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G protein signalling and G protein coupled receptor (GPCR) pathway in *Xenopus* oocyte maturation

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G protein signaling and G protein coupled receptor (GPCR) pathway in *Xenopus* oocyte maturation

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ABSTRACT

Xenopus laevis oocytes are physiologically arrested at the first meiotic prophase. Progesterone reinitiates meiosis (maturation) through inhibition of an oocyte adenylyl cyclase (AC) and reduction of intracellular cAMP. However, the mechanism by which *progesterone regulates AC activity and cAMP level* still remains unclear.

In this thesis, I summarize work I conducted that collectively helps elucidate how high levels of cAMP might be achieved in G_2 arrested oocytes. In Chapter 2, I describe our finding that inhibiting endogenous G-protein βγ subunits, through the use of two structurally distinct $G_{\beta\gamma}$ scavengers, causes hormone-independent oocyte maturation. In contrast, overexpression of *Xenopus* $G_{\beta 1}$, alone or together with bovine $G_{\gamma 2}$, inhibits progesterone-induced oocyte maturation. These results for the first time implicate that an endogenous G protein coupled receptor system releases a $G_{\beta\gamma}$ complex as the dominant meiosis inhibitor.

Chapter 3 describes my research aiming to reveal the identity of the oocyte AC responsible for generating meiosis-inhibiting cAMP. I provide further evidence here that the ability of $G_{\beta\gamma}$ to inhibit meiosis is attributed to the activation of an endogenous AC, rather than other possible $G_{\beta\gamma}$ effectors. Through molecular cloning and biochemical characterization, I discovered that the likely AC candidate is *Xenopus* AC7, an isoform that is activated by $G_{\beta\gamma}$, but only in the presence of GTP-bound $G_{s\alpha}$. The identification of xAC7 suggests that the maintenance of high levels of cAMP may require the co-operation of $G_{s\alpha}$ and $G_{\beta\gamma}$.

Finally, in Chapter 4, I describe our efforts in identifying the GPCR(s) responsible for activating the cAMP signaling in prophase-arrested oocytes. A screening of known antagonists of GPCR(s) led to the identification of ritanserin, a potent antagonist of serotonin receptors, as a potent maturation inducer in *Xenopus* oocytes. Pharmacological and molecular studies, however, have ruled out the involvement of a known serotonin receptor in meiosis arrest. Instead, the most likely candidate is a "constitutively activated" GPCR that bears structural similarities to *Xenopus* serotonin receptor 7.

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LIST OF ABBREVIATIONS

M	molar
mM	millimolar (10^{-3} molar)
μM	micromolar (10^{-6} molar)
nM	nanomolar (10^{-9} molar)
fmol	femtomol (10^{-15} mol)
mL	milliliter
μL	microlitre
nL	nanoliter
mg	milligram
ng	nanogram
pg	picogram (10^{-12} gram)
IU	international units
μCi	microcurrie
AC	adenylyl cyclase
ADP	adenosine diphosphate
AP-2	adaptor protein 2
ALP	alprenolon
ATP	adenosine 5'-triphosphate
β_2AR	β_2 adrenergic receptor
βARK	β adrenergic receptor kinase
βARK-C	C-terminus of β adrenergic receptor kinase
BSA	bovine serum albumin
cAMP	cyclic adenosine-3',5'-monophosphate
cDNA	complimentary DNA
CNBr	cyanogen bromide
EC_{50}	50% effective concentration
hCG	human chorionic gonadotropin
CHX	cycloheximide
CPEB	cytoplasmic polyadenylation element binding
CTX (CT)	cholera toxin
DMSO	dimethyl sulfoxide
ECL	enhanced chemoluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FBS	fetal bovine serum
FSK	forskolin
GAP	GTPase-activating protein
GDP	guanosine 5'-diphosphate
GFP	green fluorescence protein
G_i	inhibitory heterotrimeric GTP-binding protein
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase

G_s	stimulatory heterotrimeric GTP-binding protein
$G_{t\alpha}$	α subunit of transducin
GTP	guanosine 5'-triphosphate
GV	germinal vesicle
GVBD	germinal vesicle break down
IC_{50}	50% inhibitory concentration
HA-tag	hemagglutinin tag
5-HT	5-hydroxytryptamine (serotonin)
5-HTR	5-hydroxytryptamine receptor
IBMX	3-isobuty1-1-methylxanthine
ISO	isoproterenol
LH	luteinizing hormone
MAPK	mitogen activated protein kinase
MPF	m-phase (maturation) promoting factor
mRNA	messenger ribonucleic acid
NAD	nicotinamide adenine dinucleotide
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDE	phosphodiesterase
PI-3 K	phosphotidylinositol-3 kinase
PKA	protein kinase A, cAMP-dependent protein kinase
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PMSF	phenylmethylsulfonyl fluoride
PMSG	pregnant mare serum gonadotropin
PR	progesterone receptor
PTX	Bordetella pertussis toxin
RGS	regulator of G-protein signalling
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
TCA	trichloroacetic acid
TM	transmembrane
xG_β	*Xenopus* G-protein β subunit
x5-HT_7R	*Xenopus* 5-HT_7 receptor
xPR	*Xenopus* progesterone receptor

LIST OF FIGURES

Chapter One

General Introduction

Meiotic cell cycle consists of two consecutive divisions – Meiosis I and Meiosis II (M phases), in the absence of intervening DNA replication (S phase) (Figure 1.1). One of the best systems to study the biochemical mechanisms that regulate the meiotic cell cycle is the meiotic maturation of oocytes of the African clawed toad, *Xenopus laevis.*

Like the oocytes in many other vertebrate species, *Xenopus* oocytes are physiologically arrested at two points during meiosis (Figure 1.1) (Masui, 1985). The first arrest occurs at the prophase of meiosis I, the G_2/M boundary of the first meiotic division, also called G_2 arrest. During this time, the oocyte accumulates the components necessary for early embryogenesis, as well as substantially increases in size and pigmentation. The physical changes (size and pigmentation) are conveniently used to categorize the different stages of growth (Stages I to VI) (Figure 1.1) (Dumont, 1972). The nucleus, referred to as the germinal vesicle (GV), occupies roughly half the volume of the animal hemisphere by stage VI, at which point the oocyte is considered fully grown. Fully grown *Xenopus* oocytes remain arrested at prophase I for months, until re-entering meiosis and undergoing maturation (Sadler and Maller, 1983).

The meiotic maturation of *Xenopus* oocytes is known to be initiated by the steroid hormone progesterone, which is synthesized and released by somatic follicle cells surrounding the oocyte (Masui and Markert, 1971). When stimulated by progesterone, the oocyte re-enters meiosis, as indicated by germinal vesicle breakdown (GVBD), chromosomes condensation, spindle formation and, finally, the extrusion of the first polar body (Yamashita, 1998). The oocyte now reaches its second physiological arrest (at metaphase II) where it awaits fertilization (Sadler and Maller, 1983).

Progesterone induced-oocyte maturation in *Xenopus* laevis involves inhibition of an oocyte adenylyl cyclase (AC) and reduction of intracellular cAMP. However, the mechanism by which ***progesterone controls AC activity and cAMP level*** remains unclear. **My research project was to study G protein function in the regulation of AC activity during progesterone-induced oocyte maturation. The project also included identification and characterization of the G protein coupled receptor (GPCR) that regulates oocyte AC.**

1. Oocyte Maturation and Progesterone Signaling

Progesterone is thought via progesterone recepter (PR) to regulate intracellular G protein signaling resulting in reduction of cAMP and, therefore, inhibition of cAMP-dependent protein kinase (PKA). Inhibition of PKA leads to the activation of other protein kinase cascades that ultimately activate maturation promoting factor (MPF). MPF is the key component that catalyses the entry into M-phase of meiosis I and meiosis II. An overview of the current understanding of the pathway leading to oocyte maturation is presented in Figure 1.2.

1.1. Oocyte progesterone receptor

Our laboratory and Ruderman's laboratory recently cloned amphibian progesterone receptor, *Xenopus* progesterone receptor (xPR) and provided evidence that xPR is the long-sought oocyte progesterone receptor (Bayaa *et al.*, 2000; Tian *et al.*, 2000). Antisense oligo specific for xPR inhibited progesterone-induced oocyte maturation (Tian *et al.*, 2000). Over-expression of xPR accelerates progesterone-induced

Figure 1.1 **Oocyte maturation and early embryogenesis in *Xenopus*.** Modified from Ferrell (1999). During oogenesis, the oocytes are arrested in a G_2-like interphase while they accumulate the components necessary for early embryogenesis. The oocytes are staged according to their size and colouration. Stage VI oocytes respond to progesterone to undergo maturation. The mature oocytes, now referred to as eggs, are then fertilized and undergo embryogenesis. The red and green lines refer to the activities of MAPK, and MPF respectively during late oogenesis, oocyte maturation and early embryogenesis. The times indicated refer to both MAPK and MPF timing and cell cycle stage (yellow, blue and green bar).

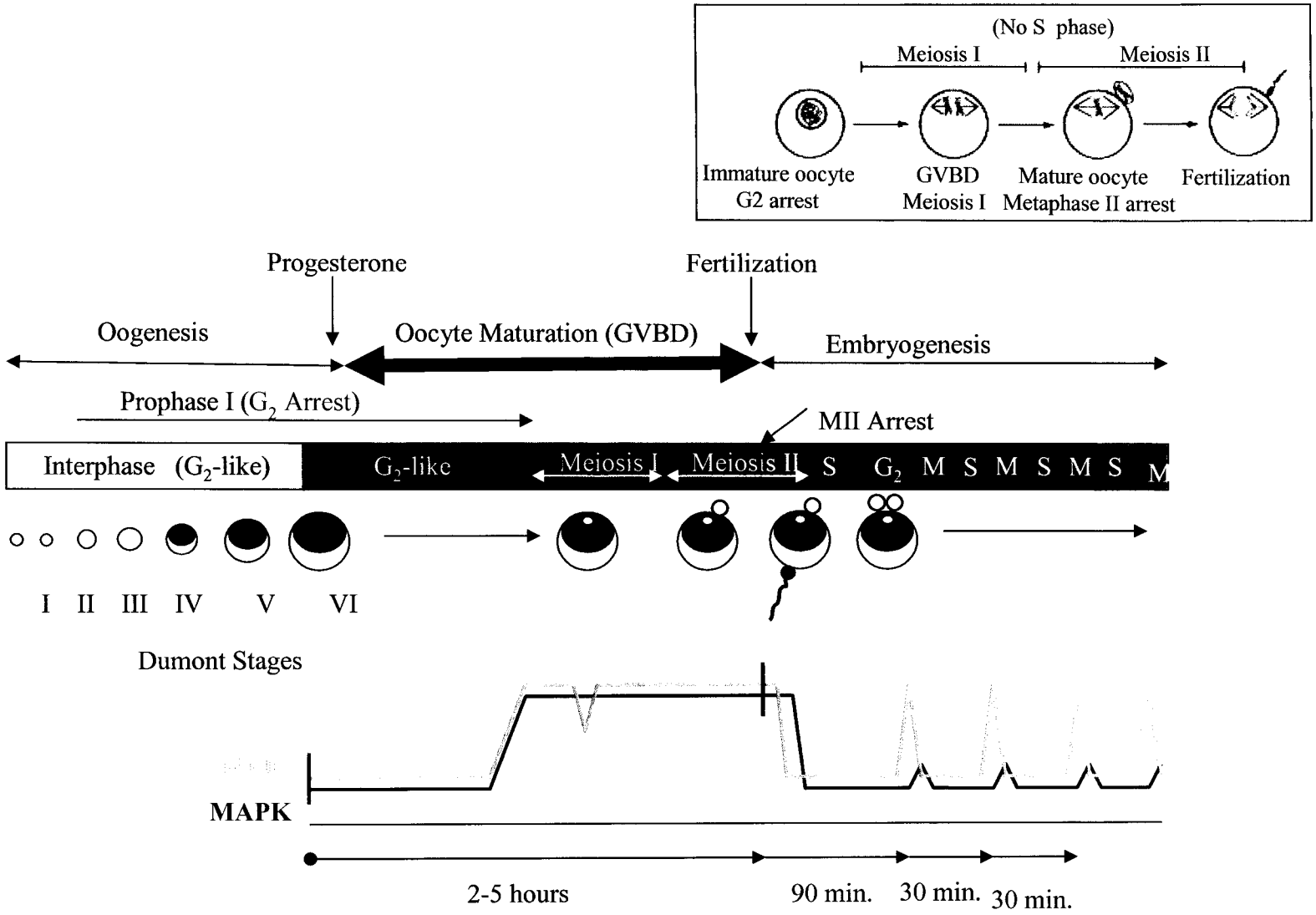
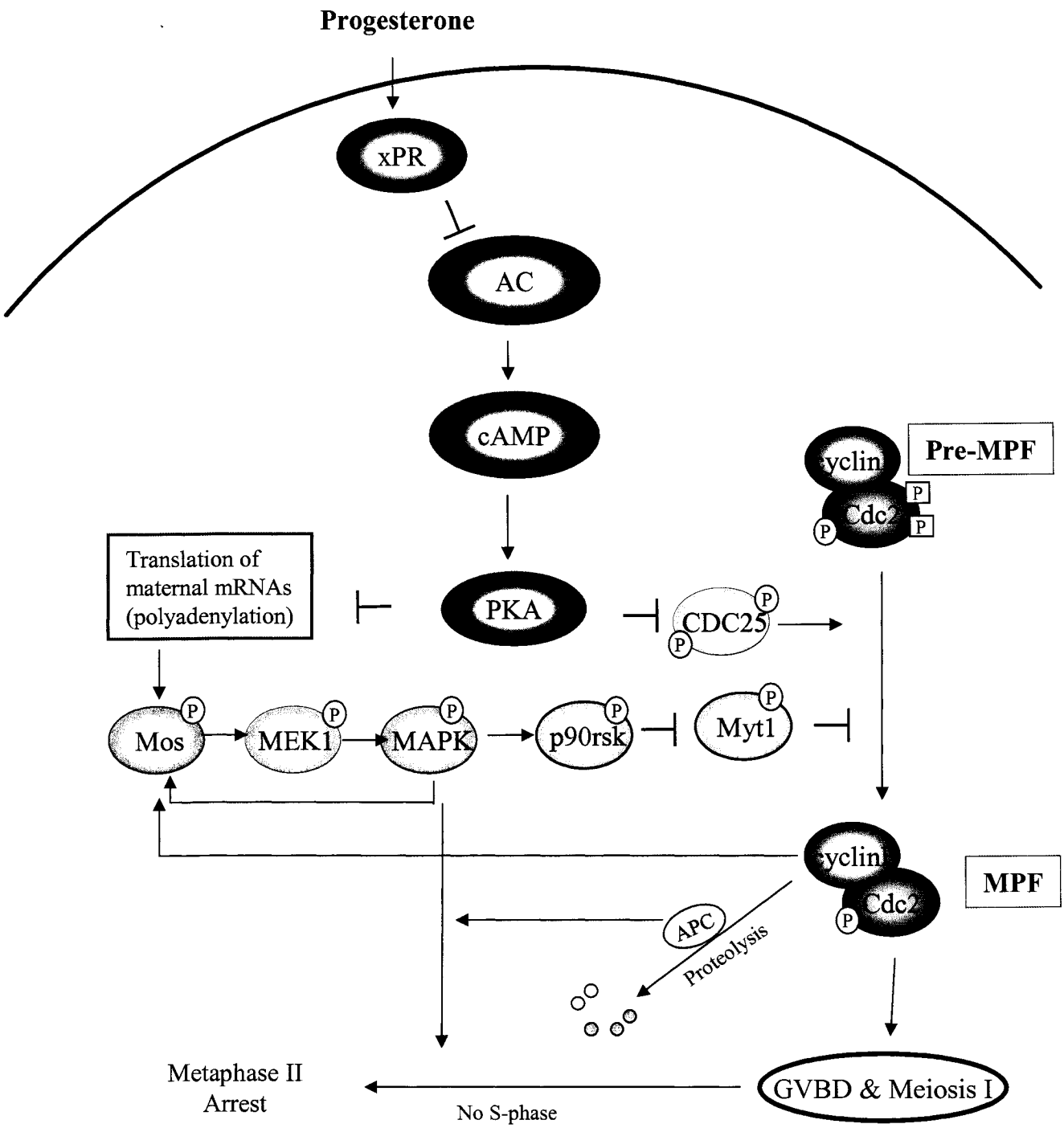

(No S phase)
Meiosis I
Meiosis II
Immature oocyte
G2 arrest
GVBD
Meiosis I
Mature oocyte
Metaphase II arrest
Fertilization
Progesterone
Fertilization
Oogenesis
Oocyte Maturation (GVBD)
Embryogenesis
Prophase I (G2 Arrest)
MII Arrest
Interphase (G2-like)
G2-like
Meiosis I
Meiosis II
S
G2
M
S
M
S
M
S
M
I II III IV V VI
Dumont Stages
MAPK
2-5 hours
90 min.
30 min.
30 min.

Figure 1.2 **Protein kinase cascades control oocyte maturation.** Modified from Schmitt (2002) and Liu and Ruderman (2004). Progesterone acting through progesterone receptor (PR), inhibits adenylyl cyclase (AC), and reduces the level of cAMP. This reduction, through inhibition of PKA activity, activates the translation of Mos protein. Mos then activates the MAPK pathway, which through Rsk and Myt1, activates MPF. Once MPF is active, it activates the anaphase promoting complex (APC) which down regulates MPF by proteolysis. MAPK remains active while the APC is active and aids in *de novo* synthesis of cyclin B and inhibition of S-phase during oocyte maturation. MAPK also aids in the maintenance of metaphase II arrest.


Progesterone
xPR
AC
cAMP
Pre-MPF
yclin
Cdc2
P
Translation of maternal mRNAs (polyadenylation)
PKA
CDC25
Mos
MEK1
MAPK
p90rsk
Myt1
yclin
Cdc2
MPF
APC
Proteolysis
Metaphase II Arrest
No S-phase
GVBD & Meiosis I

oocyte maturation and reduces the concentrations of progesterone required to induce oocyte maturation. Interestingly the endogenous xPR is restricted in the cytoplasm and mediates cytoplasmic signaling (Bayaa et al., 2000). However, recent work from Thomas' laboratory identified a putative membrane progesterone receptor (mPR) in fish oocytes (Zhu *et al.*, 2003a; 2003b). Current evidence suggested that mPR is G_i -coupled receptor. Structural analysis of the translated cDNAs suggest it encodes membrane proteins with seven transmembrane domains. Whether mPR exists in frog ocytes, or whether mPR plays a role in progesterone-mediated frog oocyte maturation, remains unknown (Hammes, 2003).

1.2. The role of cAMP and protein kinase A in oocyte maturation

Studies in species as diverse as starfish, *Xenopus*, and mammals have reached the same conclusion, that cAMP plays a critical role in maintaining G_2 arrest (Ferrell, 1999; Taieb *et al.*, 1997). Therefore, maintaining high levels of cAMP, through activation of adenylyl cyclase (with forskolin) or inhibition of cAMP-specific phosphodiesterase (with IBMX, 3-isobutyl-1-methylxanthine), inevitably inhibits hormone-induced oocyte maturation. As the main physiological effector of cAMP is PKA, it is therefore not surprising that the catalytic subunit of PKA is a potent inhibitor of meiotic maturation in vertebrate oocytes. In contrast, injection of the heat-stable PKA inhibitor PKI induces maturation in the absence of progesterone (Masui, 1985; Speaker and Butcher, 1977; Mulner, 1979; Sadler and Maller, 1981; Finidori-Lepicard, 1981; O'Connor and Smith, 1976; Maller and Krebs, 1977; Maller *et al.*, 1979; Huchon *et al.*, 1981).

PKA inhibits both branches of the MPF-activating pathway (Figure 1.2) in oocyte maturation: (i) PKA prevents the synthesis of Mos and can block Mos-induced oocyte activation, and (ii) PKA also blocks steps leading to the activation of cdc25 (Rime, 1992; Daar *et al.*, 1993; Matten *et al.*, 1994; Qian *et al.*, 2001; Schmitt and Nebreda, 2002b; Faure *et al.*, 1998; Duckworth *et al.*, 2002) (Figure 1.2).

1.3. Protein synthesis and regulation

An important downstream target of PKA pathway is the synthesis of new proteins essential in the meiotic maturation process. *Xenopus* oocytes are unable to undergo GVBD in the presence of protein synthesis inhibitors, indicating that *de novo* protein synthesis is necessary for the initiation of meiotic maturation (Wasserman and Masui, 1975).

One mechanism responsible for the translational activation of several maternal messages is cytoplasmic polyadenylation, which has been studied most extensively in *Xenopus* oocytes. Several mRNAs that are dormant in oocytes have short poly (A) tails, usually ~20 nucleotides in length. After the induction of oocyte maturation by progesterone, some of these mRNAs undergo cytoplasmic polyadenylation-induced translation (McGrew *et al.*, 1989; Sheets *et al.*, 1995; Stebbins-Boaz *et al.*, 1996). Two *cis* elements in the 3' untranslated region of responding mRNAs, the cytoplasmic polyadenylation element (CPE) and the polyadenylation hexanucleotide AAUAAA, direct cytoplasmic polyadenylation, (Fox *et al.*, 1989; McGrew *et al.*, 1989). The CPE is bound by CPEB, a zinc finger and a RNA recognition motif (RRM)-containing protein (Paris *et al.*, 1991; Hake and Richter, 1994; Hake *et al.*, 1998), while the hexanucleotide

is bound by a cytoplasmic form of cleavage and polyadenylation specificity factor (CPSF) (Bilger *et al.*, 1994; Dickson *et al.*, 1999). The stimulation of polyadenylation is most probably due to the kinase Eg2, which is activated soon after oocytes are exposed to progesterone (Andresson and Ruderman, 1998). Eg2 phosphorylates CPEB Ser174, which resides within an LDS/TR motif that is conserved among other vertebrate CPEB proteins (Mendez *et al.*, 2000a). Once phosphorylated on this residue, CPEB recruits CPSF and poly (A) polymerase into an active polyadenylation complex (Mendez *et al.*, 2000b). In addition to its role in stimulating *de nove* protein synthesis, Eg2 also functions in regulating spindle dynamics and meiosis I to meiosis II transition (Ma, *et al.*, 2003; Castro, *et al.*, 2003)

1.4. Role of the Mos/MAPK pathway

The first maternal mRNA shown to be important for *Xenopus* oocyte maturation encoded the protein kinase Mos, an oocyte-specific mitogen-activated protein kinase (MAPK) kinase kinase that can phosphorylate and activate the MAPK kinase MEK1. Activation of the MAP kinase pathway leads to the activation of the protein kinase p90Rsk, which in turn can phosphorylate and inhibit the Cdc2 inhibitory kinase Myt1 (Figure 1.2).

The MEK kinase is dual-specificity kinases that activate Erk1 and Erk2 MAPK by tyrosine and threonine phosphorylation in their activation loop. In oocytes, MEK1 phosphorylates Thr183 and Tyr185 of MAPK (Kosako *et al.*, 1993). The requirement for activation of MEK1 in oocyte maturation has been demonstated through microinjection of anti-MEK1 antibodies, which have been found to block progesterone-induced GVBD

(Kosako *et al.*, 1994), and use of the MEK1 inhibitor, PD98059, which also inhibits GVBD (Cross and Smythe, 1998). Microinjection of constitutively active MEK1 has been found to induce oocyte maturation, even in the presence of the translation inhibitor cycloheximide, demonstrating its position below Mos and upstream of MAPK in the signaling pathway (Gotoh and Nishida, 1995)

The MAPK pathway is universally activated during the meiotic maturation of vertebrate oocytes. However, the timing of, and requirement for, MAPK activation varyies in different species. In *Xenopus* oocytes, several reports support the idea that MAPK activation is required for progesterone-induced entry into meiosis. Injection of the MAPK phosphatase CL100 blocks progesterone induced GVBD (Gotoh and Nishida, 1995). MAPK activation occurs about 2 to 5 hours after stimulation, at the time of MPF activation and just preceding GVBD. MAPK in *Xenopus* oocytes is in one of two stable states, completely inactive (unphosphorylated) or completely active (phosphorylated). This presents the ability to biochemically monitor whether the oocyte maturation pathway has been activated (phosphorylated MAPK, activated pathway; unphosphorylated MAPK, inactive pathway) (Ferrell, 1999; Ohan *et al.*, 1999).

However, recent results using the Hsp90 inhibitor geldanamycin or the MEK inhibitor U0126, indicate that, in the absence of detectable MAPK activation, some oocytes still undergo MPF activation and GVBD upon progesterone stimulation (Gross *et al.*, 2000; Fisher *et al.*, 1999). Dupre *et al* showed that morpholino antisense oligo to Mos does not inhibit GVBD but inhibits meiosis I to meiosis II transition (Dupre *et al.*, 2002). These new results suggest that MAPK activation is not strictly required for GVBD in

Xenopus oocytes and that alternative MAPK-independent pathways can trigger MPF activation.

In mammalian oocytes, MAPK is activated during maturation but oocytes from c-Mos knock-out mice, in which MAPK is not activated, can undergo GVBD. In maturing starfish oocytes, MAPK is activated only after MPF activation and GVBD. These oocytes do not arrest at metaphase II but proceed to interphase and contain high levels of MAPK activity (Schmitt and Nebreda, 2002).

MAPK activation has also been reported to stimulate polyadenylation of Mos mRNA (Howard *et al.*, 1999). This probably constitutes a positive feedback loop where MAPK activation by Mos in turn stimulates the translation of Mos mRNA (Ferrell, 1999).

1.5. MPF activation in the control of meiosis

A key enzymatic activity regulating the G_2/M transition in all eukaryotic cells is the maturation-promoting factor (MPF), comprised of the serine/threonine protein kinase Cdc2 complexed with cyclin B.

In G_2-arrested oocyte, about 10% of Cdc2 is already associated with the maternal stock of cyclin B (principally, cyclins of the B2/B5 family), phosphorylated on the activating Thr161 residue, but maintained inactive by phosphorylation on Thr14 and Tyr15, forming the pre-MPF stock. The remaining 90% of the Cdc2 is monomeric and can associate with newly synthesized cyclins, giving rise either to more inactive pre-MPF molecules or to active MPF complexes, depending on the efficiency of the inhibitory Thr14/Tyr15 kinase, Myt1 (Kobayashi *et al.*, 1991; Taieb *et al.*, 1997). In response to

progesterone, a *de novo* synthesis of cyclins is induced, resulting in an accumulation of a second family of B-cyclins: the B1/B4 family (Kobayashi *et al.*, 1991; Rime *et al.*, 1994; Hochegger *et al.*, 2001). It has been shown that initiation of cyclin B1 synthesis is submitted to a negative PKA-control and is independent of MPF activity (Frank-Vaillant *et al.*, 1999).

A potential link between the MAPK pathway and the activation of pre-MPF could be $p90^{rsk}$, a well-known substrate of MAPK that was found to specifically associate with the carboxy-terminal noncatalytic region of Myt1 (Palmer *et al.*, 1998). Phosphorylation by $p90^{rsk}$ decreases the ability of Myt1 to downregulate Cdc2–cyclinB *in vitro*. Inhibition of the endogenous Myt1 can trigger meiotic maturation in the absence of progesterone stimulation (Nakajo *et al.*, 2000). Therefore, Myt1 should be responsible for maintaining the Cdc2–cyclin-B complexes in an inactive form in G_2-arrested oocytes.

Both Myt1 and Cdc25 are hypo-phosphorylated, their phosphorylation level being probably controlled by active phosphatase 2A (PP2A) (Izumi *et al.*, 1992; Jessus *et al.*, 1991; Kumagai and Dunphy, 1992; Mueller *et al.*, 1995). This pre-MPF stock can be directly activated by dephosphorylation of the Thr14/Tyr15 residues, catalysed by active Cdc25 phosphatase. Just before entry into M-phase, Cdc25 and Myt1 become hyper-phosphorylated, the former becoming active and functional, the latter being inhibited (Izumi *et al.*, 1992; Kumagai and Dunphy, 1992; Mueller *et al.*, 1995).

1.6. Regulation of MPF activity in Xenopus oocytes - feedback regulation

An important feature of meiotic maturation is an extensive network of feedback signalling (mostly downstream of MPF activation), which is responsible for the

generation of an all-or-none response ensuring that the oocyte completes meiotic progression (Schmitt and Nebreda, 2002). It has been suggested there is a brief burst of MAPK activity shortly after progesterone stimulation (Fisher *et al.*, 1999; Fisher *et al.*, 2000). This might serve to activate a small amount of MPF, which would account for the translation of Mos and MAPK/MPF full activation through a positive feedback mechanism (Figure 1.2) (Ferrell, 1999).

2. G Protein Signling and Regulation

2.1. Heterotrimeric G proteins

The heterotrimeric guanine nucleotide binding proteins (G proteins) are signal transducers that communicate signals from many hormones, neurotransmitters, chemokines, and autocrine and paracrine factors. The extracellular signals are received by members of a large superfamily of receptors with seven membrane-spanning regions (7TMRs) that activate the G proteins, which route the signals to several distinct intracellular signaling pathways. G proteins are made up of α, β, and γ subunits. Although there are many genes encoding each subunit (20 α, 6 β, and 13γ, for a maximum of 1560 possible heterotrimeric G protein combinations), four main classes of G proteins can be distinguished. $G_{\alpha s}$, whose members include $G_{\alpha s}$ and $G_{\alpha OLF}$, activates AC while $G_{\alpha i}$ ($G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$, $G_{\alpha oA}$, $G_{\alpha oB}$, $G_{\alpha t1}$, $G_{\alpha t2}$, $G_{\alpha g}$ and $G_{\alpha z}$) inhibits AC. A third class of G proteins, $G_{\alpha q}$ ($G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 15}$ and $G_{\alpha 16}$) activates phospholipase C. Finally, G12 and G13 are thought to stimulate phospholipase D, as well as several protein kinases including the tyrosine kinase c-Src and serine/threonine kinases PKC and PKB (Simon *et al.*, 1991; Clapham and Neer, 1997; Huang *et al.*, 2003; Neves, 2002).

G proteins are inactive in the GDP-bound, heterotrimeric state and are activated by receptor-catalyzed guanine nucleotide exchanges resulting in GTP binding to the α subunit. GTP binding leads to dissociation of G_{α}·GTP from its $G_{\beta\gamma}$ subunits. Both G_{α}·GTP and the free $G_{\beta\gamma}$ subunits can regulate downstream G protein effectors. G protein deactivation is rate-limiting for turnoff of the cellular response and occurs when the G_{α} subunit hydrolyzes GTP to GDP.

The βγ subunits of G protein are heterodimer, which under physiological conditions, function as a single entity in the G protein cycle. It is now recognized that the $G_{\beta\gamma}$ complex, though originally conceptualized as the passive, stabilizing binding partner for G_{α} subunit at the completion of a signalling cycle, carries an independent signal to many types of cellular effectors upon G protein activation.

There are many reports that show the involvement of the $G_{\beta\gamma}$ dimer complex in the regulation of various effectors including PLC-β2, MAPK/ERK, JNK/SAPK, phosphatidylinositol 3-kinase, and several AC isozymes (Clapham and Neer, 1997; Taussig *et al.*, 1993b; Federman *et al.*, 1992; Zhang *et al.*, 1996; Lopez-Ilasaca *et al.*, 1997; Crespo *et al.*, 1994). $G_{\beta\gamma}$, which are membrane-bound through isoprenylation of the γ subunit, also act as an anchor for βARK (β-adrenergic receptor kinase) and appear to be required for the translocation of βARK to the plasma membrane, where it phosphorylates the agonist-occupied β-adrenergic receptor, which then becomes desensitized to further stimulation (Pitcher, *et al.*, 1992).

2.2. Regulators of G protein signaling

The regulators of G protein signaling (RGS) family are GTPase-activating proteins (GAPs) that accelerate hydrolysis of GTP bound to the α subunits of certain heterotrimeric G proteins (Berman and Gilman, 1998; Dohlman and Thorner, 1997; Koelle, 1997). As such, RGS proteins can function as negative regulators of G protein-mediated signal transduction by speeding deactivation of the active form of G_{α} subunits, thereby promoting formation of inactive G protein heterotrimers ($G_{\alpha GDP\beta\gamma}$) (Posner, 1999).

RGS proteins form a superfamily of at least 25 proteins, which are highly diverse in structure, expression patterns, and function. They share a 120 amino acid homology domain (RGS domain), which exhibits GTPase accelerating activity for α-subunits of heterotrimeric G-proteins, and thus, are negative regulators of G-protein-mediated signaling. Based on the organization of the RGS genes, structural similarities, and differences in functions, they can be divided into at least six subfamilies of RGS proteins and three more families of RGS-like proteins. Many of these proteins regulate signaling processes within cells, not only via interaction with G-protein α subunits, but are G-protein-regulated effectors, $G_{\beta\gamma}$ scavenger, or scaffolding proteins in signal transduction complexes as well (Wieland and Mittmann, 2003).

Recently, a subfamily of RGS proteins has been identified in which each member possesses so-called DEP (disheveled, EGL-10, pleckstrin) and GGL (G protein gamma subunit-like) domains in addition to an RGS domain (Ponting and Bork, 1996; Snow *et al.*, 1998). Members of this group include mammalian RGS proteins (RGS6, RGS7, RGS9, and RGS11), a *Drosophila* RGS protein (dRGS7), and EGL-10, an RGS protein found in *Caenorhabditis elegans* (Snow *et al.*, 1998; Koelle and Horvitz, 1996; Elmore *et*

al., 1998) Functionally, the GGL domain was shown to specify binding of RGS11 or a fragment of RGS7 to the G protein β5 subunit (Snow *et al.*, 1998). It has also been shown that both RGS7 and RGS9 can be isolated from the brain and retina as a complex with $G_{\beta 5}$ (Makino *et al.*, 1999; Levay *et al.*, 1999; Cabrera *et al.*, 1998).

2.3. G protein signaling in oocyte maturation

Several findings suggest that the G protein mechanism is involved in meiosis-activating steroids signaling. Meiotic arrest can be released in mice by microinjecting the oocyte within the follicle with an antibody that inhibits the stimulatory heterotrimeric GTP-binding protein G_s. This indicates that G_s activity in the oocyte is required to maintain meiotic arrest within the ovarian follicle and suggests that the follicle may keep the cell cycle arrested by activating G_s (Mehlmann *et al.*, 2002). Cholera toxin (CT) catalyzes ADP-ribosylation of the α subunit of G_s, reducing GTPase, resulting in the constitutive activation of AC (Cassel and Pfeuffer, 1978; Moss and Vaughan, 1979). In naked rodent oocytes, CT has been observed to partly prevent the drop in intracellular cAMP associated with spontaneous maturation (Vivarelli *et al.*, 1983), whereas spontaneous maturation is not inhibited (Schultz *et al*, 1983; Vivarelli *et al.*, 1983; Olsiewski and Beers, 1983).

Pertussis toxin (PTX) is known to ADP-ribosylate, and inhibits, the α subunit of classical G_i proteins. Several lines of evidence suggest that a PTX-sensitive inhibitory G-protein is a component of the signaling pathway during induction of oocyte maturation in fish. First, the GTP analog GTPγS decreases specific binding of the meiosis inducing substance to its oocyte membrane receptor in rainbow trout (Yoshikuni and Nagahama,

1994). Moreover, declines in specific meiosis inducing substance binding have been observed in this species after incubation with pertussis toxin (Yoshikuni and Nagahama, 1994). In addition, pertussis toxin was also shown to catalyze ADP-ribosylation of a 40 KDa protein in rainbow trout oocyte membranes, which is recognized by an antibody to the α subunit of the inhibitory G protein, G_i (Yoshikuni and Nagahama, 1994). Furthermore, microinjection of pertussis toxin blocks maturation of seatrout oocytes as well as those in a closely related species, Atlantic croaker, in response to progestin (Thomas *et al.*, 2002). A pertussis toxin-sensitive G protein has also been implicated in the progestin induction of oocyte maturation in rainbow trout (Yoshikuni and Nagahama, 1994). Direct evidence of the role of an inhibitory G-protein in oocyte maturation was obtained in starfish by injecting the oocytes with pertussis toxin, resulting in an inhibition of oocyte maturation in response to the meiosis inducing substance, 1-methyladenine (Shilling *et al.*, 1989).

2.4. G protein signaling in *Xenopus* oocyte maturation

Earlier studies have provided some indications of the function of G protein in frog oocyte maturation. First, different $G_{\alpha s}$ stimulators such as Gpp (HN)p, GTPγS, aluminium fluoride, and cholera toxin can activate AC and inhibit progesterone-induced oocyte maturation (Sadller and Maller, 1981; Jordana *et al.*, 1984; Gallo *et al.*, 1995). Second, *Xenopus* oocytes undergo hormone-independent maturation when injected with an affinity-purified antibody against the COOH-terminal decapeptide of the α subunit of the G protein G_s, an antibody that inhibits G_s activity. These findings suggested that the endogenous *Xenopus* $G_{\alpha s}$ functions in meiosis arrest. However, it is less clear whether

progesterone-induced inhibition of AC is mediated through $G_{\alpha i}$ proteins (Simonds, 1999). For example, PTX does not block protesterone-induced oocyte maturation (Sadler *et al.*, 1984; Sheng *et al.*, 2001). Consistent with this, injection of activated α subunits of three mammalian $G_{\alpha i}$ proteins (G_{i1}, G_{i2} and G_{i3}) into *Xenopus* oocytes does not cause GVBD (Kroll *et al.*, 1991). But, Lutz *et al.* (Lutz *et al.*, 2000) demonstrated that injection of RNA encoding the *Xenopus* $G_{\alpha i2}$ protein resulted in marked hormone-independent oocyte maturation. The possible reason for the discrepancies might be the result of injection of activated $G_{\alpha i}$ proteins (Kroll *et al.*, 1991) and RNA encoding the *Xenopus* $G_{\alpha i2}$ protein (Lutz *et al.*, 2000). The newly translated $G_{\alpha i2}$ protein from RNA may in inactive status.

3. Regulation of Adenylyl Cyclases

3.1. Structure of adenylyl cyclases

Adenylyl cyclase (AC) converts intracellular ATP into cyclic-3', 5'-adenosine monophosphate (cAMP). Molecular cloning has identified at least nine isoforms of transmembrane ACs (tmACs) that respond positively or negatively to distinct sets of regulatory input (Simonds, 1999). All tmACs share a common structure: two cytoplasmic domains (C1 and C2), each flanked by N-terminal six α-helices (M1 and M2 respectively). The C1 and C2 domains have ~230 amino acid regions (C1a and C2a) that share 50% or higher similarity and form the catalytic core. C1a and C2a share homology not only with each other but also with soluble and transmembrane guanylate cyclases. Systematic mutational analysis has shown that the amino acid residues from both C1a and C2a domains contribute to ATP binding and catalysis (Tang *et al.*, 1995a; Tang *et al.*, 1995b; Yan *et al.*, 1997).

Only one adenylyl cyclase, x1AC, has been identified in *Xenopus* oocytes to date and it is most similar to the mammalian AC9 form, which is βγ-subunit insensitive (Torrejon *et al.*, 1997). Gene expression studies of the x1AC by RT PCR showed that this gene is expressed in all oogenesis stages but not during early embryogenesis. Expression of the x1AC in COS-7 cells resulted in increased basal AC activity, that was stimulated by forskolin, Gpp (NH)p and aluminium fluoride, and was insensitive to calcium and calcium-calmodulin (Ca2(+)-CaM) (Gallo *et al.*, 1995).

One soluble AC (sAC) has recently been cloned from rat testes (Buck *et al.*, 1999). Northern analysis indicates the gene encoding soluble adenylyl cyclase (sAC) is preferentially expressed in testis. The sAC cDNA encodes a 187-kD protein that is proteolytically processed to the mature 48-kD isoform. The sAC is structurally, molecularly, and biochemically distinct from G-protein-regulated tmACs (Buck *et al.*, 1999). Bicarbonate directly stimulates the sAC activity in vivo and in vitro while tmAC modulators such as G protein and forskolin do not affect sAC activity (Chen *et al.*, 2000).

3.2. Isoform-specific patterns of regulation of mammalian ACs

AC is subject to coincident regulation by both extracellular (i.e., hormones, neurotransmitters, odorants, and autocrines) and intracellular stimuli. In most tissues, the activity of AC is controlled by hormones through the action of specific receptors coupled to heterotrimeric G proteins. All of the cloned mammalian tmACs are stimulated by G_s and all but AC9 (Premont *et al.*, 1996) are also potently stimulated by forskolin. G_s and the related G_{olf} are AC-stimulating heterotrimeric G proteins (Gilman, 1995). AC3, AC5 and AC6 have been shown to be sensitive to inhibition by G_{i1} (Taussig *et al.*, 1993a,

1994) whereas AC2 was not (Taussig *et al.*, 1994). AC2, AC4 and AC7 activity are stimulated by $G_{\beta\gamma}$; $G_{\beta\gamma}$ activation of AC appears to be conditional on the presence of GTP-bound $G_{s\alpha}$ (Tang *et al.*, 1991; Taussig *et al.*, 1993b; Lustig *et al.*, 1993; Gao and Gilman, 1991). In contrast, AC1 is inhibited by $G_{\beta\gamma}$ (Tang & Gilman, 1991; Taussig *et al.*, 1993b). The $G_{\beta\gamma}$ binding site of AC2 has been mapped to the C2-α3 region (Chen *et al.*, 1995). $G_{\beta\gamma}$ is likely to modulate AC2 by binding C2a to affect its conformation, indirectly promoting optimal alignment at the catalytic site (Chen *et al.*, 1995).

The Ca^{2+} signal also regulates the enzyme activity of ACs (Cooper *et al.*, 1995). Activation of calmodulin by Ca^{2+} can directly activate AC1 and AC8 and inhibit AC3 and AC9 enzymes via calcium-dependent calmodulin kinase II and calcineurin/PP2B (protein phosphatase 2B), respectively (Tang *et al.*, 1991; Cali *et al.*, 1994; Antoni *et al.*, 1995).

3.3. Mechanism of activation by $G_{s\alpha}$.

There are several mechanisms by which $G_{s\alpha}$ could stimulate AC (Tesmer *et al*, 1997). The first of these is to direct productive formation of the $C_1 \cdot C_2$ interface. There are a number of observations that suggest that $G_{s\alpha}$ acts primarily as an allosteric activator of AC. $G_{s\alpha}$ interacts with both the C_1 and C_2 domains of AC. Of the 1800 $Å^2$ of surface area buried upon $G_{s\alpha}$ binding, 75% is with the C_2 domain and 25% is with the C_1 domain. $G_{s\alpha}$ and forskolin bind synergistically (Sunahara *et al.*, 1997) and thus stabilize the same form of the enzyme. Finally, the two activators, together and separately, promote catalysis to a degree that exceeds their effect on domain association, thus domains association *per se* is an insufficient stimulus to catalysis. Therefore, $G_{s\alpha}$, like forskolin,

appears to bind AC and induce a change in the relative orientation of the C_1 and C_2 domains that, in turn, prime the active site for catalysis (Tesmer *et al*, 1997).

The action of $G_{i\alpha}$ is not competitive with the binding of $G_{s\alpha}$ (Taussig *et al.*, 1994). The binding site for α subunit of G_i, G_o, or G_z has not been determined, but it is speculated to bind the $\alpha 2/\alpha 3$ region of C_1a on the opposite site to $G_{s\alpha}$ binding site (Yan *et al.*, 1997; Tesmer *et al.*, 1997).

3.4. Gβγ regulation of AC isoforms.

In vitro membrane reconstitution assays have clearly demonstrated that a number of AC isoforms are sensitive to $G_{\beta\gamma}$ subunits and that AC activity can either be stimulated or inhibited by $G_{\beta\gamma}$ subunits depending of the AC isoform in question. For example, the activity of AC1 is significantly inhibited while the activity of AC2, AC4, and presumably AC7 are activated by $G_{\beta\gamma}$ (Clapham and Neer, 1997; Sunahara *et al.*, 1996; Taussig *et al.*, 1993b; Tang *et al.*, 1991; Choi *et al.*, 1993; Avidor-Reiss *et al.*, 1997; Yoshimura *et al.*, 1996; Chen *et al.*, 1995). Since AC2 and AC4 are not sensitive to GTP-bound $G_{i\alpha}$, this finding has led to the understanding of how it is possible that some $G_{i/o}$-coupled GPCR (G protein coupled receptor) can actually stimulate AC activity in cells expressing primarily $G_{\beta\gamma}$-activatable ACs (Avidor-Reiss *et al.*, 1997; Yoshimura *et al.*, 1996; Federman *et al.*, 1992).

The G_β component appears to determine the functional specificities of $G_{\beta\gamma}$ complexes. For example, it has been demonstrated that AC2 is stimulated by $G_{\beta 1\gamma 2}$ but inhibited by $G_{\beta 5\gamma 2}$ (Bayewitch *et al.*, 1998). Similarly, it has been shown that PLC-β_2 is

activated equally by $G_{\beta 1\gamma 2}$ and $G_{\beta 5\gamma 2}$. In contrast, MAPK/ERK and JNK/SAPK appear to be activated much more potently by $G_{\beta 1\gamma 2}$ than $G_{\beta 5\gamma 2}$ (Zhang *et al.*, 1996).

3.5. Regulation of mammalian ACs by phosphorylation via PKA or PKC

Activation of G_q-coupled receptors results in PKC activation, which can also indirectly regulate ACs. Transfection experiments demonstrate that phorbol esters activate most ACs, including type 2, 3, 5, and 7 ACs (Yoshimura and Cooper, 1993; Jacobowitz and Iyengar, 1994; Kawabe *et al.*, 1994). Both AC2 and AC5 are phosphorylated by PKCα (Kawabe *et al.*, 1994; Zimmermann and Taussig, 1996). *In vivo* analysis using chimeric type 1/2 enzymes has mapped a region on C_2a (1034-1068), likely thr-1057, as the target of PKC phosphorylation (Levin and Reed, 1995; Bol *et al.*, 1997).

cAMP-mediated signaling is subjected to desensitization after receptor activation. GPCR desensitization by G protein coupled receptor kinase/arrestin and PKA will be discussed later. Desensitization also occurs at the level of AC. For example, AC6 can be desensitized via direct phosphorylation by PKA or PKC. PKA phosphorylation of AC6 results in a 50% reduction in $G_{s\alpha}$-stimulated activity (Chen *et al.*, 1997). The phosphorylation site probably is likely Ser674 of the C_{1b} region because mutation of this site blocks the sensitivity of AC6 to PKA (Chen *et al.*, 1997). This mechanism could explain the cAMP-dependent desensitization of glucagon stimulation in hepatocytes (Premont *et al.*, 1992; Lai *et al.*, 1997). In PC12 cells (rat pheochromocytoma cells), the predominant adenylyl cyclase is Ca^{2+}-inhibited AC6, and adenylyl cyclase activity can be desensitized on the stimulation of A2a adenosine receptor, a G_s-coupled receptor (Chen *et*

al., 1995). Interestingly, the desensitization of AC6 in PC12 cells can be blocked by treatment with pertussis toxin but not by a selective PKA inhibitor, H89 (Lai *et al.*, 1997). The desensitization in PC12 cells is mediated by a novel PKC (Ca^{2+} independent) that phosphorylates and inhibits AC6 (Lai *et al.*, 1997).

3.6. P-site inhibitors

P-site inhibitors are adenosine and adenine nucleotide analogues that bind to the catalytic core of adenylyl cyclases and inhibit AC. Adenosine alone induces *Xenopus* oocyte maturation, as well as accelerates progesterone-induced maturation (Gelerstein, 1988). P-site inhibitors are not competitive with AC activators such as Mn^{2+}, forskolin or G_s, and they are much more potent inhibitors of the activated than the basal enzyme. P-site inhibitors are now believed to form a dead-end complex with AC by occupying the site that otherwise accommodates the catalytic product cAMP. Kinetic studies of P-site inhibition and the structure of the complex itself both strongly suggest that the P-site and the substrate (ATP) binding site are the same. 2',5'-dd-3'-ATP (2',5'-dideoxyadenosine 3'-triphosphate) is a potent inhibitor. Recently, adenosine-3'-polyphosphates have been developed as a new class of P-site inhibitors with enhanced potency (Désaubry *et al.*, 1996).

4. G Protein coupled Receptor Signaling

4.1. G protein coupled receptors

G protein coupled receptors (GPCRs) represent a superfamily of proteins that are functionally dependent on heterotrimeric GTP binding and hydrolyzing proteins (G-

proteins). GPCRs are structurally characterized by their seven transmembrane domains. They link the cells to their environment by receiving stimuli, relaying the message to the cells, initiating, and regulating the response. GPCRs govern the body's reactions to a wide range of signals, from odors, to light, to neurotransmitters, and regulate vital functions in all organs (Qanbar and Bouvier, 2003). More than 1000 different GPCRs have been identified since the first receptors were cloned more than two decades ago (Kolakowski, 1994). In fact, GPCRs represent one of the largest protein superfamilies (International Human Genome Sequencing Consortium 2001).

GPCR signals are relayed through a number of secondary messenger systems that are modulated via heterotrimeric G proteins. The first step in GPCR activation is the reception of the stimulus. Whether it is a hormone, a neurotransmitter, an ion, a photon, or an odorant, the stimulus induces a conformational change in the receptor. This change, in turn, results in the engagement of the G proteins. This interaction catalyzes the replacement of the GDP bound to the G_{α}-subunit with a GTP molecule. The heterotrimer then dissociates from the receptor into "free" G_{α}- and $G_{\beta\gamma}$-subunits that modulate different downstream effectors and signaling pathways (Samama *et al.*, 1993; Wickman and Clapham, 1995). The termination of the signal is achieved by the hydrolysis of the G_{α}-bound GTP, brought about by the intrinsic GTPase activity of this protein and interactions with proteins that regulate G-protein signaling (RGS proteins). This leads to the reassociation of the inactive GDP-bound form of G_{α} and $G_{\beta\gamma}$ to form a heterotrimer that is available for another round of activation.

4.2 Classification of GPCRs

Each protein has an N-terminal extracellular domain; the seven transmembrane helices, which also define three extracellular and three intracellular loops; and an intracellular carboxyl-terminal domain. The three major subfamilies of GPCRs include the receptors related to the "light receptor" rhodopsin and the $ß_2$-adrenergic receptor (family A), the receptors related to the glucagon receptor (family B), and the receptors related to the metabotropic neurotransmitter receptors (family C). Yeast pheromone receptors make up two minor unrelated subfamilies, family D (STE2 receptors) and family E (STE3 receptors). In *Dictyostelium Discoideum* four different cAMP receptors constitute yet another minor, but unique, subfamily of GPCRs (family F) (Gether, 2000). The only residue that is absolutely conserved among all family A receptors is the arginine in the Asp-Arg-Tyr (DRY) motif at the cytoplasmic side of transmembrane segment (TM) 3 (Gether, 2000; Probst *et al.*, 1992).

4.3 Models of activation of GPCRs

Studies using constitutively active mutant GPCRs have increased our understanding of receptor function. Analysis of the pharmacological properties of constitutively active mutant adrenoceptors led to the extended ternary complex model of GPCR activation (Samama *et al.*, 1993). In this model, agonists have higher affinity for the active receptor (R^*) conformation and lower affinity for the inactive receptor (R) conformation, whereas inverse agonists (also called negative antagonists) have higher affinity for the R conformation and lower affinity for the R^* conformation, and neutral antagonists have equal affinity for R and R^* (Gether, 2000). In the absence of agonists, GPCRs are kept silent by constraining intramolecular interactions. Studies using

constitutively active mutant receptors also suggest that models based on simple R and R^* conformations cannot explain the full complexity of GPCR activation. In fact, many described constitutively active mutant GPCRs represent an intermediate level of receptor activation, which can be further stimulated by agonists (Gether, 2000). Furthermore, evidence is accumulating for the existence of multiple intermediate activated states of GPCRs (Ro, inactivated → R, Basal → R', intermediate → R'', intermediate → R*, active).

Many recent studies have provided evidence that GPCR can exist as either dimmer or higher order oligomers (Milligan, 2001, 2004; Angers *et al.*, 2002). There is also growing appreciation that GPCR dimerization occurs initially during biosynthesis and may be a requisite for function (Milligan, 2004).

4.4 GPCR cycle – desensitization, resensitization and degradation

A common feature among GPCR signaling systems is desensitization, the loss of responsiveness of a receptor when subjected to continuous stimulation. Studies into the mechanisms involved in this process have revealed that G protein coupled receptor kinases (GRKs) and arrestins are important regulators of GPCR desensitization (Krupnick and Benovic, 1998, Ferguson, 2001). Upon agonist exposure, many GPCRs are rapidly phosphorylated (seconds to minutes) by a GRK or by intracellular second-messenger regulated kinases (PKA and PKC) (Hausdorff *et al.*, 1990). GPCR phosphorylation creates binding site for arrestins, which uncouples the receptor from further G protein binding but also initiates GPCR endocytosis.

GPCR are phosphorylated by multiple serine/threonine protein kinases. PKA and PKC are both effectors of GPCR but also act to phosphorylate the upstream GPCR, often independent of the activation state of the receptors (Hausdorff *et al.*, 1990). On the other hand, GRKs preferentially phosphorylate agonist-occupied or activated forms of the GPCRs (Hausdorff *et al.*, 1990, Inglese *et al.*, 1993, Haga *et al.*, 1994). GRKs were originally described for rhodopsin (rhodopsin kinase) and the β_2-adrenergic receptor (β-adrenergic receptor kinase) (Hausdorff *et al.*, 1990, Inglese *et al.*, 1993, Haga *et al.*, 1994). Seven mammalian GRK genes have been identified, some of which undergo alternative splicing to generate different forms (Premont *et al.*, 1995; Sterne-Marr and Benovic, 1995). GRK activities are regulated by phospholipids, post-translational modifications, and G protein $\beta\gamma$ subunits (Hausdorff *et al.*, 1990, Inglese *et al.*, 1993, Haga *et al*, 1994; Kunapuli *et al.*, 1994, Pitcher *et al.*, 1995, Stoffel *et al.*, 1994).

GRK-phosphorylation of receptors is not sufficient for desensitization, but rather serves to create high affinity sites to promote the binding of arrestin proteins which in turn guarantee desensitization by preventing further coupling to G proteins (Gurevich *et al.*, 1995; Lohse *et al.*, 1990). Four distinct mammalian arrestin proteins are known, two of which (visual and cone arrestins) are restricted to the phototransduction pathway. The other two forms (non-visual arrestins), β-arrestin 1 (arrestin 2) and β-arrestin 2 (arrestin 3) are ubiquitously expressed and are thought to regulate signaling as well as internalization of many different GPCRs (Sterne-Marr and Benovic, 1995). It has been shown that non-visual arrestins act as adaptor molecules in agonist-mediated endocytosis of GPCRs by interacting with clathrin (Goodman *et al.*, 1996), adaptor protein 2 (AP-2) (Laporte *et al.*, 1999), and phosphoinositides (Gaidarov *et al.*, 1999), thereby directing

the receptor into clathrin-coated pits where it undergoes subsequent endocytosis. Once internalized, some GPCRs are dephosphorylated and subsequently recycled back to the plasma membrane where they can again respond to agonists, a process termed resensitization (Krueger *et al.*, 1997).

Once internalized, receptors are targeted to recycling or degradative pathways. Some G protein coupled receptors, including the β_2-adrenergic receptor, can be recycled back to the plasma membrane, as a fully competent receptor within minutes of being internalized (Morrison *et al.*, 1996; Pippig *et al.*, 1995; Zhang *et al.*, 1997). Other receptors such as the vasopressin type 2 receptor are detained within the cell interior for longer time period before being recycled back to the cell surface (Fonseca *et al.*, 1995; Innamorati *et al.*, 1998; Oakley *et al.*, 1999). Although many GPCRs undergo this process of endocytosis and recycling, prolonged or chronic agonist stimulation can lead to a significant decrease in the total cellular complement of a given GPCR, like the δ-opioid or thrombin receptors, a process termed down-regulation (Tsao and von Zastrow, 2000). The principal mechanism underlying down-regulation is GPCR degradation, a multistep process often involving endocytosis and subsequent delivery of the receptor to lysosomes for proteolysis (Tsao and von Zastrow, 2000).

5. Serotonin and Serotonin Receptor Signaling in the Reproductive System

Serotonin (5-hydroxytryptamine; 5-HT) is a major neurotransmitter in animals, both vertebrates and invertebrates. In the human central nervous system (CNS), 5-HT is involved in many processes, including the regulation of feeding behavior, mood, perception, anxiety, aggression and pain. In non-neuronal tissues, 5-HT also has many

roles, including smooth muscle growth and contraction and platelet aggregation (Kroeze *et al.*, 2002).

5.1 Serotonin regulation in the reproductive system

The involvement of neurotransmitters in the control of reproduction in vertebrates, through the regulation of the release of pituitary gonadotropins, is well known (Cerdà, 1998b). However, direct and indirect evidence has accumulated during the last decade suggesting that biogenic amines, and particularly serotonin also influence gonadal tissues directly, being effective regulators of oocyte maturation either by promoting the ability of follicle cells to secrete maturation-inducing steroids in response to a gonadotropin surge, or by affecting the oocyte maturation responsiveness to maturation-inducing steroids (Sirotkin and Schaeffer, 1997).

In fish and amphibians, serotonin appears to have species-specific effects on oocyte maturation. For example, in medaka (*Oryzias latipes*), 5-HT stimulates the synthesis of E_2 and the meiosis inducing substance, 17a, 20β-dihydroxy-4-pregnen-3-one (17,20βP) by the granulosa cells (Iwamatsu et al., 1992, 1993), thus promoting oocyte maturation, whereas in another teleost, *Fundulus heteroclitus*, 5-HT reversibly inhibits both gonadotropin- and meiosis inducing substance -induced meiosis reinitiation without affecting the steroidogenesis pathways in the granulosa cells (Cerdà *et al.*, 1995). The inhibitory effect by 5-HT on oocyte maturation appears to be mediated by oocyte-associated 5-HT receptors with a unique pharmacological profile (as defined by germinal vesicle breakdown (GVBD) assays (Cerdà *et al.*, 1997), whereby a cAMP-protein kinase A pathway is activated (Cerdà *et al.*, 1998). In the amphibian *Bufo viridis*, a similar

inhibitory effect on progesterone-induced meiotic maturation by 5-HT has been observed (Buznikov *et al.*, 1996; Nikitina *et al.*, 1988, 1993; Buznikov *et al.*, 1993).

5.2 Subtypes, structure and regulation of serotonin receptors

Pharmacological analyses and molecular cloning have revealed the existence of at least fourteen 5-HT receptor subtypes (Hoyer *et al.*, 1994; Lucas and Hen 1995). All 5-HT receptors, except for 5-HT3, belong to the family of G protein-coupled seven transmembrane domains receptors. 5-HT_3 receptor is a transmitter-gated Na^+/K^+ channel of pentameric subunit structure. At least five subtypes of 5-HT_1 have been identified (5-HT_{1a}, 5-HT_{1b}, 5-HT_{1d}, 5-HT_{1e} and 5-HT_{1f}), the two last being only partially characterized. 5HT1 receptors are coupled to G_i-proteins and inhibit adenylate cyclases. The three subtypes of 5-HT_2 receptor subfamily (5-HT_{2a}, 5-HT_{2b} and 5-HT_{2c}) are all coupled to G_q-proteins, and hence to phospholipase C-β activation. Other 5-HT receptor classes, namely 5-HT_4, 5-HT_6 and 5-HT_7, are linked to a G_s-protein-mediated stimulation of adenylate cyclases.

5.3 Serotonin and oocyte maturation

Serotonin (5-HT) was found to inhibit steroid (17 α, 20 β-dihydroxy-4-pregnen-3-one; 17,20 β P)-induced resumption of oocyte maturation in vitro in the teleost Fundulus heteroclitus. Serotonin inhibited both follicle-enclosed and denuded oocytes, which indicates the presence of oocyte-associated 5-HT sensitive sites. The response of oocytes to 5-HT was characterized pharmacologically, i.e., the capacity of serotonergic agonists and antagonists to mimic or block the 5-HT inhibition of the steroid-induced oocyte

maturation was assessed by the changes in the percentage of oocyte GVBD (Cerdà *et al.*, 1997).

Chapter Two

Hetrotrimeric G protein βγ subunits function in *Xenopus* oocyte meiosis arrest

SUMMARY

It is generally agreed that progesterone caused a modest (20%) reduction of oocyte cAMP levels (Smith, 1989). The importance of cAMP reduction is supported by studies demonstrating that inhibition of cAMP-dependent protein kinase (PKA) causes hormone-independent GVBD and that injection of active PKA inhibits progesterone-induced GVBD (Maller and Krebs, 1977). However, the inhibition of adenylyl cyclase or the induction of GVBD by progesterone is not blocked by Bordetella pertussis toxin (PTX) (Goodhardt *et al*, 1984: Olate *et al*, 1984: Sadler *et al*, 1984). This finding suggests that the α subunits of classical G_i proteins are not involved in the inhibition of oocyte adenylyl cyclase. Indeed, the injection of activated subunits of three mammalian G_i proteins (G_{i1}, G_{i2}, and G_{i3}) into *Xenopus* oocytes does not cause GVBD (Kroll *et al.*, 1991). Although the injection of activated $G_{o\alpha}$ can cause GVBD, $G_{o\alpha}$-induced GVBD is thought to be mediated via its activation of oocyte protein kinase C but is not related to progesterone signaling (Kroll *et al.*, 1991).

The insensitivity of oocyte maturation to PTX prompted us to explore the possibility that G protein $\beta\gamma$ subunits ($G_{\beta\gamma}$) might play a role in oocyte maturation. In this study, two well-characterized $G_{\beta\gamma}$ scavengers, the α subunit of bovine transducin ($G_{t\alpha}$) and the C-terminal $G_{\beta\gamma}$ binding domain of bovine β adrenergic receptor kinase (βARK-C), have been employed to study the potential role of endogenous $G_{\beta\gamma}$ in regulating oocyte maturation. The injection of mRNA encoding either of these $G_{\beta\gamma}$ scavengers induced oocyte maturation. The $G_{\beta\gamma}$ scavengers bound an endogenous, membrane-associated G_β subunit, indistinguishable from *Xenopus* $G_{\beta 1}$ derived from mRNA injection. The injection of *Xenopus* $G_{\beta 1}$ mRNA, together with bovine $G_{\gamma 2}$ mRNA inhibited progesterone-induced

oocyte maturation. These results suggest that G protein βγ subunits is responsible for maintaining oocyte meiosis arrest.

I was responsible for the generation of all of the experimental work described in this chapter, except for the generation of bovine $G_{\gamma 2}$ expression plasmid which was carried out by Dr. Ronald A. Booth.

MATERIALS AND METHODS

Reagents

Rabbit anti-xMAP serum was produced in house with a coupled peptide (Posada and Cooper, 1992) kindly provided by J. A. Cooper (Fred Hutchinson Cancer Research Center, Seattle, Washington). Anti-*Xenopus* β-integrin monoclonal antibodies (8C8) were purchased from Developmental Studies Hybridoma Bank at the University of Iowa. Rabbit anti-$G_{\beta 1}$ antibodies (sc378 and sc261) were purchased from Santa Cruz Biotechnology and were used at 1 μg/mL for immunoblotting. Unless otherwise indicated, sc378 was used for the anti-G_{β} antibodies. Pertussis toxin A protomer (PTX) was purchased from Calbiochem, dissolved in water to 100 μg/mL, and stored as single-use aliquots at -70°C. Other chemicals were purchased from Sigma.

Animal and oocyte manipulations

Frogs: Sexually mature, oocyte-positive *Xenopus* laevis females were purchased from NASCO and maintained according to local animal care guidelines. The frogs were injected with pregnant mare serum gonadotropin (Sigma, 50 IU/frog) 3-10 days before oocyte retrieval.

Oocytes: Stage VI oocytes (Smith *et al.*, 1991) were manually dissected in oocyte incubation medium OR2 (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, pH 7.8), supplemented with gentamicin (0.1mg/ml). These oocytes were termed manually defoliculated oocytes. All procedures involving live frog oocytes were carried out in a room maintained at between 18-20°C. Oocyte injection was carried out in

OR2 without $CaCl_2$. Unless otherwise indicated, oocytes were injected with mRNA (10nl, 1mg/ml), delivered to the cytoplasm.

In vitro GVBD assay

Oocytes were incubated in OR2 containing progesterone overnight. Oocytes that underwent GVBD had the distinct appearance of a white spot at the center of the animal hemisphere (see Figure 2.3) In some instances, GVBD was confirmed by dissecting trichloroacetic acid (TCA)(5%) fixed oocytes. The presence (GVBD negative) or absence (GVBD positive) of a germinal vesicle could be directly observed under a dissect microscope (Figure 2.3). Progesterone was typically employed at 1 μM to induce GVBD (unless otherwise indicated).

Construction of various plasmids for in vitro mRNA synthesis

The bovine $G_{t\alpha}$ subunit in pML 52 vector (Medynski *et al*, 1985) was a gift from Dr. Heidi Hamm. The $G_{t\alpha}$ construct was linearized with PstI and treated with T4 polymerase to generate a blunt end. The linearized plasmid was then digested with NcoI. The coding sequence of $G_{t\alpha}$ was then inserted into pSP64TM (Ohan *et al*, 1999) that had been previously treated with BglII/Klenow and NcoI. The resultant plasmid encoded wild-type untagged $G_{t\alpha}$. A PTX-insensitive mutant of $G_{t\alpha}$ was generated by PCR amplification from the pSP64TM-$G_{t\alpha}$ construct with SP6 primer (5') and a 3' primer that contained the C346S mutation (5'-TAT CCA TGG/TCA GAA GAG CCC GGA GTC TTT GAG G-3') (Weat *et al.*, 1985). The PCR product was digested with NcoI and then inserted into pSP64TM that had been previously digested with NcoI.

A minigene encoding the bovine β adrenergic receptor kinase (ARK) C terminus (amino acids 495–689) was a gift from Dr. Robert J. Lefkowitz (Koch *et al*, 1994). The βARK-C minigene was excised from the pRK5 vector with EcoRI and XbaI, treated with Klenow, and then ligated into pSP64TM (Ohan *et al*, 1999) that had been previously treated with EcoRV. To generate a Myc-tagged version, NcoI was used for the partial digestion of pSP64TM-ARK-C, and then the 800 bp NcoI fragment was inserted into pCS2+MT (Turner and Weintraub, 1994) that had been previously digested with NcoI. The resultant plasmid encoded five copies of the Myc tag followed by the same sequence in pSP64TM-βARK-C. To change the C-terminal ANGL of βARK into a geranylgeranylation site (CVLL) (Inglese *et al.*, 1992), we PCR-amplified the βARK sequence (minus ANGL) from pSP64TM-βARK-C by using an SP6 primer (5') and a 3' primer that included CVLL instead of ANGL (5'-TAT CCA TGG TCA AAG CAG CAC GCA ACT GCC GCG CTG GAT-3'). The PCR product was digested with NcoI and inserted into the NcoI site of pSP64TM or pCS2+MT. This generated untagged or Myc-tagged versions of βARK-C_{CAAX}, respectively.

The coding sequence of human β_2 adrenergic receptor (β_2AR) full-length cDNA was excised from a mammalian pRK5 expression vector (Lattion *et al*, 1999) (a gift from Dr. Susanna Cotecchia) by EcoRI and SalI. Klenow treatment subsequently generated blunt ends. The cDNA was then inserted into pCS2+ (Turner and Weintraub, 1994) that had been previously digested with StuI. The resulting plasmid encoded wild-type β_2AR without any sequence tag.

$xG_{\beta 1}$ sequence was PCR-amplified from an oocyte cDNA library (Rebagliati *et al*,1985) with the following primers: forward primer, 5'-TAT CTC GAG AAA ATG AGT GAA CTA GAT CAG C-3'; and reversed primer, 5'-TAT CTC GAG TTA GTT CCA GAT CTT GAG GAA G-3'. The amplified fragment was digested with XhoI and inserted into pCS2+ that had been previously digested with XhoI. The resulting plasmid encoded full-length $xG_{\beta 1}$ (Devic *et al.*, 1996). Bovine $G_{\gamma 2}$ coding sequence, a gift from Melvin I. Simon (Gautam *et al*, 1989), was excised by EcoRI and inserted into pCS2+ that had been previously digested with EcoRI. The resulting plasmid encoded full-length $G_{\gamma 2}$.

Oocyte mRNA injection

In vitro transcription was performed with Ambion's MessageMachine kit with SP6 polymerase. mRNAs were dissolved in water to 1 mg/mL. Ten nanograms mRNA (in 10 nL) was injected per oocyte unless otherwise indicated. The injected oocytes were incubated at 18°C overnight before the addition of progesterone or the injection of a second mRNA.

Protein isolation and Western Blotting

For protein isolation, the desired numbers of oocytes were lysed in ice-cold phosphate-buffered saline lysis buffer (10 mM sodium phosphate buffer, pH 7.5, 150 mM NaCl, 1% Triton X-100, and protease inhibitors: 10 μg/ml leupeptin, 100 μM phenylmethylsulfonyl fluoride; 10 μl/oocyte) by forcing through a pipette tip. To isolate the protein fraction from the homogenate, the lysates was centrifuged in an Eppendorf

centrifuge at 15,000 rpm for 15 minutes at 4°C. The middle clear fraction was collected, mixed with equal amount of 2X reduced SDS sample buffer (250 mM Tris-HCl, pH6.8; 20% glycerol; 10% β-mercaptoethanol; 6% SDS; 0.04% bromophenol blue), boiled for 3 minutes and analysed by SDS-PAGE on a 15% polyacrylamide gel. The yolk proteins, present in the top yellow layer, and the lower dense fraction were not collected. Depending on the protein assay being performed the amount of protein loaded on the gel varied as indicated. This was followed by immunoblotting with anti-xMAP kinase antiserum, used at 1:1000 dilutions. The immunoblots were then developed using an ECL kit (Amersham, Arlington Heights, IL).

Analyses of MAP kinase

Five microliter (5 µl) of undiluted oocyte extracts were mixed with 45 µl of SDS-sample buffer. The sample (10 µl or the equivalent of one half of one oocyte) were directly analysed by SDS-PAGE followed by immunoblotting with antibodies against phospho-MAP kinase (Upstate) or *Xenopus* MAP kinase (Posada *et al.*, 1991). Activated MAP kinase was recognized by antibodies specific for phosphor-MAP kinase or as the upper shifted band in *Xenopus* MAP kinase blots (Figure 2.2).

Preparation of total oocyte extracts, membrane, and cytosol fractions

To prepare total membrane and cytosol fractions, oocytes were lysed in homogenization solution (10 mM Hepes [pH 7.5], 83 mM NaCl, 1 µg/mL leupeptin, 100 µM phenylmethylsulfonyl fluoride; 10 µl per oocyte). Following two rounds of low-speed centrifugation (900 g for 5 min), the clarified supernatants were subjected to high-

speed centrifugation (100,000 g for 60 min). The pellets were designated as membrane and the supernatants as cytosol. All centrifugations were performed at 4°C. Samples were directly dissolved in SDS sample buffer for immunoblots except for conditions in which antibodies against *Xenopus* β-integrin were used, in which case samples were dissolved in SDS sample buffer without -mercaptoethanol since the antibodies (8C8) do not recognize reduced integrin proteins (Gawantka *et al.*, 1992).

Coimmunoprecipitation experiments

Ascite fluids from mice injected with 9E10 hybridoma (anti-Myc) were fractionated by 25% saturated ammonium sulfate followed by 50% saturated ammonium sulfate. The precipitates from 50% saturated ammonium sulphates were dissolved in PBS (10 mM sodium phosphate buffer [pH 7.5], 150 mM NaCl). A total of 20 mg of Myc-specific IgG (at least 80% pure judged by SDS-PAGE and Coomassie Blue staining) were coupled to 2 ml of CNBr-activated sepharose beads (Pharmacia) according to the manufacturer's instructions. Extracts (prepared in PBS lysis buffer as described above) from 20–30 control oocytes or oocytes injected with Myc-βARK-C or Myc-βARK-C_{CAAX} mRNA were incubated with 10 µl of the anti-Myc beads for 90 min at 4°C. The beads were washed three times with PBS lysis buffer. Bound proteins were eluted by boiling them in SDS sample buffer and analyzed by immunoblotting with polyclonal anti-G_{β}.

In vitro ADP-ribosylation assay

The procedure was modified from Kopf and Woolkalis (Kopf and Woolkalis, 1991). Oocytes injected with water (control) or with $G_{t\alpha}$ or $G_{t\alpha\text{-}C346S}$ mRNA were incubated

overnight to allow protein synthesis. 20 oocytes were homogenized in 1 mL ice-cold buffer (10 mM HEPES, 83 mM NaCl, 1 mM MgCl2, pH7.9, 1 μg/mL leupeptin, 100 μM PMSF). Following two rounds of low speed centrifugation (900 g for 5 minutes), the supernatants were centrifuged at 100,000 g for 1 hour. The high-speed pellets (oocyte membrane) were resuspended in 48 μL of assay buffer (0.1 M Tris-HCl [pH 7.6] and 20 mM dithiothreitol). To initiate the ADP-ribosylation reaction, 1 μl [^{32}P]NAD (1000 Ci/mmol, 10 mCi/mL) and 1 μl (100 ng) of PTX was added to the membrane extracts. The reaction was carried out at room temperature for 30 min and stopped by the addition of SDS sample buffer. Samples were analyzed by SDS-PAGE and autoradiography.

Data analysis

GVBD and cAMP data were analyzed by using SigmaPlot software, version 1.00 (Jandel Scientific). Results were presented as mean±standard error of the mean (S.E.M.).

RESULTS

$G_{t\alpha}$ induction of oocyte maturation

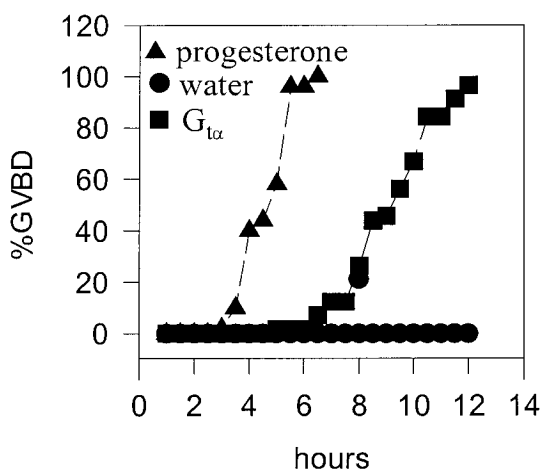
A previous study (Sadler *et al.*, 1984) has shown that incubation of oocytes with pertussis toxin does not inhibit progesterone-induced oocyte maturation, suggesting that the α subunit of inhibitory G protein ($G_{i\alpha}$) is not responsible for mediating progesterone action in frog oocytes. To examine the possible involvement of $G_{\beta\gamma}$ in regulating oocyte maturation, a well-characterized $G_{\beta\gamma}$ scavenger, the α subunit of bovine transducin ($G_{t\alpha}$) (Medynski *et al*, 1985; Crespo *et al*, 1994) was employed. $G_{t\alpha}$ mRNA injection followed by incubation of oocytes in OR2 (oocyte incubation medium) caused a time-dependent induction of GVBD (Figure 2.1A). $G_{t\alpha}$-induced GVBD lagged behind GVBD induced by the natural maturation-induction hormone progesterone. This delay presumably reflects the time required for translation and posttranslational modification of $G_{t\alpha}$. $G_{t\alpha}$ induction of GVBD was dose dependent; it required 10 ng mRNA per oocyte for efficient induction (Figure 2.1B and Figure 2.2). GVBD induced by $G_{t\alpha}$ mRNA injection was morphologically indistinguishable from that induced by progesterone (Figure 2.3), and $G_{t\alpha}$-induced GVBD was accompanied by the characteristic *Xenopus* MAP kinase phosphorylation (Figure 2.2). As shown previously (Ferrell and Machleder, 1998; Ohan *et al,* 1999), MAP kinase phosphorylation in *Xenopus* oocytes is an all-or-none event, and the ratio of phosphorylated (active) MAP kinase vs. unphosphorylated (inactive) MAP kinase therefore reflects the ratio of GVBD-positive versus GVBD-negative oocytes. Therefore, both GVBD and MAP kinase phosphorylation were used to measure oocyte maturation.

Figure 2.1 **$G_{t\alpha}$ induces oocyte GVBD**

A: Groups of 50 or more oocytes were injected with water or mRNA encoding $G_{t\alpha}$ (10 ng per oocyte). A third group of oocytes was incubated with 1 μM of progesterone. At the indicated time following injection (or following the addition of progesterone), oocytes were scored for GVBD and expressed as a percentage of total treated oocytes. Shown is a representative of three independent experiments.

B: Oocytes were injected with water or mRNA encoding $G_{t\alpha}$ (10 ng per oocyte). Injected oocytes were incubated in OR2 for 15–24 hr before being scored for GVBD. Shown are the means (with errors) of 15 independent experiments with the actual numbers of GVBD-positive/total injected oocytes indicated on top.

A


120
100
80
60
40
20
0
%GVBD
progesterone
water
$G_{t\alpha}$
0 2 4 6 8 10 12 14
hours

B

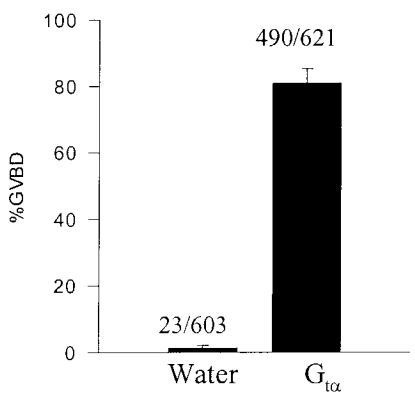

100
80
60
40
20
0
%GVBD
490/621
23/603
Water
$G_{t\alpha}$

Figure 2.2 **$G_{t\alpha}$ induces MAP kinase phosphorylation**
Groups of 20 or more oocytes were injected with water or the various amounts of $G_{t\alpha}$ mRNA (per oocyte). Injected oocytes were incubated in OR2 for 15–24 hr. oocytes were lysed and the extracts were subjected to immunoblotting with anti-xMAP kinase antiserum. Shown is a representative of three independent experiments

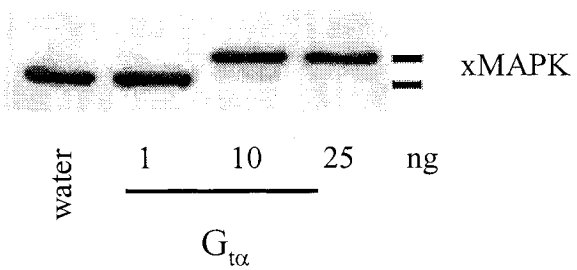
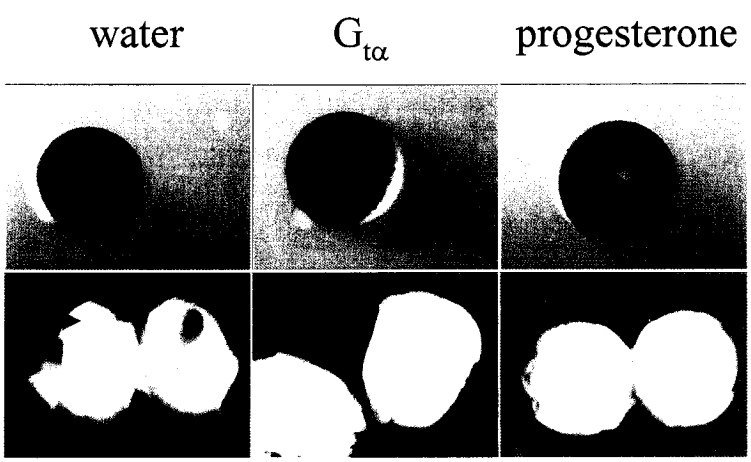

xMAPK
water
1
10
25
ng
$G_{t\alpha}$

Figure 2.3 **Morphology in $G_{t\alpha}$-induced oocyte GVBD**
Typical images of intact oocytes (top panels) and oocytes after they were fixed in 5% trichloroacetic acid and dissected (bottom panels). An oocyte that underwent progesterone-induced GVBD is shown in comparison with that $G_{t\alpha}$-induced GVBD. An arrow indicates a germinal vesicle in a GVBD-negative oocyte.


water
$G_{t\alpha}$
progesterone

Effect of pertussis toxin on oocyte maturation

To eliminate the possibility that $G_{t\alpha}$ functions as a G protein nonspecifically modulating endogenous effector system (e.g phosphodiesterases), we employed PTX which is known to ADP ribosylates $G_{t\alpha}$ and to inhibit its GTP binding (West *et al*, 1985). In vitro ADP-ribosylation assays were carried out by using cell membrane extracts from control oocytes or oocytes injected with $G_{t\alpha}$ mRNA, or mRNA for $G_{t\alpha\text{-}C346S}$, which containing a point mutation eliminating the ADP ribosylation site (West *et al*, 1985). As shown in Figure 2.4, PTX modified an endogenous protein, likely representing an α subunit(s) of the G_i class (lane 1), as reported previously (Sadler, 1984). Significantly higher levels of ADP ribosylation occurred in membrane extracts from oocytes injected with wild-type $G_{t\alpha}$ mRNA (lane 2) but not with $G_{t\alpha\text{-}C346S}$ mRNA (lane 3). This finding indicates that the wild-type $G_{t\alpha}$, in addition to the endogenous G_{α}, was modified by PTX. Like the wild-type $G_{t\alpha}$, $Gt_{\alpha\text{-}C346S}$ efficiently induced MAP kinase activation and oocyte GVBD. A typical experiment was shown in Figure 2.5. Injection of sub-optimal amounts (1-2 ng per oocyte) of $G_{t\alpha}$ mRNA resulted in little MAP kinase activation (lanes 2 and 3), but co-injection with PTX resulted in almost quantitative MAP kinase activation (lanes 7 and 8). PTX alone did not induce MAP kinase activation (lane 6). Interestingly, PTX also synergized with $G_{t\alpha\text{-}C346S}$ in inducing MAP kinase activation (lane 4 and 5 vs. lanes 9 and 10) and GVBD (Figure 2.6). These results suggest that the effect of PTX on $G_{t\alpha}$ action in oocytes is derived from its ability to modify endogenous G protein (s). Interestingly, an earlier study reported the synergistic effect of PTX on progesterone-induced GVBD, although others have reported the opposite effect (Sadler, 1984; Pellaz and Schorderet-

Figure 2.4 **ADP-ribosylation by PTX**

Groups of 20 oocytes were injected with water or with mRNA for $G_{t\alpha}$ or $G_{t\alpha\text{-}C346S}$, Following an overnight incubation in OR2, oocyte membranes were isolated and subjected to in vitro ADP-ribosylation assays. Samples were analyzed by SDS-PAGE followed by autoradiography (lanes 1–3). Shown is a representative of three independent experiments. As a control, 1 μg of each of the two types of mRNA were translated in rabbit reticulocyte lysates in the presence of [^{35}S] methionine. Samples were analyzed by SDS-PAGE followed by autoradiography (lanes 4–6).

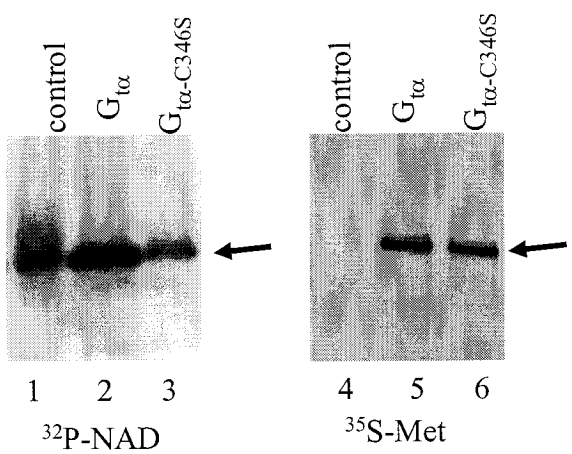

control
$G_{t\alpha}$
$G_{t\alpha\text{-}C346S}$
control
$G_{t\alpha}$
$G_{t\alpha\text{-}C346S}$
1 2 3
4 5 6
^{32}P-NAD
^{35}S-Met

Figure 2.5 **Synergism between $G_{t\alpha}$ and PTX in induction of MAP kinase activation**
Groups of at least 50 oocytes were injected with PTX, $G_{t\alpha}$ (or $G_{t\alpha\text{-}C346S}$) mRNA (1 or 2 ng per oocyte as indicated), or both PTX and mRNA. The injected oocytes were incubated in OR2 for 6–10 hr, by which time at least 50% of the oocytes coinjected with PTX and mRNA had undergone GVBD (data not shown). The oocytes were lysed for MAP kinase immunoblotting. Shown is a representative of three independent experiments.

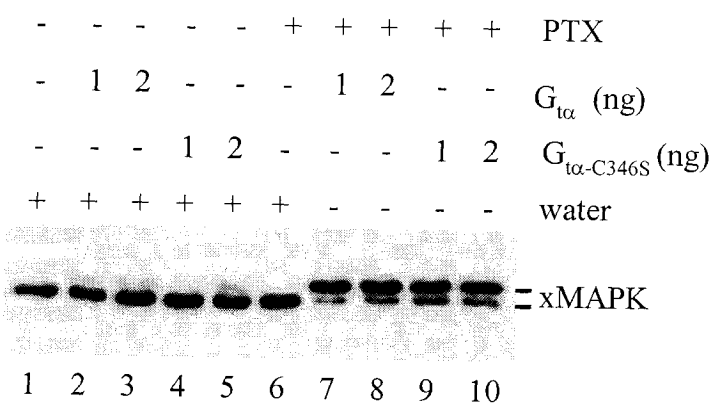
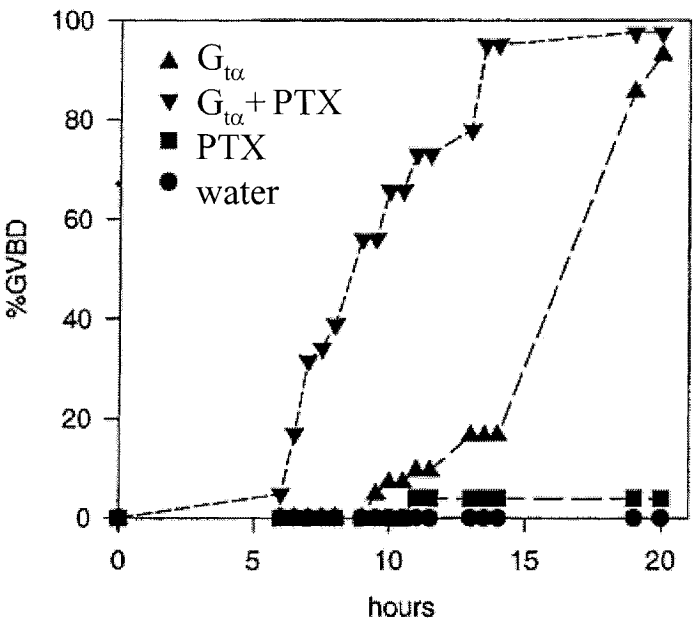
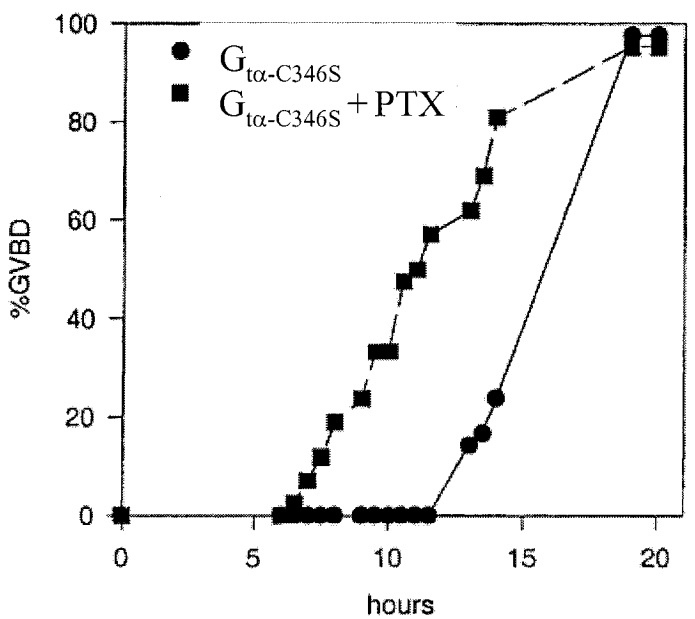

- - - - - + + + + + PTX
- 1 2 - - - 1 2 - - $G_{t\alpha}$ (ng)
- - - 1 2 - - - 1 2 $G_{t\alpha\text{-}C346S}$ (ng)
+ + + + + + - - - - water
xMAPK
1 2 3 4 5 6 7 8 9 10

Figure 2.6 **Synergism between $G_{t\alpha}$ and PTX in induction of GVBD**
Oocytes (at least 50 per group) were injected with water (control), or with PTX (0.2 ng per oocyte), or $G_{t\alpha}$ (or $G_{t\alpha\text{-}C346S}$, lower panel) mRNA, or both PTX and $G_{t\alpha}$ (or $G_{t\alpha\text{-}C346S}$, lower panel) mRNA, as indicated. All four groups were monitored for GVBD, at the indicated time following the injection. For clarity, the data are presented in two separate graphs. Shown is a representative of three independent experiments.


100
80
60
40
20
0
%GVBD
0
5
10
15
20
hours
$G_{t\alpha}$
$G_{t\alpha}$+PTX
PTX
water


100
80
60
40
20
0
%GVBD
0
5
10
15
20
hours
$G_{t\alpha\text{-}C346S}$
$G_{t\alpha\text{-}C346S}$+PTX

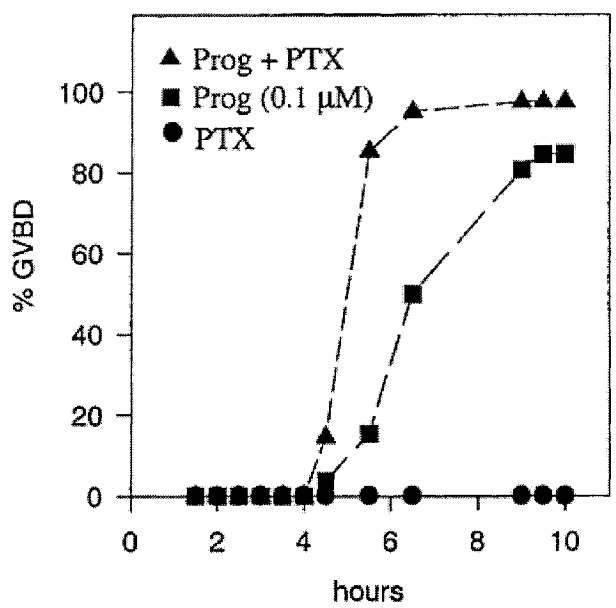
Slatkine, 1989). We have consistently observed that PTX accelerates progesterone-induced GVBD (Figure 2.7).

βARK-C_{CAAX} induces oocyte maturation

To confirm that $G_{t\alpha}$ functions as a free $G_{\beta\gamma}$ scavenger in the induction of oocyte GVBD, another known G protein $G_{\beta\gamma}$ scavenger, the noncatalytic C-terminal region of β adrenergic receptor kinase (βARK-C) (Koch *et al*, 1994; Inglese *et al*, 1994) was employed. The injection of βARK-C mRNA failed to induce oocyte GVBD (Figure 2.8A, lane 2). We reasoned that targeting βARK-C to the membrane might be necessary for its efficient binding to the membrane bound $G_{\beta\gamma}$. To create a membrane targeting βARK-C, we replaced the C-terminal four amino acids of βARK-C with a geranylgeranylation site (CVLL). We termed this construct βARK-C_{CAAX} according to the original nomenclature for isoprenylation consensus (Hancock *et al*, 1989). An identical alteration of the full-length βARK results in its constitutive association with the membranes (Inglese *et al*, 1992). Indeed, the injection of βARK-C_{CAAX} mRNA efficiently induced GVBD (Figure 2.8A, lane 3) and MAP kinase activation (Figure 2.8B, lane 3). Both βARK-C and βARK-C_{CAAX} mRNAs were translated efficiently (Figure 2.8A inset). To facilitate subcellular localization and binding studies, we generated Myc-tagged versions of both βARK-C and βARK-C_{CAAX}. The presence of the Myc tag did not alter the ability of βARK-C_{CAAX} to induce GVBD (data not shown). Subcellular fractionation experiments confirmed that a significant proportion of Myc-βARK-C_{CAAX} had been

Figure 2.7 **PTX accelerates progesterone-induced GVBD**
Oocytes injected with water or PTX were incubated for 2 hr before being divided into groups of 30 oocytes each and incubated with 0.1 μM or 1 μM of progesterone. At the indicated time following the addition of progesterone, oocytes were scored for GVBD. Shown is a representative of five independent experiments

A


Prog + PTX
Prog (0.1 µM)
PTX
% GVBD
100
80
60
40
20
0
0
2
4
6
8
10
hours

B

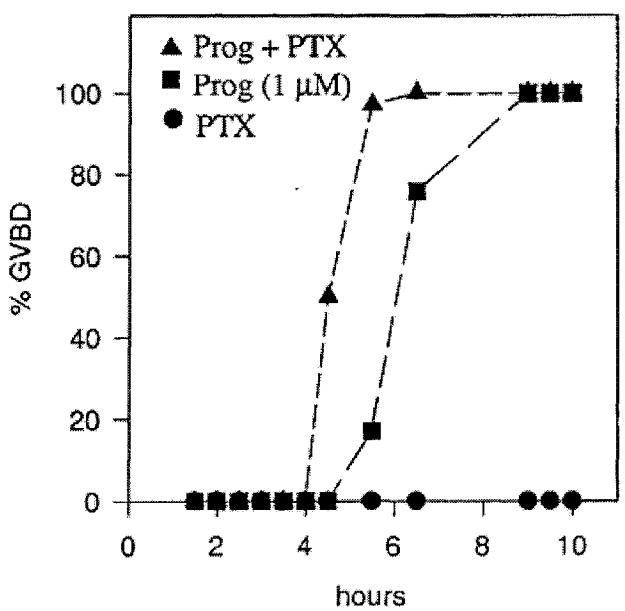
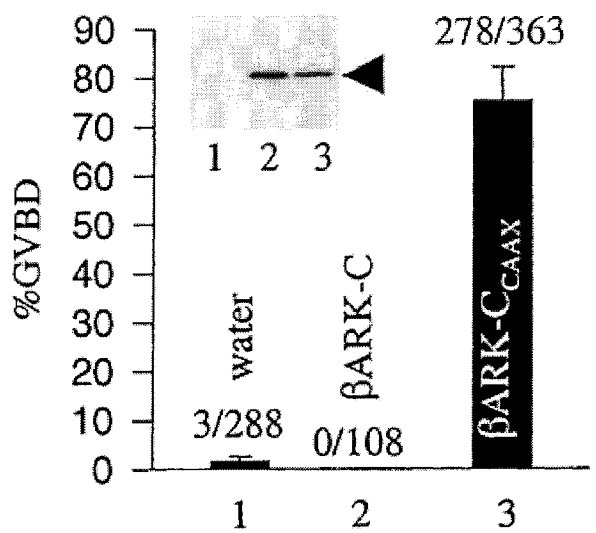

Prog + PTX
Prog (1 µM)
PTX
% GVBD
100
80
60
40
20
0
0
2
4
6
8
10
hours

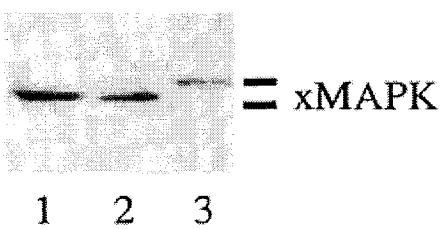
Figure 2.8 **βARK-C_{CAAX} induces oocyte maturation.**
A:Oocytes were injected with water or mRNA for βARK-C or βARK-C_{CAAX}. Injected oocytes were incubated for 15–20 hr before GVBD scoring. Shown are the means (with errors) of 3–8 independent experiments. The actual numbers of GVBD-positive oocytes/total injected oocytes are indicated. Inset is an in vitro translation experiment in which 1 μg each of the two types of mRNA was used.

B: Following GVBD scoring (as in A), oocytes were lysed and subjected to xMAP kinase immunoblotting. Shown is a representative of three independent experiments.

A


90
80
70
60
50
40
30
20
10
0
%GVBD
1 2 3
water
βARK-C
βARK-C$_{CAAX}$
3/288
0/108
278/363
1
2
3

B


xMAPK
1 2 3

targeted to the membrane, whereas Myc-βARK-C was exclusively found in the cytosol (Figure 2.9).

$G_{\beta\gamma}$ scavengers potentiate progesterone-induced GVBD

The adenylyl cyclase activator forskolin increases oocyte cAMP and inhibits progesterone-induced oocyte GVBD (Schorderet-Slatkine and Baulieu, 1982). We wished to determine whether forskolin would also inhibit $G_{t\alpha}$-induced GVBD. Indeed, forskolin effectively blocked progesterone-induced as well as $G_{t\alpha}$-induced GVBD and MAP kinase activation (Figure 2.10A).

Other characteristics of progesterone-induced GVBD are the requirement of protein synthesis and MAP kinase activation (Ferrell, 1999). We therefore tested whether a protein synthesis inhibitor cycloheximide (CHX) or a MEK inhibitor, PD98059 (Ferrel, 1999), could inhibit $G_{t\alpha}$-induced MAP kinase and GVBD. As shown in Figure 2.10B, progesterone-induced and $G_{t\alpha}$-induced MAP kinase activation and GVBD were blocked by CHX and PD98059.

The common pharmacological characteristics of $G_{t\alpha}$-induced and progesterone-induced GVBD suggest that $G_{t\alpha}$ might synergize with progesterone-induced GVBD. To test this, we first injected oocytes with low concentrations of $G_{t\alpha}$ or βARK-C_{CAAX} mRNA. Following an overnight incubation, water-injected oocytes and mRNA-injected oocytes were further incubated with a 10 nM of progesterone. Although 10 nM of progesterone is often sufficient to induce GVBD in freshly isolated oocytes (Bayaa et al., 2001), oocytes that had been stored overnight or longer (necessary to allow protein synthesis following mRNA injection) rarely respond to 10 nM of progesterone. As shown

Figure 2.9 **Myc-βARK-C_{CAAX} localized on oocyte membrane**
Oocytes injected with water or Myc-βARK-C (or Myc-βARK-C_{CAAX}) mRNA were incubated overnight. Oocyte extracts and membrane and cytosol fractions (each lane contained the equivalent of 1/2 oocyte) were immunoblotted with anti-Myc antibodies (top panel). Extracts, membrane, and cytosol derived from water-injected oocytes were also immunoblotted with anti-xMAP kinase (middle panel) or anti-*Xenopus* β-integrin (bottom panel). Shown is a representative of three independent experiments.

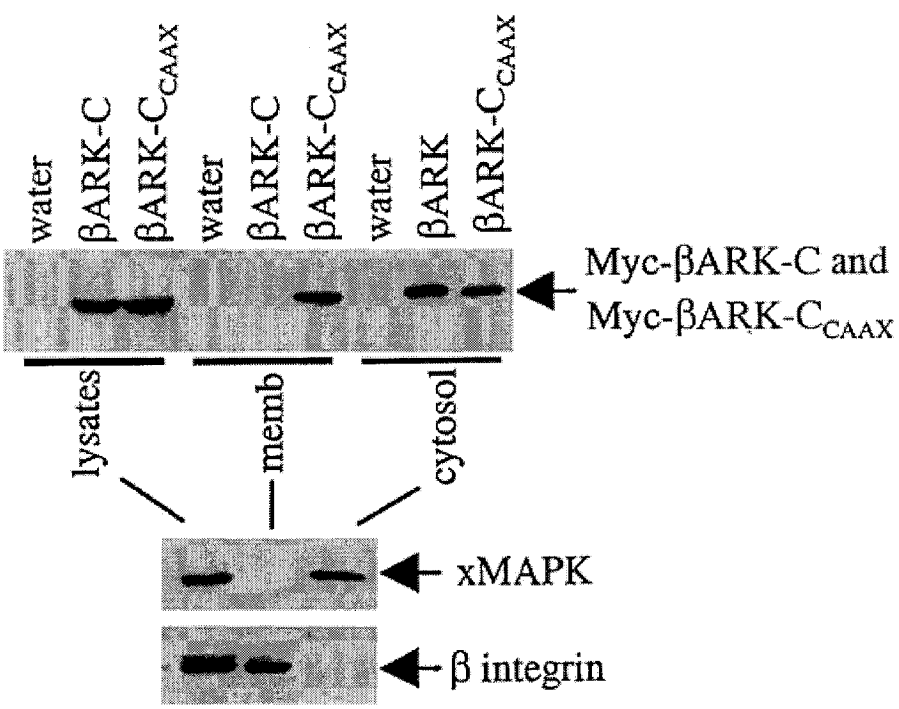
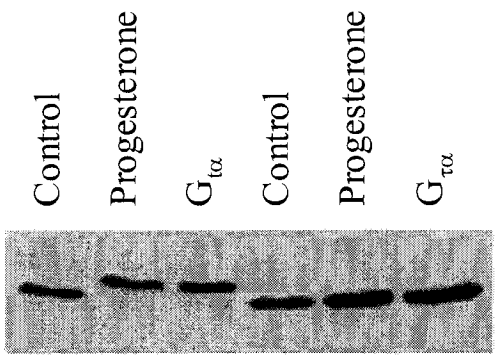

water
βARK-C
βARK-C$_{CAAX}$
water
βARK-C
βARK-C$_{CAAX}$
water
βARK
βARK-C$_{CAAX}$
Myc-βARK-C and
Myc-βARK-C$_{CAAX}$
lysates
memb
cytosol
xMAPK
β integrin

Figure 2.10 **FSK, CHX and PD98059 blocked $G_{t\alpha}$-induced oocyte maturation**
A: Oocytes were incubated in OR2 (control) or OR2 containing 1 μM progesterone, or oocytes were injected with $G_{t\alpha}$ mRNA. Either forskolin (FSK) (50 μM) or an equivalent volume of dimethylsulfoxide (DMSO) was added to each group, as indicated. Oocytes were incubated for 15 hr before GVBD scoring followed by anti-xMAP kinase immunoblotting. Shown is a representative of three independent experiments.

B: Oocytes were treated with progesterone (1 μM) or injected with $G_{t\alpha}$ mRNA. Overnight incubation was carried out in OR2 (control) or OR2 containing 10 μg/mL CHX or 50 μM PD98059. Oocytes were scored for GVBD, and then anti-xMAP kinase immunoblotting was performed. Shown is a representative of three independent experiments.

A


Control
Progesterone
$G_{t\alpha}$
Control
Progesterone
$G_{t\alpha}$
xMAPK
0 100 90 0 3 0 %GVBD
DMSO
forskolin

B

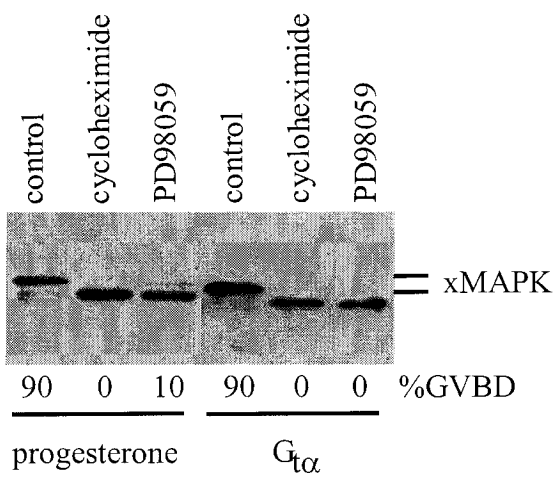
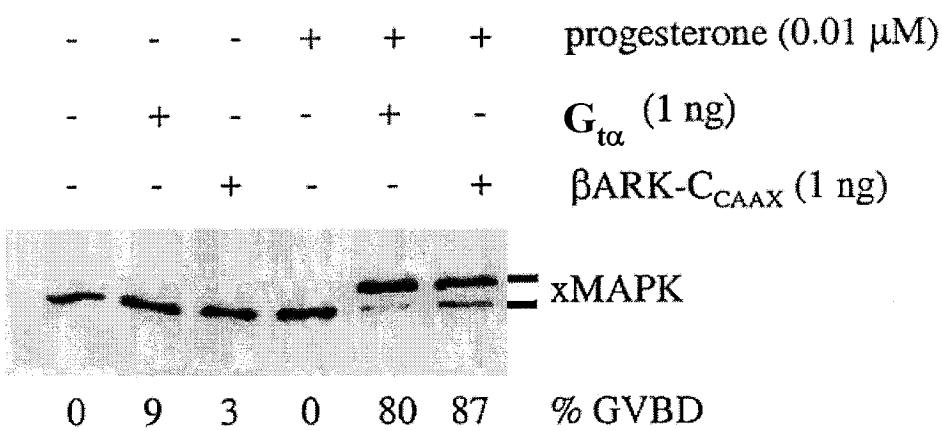

control
cycloheximide
PD98059
control
cycloheximide
PD98059
xMAPK
90 0 10 90 0 0 %GVBD
progesterone
$G_{t\alpha}$

Figure 2.11. **$G_{\beta\gamma}$ scavengers potentiate progesterone action in *Xenopus* oocytes.**
Oocytes were injected with water or mRNA (1 ng per oocyte) encoding $G_{t\alpha}$ or βARK-C_{CAAX}. Following an overnight incubation in OR2, the injected oocytes were further incubated for 10–15 hr in OR2 (lanes 1–3) or OR2 containing 10 nM progesterone (lanes 4–6) before GVBD scoring and anti-xMAP kinase immunoblotting were performed. Shown is a representative of three independent experiments.


- - - + + + progesterone (0.01 μM)
- + - - + - $G_{t\alpha}$ (1 ng)
- - + - - + βARK-C_{CAAX} (1 ng)
xMAPK
0 9 3 0 80 87 % GVBD

in Figure 2.11, neither progesterone (10 nM) nor the scavenger mRNA (1 or 2 ng per oocyte) injection alone was sufficient to induce significant GVBD. A combination of both however, caused > 80 % GVBD.

Overexpression and activation of human β_2AR inhibits progesterone- and $G_{\beta\gamma}$ scavenger–induced GVBD

Our data implicate the involvement of an endogenous G protein, in particular $G_{\beta\gamma}$ complex as a key inhibitor of oocyte meiosis. How is this $G_{\beta\gamma}$ generated in G_2-arrested oocytes? We reasoned that there might be a constitutively active G protein–coupled receptor (GPCR) in G_2 oocytes that is responsible for activating a trimeric G protein and releasing the inhibitory $G_{\beta\gamma}$. The inhibition of this putative GPCR might therefore inhibit the release of the $G_{\beta\gamma}$ and hence cause GVBD. A survey of well-characterized GPCR antagonists (those specific for β adrenergic receptors, acetylcholine receptors, dopamine receptors, or serotonin receptors) found that only very high and nonphysiological concentrations (500 μM to 1 mM) of propranolol or alprenolon, both antagonists for β adrenergic receptors, were able to induce GVBD (data not shown). Interestingly, an earlier study (Baulieu *et al*, 1978) has reported similar findings, although it has concluded that the ability of these antagonists to induce GVBD is the result of their nonspecific interaction with other membrane components (Baulieu *et al*, 1978). Indeed, whereas the agonist for β adrenergic receptor, isoproterenol (ISO), had no effect on progesterone-induced GVBD or MAP kinase activation (Figure 2.12, lane 3), ISO blocked both in oocytes that had been injected with human β_2 adrenergic receptor (β_2AR) (Simonds, 1999) mRNA (lane 4). The inhibitory effect of β_2AR/ISO was completely reversed by 10

μM alprenolon (ALP, Figure 2.13, lane 3), which otherwise had no effect on oocyte maturation (Figure 2.13, lane 4). Similarly, the β_2AR/ISO combination also effectively blocked $G_{\beta\gamma}$ scavenger–induced MAP kinase activation (Figure 2.14) and GVBD (data not shown). These results indicate that oocytes do not have a functional β adrenergic–receptor system.

Xenopus G_β1

Our results suggest that the $G_{t\alpha}$ and βARK-C_{CAAX} induced GVBD by binding and sequestering an endogenous $G_{\beta\gamma}$ complex. Therefore over-expression of $G_{\beta\gamma}$ subunits may counteract these $G_{\beta\gamma}$ scavengers. Although multiple G protein α subunits have been detected in or cloned from *Xenopus* oocytes (Gallo *et al.*, 1995; Olate *et al*, 1990), only one G_β subunit (xG_β1 (Devic *et al.*, 1996)), and no G_γ subunit, have been identified or cloned in *Xenopus*. Furthermore, xG_β1 has been detected in immature oocytes (Devic *et al.*, 1996). I PCR-amplified xG_β1 (Devic *et al.*, 1996) from an oocyte cDNA library and constructed an expression plasmid for mRNA synthesis. Two commercial rabbit polyclonal anti-G_β antibodies were tested in an attempt to detect endogenous protein and G_β1 derived from mRNA injection. Sc378, which was raised against the C terminus of mouse $G_{\beta1}$, detected a prominent protein of relative molecular mass of about 35 kDa in oocyte extracts (Figure 2.15A, lanes 1–3). This finding is consistent with the protein being an endogenous *Xenopus* G_β (xG_β). The identity of a second protein, of slightly greater molecular mass, is unknown. Sc261, which was raised against the N terminus of bovine $G_{\beta1}$, did not detect either of these proteins (Figure 2.15).

Figure 2.12 **β_2AR/ISO inhibits oocyte GVBD**

Oocytes were injected with water (lanes 1, 3, and 5) or human β_2AR mRNA (lanes 2, 4, and 6) and then incubated overnight in OR2. ISO (final 1 μM) was added to lanes 3 and 4. One hour following the addition of ISO, progesterone was added to the indicated groups. All groups were further incubated for at least 15 hr before GVBD scoring. Shown are means of three to seven independent experiments; the actual numbers of GVBD-positive oocytes/total treated oocytes are indicated. A typical xMAP kinase immunoblot is shown below.

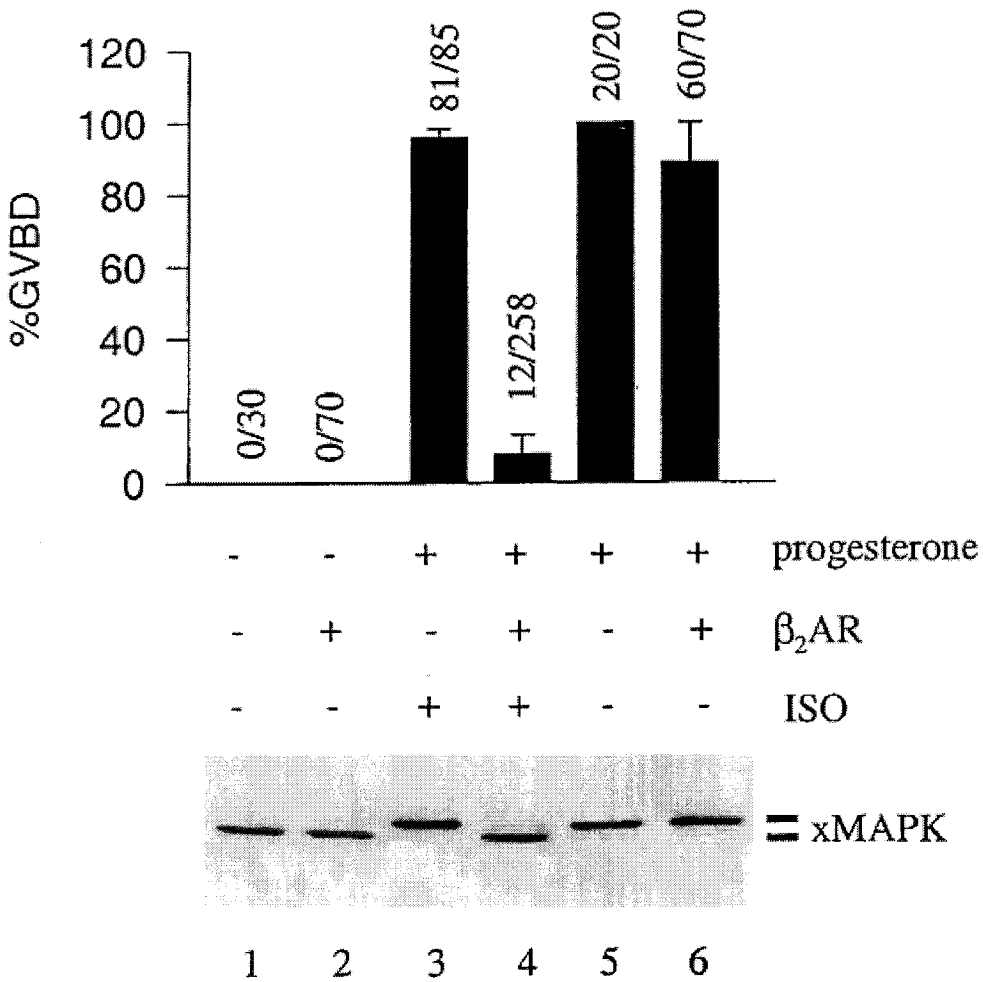

%GVBD
120
100
80
60
40
20
0
0/30
0/70
81/85
12/258
20/20
60/70
- - + + + + progesterone
- + - + - + β_2AR
- - + + - - ISO
xMAPK
1 2 3 4 5 6

Figure 2.13 **Alprenolon (ALP), antagonist of β_2AR reversed β_2AR inhibitory function**

Groups of at least 20 oocytes were injected with human β_2AR mRNA and then incubated overnight in OR2. Where indicated, ISO (1 μM), ALP (10 μM), or both were added. Oocytes were further incubated for at least 15 hr before being scored for GVBD (data not shown) and being lysed for anti-xMAP kinase immunoblotting.

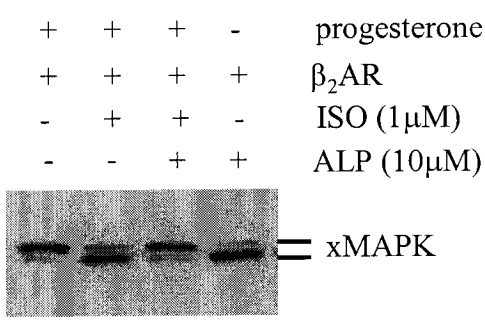

+ + + - progesterone
+ + + + β_2AR
- + + - ISO (1μM)
- - + + ALP (10μM)
xMAPK

Figure 2.14 **β_2AR/ISO blocked $G_{\beta\gamma}$-scavenger-induced MAP kinase activation**
Oocytes were injected with water or β_2AR mRNA followed by incubation in OR2 for 6 hr. Oocytes were injected again with water, $G_{t\alpha}$ mRNA, or βARK-C_{CAAX} mRNA. Following the second injection, oocytes were immediately transferred to OR2 containing the indicated concentrations of ISO, and incubation continued overnight. Oocytes were scored for GVBD (data not shown) and lysed for anti-xMAP kinase immunoblotting. Shown is a representative of three independent experiments. Each group contained at least 20 oocytes.

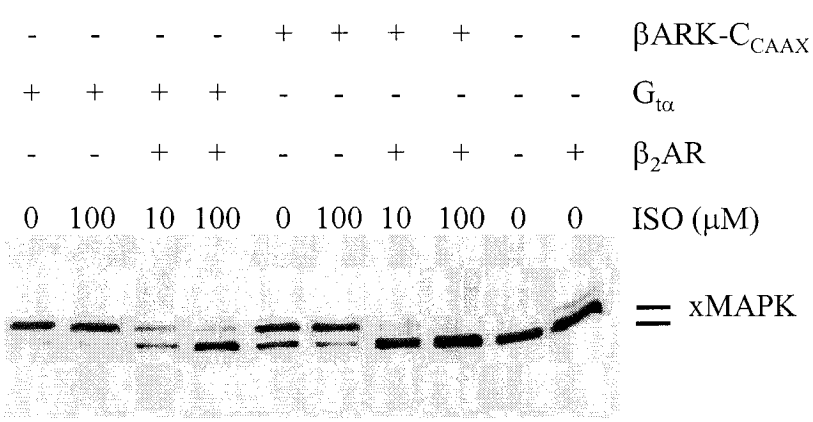
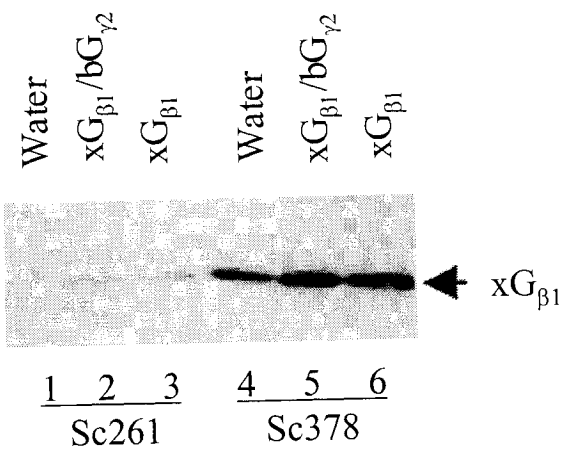

- - - - + + + + - - βARK-C$_{CAAX}$
+ + + + - - - - - - $G_{t\alpha}$
- - + + - - + + - + β_2AR
0 100 10 100 0 100 10 100 0 0 ISO (μM)
xMAPK

Figure 2.15. **Immunodetection of overexpressed $xG_{\beta 1}$**
Oocytes were injected with water (lanes 1 and 4), $xG_{\beta 1}$ mRNA (lanes 3 and 6), or a combination of $xG_{\beta 1}$ mRNA and bovine $G_{\gamma 2}$ mRNA (lanes 2 and 5). Following an overnight incubation in OR2, oocyte extracts were prepared and immunoblotted with Sc261 (lanes 1–3) or Sc378 (lanes 4–6) anti-$G_{\beta 1}$ antibodies. Shown is a representative of three independent experiments.


Water
xG$_{\beta1}$/bG$_{\gamma2}$
xG$_{\beta1}$
Water
xG$_{\beta1}$/bG$_{\gamma2}$
xG$_{\beta1}$
xG$_{\beta1}$
1 2 3
Sc261
4 5 6
Sc378

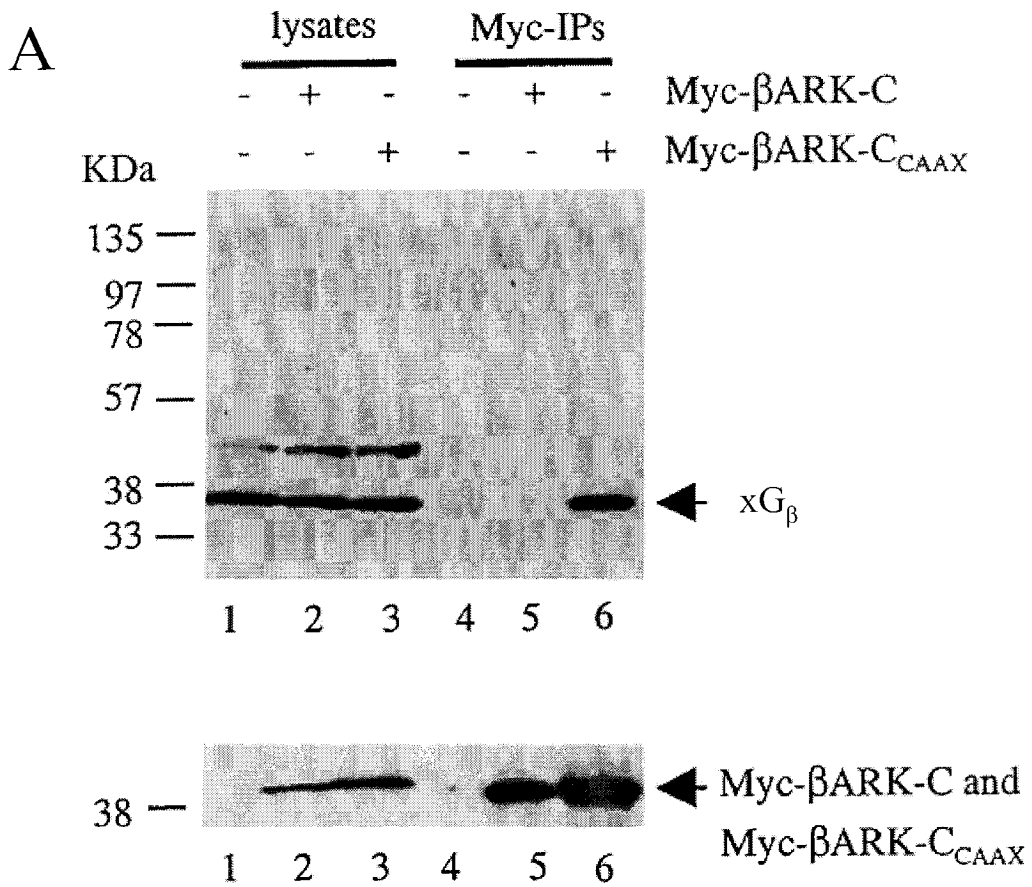
As expected, xG_{β} was associated with oocyte membrane (Figure 2.16). To determine whether Myc-βARK-C_{CAAX} bound xG_{β}, coimmunoprecipitation experiments were performed. Anti-Myc antibodies pulled down similar amounts of Myc-βARK-C (Figure 2.16A, lower panel, lane 5) or Myc-βARK-C_{CAAX} (lane 6) from oocytes injected with the respective mRNA. However, xG_{β} was only pulled down with Myc-βARK-C_{CAAX} (Figure 2.16A, upper panel, lane 6) but not with Myc-βARK-C (lane 5). Anti-Myc also did not pull down xG_{β} in extracts made from water-injected oocytes (lane 4). Coimmunoprecipitation experiments (Figure 2.17) indicated that an increased level of G_{β} became associated with Myc-βARK-C_{CAAX} in oocytes injected with $xG_{\beta}1$ mRNA (Figure 2.17; compare lane 5 to 4). These results further support the notion that the endogenous xG_{β} is $xG_{\beta}1$.

$G_{\beta\gamma}$ subunits inhibited $G_{\beta\gamma}$ scavenger– or progesterone-induced GVDB

Injection of $xG_{\beta 1}$ mRNA significantly reduced the ability of $G_{t\alpha}$ (Figure 2.18) or βARK-C_{CAAX} (data not shown) to induce GVBD. Coinjection of bovine $G_{\gamma 2}$ mRNA further enhanced the inhibitory effect of $xG_{\beta 1}$. Importantly, the injection of $xG_{\beta 1}$ mRNA, alone or in combination with bovine $G_{\gamma 2}$ mRNA, also reduced the ability of progesterone to induce GVBD (Figure 2.19). The possibility of nonspecific effects on oocyte protein synthesis due to mRNA injection was eliminated since injection of an equal amount of a control mRNA, green fluorescence protein (GFP) (Ohan *et al*, 2000), did not affect progesterone-induced GVBD (Figure 2.20).

Figure 2.16 **Association of Myc- βARK-C_{CAAX} with an endogenous G_{β}**
A: Extracts (lanes 1–3, one oocyte equivalent) or anti-Myc immunoprecipitates (lanes 4–6, each derived from 20 oocytes) from oocytes injected with water (lanes 1 and 4) or mRNA encoding Myc-βARK-C (lanes 2 and 5) or Myc-βARK-C_{CAAX} (lanes 3 and 6) were immunoblotted with anti-$G_{\beta 1}$ (upper panel) or anti-Myc (lower panel). Shown is a representative of four independent experiments.

B: Extracts (one oocyte), supernatant (cytosol, two oocytes), or pellet (membrane, two oocytes), following centrifugation at 100,000 g, were immunoblotted with anti-$G_{\beta 1}$. Shown is a representative of three independent experiments.


A
lysates
Myc-IPs
- + - - + - Myc-βARK-C
- - + - - + Myc-βARK-C$_{CAAX}$
KDa
135
97
78
57
38
33
xG$_\beta$
1 2 3 4 5 6
38
Myc-βARK-C and
Myc-βARK-C$_{CAAX}$
1 2 3 4 5 6

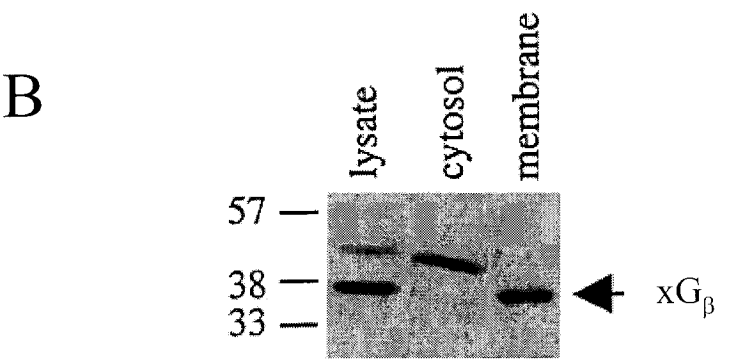

B
lysate
cytosol
membrane
57
38
33
xG$_\beta$

Figure 2.17 **Binding of Myc-βARK-C_{CAAX} and injected $xG_{\beta 1}$**
Oocytes were injected with Myc-βARK-C_{CAAX} mRNA together with water (lanes 1 and 4), $xG_{\beta 1}$ mRNA (lanes 3 and 6), or a combination of $xG_{\beta 1}$ mRNA and bovine $G_{\gamma 2}$ mRNA (lanes 2 and 5). Following an overnight incubation in OR2, oocyte extracts were prepared and immunoprecipitated with anti-Myc. The extracts (lanes 1–3) and immunoprecipitates (lanes 4–6) were blotted with anti-$G_{\beta 1}$ antibodies. Shown is a representative of two independent experiments.

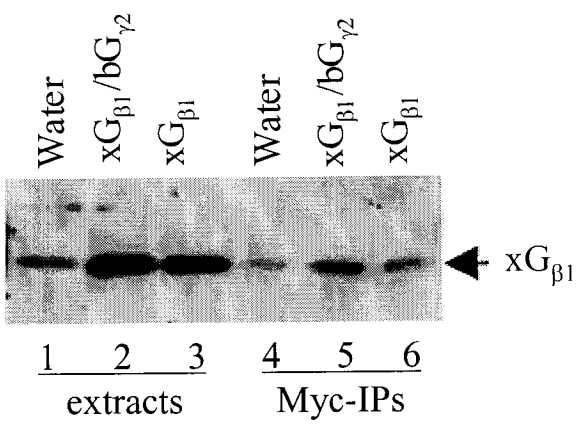

Water
xG$_{\beta1}$/bG$_{\gamma2}$
xG$_{\beta1}$
Water
xG$_{\beta1}$/bG$_{\gamma2}$
xG$_{\beta1}$
xG$_{\beta1}$
1 2 3
4 5 6
extracts
Myc-IPs

Figure 2.18 **Overexpression of $G_{\beta\gamma}$ inhibits $G_{t\alpha}$-induced GVBD**
Oocytes injected with water or the indicated mRNA ($xG_{\beta 1}$, $bG_{\gamma 2}$, or both) were incubated overnight in OR2 before a second injection with Gtα mRNA. At the indicated time following $G_{t\alpha}$ mRNA injection, oocytes were scored for GVBD. Each group contained at least 30 oocytes. Shown is a representative of three independent experiments.

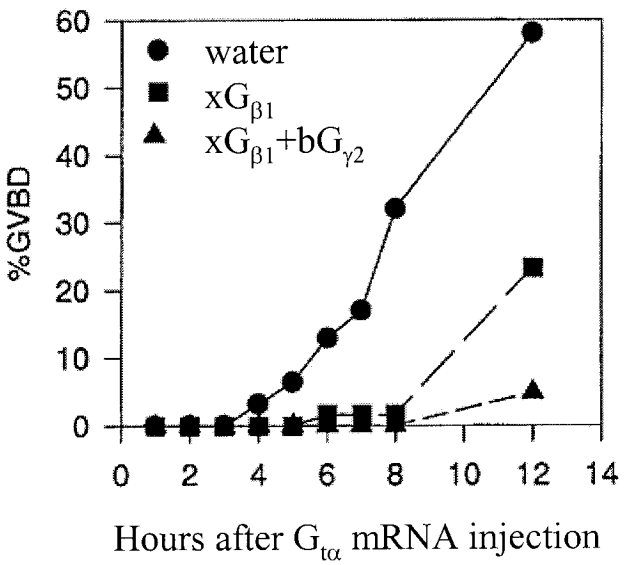

60
50
40
30
20
10
0
%GVBD
water
xG$_{\beta 1}$
xG$_{\beta 1}$+bG$_{\gamma 2}$
0
2
4
6
8
10
12
14
Hours after G$_{t\alpha}$ mRNA injection

***Figure 2.19* Overexpression of $G_{\beta\gamma}$ inhibits progesterone-induced GVBD**
Oocytes injected with water or the indicated mRNA ($xG_{\beta 1}$, $bG_{\gamma 2}$, or both) were incubated overnight in OR2 before the addition of progesterone (100 nM). At the indicated time following the addition of progesterone, oocytes were scored for GVBD. Shown is a representative of three independent experiments.

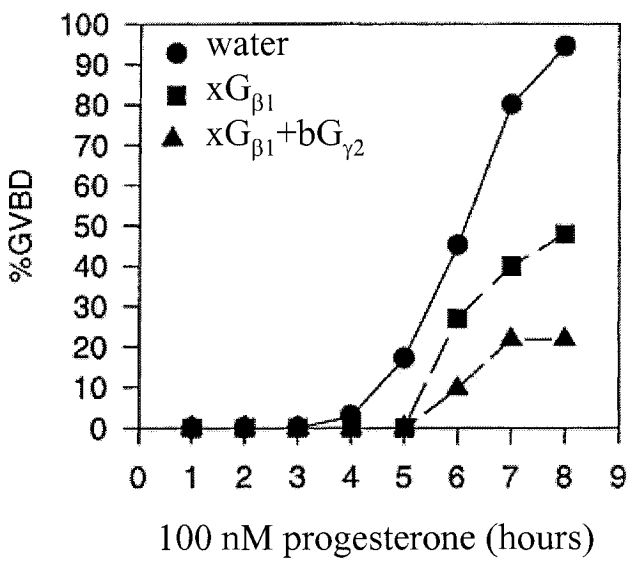
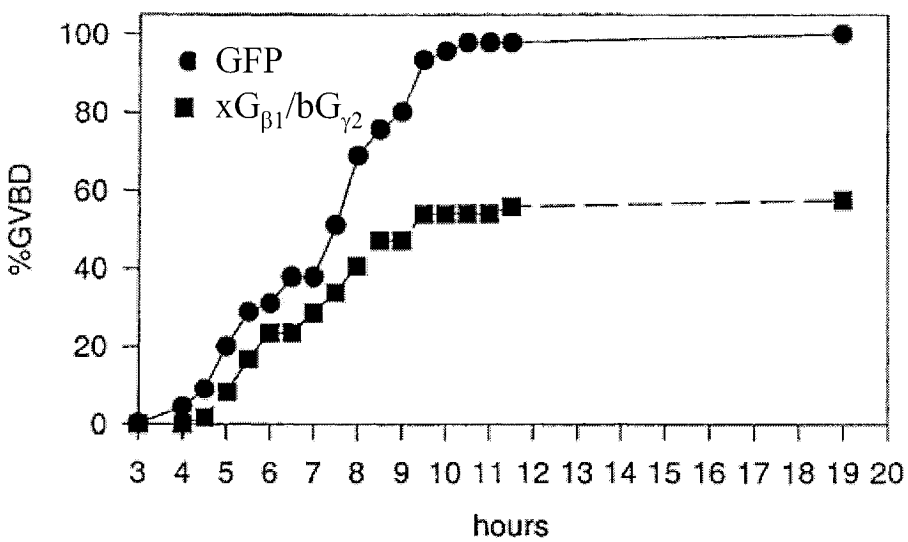

100
90
80
70
60
50
40
30
20
10
0
%GVBD
water
xG$_{\beta 1}$
xG$_{\beta 1}$+bG$_{\gamma 2}$
0 1 2 3 4 5 6 7 8 9
100 nM progesterone (hours)

Figure 2.20 **Control mRNA, GFP did not affect progesterone-induced GVBD**
Oocytes injected with a control mRNA (GFP, 10 ng per oocyte) or a mixture of $xG_{\beta 1}$ and $bG_{\gamma 2}$ (5 ng each per oocyte) were incubated overnight in OR2 before the addition of progesterone (1 μM). At the indicated time following the addition of progesterone, oocytes were scored for GVBD. Shown is a representative of two independent experiments.


100
80
60
40
20
0
%GVBD
GFP
xG$_{\beta 1}$/bG$_{\gamma 2}$
3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
hours

DISCUSSION

In this study, the role of G protein βγ subunits in regulating *Xenopus* oocyte maturation was examined. We demonstrated that two structurally distinct $G_{\beta\gamma}$ scavengers, $G_{t\alpha}$ and a membrane-targeting βARK-C (βARK-C_{CAAX}), were able to induce hormone-independent oocyte maturation. $G_{t\alpha}$ and βARK-C are thought to specifically inhibit G protein function that involves $G_{\beta\gamma}$ heterodimers as regulators of cellular effector systems. The specificity of $G_{\beta\gamma}$ scavengers in blocking $G_{\beta\gamma}$ function is supported by studies in which they do not interfere with the signaling of many classical G proteins (G_s (Federman *et al*, 1992), G_q, or G_o (Tsu and Wong, 1996; van Biesen *et al*, 1996)) that are dependent on GTP binding to the α subunits. The results of this study therefore implicate an endogenous $G_{\beta\gamma}$ heterodimer in maintaining meiosis arrest. In a recent study (Lutz *et al*, 2000), Lutz *et al.* demonstrated that the injection of mRNA encoding $G_{t\alpha}$ or the GRK minigene (identical to βARK-C) accelerates progesterone-induced GVBD but does not induce hormone-independent GVBD. Whereas the inability of the GRK minigene to induce GVBD (Lutz *et al*, 2000) can be explained by its lack of membrane association, compard to βARK-C_{CAAX} (Figure 2.8, Figure 2.9), the reasons for the discrepancy concerning $G_{t\alpha}$ are not known. It is possible that Lutz *et al.* did not obtain the relatively high concentrations of $G_{t\alpha}$ required to induce GVBD (see Figure 2.2). It is also possible that oocytes that were isolated via collagenase treatment, as was the case in the study by Lutz *et al.* (Lutz *et al*, 2000), were less responsive in GVBD induction than were manually isolated oocytes (in this study). Treating oocytes with commercial collagenase preparations is known to significantly reduce the oocytes' metabolic capacities (Smith, 1991).

We have further identified an endogenous xG_{β} that bound Myc-βARK-C_{CAAX} but not Myc-βARK-C. The lack of coimmunoprecipitation between the endogenous, membrane bound xG_{β} and the cytoplasmic Myc-βARK-C argues strongly that the coimmuno-precipitation of the xG_{β} and Myc-βARK-C_{CAAX} was the result of the two forming a complex at the membrane in intact oocytes. Although our data are consistent with assigning the endogenous xG_{β} as $xG_{\beta 1}$, we cannot rule out the possible involvement of other G_{β} isoforms since the anti-$G_{\beta 1}$ antibodies (both sc378 and sc261) are known to cross-react with other mammalian G_{β} proteins.

Consistent with the notion that endogenous free $G_{\beta\gamma}$ dimer functions to inhibit oocyte maturation, we have shown that the overexpression of xG_{β} alone or in combination with bovine $G_{\gamma 2}$ significantly reduced the ability of $G_{\beta\gamma}$ scavengers or progesterone to induce MAP kinase activation or GVBD. Similarly, Lutz *et al.* (Lutz *et al*, 2000) have demonstrated that the injection of mRNA encoding bovine $G_{\beta 1}$ and $G_{\gamma 2}$ partially inhibits progesterone-induced GVBD. Although $G_{\beta\gamma}$ dimers function as single entities in all systems that have been studied, others have shown that overexpression of G_{β} subunits alone have similar effects as overexpression of both G_{β} and G_{γ} subunits (Pellegrino *et al*, 1997; Hawes *et al*, 1995). The overexpressed $G_{\beta\gamma}$ subunits may mimic native $G_{\beta\gamma}$ dimers in interacting with cellular effectors (Buck *et al*, 1999), or they may increase the level of $G_{\beta\gamma}$ dimers by promoting dimer formation, increasing G_{γ} protein synthesis, and/or increasing G_{γ} protein stability (Wang *et al*, 1997).

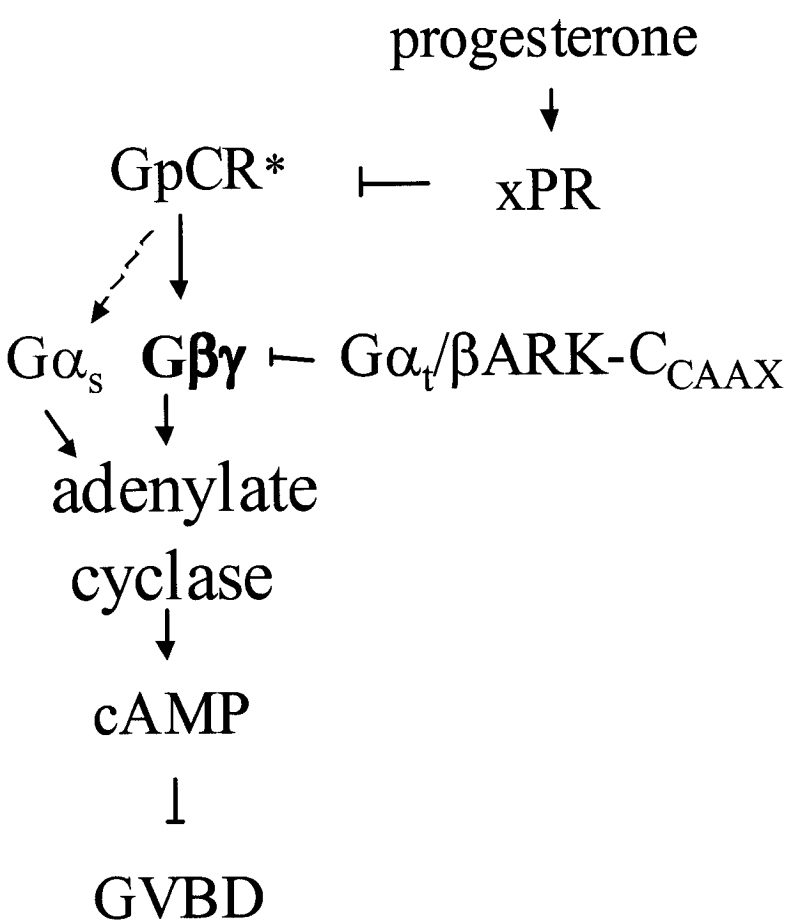
Our data implicate endogenous G protein βγ subunits as the natural inhibitors of oocyte meiosis in *Xenopus laevis* (Figure 2.21). Interestingly, earlier studies by others

(Jaffe *et al*, 1993; Chiba *et al*, 1993) have suggested that an endogenous G protein βγ dimer functions to promote oocyte maturation in starfish. Whereas progesterone is the natural inducer of *Xenopus* oocyte maturation, starfish oocyte maturation is induced by the ovarian hormone 1-methyladenine (Kanatani *et al*, 1969). Receptors for 1-methyladenine, although not yet identified, are thought to signal through a PTX-sensitive starfish G_i protein, in particular its $G_{\beta\gamma}$ (Jaffe *et al*, 1993; Chiba *et al*, 1993, Tadenuma *et al*, 1992). Furthermore, the main effector for the starfish G appears to be phosphatidylinositol 3'-kinase (Nakano *et al*, 1999; Sadler and Ruderman, 1998), which is not required for progesterone-induced GVBD (Liu *et al*, 1995; Deuter-Reinhard *et al*, 1997).

The synergistic effect of PTX in induction of oocyte maturation may be attributed to its ability to convert endogenous G_i or G_o into $G_{\beta\gamma}$ inhibitor since ADP ribosylation of the α subunit prevents its dissociation from $G_{\beta\gamma}$ (Simon *et al*, 1991). Alternatively, PTX may target the α subunit of the G protein depicted in Figure 2.22 and therefore inhibit the release of the inhibitory $G_{\beta\gamma}$.

As shown in Figure 2.21, we suggest that an endogenous G protein coupled receptor (GPCR) regulates the release of $G_{\beta\gamma}$. The released $G_{\beta\gamma}$ activates adenylyl cyclase, which in turn maintains elevated cAMP levels and G_2 arrest. In this scenario, the GPCR is constitutively active (GPCR*) in G_2 oocytes (e.g., by an autocrine loop in which its ligand is secreted by the oocytes or via a ligand-independent mechanism). Our lab recently cloned the first amphibian progesterone receptor (xPR). We speculate that xPR negatively regulates this oocyte GPCR signaling function and therefore results in adenylyl cyclase inhibition and the release of G_2 arrest or GVBD.

Figure 2.21. **A working model.**
An endogenous $G_{\beta\gamma}$ dimer is responsible for maintaining *Xenopus* oocyte G_2 arrest. The binding and sequestering of this $G_{\beta\gamma}$ dimer by $G_{t\alpha}$ or βARK-C_{CAAX} releases G_2 arrest and results in oocyte maturation. The nongenomic action of progesterone, mediated via the extranuclear xPR, is to interfere with the activation of the trimeric G protein and hence the release of $G_{\beta\gamma}$ dimer.


progesterone
GpCR*
xPR
Gαs
Gβγ
Gαt/βARK-CCAAX
adenylate
cyclase
cAMP
GVBD

Chapter Three

Co-operation of $G_{s\alpha}$ and $G_{\beta\gamma}$ in maintaining G_2 arrest in *Xenopus* oocytes

--A $G_{\beta\gamma}$-regulated adenylyl cyclase in frog oocytes

SUMMARY

Tang and Gilman (Tang and Gilman, 1991) first demonstrated that mammalian AC2 (and AC4) could be activated in vitro by purified $G_{\beta\gamma}$ complexes derived from several sources. However, the activation of AC2 and AC4 by $G_{\beta\gamma}$ is conditional on the presence of GTP-$G_{s\alpha}$. The physiological relevance of this regulatory mechanism was verified in intact cells in which AC2 (not sensitive to GTP-$G_{i\alpha}$) was activated by $G_{\beta\gamma}$ released from activation of a G_i-coupled receptor (Federman *et al.*, 1992). In *Xenopus* oocytes, that both $G_{s\alpha}$ (Gallo *et al.*, 1995) and $G_{\beta\gamma}$ (Lutz *et al.*, 2000; Sheng *et al.*, 2001) appear to play critical roles in maintaining G_2 arrest suggests a mechanism in which both $G_{s\alpha}$ and $G_{\beta\gamma}$ co-operate in activating the cAMP signaling pathway. In this chapter, I demonstrated that frog oocytes contained a $G_{s\alpha}$ and $G_{\beta\gamma}$ co-activated cAMP regulatory system that is responsible for the maintenance of G_2 arrest. I demonstrated that overexpression and activation of human β2 adrenergic receptor (β_2AR) enhanced cAMP levels in oocytes. Similarly, over-expression of *Xenopus* $G_{\beta 1}$ (x$G_{\beta 1}$), alone or together with bovine $G_{\gamma 2}$, elevates oocyte cAMP. Purified brain $G_{\beta\gamma}$ complexes significantly activated membrane-bound adenylyl cyclase activities. These results suggest the presence of a Gβγ-responsive adenylyl cyclase (AC) in frog oocytes. To identify this putative AC, I carried out PCR cloning of oocyte AC using degenerate primers corresponding to highly conserved catalytic amino acid sequences of all ACs. Among these a putative *Xenopus* adenylyl cyclase 7 (xAC7) that was 56% identical in amino acid sequence to human AC7, was identified. A dominant-negative mutant of xAC7 induced hormone-independent oocyte maturation and accelerated progesterone-induced oocyte maturation.

These findings suggest that xAC7 is a major component of the G_2 arrest mechanism in *Xenopus* oocytes.

I was responsible for all experimental work described in this chapter except for experiments depicted in Figures 3.10, 3.11 and 3.12., which were carried out by Ms. Veronique Montplaisir.

MATERIALS AND METHODS

Materials

Rabbit anti-$G_{\beta 1}$ (Sc378) antibodies were purchased from Santa Cruz Biotechnology and were used at 1 μg/mL for immunoblotting. Antibodies against *Xenopus* MAP kinase was a gift from Jonathan Cooper (Posada *et al.*, 1991). Antibodies against cyclin B2 was a gift from James Maller (Taieb *et al.*, 2001). Antibodies against phosphor-MAP kinase were purchased from Upstate. All radioactive compounds were from Amersham. Cholera toxin A subunit was purchased from Sigma, dissolved in water (1 mg/mL) and stored at -70^0C in single-use aliquots. An aliquot was thawed and diluted in water just before injection. Other chemicals were purchased from Sigma unless otherwise stated.

Animal and oocyte manipulation

Refer to: Chapter 2 Materials and Methods

cAMP assays

The following procedures of ^{125}I radioimmunoassay for cAMP were modified from Noh and Han (Noh and Han, 1998). 20 oocytes were lysed, by forcing them through pipette tips, in 500 μl of ice-cold 95% ethanol. Extracts were centrifuged at 15,000 g for 20 min. The ethanol extracts were transferred to new tubes and dried under vacuum. Samples were resuspended in 1 mL of 10 mM sodium acetate buffer, pH 4.75. The cAMP samples were mixed with 20 μl of triethylamine and 10 μl of acetic anhydride (in that order). Acetylation was carried out at room temperature for 2-3 hours. Fifty microliters of

the acetylated cAMP were mixed with 25 μl of ^{125}I-cAMP (approximately 12,000 cpm diluted in 50 mM sodium acetate buffer, pH 4.5 containing 1 mg/mL of human γ-globulin) and 25 μl of rabbit anti cAMP antibody (CALBIOCHEM, diluted 1:3,000 in the same γ-globulin-containing buffer). The mixture was incubated overnight at 4°C. Fifty microliters of goat anti-rabbit antibody with magnetic beads (Qiagen) was added and incubated with mixture for 1 h at 4°C. The beads were washed twice with 50 mM sodium acetate buffer, pH 6.2, 12% polyethylene glycol (Mr. 8000). Separation of bound from free was achieved by centrifugation, and bound radioactivity was counted by using BECKMAN Gamma 5500B Counting System and compared with a standard curve. The high levels of cAMP correspond with lower levels of ^{125}I cAMP bound to beads.

A standard curve was generated by plotting known concentrations of cAMP vs. B/B_o (B=binding at a given concentration of standard cAMP; B_o=maximum binding with no cold cAMP). Under these conditions, the amount of cAMP from each oocyte was determined to be 9.3 ± 2.3 fmol or 0.55 ± 0.14 μM (four determinations; given 1.7 μl as oocyte volume calculated based on 1.5 mm diameter sphere).

Plasmid construction and in vitro mRNA synthesis

Human $G_{\beta 5L}$ cDNA, cloned in the vector of pcDNAIII, was a gift from Dr. M. I. Simon (Watson *et al.*, 1996) and used directly for in vitro mRNA synthesis. All plasmid DNAs were linearized prior to serving as template for in vitro mRNA synthesis. mRNA synthesis was carried out using Ambion's mMESSAGE mMACHINE^{TM} kit and the mRNA was not capped. We usually dissolved mRNA in water to 1 mg/mL and injected 10 nL per oocyte.

$xG_{\beta 1}$ mutants were generated by the two-step PCR procedure (Vallette *et al.*, 1989) from pCS2+$xG_{\beta 1}$ (Sheng *et al.*, 2001). The mutagenesis primers for $xG_{\beta 1 W99A}$ were: forward, 5'CCT CTG CGT TCC TCT GCG GTG ATG ACC TGT GC3', and reverse, 5'GC ACA GGT CAT CAC CGC AGA GGA ACG CAG AGG3' (the underlined codon changed Trp-99 to Ala). For $xG_{\beta 1 D228G}$, the primers were: forward, 5'CT GGA CAT GAG TCG GGC ATA AAT GCC ATC TGT TT3', and reverse, 5'AA ACA GAT GGC ATT TAT GCC CGA CTC ATG TCC AG3' (the underlined codon changed Asp-228 to Gly).

C1b region (Yan *et al.*, 2001) of xAC7 (GenBank accession: AY283437) was PCR-amplified using the following two primers: C1b5': 5'TATGAATTCTCCAAGGGCAAAGAACCGTCGTG3' (underlined nucleotides indicate xAC7 coding sequences); and C1b3': 5'TATGAATTCGGATACAAGGTATTCTGAAGGTG3' (underlined nucleotides indicate xAC7 coding sequences). The amplified cDNA was digested with EcoRI and ligated into pCS2+HA (Booth *et al.*, 2002) previously digested with EcoRI.

Adenylyl cyclase assay

The procedure was modified from Kobilka et al (Kobilka *et al.*, 1987). Briefly, 100 oocytes were homogenized in 200µl of ice-cold assay buffer (75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA) containing 30% (w/v) sucrose. The homogenate was centrifuged at 3,000 *g* for 10 min to remove cell debris. The supernatant was centrifuged at 100,000 *g* for 60 minutes. The pellet (total membranes) was resuspended in the same assay buffer (100 µl). Protein concentrations were determined by Bradford procedures

(Bio-Rad) using bovine serum albumin as standards. For in vitro cyclase assays, 20 µl of oocyte membrane preparations (40 ~ 60 µg proteins) were mixed with 20 µl of assay mix (2.7 mM phosphoenol pyruvate, 53 µM GTP, 0.1 mM cAMP, 0.12 mM ATP, 0.2 IU pyruvate kinase and 1.0 IU myokinase, 1.5 µCi of [α-^{32}P] ATP). Incubations were for 40 min at 37 °C. Reaction was terminated by the addition of 1 ml stop solution (0.5 mM ATP and 0.25 mM cAMP). [^{32}P]- cAMP was purified by sequential chromatography using Dowex and alumina columns according to Johnson and Salomon (Johnson and Salomon, 1991) and counted following the addition of scintilation fluids.

PCR amplification of Xenopus adenylyl cyclases

Adenylyl cyclase fragments were amplified from an oocyte cDNA library (Rebagliati *et al.*, 1985) by using the polymerase chain reaction (PCR). These initial PCR experiments employed degenerate primers encoding two highly conserved regions of amino acid sequences within the catalytic C1 domain: GDCYYC and WQ (Y/F)DVW (Premont, 1994). Sense primer: 5`-atcaagctcgagggIga(c/t)tg(c/t)ta(c/t)ta(c/t)tg, encoding GDCYYC, antisense primer: 5`-cacgtcctcgagccaIac(a/g)tc(a/g)(c/g)a(c/t)tgcca, encoding WQ(Y/F)DVW. Briefly, 200 ng of cDNA was subjected to amplification in 100-µl reaction containing 1 x polymerase buffer, 1.5 mM $MgCl_2$, 200 µM each dNTP, 500nM each oligonucleotide primer, and 2.5 units of *Taq* DNA polymerase (Invitrogen). Reaction with all were heated 10 min at 94 °C. Reactions were cycled 35 times for 30 second at 94 °C, 1 min at 50 °C, and 1 min at 73 °C followed by a final 1 min at 70 °C. Products were separated on 1 % or 2% agarose gels, and appropriate size bands were excised and directly TA-subcloned into PCR2.1 vector (Invitrogen). Further PCR experiments were carried out to clone the remaining coding sequences of xAC7. xAC7-

specific primers were used in conjunction with "anchor" primers derived from the vector sequences (Rebagliati *et al.*, 1985).

The GenBank accession numbers for the three partial *Xenopus* AC described in the paper are AY283436 (xAC8), AY283437 (xAC7), and AY283438 (xAC6).

RESULTS

Regulation of oocyte cAMP by $G_{t\alpha}$ and $G_{\beta\gamma}$

Despite the common belief that progesterone inhibits adenylyl cyclase and reduces oocyte cAMP levels (Maller, 1998; Ferrell, 1999), the biochemical demonstration of a cAMP reduction by progesterone stimulation has been difficult and controversial (Smith, 1989). One exception is the report by Schorderet-Slatkine and Baulieu (Schorderet-Slatkine and Baulieu, 1982) who demonstrate that when oocytes are first treated with forskolin, which significantly raises cAMP levels, progesterone reproducibly blunts the forskolin-dependent cAMP elevations. Taking a similar approach, we used overexpression and activation of human β_2AR (Simonds, 1999) to elevate oocyte cAMP (Figure 3.1, lane 2). Under these conditions, $G_{t\alpha}$ (lane 4) or progesterone (lane 3) clearly reduced oocyte cAMP levels. In contrast, the injection of $xG_{\beta 1}/bG_{\gamma 2}$ significantly increased oocyte cAMP levels in the presence of cAMP phosphodiesterase inhibitor 3-isobutyp-1-methylxanthine (IBMX) (Figure 3.2). IBMX alone did not change oocyte cAMP levels (data not shown). These results suggest that *Xenopus* oocytes contain a $G_{\beta\gamma}$-responsive adenylyl cyclase.

Co-operation of GTP-$G_{s\alpha}$ and $G_{\beta\gamma}$ in elevating oocyte cAMP

Activation of cAMP by injection of $G_{\beta 1}G_{\gamma 2}$ mRNA alone suggests that, if activation of endogenous adenylyl cyclase requires co-activation of $G_{s\alpha}$, G_2-arrested oocytes must already contain activated GTP-bound $G_{s\alpha}$. To demonstrate co-operation of GTP-$G_{s\alpha}$ and $G_{\beta\gamma}$ in elevating oocyte cAMP levels, we injected oocytes with cholera toxin A subunit, which ADP-ribosylates and activates $G_{s\alpha}$ (Bokoch *et al.*, 1983). Maller *et al.* (Maller *et*

al., 1979) have previously reported that cholera toxin A subunit potently inhibits progesterone-induced oocyte maturation with EC_{50} of 10^{-7} M (internal concentrations when injected). We found that cholera toxin injection significantly inhibited progesterone-induced GVBD at as little as 10 pg per oocytes or approximately 3 X 10^{-10} M internal concentration (Figure 3.3, lane4). This difference could simply be attributed to the potencies of the toxins used in the two labs (performed decades apart!). In addition, it completely blocked phosphorylation of MAP kinase and of cyclin B2 (Figure 3.4). As previously reported (Maller *et al.*, 1979) cholera toxin significantly elevated oocyte cAMP (Figure 3.5, comparing lane 2 to lane 1). Co-injection of $G_{\beta 1/\gamma 2}$ with cholera toxin further elevated oocyte cAMP (Figure 3.5, lane 3).

$xG_{\beta 1(D228G)}$ failed to increase cAMP levels and failed to inhibit GVBD

To further investigate the correlation between $G_{\beta\gamma}$ stimulation of oocyte cAMP pathway and the inhibition of progesterone-induced oocyte maturation, we explored site-directed mutagenesis of $xG_{\beta 1}$. We constructed two $xG_{\beta 1}$ mutants ($xG_{\beta 1D228G}$ and $xG_{\beta 1W99A}$) in the "switch interface" (referring to contact with region of $G_{t\alpha}$ that undergoes nucleotide-dependent conformational changes) (Lambright *et al.*, 1996). Equivalent mutations in mammalian $G_{\beta 1}$ have been shown to affect interaction of $G_{\beta\gamma}$ with its effectors, including AC2 (Ford *et al.*, 1998). Indeed, we found that, the complex of $xG_{\beta 1D228G}/G_{\gamma 2}$ had no effect on oocyte cAMP levels beyond those in cholera toxin-treated oocytes (Figure 3.5, lane 4). The other mutant, $xG_{\beta 1W99A}$, elevated cAMP as effectively as wild-type $xG_{\beta 1}$ when co-injected with $G_{\gamma 2}$ (Figure 3.5, lane 5).

Figure 3.1. **Regulation of oocyte cAMP by $G_{t\alpha}$.**
Oocytes were injected with water (lane 1) or β_2AR mRNA (lanes 2–4). Six hours later, one group (lane 4) was further injected with $G_{t\alpha}$ mRNA. Two hours following the $G_{t\alpha}$ injection, all groups were treated with ISO (1 μM) except for group 3, which was treated with a combination of ISO and 1 μM progesterone. One hour later, oocytes were lysed and subjected to cAMP assays. Shown are means of two independent experiments done in duplicate determinations.

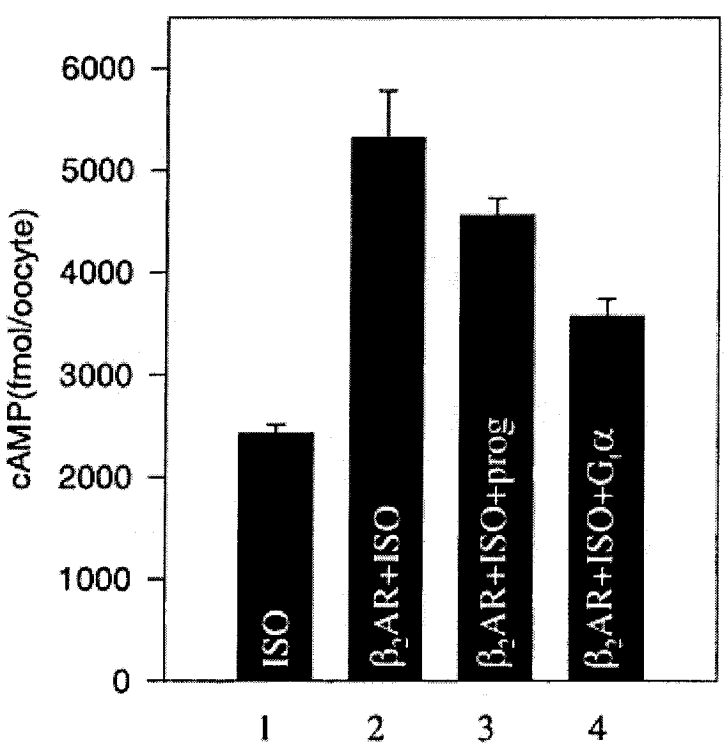

6000
5000
4000
3000
2000
1000
0
cAMP(fmol/oocyte)
ISO
β2AR+ISO
β2AR+ISO+prog
β2AR+ISO+Giα
1
2
3
4

Figure 3.2 **Regulation of oocyte cAMP by $G_{\beta\gamma}$.**
Oocytes were injected with water or a mixture of $xG_{\beta 1}$ and $bG_{\gamma 2}$ mRNA (5 ng each mRNA per oocyte). Following incubation in OR2 for the indicated period of time, IBMX (0.5 mM) was added, and the incubation continued for the indicated length of time. Oocytes were than lysed and subjected to cAMP assays. The levels of cAMP are expressed as a percentage of levels in water-injected oocytes. Shown are means (with standard errors) of three to four independent experiments of duplicate determinations. The sign * denotes $p < 0.05$ in pair-wise Student's test.

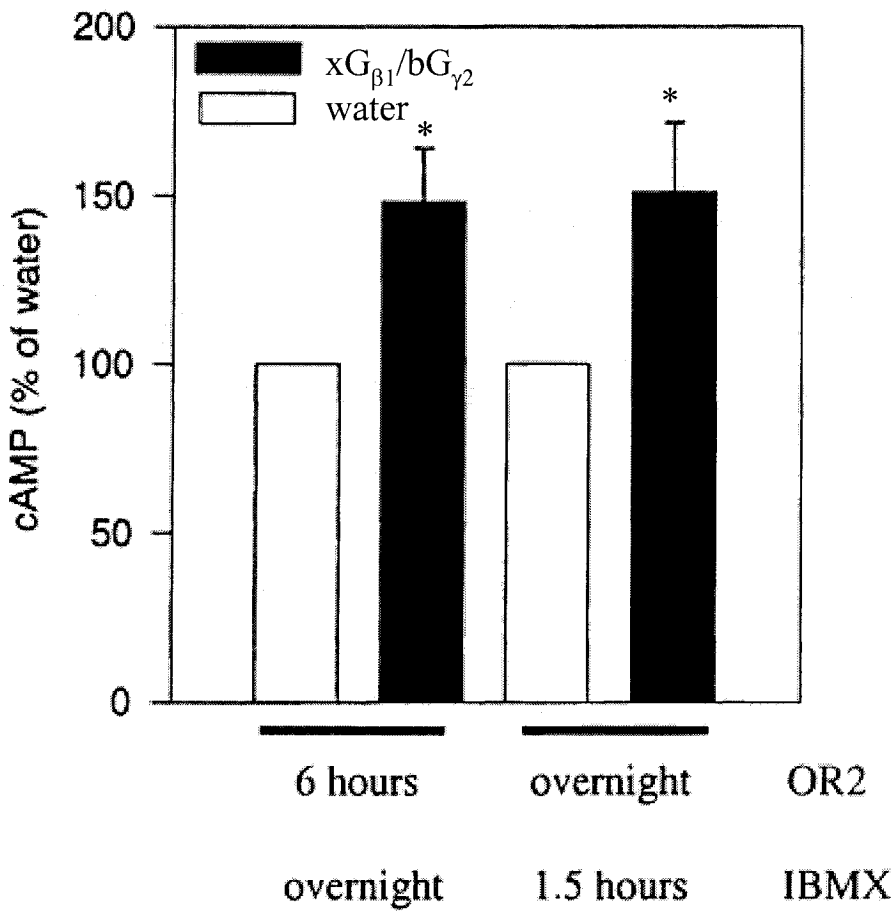

200
150
100
50
0
cAMP (% of water)
xG$_{\beta 1}$/bG$_{\gamma 2}$
water
*
*
6 hours
overnight
OR2
overnight
1.5 hours
IBMX

***Figure3.3* Cholera toxin A inhibited progesterone-induced GVBD**
Oocytes (20 or more each group) were injected with water (10 nL per oocyte, lane 1 and 2) or 10 nL of cholera toxin A (lanes 3-6) containing the indicated amount of protein per oocyte. Following overnight incubation, oocytes were scored for GVBD (upper panel). Extracts were prepared from the same ocytes for immunoblotting using anti-cyclin B2 antibodies (lower panel). Shown is a representative of three independent experiments.

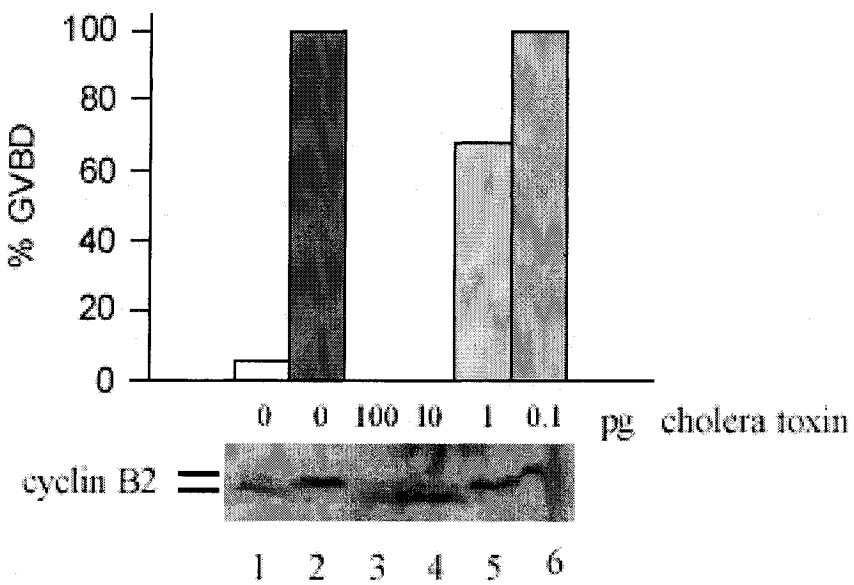

100
80
60
40
20
0
% GVBD
0 0 100 10 1 0.1 pg cholera toxin
cyclin B2
1 2 3 4 5 6

Figure 3.4 **Cholera toxin A inhibited MAP kinase phosphorylation**
Oocytes (20 or more each group) were injected with water (lanes 1 and 2) or the indicated amounts of cholera toxin A subunit. Following overnight incubation, oocytes were lysed and the resultant extracts were subjected to immunoblotting using anti-xMAP kinase antibodies. Shown is a representative of three independent experiments.

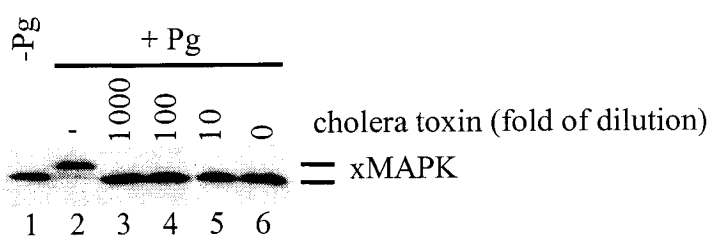
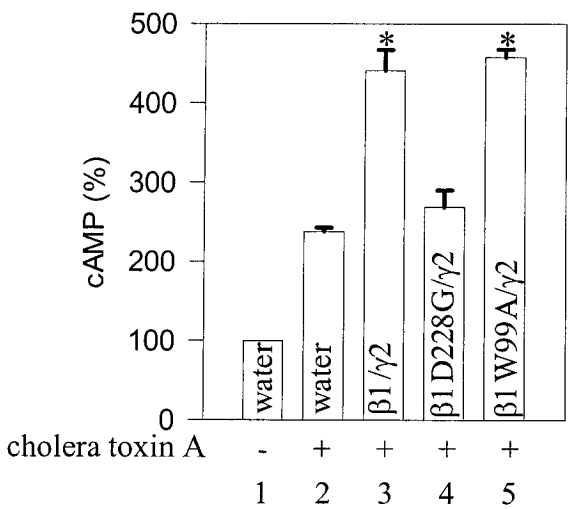

-Pg
+ Pg
-
1000
100
10
0
cholera toxin (fold of dilution)
xMAPK
1 2 3 4 5 6

Figure 3.5. **$xG_{\beta 1(D228G)}$ failed to increase cAMP levels**
Oocytes were injected with water or the indicated mRNA. Following overnight incubation, each group received a second injection of either water (-) or cholera toxin A (100 pg per oocyte). Oocytes were analyzed for cAMP. The levels of cAMP are expressed as percentages of levels in water-injected oocytes. Shown are means (with standard errors) of three independent experiments. The sign * denotes $p < 0.05$ in pair-wise Student's test.


500
400
300
200
100
0
cAMP (%)
water
water
β1/γ2
β1D228G/γ2
β1W99A/γ2
*
*
cholera toxin A
-
+
+
+
+
1
2
3
4
5

To determine the effects of the mutant $xG_{\beta 1}$ on progesterone-induced oocyte maturation, we injected each mRNA together with mRNA encoding $G_{\gamma 2}$ followed by progesterone treatment. As shown in Figure 3.6A, wild-type $xG_{\beta 1}$ significantly inhibited progesterone-induced GVBD, as the data demonstrated previously (Sheng *et al.*, 2001). $xG_{\beta 1W99A}$ was similarly effective in inhibiting progesterone-induced GVBD. In contrast, $xG_{\beta 1D228G}$-injected oocytes behaved much more similarly to control oocytes. Immunoblotting of extracts derived from the variously injected oocytes indicated that the wild type and mutant forms of $xG_{\beta 1}$ were expressed at very similar levels (Figure 3.6B). The differential abilities of the two point mutants in elevating oocyte cAMP (Figure 3.5) correlated with their abilities to inhibit progesterone-induced GVBD (Figure 3.6A).

Activation of membrane-bound adenylyl cyclase by $G_{\beta\gamma}$ in vitro

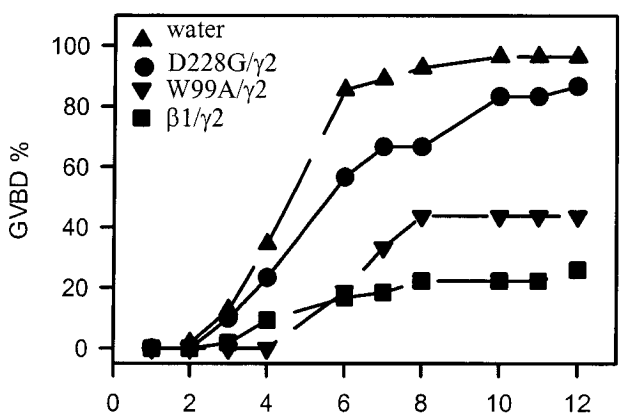
To directly demonstrate that oocytes contained a $G_{\beta\gamma}$-responsive adenylyl cyclase, we measured the ability of oocyte membranes to convert [^{32}P] α-ATP into cAMP (adenylyl cyclase assays) in the presence or absence of purified $G_{\beta\gamma}$ (Casey *et al.*, 1989) (a generous gift from R. J. Lefkowitz). To achieve synchronized activation of endogenous $G_{s\alpha}$, we first injected oocytes with β_2 adrenergic receptor (β_2AR) mRNA and incubated overnight to allow receptor protein expression before they are treated with β_2AR agonist isoproterenol. Then, membranes were isolated and assayed for adenylyl cyclase activities in the presence or absence of brain-derived $G_{\beta\gamma}$ (Figure 3.7). Whereas injection of β_2AR mRNA alone did not alter the membrane adenylyl cyclase activities, activation of β_2AR with isoproterenol resulted in a significant increase of membrane adenylyl cyclase

Figure 3.6. **$xG_{\beta 1(D228G)}$ failed to inhibit GVBD**

A. Oocytes (50 or more each group) were injected with water or the indicated mRNA. Following overnight incubation, progesterone (100 nM) was added and oocytes were observed for GVBD at the indicated time after the addition of progesterone. Shown is a representative of three independent experiments (each with a different donor frog).

B. A typical anti-$G_{\beta 1}$ immunoblot of extracts derived from the variously injected oocytes.

A


water
D228G/γ2
W99A/γ2
β1/γ2
GVBD %
100
80
60
40
20
0
0
2
4
6
8
10
12

B

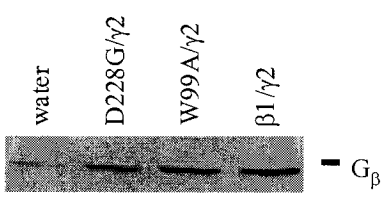
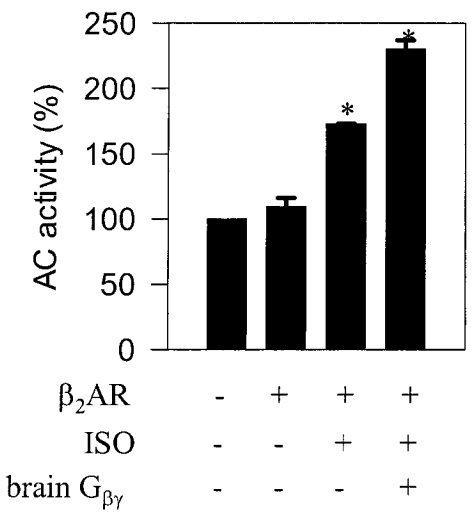

water
D228G/γ2
W99A/γ2
β1/γ2
Gβ

Figure 3.7. **Activation of membrane-bound adenylyl cyclase by $G_{\beta\gamma}$ *in vitro***
Oocytes were injected with water or with mRNA encoding β_2AR. Following overnight incubation, oocytes were treated with isoproterenol (ISO, 100 μM) or not before membrane isolation. Adenylyl cyclase assays were carried out in the presence or absence of purified $G_{\beta\gamma}$ (70 nM). The amounts of [^{32}P] cAMP formed with control oocyte membranes were habitually set at 100% in each experiment. Shown are means of three independent experiments with standard errors. The sign * denotes $p < 0.05$ in pair-wise Student's test.


250
200
150
100
50
0
AC activity (%)
*
*
β_2AR - + + +
ISO - - + +
brain $G_{\beta\gamma}$ - - - +

activities. The addition of purified $G_{\beta\gamma}$ further increased membrane adenylyl cyclase activities. These results (Figure 3.7) clearly indicate that frog oocytes contain a membrane-bound, $G_{\beta\gamma}$-activated adenylyl cyclase. These studies suggest that oocytes contain a $G_{\beta\gamma}$-simulated adenylyl cyclase and that this adenylyl cyclase plays a critical role in maintaining meiosis arrest.

Cloning of Xenopus adenylyl cylases

Among the 9 known known mammalian adenylyl cyclases (AC), AC2, AC4 and AC7 are activated by $G_{\beta\gamma}$ (Yoshimura *et al.*, 1996; Tang and Gilman, 1991; Gao and Gilman, 1991). Currently only one adenylyl cyclase, x1AC (Torrejon *et al.*, 1997), has been identified and cloned in *Xenopus laevis*. x1AC is most similar to mammalian AC9, which is $G_{\beta\gamma}$-insensitive (Simonds, 1999). To identify the putative $G_{\beta\gamma}$-responsive oocyte adenylyl cyclase, we carried out PCR-amplification of *Xenopus* AC by using degenerate primers derived from conserved sequences within the catalytic regions of all mammalian AC (Premont *et al.*, 1994) and x1AC (Torrejon *et al.*, 1997). So far, three distinct partial cDNA encoding 69-70 amino acids corresponding to the highly conserved region of the C1 domain (cytoplasmic domain 1) of all adenylyl cyclases have been identified (Genbank accessions: AY283436, AY283437 and AY283438). Among the three partial cDNAs, one (represented by two PCR clones) was highly similar to human AC7 (Nomura *et al.*, 1994; Hellevuo *et al.*, 1995) and therefore represents a likely candidate for being the endogenous $G_{\beta\gamma}$-activated adenylyl cyclase. The other two (one PCR clone each) were most similar to mammalian AC6 and AC8, respectively, which are $G_{\beta\gamma}$-insensitive (Simonds, 1999). Further PCR amplification was performed in an attempt to isolate the entire coding sequence of the putative *Xenopus* AC7 (xAC7). These

experiments have resulted in the isolation of a partial cDNA encoding an open reading frame of 667 amino acids (GenBank accession: AY283437). Following the submission of the original manuscript, another partial cDNA for the same gene (*xac7*) appeared in GenBank (accession: AY462195). The two partial xAC7 cDNA overlaps by 406 conceptual amino acids (405 of which are identical). The combined partial xAC7 cDNA therefore encodes 956 amino acids that are 65% identical to the corresponding region of hAC7 (accession: D25538). The two cytoplasmic domains (C1 and C2) (Watson *et al.*, 1994; Simonds, 1999) are particularly similar between hAC7 and xAC7 (Figure 3.8 and Figure 3.9).

xAC7 C1b domain induced GVBD

To determine the possible function of the endogenous xAC7, we explored the potential dominant-negative effect of C1b domain (Yan *et al.*, 2001). C1b is defined as the carboxy-terminal region of C1 that is not essential for catalytic function of C1 domain. In vitro studies have demonstrated that recombinant AC7 C1b inhibits both membrane-bound AC7 as well as soluble AC7 C1/C2 domains, suggesting that AC7 C1b domain functions as a dominant-negative mutant (Yan *et al.*, 2001). We subcloned the equivalent region of xAC7 (Materials and Methods) for mRNA synthesis. Injection of HA-tagged xAC7 C1b into oocytes caused low, but significant, percentages of oocytes to undergo GVBD (Figure 3.10). To determine whether C1b also synergizes with progesterone-induced GVBD, we injected a reduced amount (5 ng per oocyte) of C1b mRNA such that the mRNA alone did not cause significant GVBD. Oocytes injected with C1b mRNA exhibited significantly accelerated GVBD response when treated with

Figure 3.8. **Schematic representation of partial cDNA sequence of xAC7 and its comparison with hAC7.**
Numbers indicate positions of conceptual amino acid within each cDNA. Homologies between the indicated regions are shown as amino acid sequence identities. Also indicated is the approximate position of C1b. TM1-6 and TM7-12 are the regions encompassing the N-terminal and C-terminal, respectively, six transmembrane domains.

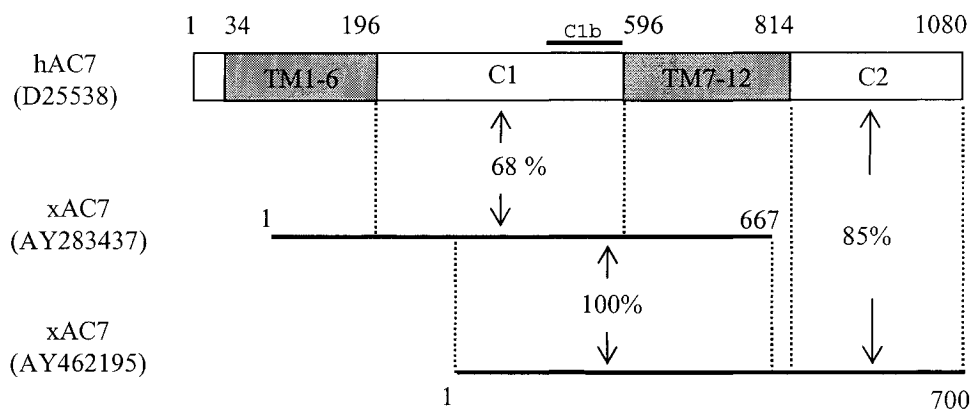

1
34
196
C1b
596
814
1080
hAC7
(D25538)
TM1-6
C1
TM7-12
C2
68 %
xAC7
(AY283437)
1
667
85%
100%
xAC7
(AY462195)
1
700

Figure 3.9 **Amino acid comparison of xAC7 and hAC7**
A. Sequence comparison between the C1 region of hAC7 and its counterpart in xAC7, generated by PAIRWISE BLAST program. Sequence identities are highlighted. xAC7 C1b sequence is underlined.

B. Sequence comparison between the C2 region of hAC7 and its counterpart in xAC7, generated by PAIRWISE BLAST program. Sequence identities are highlighted.

A

```
hAC7 197 HKHQMQDASRDLFTYTVKCIQIRRKLRIEKRQQENLLLSVLPAHISMGMKLAIIERLKEH 256
xAC7 89  HKYHMQDASRDLYSYTVKCIRIKMKLELKKRQQESLLLSVLPVYISMGDELAIQDRLTET 148

hAC7 257 GDRRCMPDNNFHSLYVKRHQNVSILYADIVGFTQLASDCSPKELVVVLNELFGKFDQIAK 316
xAC7 149 NDNR-QPDNNFTALYVKRHQNVSILYADIVGFTRLASDCSPKELVVMLNELFGKFDQIAK 207

hAC7 317 ANECMRIKILGDCYYCVSGLPVSLPTHARNCVKMGLDMCQAIKQVREATGVDINMRVGIH 376
xAC7 208 ENECMRIKILGDCYYCVSGLPVSLPNNAKNCVKMGLDICESIKQVREATGADINMRVGVH 267

hAC7 377 SGNVLCGVIGLRKWQYDVWSHDVSLANRMEAAGVPGRVHITEATLKHLDKAYEVEDGHGQ 436
xAC7 268 SGNVLCGVIGLRKWQFDVWSHDVSLANRMESAGLPGRVHITEATLKHMNGAYDVEEGHGE 327

hAC7 437 QRDPYLKEMNIRTVLVIDPRSQQPPPPSQHLPRPKGDAALKMRASVRMTRYLESWGAARP 496
xAC7 328 LRDPYLKEMNIKTYLVIDPRAKNRRVQNIHLPKHRVNDGLKVRPSVRMTRYLESWGAARP 387

hAC7 497 FAHLNHRESVSSGETHVPNGRRPKSVPQRHR-RT--PDRSMSPKGRSEDDSYDDEMLSAI 553
xAC7 388 FSHFDQSDA-SSAEVPVVNGKPKLDIPLKGMGRTVKTERNVSQK-RNQEEELHDRMMNII 445

hAC7 554 EGLSSTRPCCSKSDDFYTFGSIFLEKGFEREYRLAPIPRAR 594
xAC7 446 DGLSSAKPWFGKTDDFVGFLLFFTADGLEKEYRTFRIPCIR 486
```

B

```
hAC7  815 RQIDYYCRLDCLWKKKFKKEHEEFETMENVNRLLLENVLPAHVAAHFIGD-KLNEDWYHQ 863
xAC7  434 RQVEYYSRLDCLWKRKFRKEDEEIETMENLNQLLLENVLPAHVAAYFIGDNKSNEDLYHQ 493

hAC7  874 SYDCVCVMFASVPDFKVFYTECDVNKEGLECLRLLNEIIADFDELLLKPKFSGVEKIKTI 933
xAC7  494 SYDCVCVMFASVPEFKVFYTECDVNKEGLECLRLLNEIIADFDELLMKPKFSGVEKIKTI 553

hAC7  934 GSTYMAAAGLSVASGHENQELERQHAHIGVMVEFSIALMSKLDGINRHSFNSFRLRVGIN 993
xAC7  554 GSTYMAATGLTATPGQENQDQEKQHALIGITVEYAMALMSKLDGINRHSFNNFSLRVGIN 613

hAC7  994 HGPVIAGVIGARKPQYDIWGNTVNVASRMESTGELGKIQVTEETCTILQGLGYSCECRGL 1053
xAC7  614 HGPVIAGVIGAKKPQYDIWGNTVNVASRMESTGELGKIQVTEETCQILEGLGYSCECRGF 673

hAC7 1054 INVKGKGELRTYFVCTDTAKFQGLGLN 1080
xAC7  674 INVKGKGELRTYFVCTDMAKSQSIGMN 700
```

Figure 3.10 **xAC7 C1b domain induced GVBD**
Oocytes (at least 50 per group) were injected with water or mRNA for HA-xAC7 C1b. Injected oocytes were incubated overnight in OR2 before being scored for GVBD. Shown are means of percentages of GVBD (with standard errors) of three independent experiments.

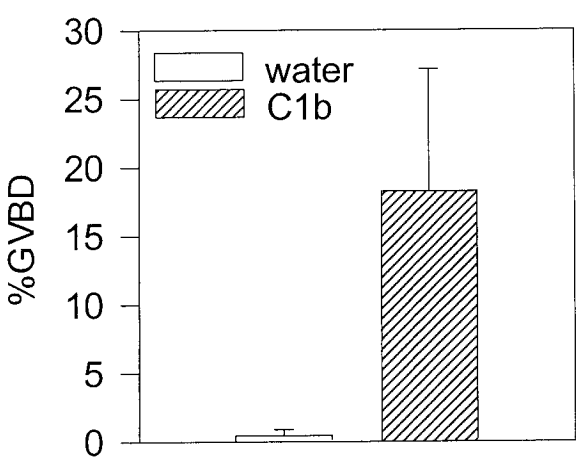
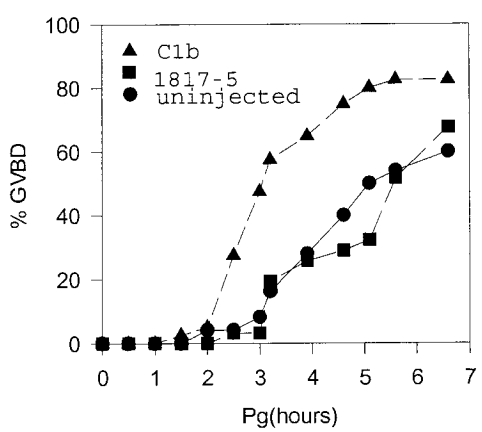

30
25
20
15
10
5
0
%GVBD
water
C1b

Figure 3.11 **xAC7 C1b domain accelerated progesterone-induced GVBD**
Oocytes (at least 50 per group) were injected with water; control mRNA (1817-5, 5 ng per oocyte) or mRNA for HA-xAC7 C1b (5 ng per oocyte). Following overnight incubation, progesterone was added and the incubation continued. Oocytes were scored for GVBD at the indicated time following the addition of progesterone. Shown is a representative of two independent experiments.

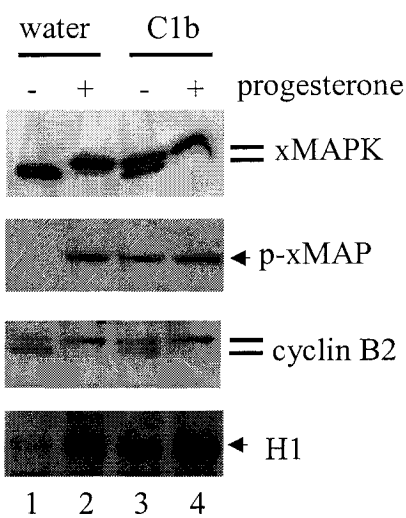

100
80
60
40
20
0
% GVBD
C1b
1817-5
uninjected
0
1
2
3
4
5
6
7
Pg(hours)

progesterone, compared with uninjected oocytes or oocytes injected with a control mRNA (Figure 3.11, 1817-5, 5 ng per oocyte). We used this control mRNA for the reason that it encodes an HA-tagged protein of similar size (15 kDa) to HA-C1b (not shown). Furthermore, 1817-5 mRNA, which has no apparent sequence homology with any proteins in the databases, is one of many *Xenopus* oocyte cDNAs isolated in an unrelated project that do not affect oocyte maturation (C. Ma and X. J. Liu, unpublished). To confirm that the C1b construct was able to activate maturation-specific protein kinases, we analyzed cell extracts for activation of *Xenopus* MAP kinase (Posada and Cooper, 1992) and maturation promoting factor or MPF (Masui and Markert, 1971; Gautier *et al.*, 1988; Gautier *et al.*, 1990). As shown in the top two panels of figure 3.12, whereas water-injected oocytes contained only unphosphorylated (inactive) xMAP kinase (lane 1), a significant percentage of C1b-injected oocytes contained phosphorylated (activated) xMAP kinase (lane 3). Similarly, C1b injection also activated MPF, as indicated by both cyclin B2 phosphorylation and by in vitro MPF assays using histone H1 as substrate (Figure 3.12, the bottom two panels, comparing lane 3 vs. lane 1). In the presence of progesterone, water-injected oocytes (lane 2) and C1b-injected oocytes (lane 4) all contained activated MAP kinase and MPF, consistent with the GVBD data.

$G_{\beta 5L}$ enhanced progesterone-induced GVBD

Among the 9 known mammalian ACs, AC2, AC4 and AC7 are conditionally activated by $G_{\beta\gamma}$ (Yoshimura et al., 1996; Tang and Gilman, 1991; Gao and Gilman, 1991). Although a variety of G_{β} subunits can replace $G_{\beta 1}$ to form $G_{\beta\gamma}$ complexes capable of activating adenylyl cyclases in a fashion that is dependent on the presence of activated

Figure 3.12 **xAC7 C1b domain induced activation of MAPK, cyclin B2 and MPF**
Oocytes injected with water or xAC7 C1b were incubated overnight in the presence or absence of progesterone. Extracts were made and were subjected to immunoblotting with antibodies against, from the top panel, xMAP kinase, phosphor-MAP kinase or cyclin B2, or subjected to MPF kinase assays using histone H1 as substrate (bottom panel). Shown is a representative of three independent experiments.

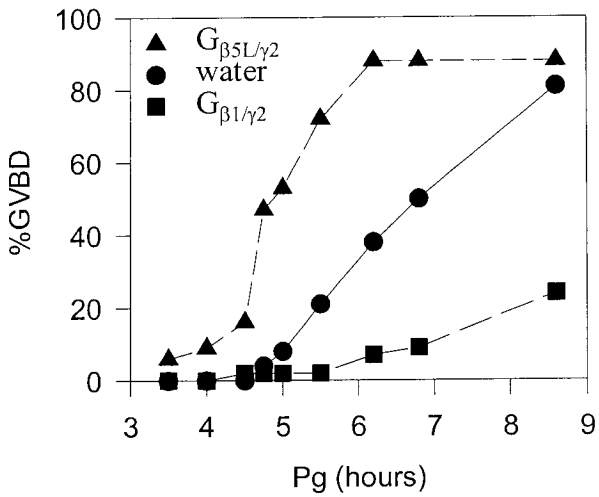

water
C1b
- + - +
progesterone
xMAPK
p-xMAP
cyclin B2
H1
1 2 3 4

$G_{s\alpha}$, $G_{\beta 5L}$, a retina-specific isoform of $G_{\beta 5}$ with an N-terminal extension of 43 amino acids (Watson *et al.*, 1996), inhibits this group of adenylyl cyclases when co-expressed with $G_{\gamma 2}$ (Bayewitch *et al.*, 1998). We reasoned that if the endogenous AC7 is functionally important in the maintenance of G_2 arrest, over-expression of $G_{\beta 5L}$ and $G_{\gamma 2}$ may decrease cAMP and enhance oocyte maturation. Co-injection of $G_{\beta 5L}$ and $G_{\gamma 2}$ mRNAs did not cause hormone-independent oocyte maturation, as determined by germinal vesicle breakdown (GVBD) (not shown). However, oocytes injected with $G_{\beta 5L/\gamma 2}$ exhibited accelerated GVBD responses (compared to water-injected oocytes) when exposed to progesterone (Figure 3.13A). In contrast, oocytes injected with $G_{\beta 1/\gamma 2}$ mRNAs showed little GVBD responses under the same conditions (Figure 3.13A), as reported previously (Sheng *et al.*, 2001). To compare the relative levels of $G_{\beta 1}$ and $G_{\beta 5L}$ proteins in mRNA-injected oocytes, we carried out metabolic labeling of oocyte proteins with [^{35}S]-methionine. In our experience, proteins derived from exogenous mRNAs can be readily identified because they are often the most prominently labeled proteins in total oocyte extracts. As shown in figure 3.13B, the 37 kDa $xG_{\beta 1}$ (Devic *et al.*, 1996), as previously identified by anti-$G_{\beta 1}$ immunoblotting (Sheng *et al.*, 2001), and a slightly larger protein, consistent with being the 43 kDa $G_{\beta 5L}$ (Bayewitch *et al.*, 1998; Watson *et al.*, 1996), were both prominently expressed in mRNA-injected oocytes. The 71 amino acid $G_{\gamma 2}$ protein (Gautam *et al.*, 1989) was not consistently detected, presumably due to the inability of our gel system to resolve it from the buffer front. These results clearly demonstrated that $G_{\beta 1}$ and $G_{\beta 5L}$ had opposite effects on progesterone-induced GVBD. Furthermore, injection of $G_{\beta 5L/\gamma 2}$ significantly reduced oocyte cAMP (Figure 3.14), in contrast to $xG_{\beta 1/\gamma 2}$ injection which elevated oocyte cAMP (Figure 3.2)

Figure 3.13 **$G_{\beta 1}$ inhibited but $G_{\beta 5L}$ enhanced progesterone-induced GVBD**
A. Oocytes (50 or more in each group) were injected with water or $xG_{\beta 1}/G_{\gamma 2}$ or $G_{\beta 5L}/G_{\gamma 2}$ mRNAs. Following an overnight incubation in OR2, progesterone (100 nM) was added. At the indicated time following the addition of progesterone, oocytes were scored for GVBD. Shown is a representative of three independent experiments.

B. Oocytes injected with wateror the indicated mRNA were incubated overnight in OR2 containing [^{35}S]-methionine (50 μCi/ml). Metabolically labeled oocytes were lysed and extracts were analyzed by SDS-PAGE followed by autoradiography.

A


100
80
60
40
20
0
%GVBD
3
4
5
6
7
8
9
Pg (hours)
$G_{\beta 5L/\gamma 2}$
water
$G_{\beta 1/\gamma 2}$

B

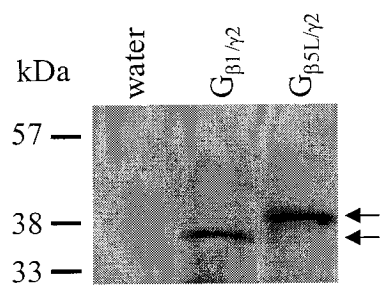
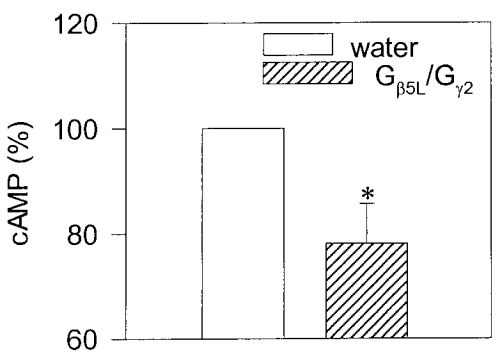

kDa
water
$G_{\beta 1/\gamma 2}$
$G_{\beta 5L/\gamma 2}$
57
38
33

Figure 3.14 **Injection of $G_{\beta 5L/\gamma 2}$ reduced oocyte cAMP**
Oocytes were injected with water or a mixture of mRNA for $G_{\beta 5L}$ and $G_{\gamma 2}$. Following overnight incubation, cAMP levels were measured. cAMP levels in water-injected oocytes were habitually set as 100%. Shown are means (with standard errors) of six independent experiments each in triplicate determinations. The sign * denotes $p < 0.05$ in unpair-wise Student's test.


120
100
80
60
cAMP (%)
water
$G_{\beta 5L}/G_{\gamma 2}$
*

DISCUSSION

Although signal transduction pathways mediating progesterone action in frog oocytes remain poorly understood, the role of cAMP is undeniable. Progesterone-induced GVBD is blocked by the activator of adenylyl cyclases (forskolin) (Schorderet-Slatkine and Baulieu, 1982) or by the inhibitor of cAMP-specific phosphodiesterases (isobutylmethylxanthine) (Sadler and Maller, 1987). In contrast, injection of cAMP-specific phosphodiesterase (PDE3) mRNA induces robust GVBD in the absence of any hormones (Andersen *et al.*, 1998). Clearly, progesterone-induced oocyte maturation requires a reduction of intracellular cAMP. This could be achieved by inhibition of adenylyl cyclases or activation of phosphodiesterases, or both. Earlier work has indicated that progesterone inhibits membrane-bound adenylyl cyclases (Finidori-Lepicard *et al.*, 1981; Sadler and Maller, 1981) but does not appreciably activate phosphodiesterases (Sadler and Maller, 1987). Substantial bodies of evidence have indicated that the classical $G_{i\alpha}$ proteins are not involved in progesterone-induced cAMP reduction. As mentioned above, progesterone-mediated adenylyl cyclase inhibition or oocyte maturation is not blocked by the classical $G_{i\alpha}$ inhibitor, pertussis toxin (Sadler *et al.*, 1984; Sheng *et al.*, 2001). In addition, over-expression of classical G_i-coupled receptors, serotonin receptor 1A (Noh and Han, 1998), *Xenopus* muscarinic m4 receptor (Romo *et al.*, 2002), or human A1 adenosine receptor (Kalinowski *et al.*, 2003), fails to induce oocyte maturation. Furthermore, injection of activated (GTP-bound) $G_{i\alpha}$ proteins (G_{i1}, G_{i2} or G_{i3}) does not induce GVBD (Kroll *et al.*, 1991). Therefore, the reduction of cAMP is likely the result of inhibition of AC-activating G proteins.

Do frog oocytes represent a physiological system in which $G_{s\alpha}$ and $G_{\beta\gamma}$ co-operate to activate AC7? Evidence in support of this notion include previous studies demonstrating that antibodies against $G_{s\alpha}$ (Gallo *et al.*, 1995) and $G_{\beta\gamma}$ scavengers (Sheng *et al.*, 2001) are both capable of inducing spontaneous GVBD. Presumably, both activated G_{sa} and free $G_{\beta\gamma}$ complexes are required to achieve the desired levels of AC activation and cAMP to maintain G_2 arrest. On the other hand, artificially activating $G_{s\alpha}$ (by cholera toxin (Maller *et al.*, 1979) or by a combination of β_2AR and its agonist (Sheng *et al.*, 2001)) or over-expression of $G_{\beta\gamma}$ (Lutz *et al.*, 2000; Sheng *et al.*, 2001) is sufficient to achieve the inhibitory levels of cAMP. The direct demonstration that oocyte membranes contained a $G_{\beta\gamma}$ responsive AC provide further evidence that $G_{s\alpha}$ and $G_{\beta\gamma}$ synergism, discovered a decade ago (Tang *et al.*, 1991) , indeed represents physiological response.

Several lines of evidence presented here support the role of a $G_{\beta\gamma}$-regulated adenylyl cyclase in maintaining G_2 arrest in *Xenopus* oocytes. First, whereas $G_{\beta1\gamma2}$ increased oocyte cAMP and inhibited progesterone-induced GVBD, $G_{\beta5L\gamma2}$ decreased oocyte cAMP and enhanced progesterone response. Second, a single amino acid substitution of $xG_{\beta1}$ (D228G) virtually eliminated the ability of the $G_{\beta1\gamma2}$ complex to increase cAMP and similarly diminished the ability of $G_{\beta1\gamma2}$ to inhibit progesterone-induced GVBD. In contrast, another substitution (W99A) had no effect on cAMP elevation or GVBD inhibition. Third, we have demonstrated that oocyte membranes contained adenylyl cyclase activities that were activated by purified $G_{\beta\gamma}$ derived from bovine brains (Casey *et al.*, 1989). Fourth, a partial cDNA highly similar to mammalian AC7 (a $G_{\beta\gamma}$-activated adenylyl cyclase) has been identified in an oocyte cDNA library. Finally, and most

importantly, injection of xAC7 C1b domain induced hormone-independent GVBD and accelerated progesterone-induced GVBD.

The presence and the function of xAC7 in oocytes would explain the seemingly paradoxical roles of $G_{s\alpha}$ and free $G_{\beta\gamma}$ in maintaining G_2 arrest (paradoxical because traditionally $G_{\beta\gamma}$ is thought to combine with, and hence inhibit, G_{α}). Presumably, both GTP-$G_{s\alpha}$ and free $G_{\beta\gamma}$ co-operate to activate endogenous xAC7 and maintain high levels of cAMP. Inhibition of endogenous $G_{s\alpha}$, by either neutralizing antibodies (Gallo *et al.*, 1995) or by antisense oligonucleotides (Romo *et al.*, 2002), would release G_2 arrest. Similarly, inhibition of $G_{\beta\gamma}$, by $G_{\beta\gamma}$ scavengers, would also release G_2 arrest (Sheng *et al.*, 2001). Therefore, it appears that physiological maintenance of G_2 arrest may require both activated $G_{s\alpha}$ and $G_{\beta\gamma}$. However, artificially and persistently activating either $G_{s\alpha}$ or $G_{\beta\gamma}$ is sufficient to sustain G_2 arrest, even in the presence of progesterone. For example, cholera toxin, which irreversibly activates $G_{s\alpha}$ by covalent modification, is a potent inhibitor of progesterone-induced oocyte maturation (Maller *et al.*, 1979). Similarly, injection of mRNA for a constitutively activated $G_{s\alpha}$ (Romo *et al.*, 2002), or over-expression of a G_s-coupled receptor (β_2AR) with continuous presence of the cognate ligand (Sheng *et al.*, 2001), also potently blocks progesterone-induced oocyte maturation. On the other hand, over-expression of $G_{\beta\gamma}$ (Sheng *et al.*, 2001; Lutz *et al.*, 2000) also potently inhibits progesterone-induced oocyte maturation. More intriguingly, over-expression of G_i- or G_q-coupled receptors also inhibits progesterone-induced oocyte maturation (Romo *et al.*, 2002). The most likely explanation for these phenomena is that the $G_{\beta\gamma}$ released from $G_{i\alpha}$ or $G_{q\alpha}$ is responsible for the inhibitory effects (Romo *et al.*, 2002). In this regard, it is interesting to note that pertussis toxin, which clearly ADP-ribosylates endogenous G_{α}

proteins (Sheng *et al.*, 2001), actually facilitates progesterone-induced oocyte maturation (Mulner *et al.*, 1985; Sheng *et al.*, 2001). This can be readily explained if the $G_{\beta\gamma}$ complexes are derived from endogenous pertussis toxin-sensitive G proteins, as pertussis toxin-catalyzed ADP-ribosylation of G_{α} subunits inhibits G protein activation and therefore prevents the release of $G_{\beta\gamma}$ subunits (West *et al.*, 1990).

The conditional activation of adenylyl cyclases by $G_{\beta\gamma}$ (conditional on the presence of GTP-$G_{s\alpha}$) was first discovered a decade ago in *in vitro* studies using purified proteins (Tang and Gilman, 1991). This regulatory mechanism has since been demonstrated in cultured cells over-expressing AC2 or AC7 (Lustig *et al.*, 1993; Yoshimura *et al.*, 1996). In these cells, activation of the G_i-coupled receptor was found to synergise with G_s-coupled receptor in elevating cAMP (Lustig *et al.*, 1993; Yoshimura *et al.*, 1996). This unique phenomenon is explained by the fact that AC2, or AC7, is not inhibited by GTP-$G_{i\alpha}$ but instead is activated by the $G_{\beta\gamma}$ released from $G_{i\alpha}$ (Simonds, 1999). It seems therefore that in cells predominantly expressing AC2 or AC7, G_s- and G_i-coupled receptors can work in concert, rather than cancel each other, in generating cAMP. The best physiological example may be the mammalian hippocampus where in vitro studies using brain slices have shown that activation of G_i/G_o coupled receptors (5-hydroxytryptamine 1 and γ-aminobutyric acid B receptors) enhances β-adrenergic response (Andrade, 1993; Gereau and Conn, 1994). Interestingly, several previous studies have demonstrated that cAMP-regulated ion channels, the cystic fibrosis transmembrane conductance regulator (Uezono *et al.*, 1993; Kaneko *et al.*, 1994; Birnbaum *et al.*, 1995) and the HCN2 pacemaker channel (Ulens and Tytgat, 2001), are both co-activated by G_s- and G_i-coupled receptors in frog oocytes injected with the channels and the G protein

coupled receptors (GPCRs). These studies (Uezono *et al.*, 1993; Kaneko *et al.*, 1994; Birnbaum *et al.*, 1995; Ulens and Tytgat, 2001) are consistent with our finding that the oocytes contain a functional $G_{\beta\gamma}$-activated adenylyl cyclase, xAC7. We believe that xAC7 functions as the signal integrator for both GTP-$G_{s\alpha}$ and $G_{\beta\gamma}$ in maintaining meiotic G_2 arrest in *Xenopus* oocytes. The GTP-$G_{s\alpha}$ and $G_{\beta\gamma}$ may arise, respectively, from two upstream GPCRs that are tightly regulated by their respective ligands. The presence, and the proposed function, of these GPCRs is supported by our recent demonstration that injection of mammalian G protein coupled receptor kinases, or β-arrestins, caused hormone-independent oocyte maturation (Wang and Liu, 2003). Presumably, the G protein coupled receptor kinases and β-arrestins disrupt the tonic regulation of these GPCRs by promoting desensitization and/or endocytosis of the receptors (Wang and Liu, 2003).

Chapter Four

G protein coupled receptor in meiotic arrest

SUMMARY

Accumulating evidence has indicated that vertebrate oocytes are arrested at late prophase (G_2 arrest) mediated by a G protein coupled receptor (GPCR) that activates adenylyl cyclases. However, the identity of this GPCR or its regulation in G_2 oocytes is unknown. Our previous study proposed the presence of a constitutively active G protein coupled receptor (GPCR*; perpetual activation can be achieved either through an autocrine loop or through a ligand-independent mechanism) in G_2-arrested frog oocytes (Sheng *et al.*, 2001). We hypothesize that this GPCR*, through its coupling to adenylyl cyclase-activating G proteins, is responsible for maintaining high levels of cAMP. Our lab has recently demonstrated that over-expression of a G protein coupled receptor kinase (GRK3) or β-arrestin-1 causes spontaneous oocyte maturation in the frog. Presumably, GRK3 and β-arrestin-1 cooperate to desensitize and/or internalize the frog GPCR* and hence releases G_2 arrest (Wang and Liu, 2003).

In this study, we attempted to identify the frog GPCR by testing specific GPCR antagonists for their ability to induce oocyte maturation. I demonstrated here that ritanserin, a known antagonist for several types of serotonin (5-hydroxytryptomine or 5-HT) receptors, is a potent inducer of oocyte maturation in frogs. In contrast to ritanserin, several other serotonin receptor antagonists (mesulergine, methiothepin and risperidone) had no effect on oocyte maturation. The unique ability of ritanserin, among serotonin receptor antagonists, to induce GVBD did not match the antagonist profile of any known serotonin receptors including *Xenopus* 5-HT_7R. Unexpectedly, injection of x5-HT_7R mRNA in frog oocytes resulted in hormone-independent frog oocyte maturation. The addition of exogenous serotonin abolished x5-HT_7R-induced oocyte maturation.

Furthermore, the combination of x5-HT_7R and exogenous serotonin potently inhibited progesterone-induced oocyte maturation. These results provide the first evidence that a G protein coupled receptor related to 5-HT_7R may play a pivotal role in maintaining G_2 arrest in vertebrate oocytes.

I was responsible for all work described here.

MATERIALS AND METHODS

Materials

Antibodies against cyclin B2 was a gift from James L. Maller. Antibodies against phosphor-MAP kinase was purchased from Upstate. Antibodies against *Xenopus* MAP kinase have been described previously (Posada *et al.*, 1991; Bayaa *et al.*, 2000). All radioactive compounds were from Amersham. Other chemicals were purchased from Sigma unless otherwise stated.

Animal and oocyte manipulations

Refer to: Chapter 2 Materials and Methods

Plasmid construction and in vitro mRNA synthesis

The coding sequence of x5-HT_7R (Nelson *et al.*, 1995) was PCR-amplified from a frog oocyte cDNA library (Rebagliati *et al.*, 1985). The primers were: 5'-TAT AGG CCT ATG CTG ATC CAG GTC CAG CC (5' primer); and 5'-TAT AGG CCT CTA CAG AAT GCC CTC GTG CC (3' primer). The PCR product was digested with StuI and inserted into pCS2+ (Turner and Weintraub, 1994) previously digested with StuI.

Morpholino experiments

A morpholino oligonucleotide (5'-GGCTGGGCTGGACCTGGATCAGCAT 3') antisense to the first 25 nucleotides starting from the ATG initiation codon of *Xenopus* 5-HT_7R (Nelson *et al.* 1995) was purchased from GENE TOOLS (Philomath, OR). The Standard Control Oligo has the following sequence: 5'-

CTCTTACCTCAGTTACAATTTATA 3' (GENE TOOLS). Both oligo were dissolved in water to 1 mM (~8.3 mg per ml) and stored in single-use aliquots. Oocytes were injected with 5-HT_7R mRNA (5 ng per oocyte; this reduced amount of 5-HT_7R mRNA rarely induced GBVD in the absence of exogenous 5-HT but potently inhibited progesterone-induced GVBD in the presence of 5-HT, see Figure. 4.7). Oocytes were placed immediately in OR2 containing 20 μM 5-HT, where indicated. Six hours later, progesterone was added and incubation continued overnight.

MPF assay

The procedure followed the protocol of Nebreda and Hunt (Nebreda and Hunt, 1993). Oocytes were lysed in extraction buffer (20 mM Hepes, pH7.3, 80 mM glycerophosphate, 20 mM EGTA, .5 mM $MgCl_2$, 1 mM DDT, 10 μM ATP, 150 μM NaF, 10 mg/mL leupeptin, 200 μM PMSF, 25 μg/mL benzamidine; 5μL per oocyte). Following centrifugation at 15,000 rpm for 10 minutes, an aliquot of the clear supernant was diluted 10 times with extraction buffer; 8 μL of the diluted extract was added to 4 μL of the same extraction buffer containing 2 μg histone H1, 3.2 μCi of [γ-^{32}P] ATP, and 100 μM ATP. Kinase reactions were carried out for 15 minutes at room temperature, and stopped by the addition of 12 μL of SDS sample buffer.

cAMP assay

Refer to: Chapter 3 Materials and Methods

Statistical analysis

Data were analyzed by using Mean, error, and Student test (SigmaPlot software, version 1.00, Jandel Scientific). $P < 0.05$ was considered statistically significant.

RESULTS

Ritanserin induced oocyte maturation

Searching the literature for possible clues on GPCR function in controlling G_2 arrest in frog oocytes, we came upon the English abstract of a Russian paper (Nikitina *et al.*, 1993) describing an in-house synthesized antagonists (inmecarb hydrochloride and inmecarb methiodide, not commercially available) of 5-HT receptors that were capable of inducing GVBD in *Xenopus* laevis oocytes. In addition, several studies have suggested that the "neurotransmitter" serotonin may have a role in controlling oocyte maturation and/or early embryonic development in several marine species (Stricker and Smythe, 2001; Buznikov *et al.*, 2003; Yi *et al.*, 2002). To determine whether serotonin had a role in frog oocyte G_2 arrest, we tested several antagonists of serotonin (methiothepin, mesullergine, risperidone, NAN190 and ritanserin) for their ability to induce GVBD. Indeed, of these, ritanserin, but not any others, induced robust GVBD in frog oocytes (Figure 4.1). Time course experiments indicated that ritanserin-induced GVBD occurred almost simultaneously with progesterone-induced GVBD (Figure 4.2). Ritanserin-induced GVBD was also accompanied by the activation of maturation-specific protein kinases such as MAP kinase and MPF (maturation-promoting factor), and by phosphorylation of cyclin B (Figure 4. 3 and Figure 4.4). Like progesterone, ritanserin also induced reduction of intracellular cAMP (Figure 4.5).

Xenopus 5-HT$_7$R characterization

Among the 7 known subgroups of serotonin receptors, 5-HT$_4$R, 5-HT$_6$R and 5-HT$_7$R are known to couple to G_s (Kroeze *et al.*, 2002), the form of G protein implicated in

Figure 4.1 **Ritanserin induces hormone-independent GVBD**
Groups of 20 or more oocytes were incubated overnight in OR2 (ctrl), or OR2 containing 0.5 μM progesterone (Pg), or 6 μM ritanserin (Rit) or the indicated other serotonin receptor antagonists (each at 50μM). Oocytes were scored for GVBD. Shown are averages of two (MES (mesullergine), MET (methiothepin), RIS (risperidone), and NAN (NAN190)) or four (the rest) independent experiments. The sign * denotes $p < 0.05$ in pair-wise Student's *t* test.

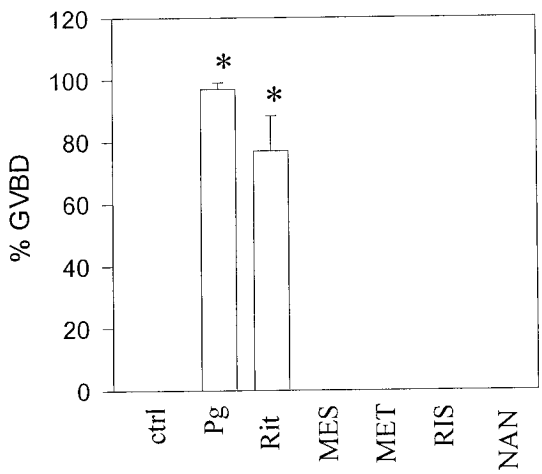

120
100
80
60
40
20
0
% GVBD
*
*
ctrl
Pg
Rit
MES
MET
RIS
NAN

Figure 4.2 **Time course of ritanserin-induced GVBD**
Groups of 40 or more oocytes were incubated with progesterone (1μM) or ritanserin (1, 2 or 5 μM). At the indicated time following the addition of progesterone or ritanserin, oocytes were scored for GVBD and expressed as a percentage of total treated oocytes. Shown is a representative of three independent experiments.

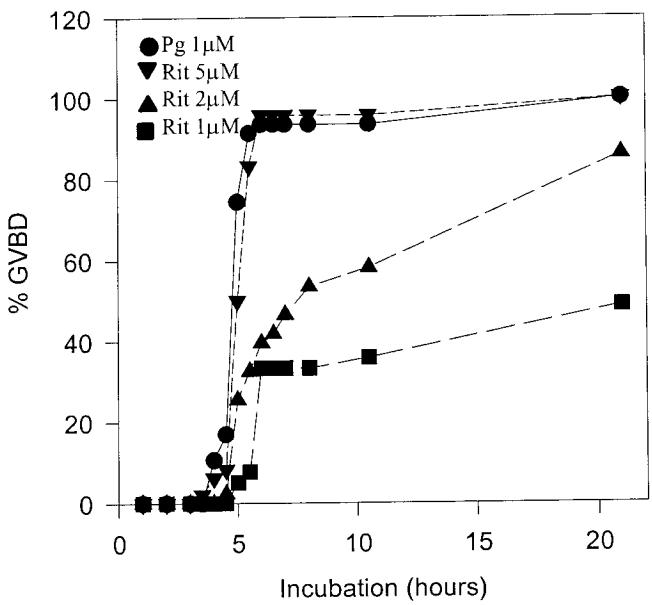

120
100
80
60
40
20
0
% GVBD
Pg 1μM
Rit 5μM
Rit 2μM
Rit 1μM
0
5
10
15
20
Incubation (hours)

Figure 4.3 **Ritanserin induces activation of MAPK and phosphorylation of cyclin B in *Xenopus* oocyte.**
Groups of 20 oocytes were incubated with indicated concentration of ritanserin or 0.5 μM progesterone (Pg) for overnight before being lysed and subjected to immunoblotting with anti-xMAP kinase antiserum, anti-phosphor MAP kinase antiserum or anti-cyclin B2 antibody. Shown is a representative of three independent experiments.

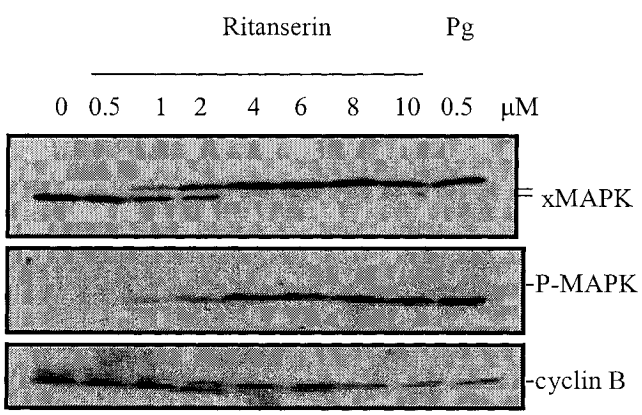

Ritanserin
Pg
0 0.5 1 2 4 6 8 10 0.5 μM
xMAPK
P-MAPK
cyclin B

Figure 4.4 **Ritanserin induces activation of MPF**
Oocytes were incubated with OR2, or OR2 containing progesterone (Pg) or the indicated concentrations of ritanserin. Oocytes were lysed for xMAP kinase immunoblotting (upper panel) or MPF assays using histone H1 as a substrate (lower panel).

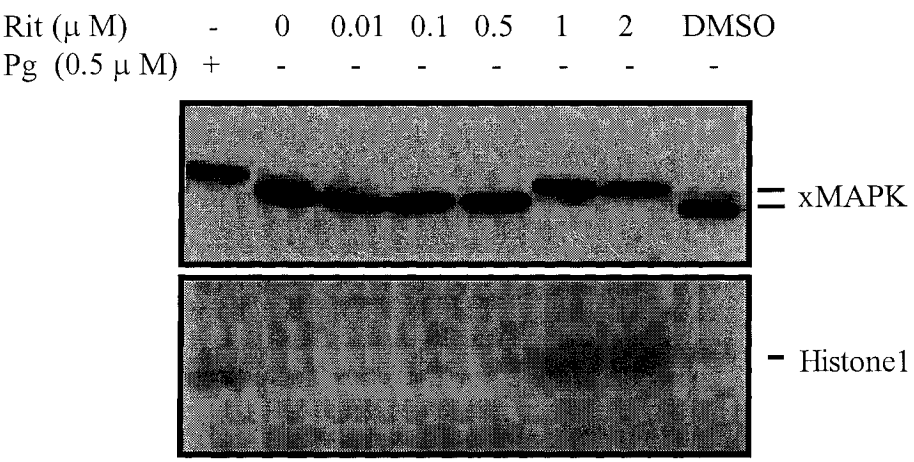
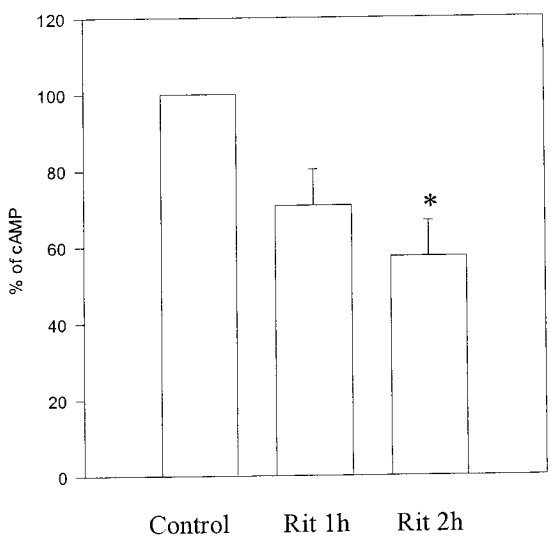

Rit (μ M) - 0 0.01 0.1 0.5 1 2 DMSO
Pg (0.5 μ M) + - - - - - - -
xMAPK
Histone1

***Figure 4.5* Ritanserin induces reduction of cAMP in *Xenopus* oocyte**
Oocytes (20 per group) were incubated in OR2 (Control) or OR2 containing 6 μM ritanserin (Rit) for 1 or 2 hours. Oocytes were immediately lysed in 95% ethanol. cAMP levels in ethanol lysates were determined and expressed % of those in derived from control oocytes, which were habitually set at 100% in each experiment. Shown are averages (with SEM) of 3 independent experiments of duplicate determinations. The sign * denotes $p < 0.05$ in unpair-wise Student's test.


120
100
80
60
40
20
0
% of cAMP
*
Control
Rit 1h
Rit 2h

maintaining meiotic G_2 arrest. 5-HT_7R was of particular interest to us, as the putative *Xenopus* 5-HT_7R had been cloned (Nelson *et al.*, 1995). Furthermore, ritanserin, which was discovered as a high affinity antagonist for 5-HT_2R (Leysen *et al.*, 1985), is also a potent antagonist of both mammalian (Lovenberg *et al.*, 1993) and *Xenopus* (Nelson *et al.*, 1995) 5-HT_7R.

To identify the possible target of ritanserin in oocytes, we PCR-amplified cDNA encoding sequence of *Xenopus* 5-HT_7 receptor from an oocyte cDNA library (Rebagliati et al., 1985). Injection of x5-HT_7R mRNA into *Xenopus* oocytes, surprisingly, induced spontaneous GVBD (Figure 4.6). In the presence of exogenous serotonin, however, oocytes injected with x5-HT_7R mRNA failed to undergo GVBD (Figure 4.7, lane 7). Furthermore, the combination of x5-HT_7R mRNA and exogenous serotonin potently inhibited progesterone-induced GVBD (Figure 4. 7, lane 8). The ability of 5-HT_7R to induce GVBD was in contrast to another G_s-coupled receptor, β_2-adrenergic receptor (β_2AR), which, when expressed alone, did not induce GVBD (Figure 2.17, lane 2). Instead, β_2AR alone actually showed modest inhibition of progesterone-induced GVBD (Figure 2.17, lane 6). In the presence of its agonist isoproterenol, β_2AR was a potent inhibitor of progesterone-induced GVBD, as shown previously (Figure 2.17, lane 4).

The ability of unliganded 5-HT_7R to induce GVBD also appeared to be unique among serotonin receptors. For example, rat 5-HT_{1A}R (Albert *et al.*, 1990), a G_i-coupled receptor was unable to induce GVBD (Figure 4.9, lane 9). In the presence of exogenous serotonin, 5-HT_7R potently inhibited progesterone-induced oocyte maturation (Figure 4.7, lane 8 and Figure 4.8 lane 8), whereas 5-HT_{1A}R had no effect (Figure 4.9, lane 12). The results obtained with injection of 5-HT_{1A}R (Figure 4.9) were in agreement with an

Figure 4.6 **Unliganded x5-HT$_7$R induces *Xenopus* oocyte maturation**
Control oocytes and ocytes injected with x5-HT$_7$ receptor mRNA were incubated in OR2. Following overnight incubation, oocytes were scored for GVBD and expressed as a percentage of total treated oocytes. Shown are the means (with SEM) of 17 independent experiments. Shown above the bars are actual numbers of GVBD-positive oocytes over those of treated oocytes. The sign * denotes $p < 0.05$ in pair-wise Student's t test.

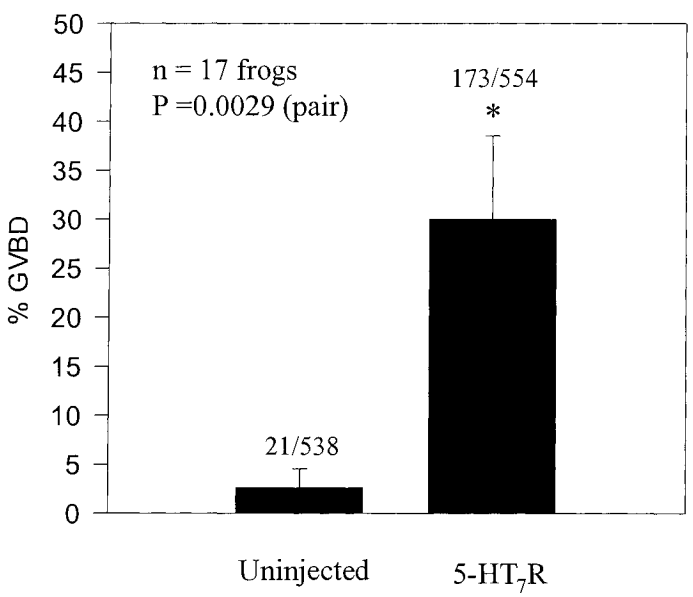
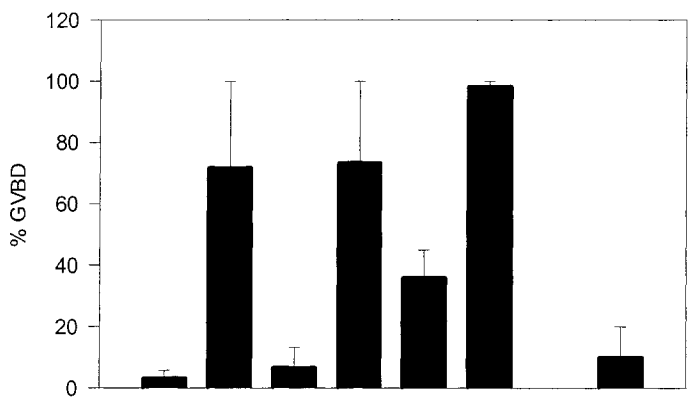

50
45
40
35
30
25
20
15
10
5
0
% GVBD
n = 17 frogs
P =0.0029 (pair)
173/554
*
21/538
Uninjected
5-HT7R

***Figure 4.7* x5-HT$_7$R/serotonin inhibits progesterone-induced GVBD.**
Oocytes injected with water or with mRNA encoding x5-HT$_7$ receptor were incubated in OR2 for 30 min – 4 hours, before threatment with 10 μM serotonin. Three to five hours following incubation with serotonin, oocytes were treated with or without progesterone (0.5 μM). Following overnight incubation, oocytes were scored for GVBD and expressed as a percentage of total treated oocytes. Shown are the means (with SEM) of three to ten independent experiments.



lane	1	2	3	4	5	6	7	8
Progesterone(0.5μ M)	-	+	-	+	-	+	-	+
5-HT(μ M)	-	-	+	+	-	-	+	+
x5-HT$_7$R mRNA	-	-	-	-	+	+	+	+

Figure 4.8 **x5-HT$_7$R/serotonin inhibits progesterone- and ritanserin-induced activation of MAPK.**
Oocytes injected with water or with mRNA encoding x5-HT$_7$ receptor were incubated in OR2 for 30 min – 4 hours, before treatment with serotonin where indicated. Three to five hours following incubation with serotonin, oocytes were treated with or without ritanserin (Rit) (2 μM) or progesterone (Pg) (0.5 μM). Following overnight incubation, oocytes were lysed and the resultant extracts were analysis for xMAPK and xMAP kinase phosphorylation. Shown is a representative of three independent experiments.

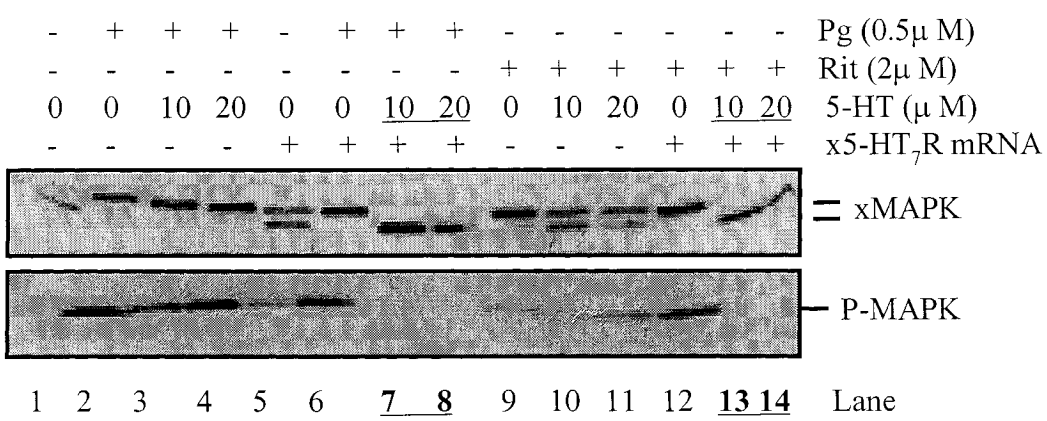
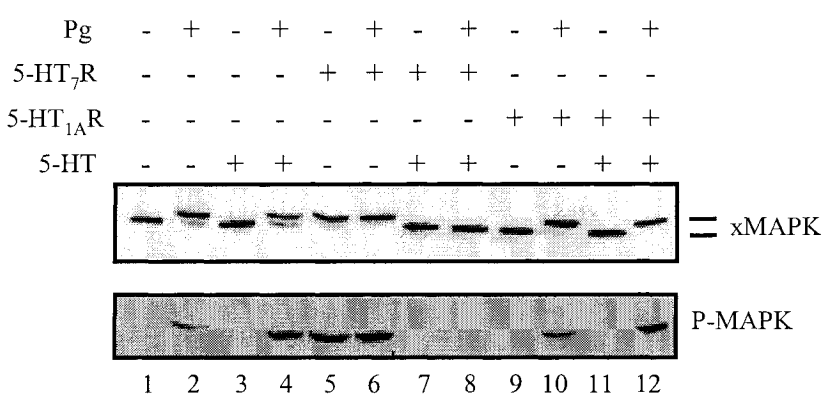

- + + + - + + + - - - - - - Pg (0.5μ M)
- - - - - - - - + + + + + + Rit (2μ M)
0 0 10 20 0 0 10 20 0 10 20 0 10 20 5-HT (μ M)
- - - - + + + + - - - + + + x5-HT7R mRNA
xMAPK
P-MAPK
1 2 3 4 5 6 7 8 9 10 11 12 13 14 Lane

Figure 4.9 **Effect of 5-HT_{1A}R on MAPK activation**
Oocytes were injected with water or the indicated mRNA and immediately placed in OR2 containing 5-HT (where indicated). Seven hours later, progesterone (Pg) was added as indicated and incubation continued overnight. Extracts were prepared and analyzed by immunoblotting with antibodies against MAP kinase (upper) and phospho-MAP kinase (lower). Shown is representative of three independent experiments.


Pg - + - + - + - + - + - +
5-HT7R - - - - + + + + - - - -
5-HT1AR - - - - - - - - + + + +
5-HT - - + + - - + + - - + +
xMAPK
P-MAPK
1 2 3 4 5 6 7 8 9 10 11 12

early study by Noh and Han who reported that injection of mammalian 5-HT_{1A}R or 5-HT_{2C}R did not induce GVBD (Noh and Han, 1998).

Antisense morpholino blocked the activities of x5-HT7R mRNA

Unfortunately, we were not able to determine whether frog oocytes expressed endogenous x5-HT_7R protein, as our attempt to contract commercial anti-peptide antibodies against x-5HT_7R (CLSAASMHEALKVTERHEGIL; sequence derived from the C-terminus of x5-HT_7R (Nelson *et al.*, 1995)) did not produce useful antibodies (not shown). Nevertheless, we wished to explore the possibility that morpholino oligo (Summerton, 1999) antisense to x5-HT_7R might affect oocyte maturation. Morpholinos block translation by an RNase-H-independent mechanism (Summerton and Weller, 1997). As a positive control, we took advantage of the ability of exogenous x5-HT_7R, together with 5-HT, to inhibit progesterone-induced GVBD (Figure 4.7, lane 8). Injection of x5-HT_7R-specific morpholino oligo, but not a control morpholino oligo, reversed x5-HT_7R mRNA-mediated inhibition of progesterone-induced GVBD and MAPK activation (Figure 4.10 and Figure 4.11). These control experiments clearly demonstrated the effectiveness of the morpholino oligo to inhibit x5-HT_7R translation. To test whether the morpholino oligos affect oocyte maturation, uninjected oocytes or oocytes injected with either the control morpholino oligo or x5-HT_7R-specific morpholino oligo were incubated for as long as five days. No spontaneous GVBD was observed in any group of oocytes (Figure 4.12, lane 3). Furthermore, all groups underwent progesterone-induced GVBD following the 5-days incubation (Figure 4.12). These results would argue against a prominent role for endogenous x5-HT_7R in maintaining G_2 arrest.

Figure 4.10 **Antisense morpholino blocked the activities of x5-HT$_7$R mRNA on GVBD**

Oocytes were injected with the indicated amounts of a control morpholino (ctrl-morph) or a morpholino oligo antisense to x5-HT$_7$R (7-morph). All groups were injected again with x5-HT7R mRNA (5 ng per oocyte) and treated with 5-HT (where indicted) and with progesterone. Shown are means (with SEM) of three independent experiments.

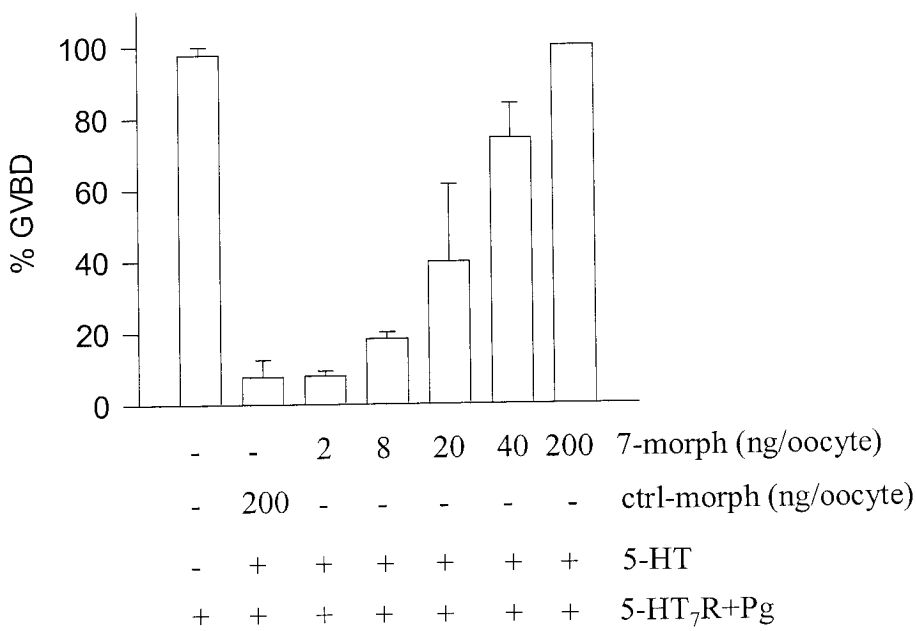

% GVBD
100
80
60
40
20
0
- - 2 8 20 40 200 7-morph (ng/oocyte)
- 200 - - - - - ctrl-morph (ng/oocyte)
- + + + + + + 5-HT
+ + + + + + + 5-HT7R+Pg

Figure 4.11 **Antisense morpholino blocked the activities of x5-HT$_7$R mRNA on MAPK**

Oocytes were treated similarly as in Figure 4.10. Extracts were prepared and analyzed by immunoblotting with anti-xMAP kinase. Shown is a representative of three independent experiments.

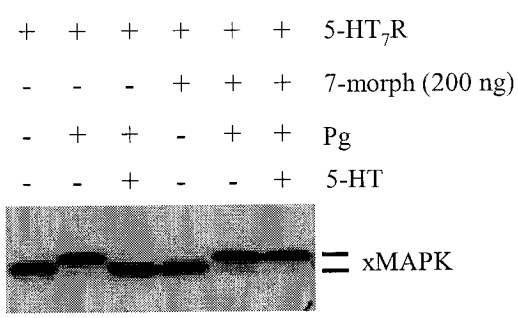
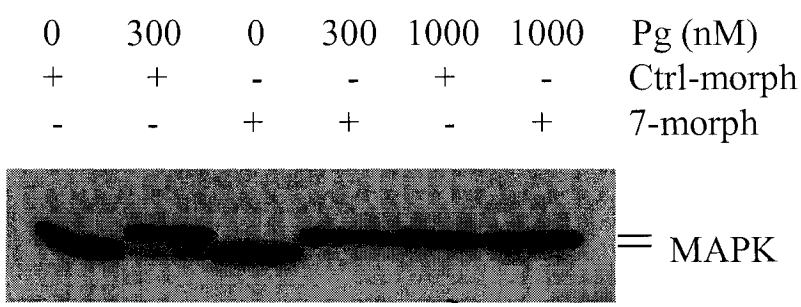

+ + + + + + 5-HT7R
- - - + + + 7-morph (200 ng)
- + + - + + Pg
- - + - - + 5-HT
xMAPK

Figure 4.12 **Antisense morpholino does not induce oocyte maturation**
Oocytes injected with control morpholino (ctrl-morph) or a morpholino oligo antisense to x5-HT_7R (7-morph) were incubated in OR2 for five days. Oocytes were then incubated with progesterone (concentration as indicated). Following overnight incubation, oocytes were lysed for MAPK analysis.


0 300 0 300 1000 1000 Pg (nM)
+ + - - + - Ctrl-morph
- - + + - + 7-morph
= MAPK

DISCUSSION

Ritanserin was first described as a high affinity (IC_{50} = 0.9 nM; measured by in vitro radioligand binding assays) antagonist of serotonin-S_2 sites (5-HT_2 receptors) in rat frontal cortex (Leysen *et al.*, 1985). With significantly reduced affinities, it also inhibits histamine H_1 sites (histamine H_1 receptors, IC_{50} = 35 nM) and dopamine D_2 sites (dopamine D_2 receptors, IC_{50} = 70 nM). Significantly, it shows very low affinity (IC_{50}>1000 nM) for serotonin S_1 sites (5-HT_1 receptors)(Leysen *et al.*, 1985). Ritanserin is also known to be a potent antagonist for 5-HT_7R, with IC_{50} of 45 nM by similar radioligand binding assays (Lovenberg *et al.*, 1993; Bard *et al.*, 1993). However, in intact cells expressing recombinant 5-HT_7R, the reported effective concentrations (IC_{50} or EC_{50} measured by inhibition of 5-HT_7R-mediated cAMP production or other biological readouts) of ritanserin are between 500 nM to 5 uM (Krobert and Levy, 2002; Nelson *et al.*, 1995). In fact, most *in vitro* studies employed 10 to 20 uM ritanserin as 'specific antagonist' for 5-HT_7R. The EC_{50} for ritanserin in GVBD induction, therefore, was in line with these earlier studies. It should also be pointed out that this concentration was also in agreement with several studies in which ritanserin is reported to inhibit cleavage of early Tritonia domedea embryos (Buznikov *et al.*, 2003).

The unique ability of ritanserin to induce GVBD ruled out the possibility that the endogenous oocyte target of ritanserin is x5-HT_7R, as we demonstrated that x5-HT_7R was inhibited by other serotonin antagonists (Sheng *et al.*, 2004b). Despite the uncertainty of the endogenous ritanserin target, the resemblance of ritanserin-induced GVBD to progesterone-induced GVBD was striking. Ritanserin-induced GVBD in the frog proceeded with a time course indistinguishable from that of progesterone-induced

GVBD. Like progesterone, ritanserin induced a reduction of cAMP. These comparisons would suggest that ritanserin acted at an early step in the physiological maturation pathway. The basal cAMP concentration in G_2 oocytes was determined as 0.55 μM (Maller *et al.*, 1979). This value is identical to the apparent activation constant of type II protein kinase (or PKA II, determined by the regulatory subunits RII) for cAMP (In contrast, the activation constant of PKA I for cAMP is about 0.1 μM) (Dostmann and Taylor, 1991). Significantly, PKA II is the predominant form of PKA in frog oocytes (Masaracchia *et al.*, 1979; Schmitt and Nebreda, 2002). The modest reduction of cAMP therefore could have a significant impact on the activities of endogenous PKA.

The ability of unliganded x5-HT_7R, but not β_2AR or 5-$HT_{1A,}$ to induce GVBD suggests that x5-HT_7R, but not these other GPCRs is able to act dominant negatively over the endogenous GPCR*. Dimerization or oligomerization of GPCR is increasingly recognized as an important regulatory mechanism (Xie *et al.*, 1999; Rocheville *et al.*, 2000; Milligan, 2001). Of particular interest are the altered agonist/antagonist specificities exhibited by heterodimers of two isotypes of opioid receptors, distinct from those exhibited by either contributing partners (George *et al.*, 2000; Jordan and Devi, 1999). If x5-HT_7R forms heterodimers with the endogenous GPCR and renders the GPCR unresponsive to its physiological ligand (due to reduced affinities and/or altered ligand specificities), the constitutive GPCR* circuit would be inactivated. This clearly represents a plausible explanation for the ability of x5-HT_7R to release GPCR*-induced G_2 arrest in frog oocytes. In the presence of exogenous serotonin, x5-HT_7R potently activated adenylyl cyclases in the oocytes and therefore inhibited progesterone-induced oocyte maturation, much the same way ligand/β_2AR did.

In summary, data presented here further substantiate the notion that a constitutively activated, adenylyl cyclase-activating GPCR is responsible for maintaining G_2 arrest in the frog. The unique ability of unliganded x5-HT_7R, but not 5-HT_{1A}R, to induce frog oocyte maturation, presumably through dimerization with this GPCR in frog oocytes, combined with the unusual biochemical stability of GPCR dimers (Milligan, 2001), clearly offers an interesting strategy for its eventual identification.

GENERAL CONCLUSIONS

It has been known for three decades that hormonal induction of oocyte maturation in the frog, and in mammals as well, requires reduction of intracellular cAMP. As the "classical" theory of cAMP signaling would suggest the involvement of inhibitory G protein (G_i) in the reduction of cAMP, it has been puzzling that Bordetella pertussis toxin, which blocks the action of G_i through ADP-ribosylation of the α subunits, does not block hormonal induction of oocyte maturation in the frog (Sadler *et al.*, 1984; Sheng *et al.*, 2001).

My original research project of focusing on the $\beta\gamma$ subunits (which are not direct targets of pertussis toxin) of trimeric G protein revealed very surprising results. I demonstrate, in chapter 2, that an endogenous $G_{\beta\gamma}$ plays a dominant role in oocyte G_2 arrest: inhibition of this complex by two structurally unrelated proteins results in hormone-independent oocyte maturation. I further demonstrate that over-expression of $G_{\beta\gamma}$ inhibits progesterone-induced oocyte maturation. These results demonstrate for the first time that frog oocytes contain a $G_{\beta\gamma}$-regulated cAMP pathway which plays a critical role in maintaining G_2 arrest.

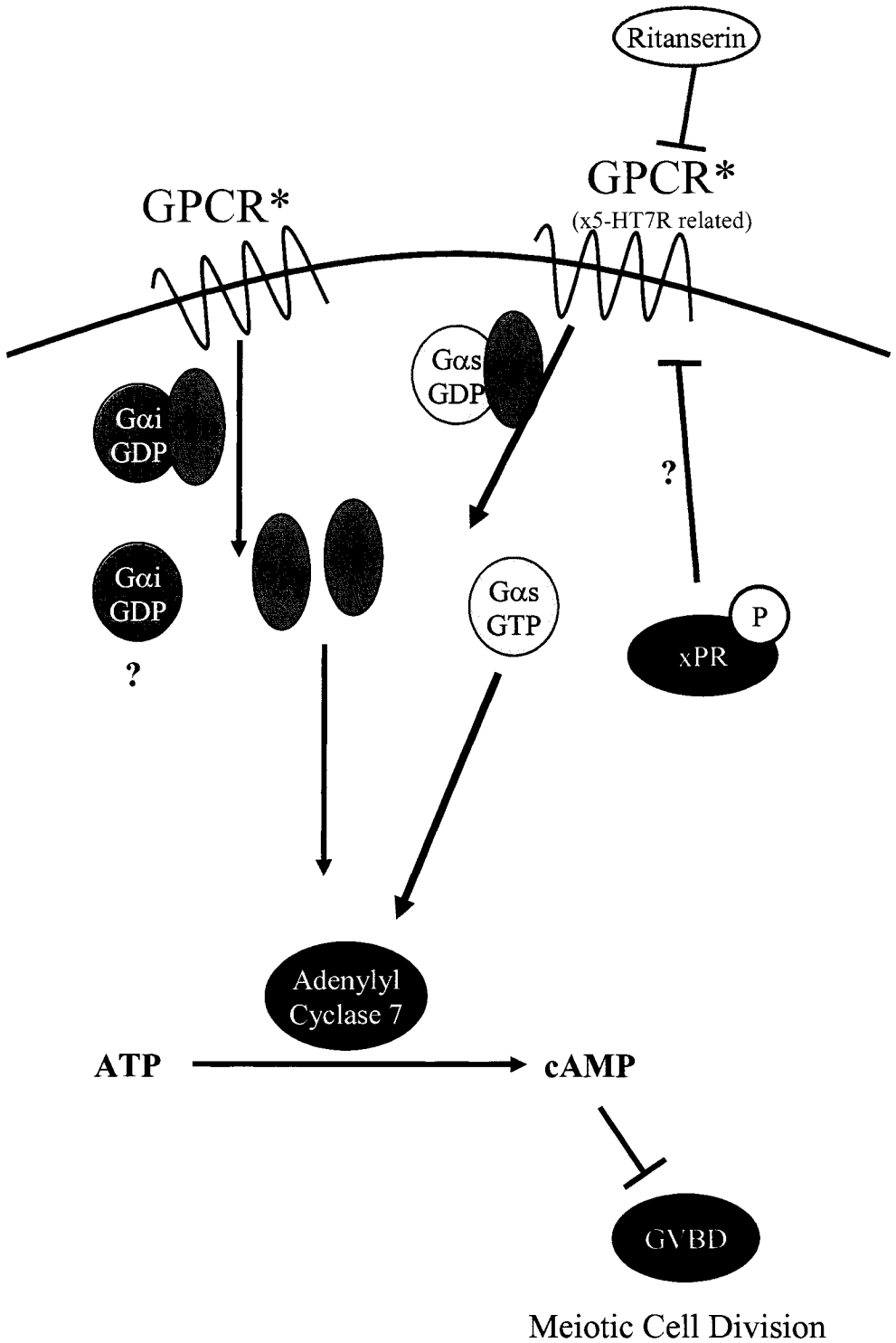
Among the nine membrane-bound adenylyl cyclases, only three (AC2, AC4 and AC7) are known to be activated by $G_{\beta\gamma}$ complexes. To determine whether one of these AC is present and functional in frog oocytes, I undertook a cloning project. In chapter 3, I describe the identification and functional characterization of *Xenopus* AC7. I demonstrate that a dominant negative mutant of xAC7 is able to induce hormone-independent oocyte maturation, as well as significantly accelerate progesterone-induced oocyte maturation.

These results indicate that xAC7 is the $G_{\beta\gamma}$-regulated adenylyl cyclase, which is responsible for the G_2 arrest in *Xenopus* oocytes.

Based on my own work and those of others in our laboratory as well as several other laboratories, we propose that a constitutively activated G protein coupled receptor(s) (GPCR*) is responsible for activating adenylyl cyclases (e.g. xAC7) and maintaining G_2 arrest. Towards the identification of such a GPCR*, I searched the literature for possible candidate GPCR in frog oocytes. Earlier work has suggested the possible presence of serotonin receptor in oocytes of amphibian and marine species. I have discovered that ritanserin, a potent antagonist of serotonin receptors, induces hormone-independent oocyte maturation. As described in detail in Chapter 4 and in a manuscript (Sheng, *et al.*, 2005), we do not believe the endogenous GPCR* is regulated by serotonin. Rather, we believe that a novel GPCR* with structural similarities to x5-HT_7R may be responsible for maintaining G_2 arrest.

Figure 5 depicts one model regarding trimeric G protein signaling in regulating meiosis. In this model, AC7 is activated by the coorperation of GPCRs*. One GPCR* activates G_s, releasing $G_{s\alpha}$GTP. The other GPCR* activates anther G protein, releasing $G_{\beta\gamma}$. In this model, inhibition of either GPCR* would release G_2 arrest. My PhD thesis work contributes significantly to this scheme. Our contribution (Sheng *et al.*, 2001) has been highlighted in *Science*'s on-line STKE (Signal Transduction Knowledge Environment) under TWIST (This Week in Signal Transduction, March 27, 2001).

Figure 5 **Proposed mechanism of regulation of AC in *Xenopus* oocytes**
See text for details.


Ritanserin
GPCR*
(x5-HT7R related)
GPCR*
Gαs
GDP
Gαi
GDP
?
Gαi
GDP
?
Gαs
GTP
P
xPR
Adenylyl
Cyclase 7
ATP
cAMP
GVBD
Meiotic Cell Division

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PROFESSIONAL EXPERIENCE

1998/11 – 1999/4: **Vice Director**
Department of Pathology, Ningxia Medical College; Department of Pathology, Affiliated Hospital of Ningxia Medical College, China

1997/11 – 1998/7: **Research Associate**
Reproductive Biology Unit and Division of Gynaecologic Oncology, Department of Obstetrics and Gynaecology and Cellular and Molecular Medicine, University of Ottawa, Hormones, Growth and Development Unit, Loeb Health Research Institute.
Project: Down-regulation of X-linked Inhibitor of Apoptosis Protein with adenovirus antisense XIAP induces apoptosis in chemoresistant human ovarian cancer cells

1997/7 – 1997/10: **Research Associate** University of Ottawa, Ottawa, Canada.
Project: Correlation between thymidylate synthase (TS) and resistance of 5- fluorouracil in colon cancer

1995 – 1999: **Associate Professor** Dept. of Pathology, Ningxia Medical College

1990 – 1995; 1982 – 1990: Lecturer; Assistant Professor
Dept. of Pathology, Ningxia Medical College, China

1981/06-1982/06: **Intern.** Affiliated Hospital, Ningxia Medical College, China

AWARDS AND SCHOLARSHIPS:

2001 - 2004 Canadian Institutes of Health Research (CIHR) Postdoctoral Fellowship
2001 - 2004 Excellence Scholarships, University of Ottawa

2001	Outstanding achievements in obtaining a CIHR Postdoctoral Fellowship Award, Ottawa Health Research Institute, Ottawa Hospital & University of Ottawa,
1999 - 2001	Ontario Graduate Scholarship in Science and Technology (OGSST), Government of Ontario and Loeb Health Research Institute, Canada
1999 - 2001	Admission scholarship, University of Ottawa
1999	Best poster presentation, 18th Annual Ottawa Reproductive Biology Workshop, Ottawa, Canada,
1997 – 1998	Visiting scholar fellowship, Chinese Scholarship Council
1994	The third prize award for outstanding research paper presentation, the 9th Congress of Chinese Ophthalmology of the Middle and Young
1994	The third prize award for outstanding research paper, Scientific & Technical Committee of Ningxia,
1992	The second prize award for outstanding research paper, Ningxia Scientific & Technical Association

PUBLICATIONS

Works published in refereed publications

1. **Sheng Y**, Wang L, Liu X, Montplaisir V, Tiberi M, Baltz JM and Liu X J (2005b)A serotonin antagonist induces oocyte maturation in both frogs and mice: evidence that the same G protein-coupled receptor is responsible for maintaining meiosis arrest in both species. *J Cell Physiol.* **202**:777-76.
2. **Sheng Y**, Montplaisir V and Liu X J (2005a) Co-operation of $G_{s\alpha}$ and $G_{\beta\gamma}$ in maintaining G2 arrest in *Xenopus* oocytes-- A $G_{\beta\gamma}$-regulated adenylyl cyclase in frog oocytes. *J Cell Physiol.* **202**:32-40
3. Ohashi S, Naito K, Liu J, **Sheng Y**, Yamanouchi K and Tojo H. (2001) Expression of exogenous proteins in porcine maturing oocytes after mRNA injection: kinetic analysis and oocyte selection using EGFP mRNA. *J Reprod Dev.* **47**: 351-357.
4. **Sheng Y**, Tiberi M, Booth RA, Ma CQ and Liu X J (2001) Regulation of Xenopus oocyte meiosis arrest by G protein βγ subunit. *Current Biology* **11**:405-416. highlighted in *Science*'s on-line STKE (Signal Transduction Knowledge Environment) under TWIST (This Week in Signal Transduction, March 27, 2001).
5. Bayaa M, Booth RA, **Sheng Y** and Liu XJ (2000) The classical progesterone receptor mediates *Xenopus* oocyte maturation through a nongenomic mechanism. *Proc Natl Acad Sci USA* **97**: 12607-12612.
6. Sasaki H*, **Sheng Y***, Kotsuji F and Tsang BK (2000) Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. *Cancer Research* **60**:5659-5666.
   *H.S. and Y. S. are equal contributing authors
7. Sheng XL, Tanaka Y and **Sheng Y** (1996). Immunohistochemical ferritin in relation to retinoblastoma – Associated characteristics. *Ophthalmics Practices* (Canada) **14** (6): 265-270.
8. Zhao L, **Sheng Y**(1997). Autopsy analysis of children in Ningxia. *Med J Ningxia*

19 : 114.

9. Lui A, Gao F, Zheng QX and **Sheng Y**(1995). Relation of DNA ploidy to histologic types and prognosis in retinoblastoma. *Chinese J Ophthalmology* **31**: 91-92.
10. **Sheng Y**, Zheng QX, Guo FY, Lui B, Zhang JY and Li YB (1994). Pathological studies on alveolar hydatid in gerbils treated with albendazole and preziquantel. *Chinese J Parasitology & Parasitic Diseases* **12**: 178-181.
11. **Sheng Y** and Zheng QX (1993). An immunohistochemical study of ferritin and carcinoembryonic antigen in germ cell tumors of the ovary and testis. *Aata Ontological Sinica* **3**: 11- 13.
12. Sheng XL and **Sheng Y** (1993). Clinical pathological and prognostic analysis of retinoblastoma. *Acta Oncological Sinica*. **3**: 65-66.
13. **Sheng Y**, Zheng QX and Hou ZW (1992). An immunohistochemical study of ferritin in ovarian epithelial tumors. *Chinese J. Pathology* **21**: 95-97.
14. Zhao H, Liu AP and **Sheng Y** (1992). Clinical and pathological analysis of hemangioma and vascular malformation on the oral and maxillofacial region. *J Ningxia Med Coll.*
15. Sheng XL and **Sheng Y** (1992). Clinical and pathological analysis of 470 cases of eye biopsy. *Ningxia Med J* **14**: 156-158.
16. **Sheng Y**, Li Z, Zheng QX and Hou ZW (1991). An immunohistochemical study of ferritin and carcinoembryonic antigen in ovarian neoplasm. *Chinese J Cancer* **10** : 395-398.
17. **Sheng Y** (1988). Study of CEA in ovarian tumors. *J Ningxia Med Coll.* **10**: 106-108.
18. Li Z, **Sheng Y** and Hu SP (1988). Pathological analysis of 3433 cases of gastric mucosal biopsy. *J Ningxia Med Coll.* **10**: 23-25.
19. **Sheng Y** and Hou ZW (1987). Two cases of tonsil chondroitic metaplasia. *Med J Ningxia* **9**: 114.
20. Sheng X.L. Qi M..and **Sheng Y** (1985). Malignant melanoma of bulbar conjunctiva: A case report. *J Ningxia Med Coll.* **3**: 87-88

Meeting abstracts

1. **Sheng Y**, Tiberi M and Liu X J. Membrane-bound Type II- like adenylyl cyclase function in G protein signalling regulation of Xenupos oocyte maturation July 8-13, 2001.Gordon Research Conferences-Hormonal Carcinogenesis, Meriden, NH, USA
2. **Sheng Y**, Tiberi M, Booth RA, Ma C.Q and Liu X.J. Regulation of Xenopus oocyte meiosis arrest by G protein $\beta\gamma$ subunit. March 2-6, 2001. The Fifth conference on Signalling in Normal and Cancer cells. Banff, Alberta, Canada
3. Bayaa M, Booth RA, **Sheng Y** and Liu XJ. The classical progesterone receptor mediates Xenopus oocyte maturation via a nongenomic mechanism. March 2-6, 2001. The Fifth conference on Signalling in Normal and Cancer cells. Banff, Alberta, Canada
4. Sasaki H, **Sheng Y**, Kotsuji F and Tsang BK(2000) Down-regulation of X-linked Inhibitor of apoptosis protein by adenoviral antisense expression induces apoptosis in chemo-resistant human ovarian cancer cells *in vitro*----- A pivotal role of p53. 47th Annual Society for Gynecologic Investigation. Chicago, USA

5. Liu XJ, Bayaa M, Booth RA and **Sheng Y**. (2000) Non-genomic action of progesterone in inducing amphibian oocyte maturation. 19th Annual Ottawa Reproductive Biology Workshop, Ottawa, Canada
6. **Sheng Y**, Sasaki H, Li J, Schneiderman D, Kim J-M, Lison P, Fung Kee Fung M., Faught W, Senteman M, Korneluk R, Kotsuji F and Tsang BK (1999). X-linked inhibitor of apoptosis protein in human ovarian cancer: Chemoresistance and a possible target for gene therapy. 18th Annual Ottawa Reproductive Biology Workshop, Ottawa
7. Tsang BK, **Sheng Y**, Sasaki H., Li J, Schneiderman D, Kim J-M, Lison P, Fung Kee Fung M, Faught W, Senteman M, Korneluk R and Kotsuji F. (1999). Xiap expression in human ovarian cancer: implications in Chemoresistance and gene therapy. Southern Ontario Reproductive Biology Meeting London, Canada
8. Tsang BK, **Sheng Y**, Sasaki H., Li J, Schneiderman D, Kim J-M, Lison P, Fung Kee Fung M, Faught W, Senteman M, Korneluk R, and Kotsuji F. (1999). X-linked inhibitor of apoptosis protein in human ovarian cancer: Chemoresistance and a possible target for gene therapy, Ovarian cancer forum `99 Toronto, Canada
9. **Sheng Y** and Tsang BK (1998). Down-regulation of Xiap induces apoptosis in chemoresistant human ovarian cancer cells. 17th Annual Ottawa Reproductive Biology Workshop, Ottawa, Canada
10. Sasaki H, Li J, Schneiderman D, **Sheng Y**, Kim J-M, Feng Q, Kotsuji F and Tsang BK. Life, death and immortality: cellular and molecular perspectives in human ovarian cancer cell growth. IV Sapporo International Symposium on Ovarian Function. Japan 1998.
11. Sheng XL, **Sheng Y** and Tanaka Y. (1996). Clinical significance and immunohistochemical characterization of ferritin in human retinoblastoma. XII International Congress of Eye Research. Yokohama, Japan.
12. Sheng, X.L., **Sheng Y.** and Tanaka Y (1996). Clinical significance and immuno-histochemical characterization of ferritin in human retinoblastoma. XII International Congress of Eye Research. Yokohama, Japan.
13. Liu A., Gao F., Zheng Q.X. and **Sheng Y.** (1994). Relation of DNA ploidy to histologic types and prognosis in retinoblastoma. 6th Congress of Chinese Medical Association of Ocular Fundus Disease and International Symposium. Shenzhen, China.
14. **Sheng Y.**, Zheng Q.X.,. Guo F.Y, Lui B., Zhang J.Y. and Li Y.B. (1994). Pathological studies on alveolar hydatid in gerbils treated with albendazole and prziquantel. 6th International Congress of Hydatidology. Beijing.
15. **Sheng Y.** and Zheng Q.X. (1993). An immunohistochemical study of ferritn and carcinoembryonic antigen in germ cell tumors of the ovary and testis. 1st Chinese Medical Association of Pathological Symposium on Ovarian Tumors. Yangzhou, China
16. **Sheng Y.**, Zheng Q.X. and Hou Z.W. (1992). An immunohistochemical study of ferritin in ovarian epithelial tumors. 5th Chinese Pathological Congress. Chengdu, China.

Book Chapters and Review Articles

1. Li J, Sasaki H, **Sheng Y**, Schneiderman D, Xiao CW, Kotsuji F and Tsang BK (2000) Apoptosis and Chemoresistance in Human Ovarian Cancer: is Xiap a determinant? Biol Signals Recept. 9: 122-130
2. Sasaki H, Li J, Schneiderman D, **Sheng Y**, Kim J-M, Feng Q, Kotsuji F and Tsang BK (1999). Life, death and immortality: cellular and molecular perspectives in human ovarian cancer cell growth. In "Frontiers in Endocrinology: Ovarian function Research: present and future" (ed. Seiichiro Fujimoto), Serono Symposia Publications 21:349-358
3. Cheng CZ and **Sheng Y** (1995). The principle of Diseases. Chinese Pharmaceutical Scientific & technical Publishing house. Beijing. P. 146-60.