorylated form, as demonstrated by 2-D gel analysis, clearly indicates the sensitivity of this strategy for measuring PKA in live oocytes.

In addition to be the first to demonstrate PKA activity dynamic in frog oocytes, my thesis work also advanced knowledge about mechanisms by which PKA helps to maintain prophase arrest. Recent studies identify two physiological substrates for PKA, cdc25C and wee1B (Duckworth et al., 2002; Han et al., 2005). Both are immediate

regulators of cdc2, the catalytic subunit of MPF. Cdc25 is a dual specificity phosphatase that dephosphorylates and activates cdc2. Wee1B is a dual specificity protein kinase that phosphorylates and inhibits cdc2. Therefore, these studies suggest that PKA maintains prophase arrest by simultaneously inhibiting cdc25C and activating wee1, thus resulting in "double" inhibition of MPF. My thesis work suggests that other physiological substrates of PKA, in addition to cdc25C and wee1, must exist in oocytes that are important for maintaining prophase arrest. Specifically, I show that in the presence of cycloheximide, an inhibitor of protein synthesis, progesterone causes inhibition of PKA, but does not cause activation of cdc25C and MPF. Therefore, following PKA inhibition, *de novo* synthesis of certain proteins is required before cdc25C activation. We propose that PKA maintain prophase arrest by phosphorylation and inhibition of key component(s) of oocyte protein synthesis machinery. Possible PKA substrates are proteins related to cytoplasmic polyadenylation, which is a key mechanism to regulate protein synthesis during oocyte maturation (Kuge and Inoue, 1992; McGrew et al., 1989). Certainly, future work is required to identify these PKA substrates.

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WORK EXPERIENCE:

- *May 2001 - present:* Research assistant, Dept. of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada. Studying signaling pathways involved in frog oocyte maturation;
- *Sept. 1998 - Apr. 2001:* Research assistant, Dept. of Physiology, McGill University, Montreal, Quebec, Canada. Studied mechanisms involved in antinociceptive action of delta opioid receptors at the spinal level with both behavioral and electrophysiological methods;
- *Jul. 1997 - Jul. 1998:* Lecturer and research assistant in Dept. of Physiology, Xi'an Medical University, Xi'an, Shaanxi, P.R.China. Gave lectures and supervised lab courses in physiology; Participated in the neurophysiological research (pain mechanism) with graduate students;
- *Jul. 1996 - Jun. 1997:* Intern Doctor, Hospital attached to Xi'an Medical University, Xi'an, Shaanxi, P.R.China. Practiced in Divisions of Medicine, Surgery, GYN and OBS, and Infect disease as a clinic doctor. Gained experience in the precaution, diagnosis and curing of a variety of clinic diseases.

PUBLICATIONS:

- Protein kinase A (PKA) restrictive and PKA permissive phases of oocyte maturation, **Jing Wang**, Winnie L Cao and X. Johné Liu, **Cell cycle**, 5(2) 213-7, 2006;
- Monitoring protein kinase A activities using expressed substrate in live cells, **Jing Wang** and X. Johné Liu, chapter 30 in the book called **Xenopus protocols**: *Cell Biology and Signal transduction* in the series, Methods in Molecular Biology, the humana Press Inc;
- Progesterone inhibits protein kinase A (PKA) in *Xenopus* oocytes: demonstration of endogenous oocyte PKA activities using expressed substrate, **Jing Wang** and X. Johné Liu, **Journal Cell Science**, 117(Pt 21):5107-16,2004;
- A G protein-coupled receptor kinase induces Xenopus oocyte maturation, **Jing Wang** and X. Johné Liu, **J Bio Chem**, 278 (18) 15809-14,2003;
- Modulation by delta-opioid receptor agonists of the response to pro-nociceptive transmitters at the spinal level in the rat, **Jing Wang** and James L. Henry, No.823.9 in abstract of 30th **Society for Neuroscience** in 2000;

SCIENTIFIC PRESENTATIONS:

- Protein kinase A (PKA) restrictive and PKA permissive phases of oocyte maturation **Jing Wang**, Winnie L Cao, and X. Johné Liu, **oral presentation**. Ottawa Reproductive Biology Workshop, May 2, 2005, Ottawa, Ontatio, Canada
- Dynamic of protein kinase A activity during Xenopus oocyte maturation **Jing Wang**, Winnie L Cao, and X. Johné Liu, **Poster presentation**. EMBO Workshop 2005 " meiotic divisions and checkpoints", Mar.16-20, 2005, Cargese, Corsica, France
- Progesterone inhibits protein kinase A (PKA) in *Xenopus* oocytes: demonstration of endogenous oocyte PKA activities using expressed substrate **Jing Wang** and X. Johné Liu, **Poster presentation**. International Xenopus Meeting, Sept.14-18, 2004, Woods Hole Marine Biology Laboratory, Massachusetts, USA
- A new approach for monitoring endogenous activation of protein kinase A using two dimension gel electrophoresis and in vivo substrate during Xenopus oocyte maturation **Jing Wang** and X. Johné Liu, **Poster presentation**. Canadian Human Reproduction and Reproductive Biology Workshop, May 3-4, 2004, Ottawa, Ontario, Canada
- A G-protein coupled receptor kinase induces oocyte maturation **Jing Wang** and X. Johné Liu, **Poster presentation**. Keystone Symposia: cell cycle, Jan6-11, 2004, Snowbird, Utah, USA
- A G-protein coupled receptor kinase induces oocyte maturation **Jing Wang** and X. Johné Liu, **Poster presentation**. Ottawa Reproductive Biology Workshop, May12, 2003, Ottawa, Ontario, Canada
- Modulation by delta-opioid receptor agonists of the response to pro-nociceptive transmitters at the spinal level in the rat, **Jing Wang** and James L. Henry, **Poster presentation**. 30th Society for Neuroscience, Nov. 4-9, 2000, New Orleans, Louisiana, USA

AWARDS RECEIVED:

- "CIHR Doctoral Research Award" 2004-2006;
- "Excellence scholarship" awarded by Faculty of Graduate and postdoctoral studies (FGPS) of University of Ottawa, 2005-2006;
- "Keystone travel scholarship" awarded by Keystone Symposia: cell cycle, Snowbird, 2004;
- "Admission scholarship" awarded by Faculty of Graduate and postdoctoral studies (FGPS) of University of Ottawa, 2001-2005;
- "Department entrance scholarship" awarded by Dept. of Biochemistry in University of Ottawa, 2001-2003;
- "Graduate with distinction" title awarded by Shaanxi Province, P.R.China, 1997;
- "Most Outstanding Student" title and first grade scholarship, awarded by Xi'an Medical University, Xi'an, P.R.China, 1993-1996 ;
- 505 Scholarship, awarded by Xi'an Medical University, Xi'an, P.R.China, 1993.