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RAF-1 KINASE INHIBITOR PROTEIN-MEDIATED CHOLECYSTOKININ-2 RECEPTOR DESENSITIZATION AND EXTRACELLULAR SIGNAL-REGULATED KINASE ACTIVATION

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by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas Medical Branch

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas Medical Branch

August, 2009

Dedication

This dissertation is dedicated to my father, Hae-Seok Park, in heaven, and my mother, Myoung-Seok Kong.

Acknowledgements

First, I want to express my gratitude to my mentor Dr. Mark Hellmich for his patience, inspiration, and support through this dissertation. He made me capable enough to understand the scientific aspects of my research and present it in the best possible manner. I also wish to thank my dissertation committee including Dr. Celia Chao, Dr. Lisa Elferink, Dr. Robert Davey, and Dr. Glenn Kroog. Also I appreciate all Dr. Hellmich's lab members- Kirk Ives, Xiadong Wen, and Sung Ok Kim for their coorperation and help during this research. I would especially like to thank Kirk Ives for his expertise in calcium imaging. Stephen Schuenke helped me proofread this manuscript. I would like to acknowledge all my friends and my relatives in Korea and Galveston for keeping me on the track for a long time.

RAF-1 Kinase Inhibitor Protein-mediated Cholecystokinin-2 Receptor Desensitization and Extracellular Signal-regulated Kinase Activation

Publication No._____

Jeseong Park, Ph.D.

The University of Texas Medical Branch, 2009

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Raf-1 kinase inhibitor protein (RKIP) is initially known as a suppressor for Raf-1-mediated ERK activation. Moreover, recent findings indicate that RKIP also has a role in G-protein-coupled receptor (GPCR) desensitization. Protein kinase C (PKC)-mediated phosphorylation at Serine 153 (S153) on RKIP switches RKIP association from Raf-1 to GPCR kinase-2 (GRK2) for inhibiting GRK2-mediated G-protein-coupled receptor (GPCR) desensitization. As a member of the GPCR superfamily, Cholecystokinin-2 receptor (CCK2R) is a physiological receptor for gastrin (G17) and activates extracellular signal-regulated kinase (ERK) via the PKC activity. The inhibition of classical PKCs (cPKC, PKC-α,-β, and -γ) by Gö6976 indicated the augment in ERK activation compared to vehicle control, suggesting cPKC's involvement in CCK2R desensitization. CCK2R-mediated ERK activation was significantly decreased when PKC-δ was selectively silenced by siRNAs, indicating that PKC-δ, a member of the novel PKC family, mediates CCK2R-induced ERK activation. Furthermore, the data for CCK2R desensitization showed that inhibited cPKC activity by Gö6976 facilitated CCK2R desensitization. However, the silencing for PKC-α,-β, or –δ by siRNAs indicated that each knockdown of PKC isozymes attenuated CCK2R desensitization. The PKC involvement in CCK2R desensitization and ERK activation also suggested a potential role of RKIP in regulation of CCK2R activity. By either silencing or overexpressing RKIPs, I proved that RKIP acts as a suppressor for CCK2R desensitization, and the phosphorylation at S153 on RKIP plays a crucial role for inhibiting desensitization. The RKIP-mediated inhibition of CCK2R desensitization also resulted in augmentation of receptor-induced ERK activation, and this finding indicates that RKIP acts as a modulator for CCK2R-mediated signaling. The mechanism for RKIP-mediated receptor desensitization was investigated by co-immunoprecipitation of GRK2 with RKIP. The data indicated that RKIP strongly associated onto GRK2 when PKC was activated by phorbol 12-myristate 13-acetate

(PMA) treatment, but either G17 stimulation or Gö6976 did not affect on the association. It suggests that PMA-sensitive PKC isozymes are responsible for RKIP phosphorylation; however, CCK2R-mediated PKC isozymes are not involved in RKIP phosphorylation directly, rather PKC activation by other cellular mechanisms mediate RKIP phosphorylation resulting in GRK2 association. Therefore, I conclude that RKIP mediates CCK2R desensitization and ERK activation through PKC activation.

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List of Abbreviations

$[Ca^{2+}]i$, Intracellular calcium concentration

CCK, Cholecystokinin

CCK2R, Cholecystokinin 2 receptor

DAG, 1,2,-diacylglycerol

DMEM, Dulbecco's Modified Eagle Medium

ERK, Extracellular signal-regulated kinase

FBS, Fetal bovine serum

GPCR, Guanine nucleotide-binding protein coupled receptor

GRK, GPCR kinase

IP, Immunoprecipitation

IP_3, inositol-1,4,5-triphosphate

PKA, Protein kinase A

PKC, Protein kinase C

cPKC, classical PKC

nPKC, novel PKC

aPKC, atypical PKC

PMA, phorbol 12-myristate 13-acetate

PLC, phospholipase C

RKIP, Raf-1 kinase inhibitor protein

RT, room temperature

RT-PCR, reverse transcriptase polymerase chain reaction

Chapter 1: General Introduction

I. Gastrin and Its Physiological Receptors

In 1905, Edkins observed the secretion of gastric juice (acid) by injecting the extract from pyloric mucous membrane in rabbit, and he coined the term gastrin as an active substance for gastric acid secretion (Edkins 1905). Approximately 60 years later, two peptides for gastrin were isolated and sequenced (Gregory 1964; Gregory 1969), then cDNA for human gastrin was revealed in 1983 (Boel 1983). Gastrin, which is synthesized from G cells, has been known to mediate several roles in the human body - stimulation of gastric acid, release of histamine, and growth and repair of the gastric mucosa. The excessive secretion of gastrin is observed in gastrinoma and gastrititis. There are two identified receptor families for gastrin: Cholecystokinin (CCK) 1 receptor (CCK1R) and CCK2 receptor family, which are members of seven transmembrane G-protein coupled receptors (GPCRs). Both receptors share approximately 50% of homology in amino acid sequence, and they are classified in the rhodopsin family, which is called the family I of GPCRs. CCK1R shows a high affinity for CCK, but it shows lower affinity (~1000-fold less) for gastrin; whereas, CCK2R shows almost the same high affinity for either CCK or gastrin, since CCK and gastrin share the last five C-terminal amino acids with amidated modification (Gly-Trp-Met-Asp-Phe-CO-NH_2), and this homology allows the recognition of CCK1R and CCK2R.

I. I. CCK2R Variants

The first cDNA for gastrin receptors was cloned in canine parietal cells, and it showed an open reading frame encoding a 453 amino acid protein for putative GPCR. The overexpression of cloned cDNA in Cos-7 cells indicated specific binding for ^{125}I-CCK-8, and this specific binding was inhibited by CCK and gastrin peptides, indicating this clone encodes for gastrin receptor (Kopin 1992). Then several months later, Wank *et al.* cloned the cDNA for human CCK2R in brain and stomach. It encodes a 447 amino acid protein, which shares 90% homology with rat CCK2R (Pisegna 1992). The human CCK2R gene is located on chromosome 11, and it has five exons and four introns shown in Figure 1.1. There are four isoforms of CCK2R by alternative splicing of mRNA from the same gene for CCK2R. A truncated CCK2R isoform, ΔCCK2R, was reported in human brain, stomach, pancreas, and stomach cancer cell line AGS (Miyake 1995). The N-terminus of ΔCCK2R is encoded by exon 1b resulting in truncation of 66 amino acid residues of the N-terminal domain of CCK2R, which shows lower affinity for gastrin. Three other variants are encoded by exon 1a, which generates full length of N-terminal domain of CCK2R. However, these variants are distinctive in the third intracellular loop (3il) domain by different splicing events. First, CCK2R is considered a prototype for all other variants because of predominant expression levels through various tissues. The putative amino acid analysis indicates that CCK2R is composed of a 55 amino acid of N-terminus followed by a series of seven transmembrane (TM) domains (TM domain I through VII), which are connected by three extracellular loop domains and an intracellular C-terminal tail domain (Figure 1.2). Two other variants for CCK2R have the exact same amino acid composition as CCK2R, except they have longer 3il domains by alternative splicing events. The gene for CCK2R is also known to express CCK2LR, which has an additional five amino acid residues (GGAGP) on the end

of TM V by an alternative splicing event that transcripts the 5' splicing site at the end of exon 4 (Song 1993). However, the specific mechanism has not been addressed. The specific function of CCK2LR is not clear, even though it has been identified in some human cancers (Biagini 1997). CCK2i4svR, a splice variant of CCK2R, was initially cloned in human colorectal cancers in 2000 (Hellmich 2000). It has been identified in other malignant human tissues including pancreatic cancers (Ding 2002; Smith 2005), gastric cancers (Zhou 2004), and premalignant Barrett's esophagus (Harris 2004). Compared to CCK2R, CCK2i4svR has an additional 69 amino acid residues in its third intracellular loop (3il) domain, generated by intron 4 retention; it shows unique signaling properties compared to CCK2R including cell growth (Hellmich 2000), tumor growth in nude mice (Chao 2007), agonist-independent (constitutive) activation of the proto-oncogene Src kinase (Olszewska-Pazdrak 2004), Src kinase-mediated constitutive internalization and a fast rate of receptor resensitization (Chao 2005).

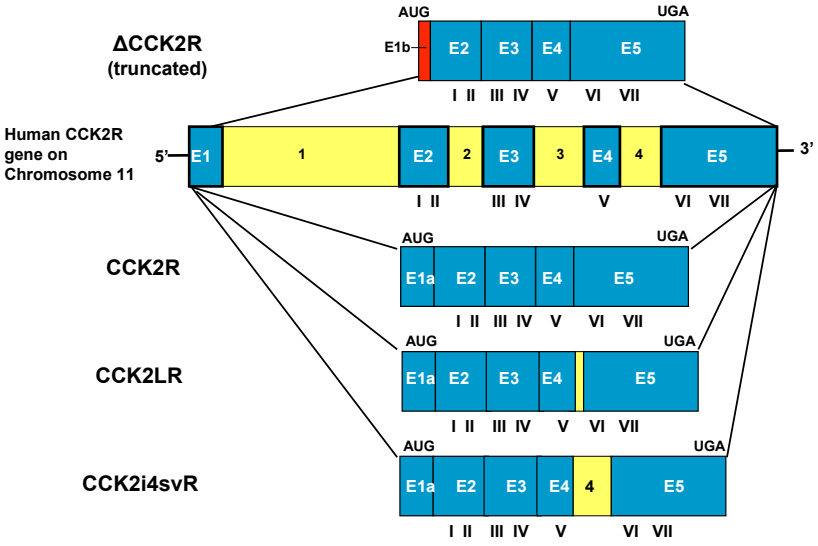

Figure 1.1: Schematic representation of the structure of CCK2R gene and its four splice variants. The human CCK2R gene is located on chromosome 11, and it has five exons (blue) and four introns (yellow). Roman numerals indicate transmembrane domains.

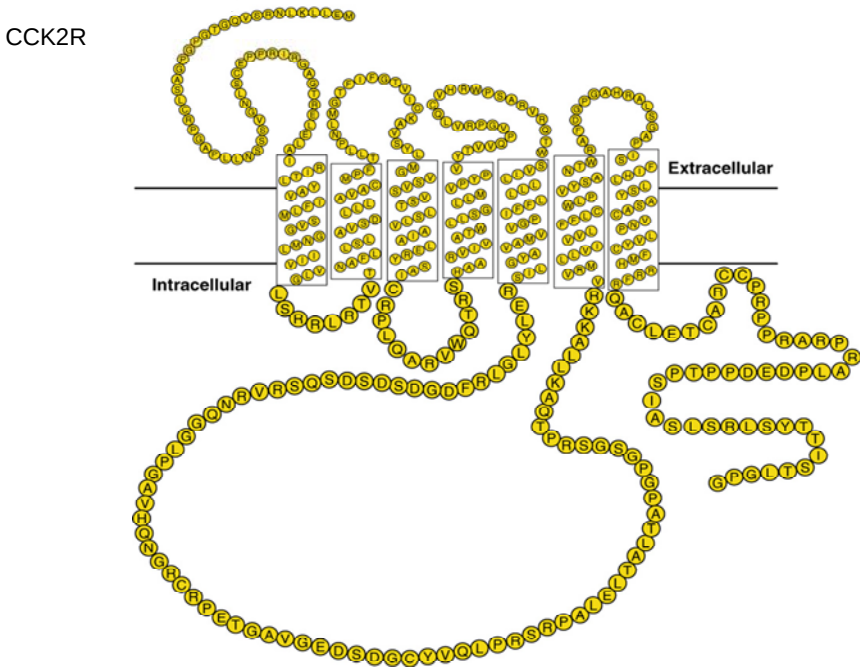

Figure 1.2: Schematic representation of the deduced structure and membrane topology of human CCK2R. The yellow circles with black letters designate amino acid sequences.

I. II. Tissue Distribution and Physiological Functions of CCK2R

Stomach

The stomach is the first organ that the existence of gastrin was confirmed in by Edkins (Edkins 1905). The main physiological action of gastrin from G cells is gastric-acid secretion. The predominant expression of CCK2R was reported in human midglandular region of the fundic mucosa by *in vitro* autoradiography with ^{125}I-labeled gastrin (Reubi 1997). The cDNA for CCK2R was cloned in smooth muscle cells in the stomach in guinea pigs by screening cDNA library using rat CCK2R probes (de Weerth 1997). Enterochromaffin-like (ECL) cells, which synthesize and secrete histamine by

gastrin stimulation, are believed to express CCK2R. Because the treatment of CCK2R-specific antagonist inhibited histamine secretion from rat ECL cells (Håkanson 1997). mRNA expression of CCK2R was identified in human gastric mucosa (Schmitz 2001), and gastric parietal cells and ECL cells (Kulaksiz 2000), supporting the hypothesis that CCK2R promotes gastric acid secretion from paritetal cells and ECL cells.

In CCK2R knockout mice studies, the level of histamine in gastric mucosa was significantly reduced compared to wild type mice, and there were no secretory vesicles in ECL cells in CCK2R knockout mice (Chen 2002). CCK2R deficiency also caused marked decrease in gastric acid secretion (Langhans 1997); moreover, it resulted in significant atrophy of gastric mucosa, indicating the role of CCK2R in the proliferation of gastric mucosal cells (Nagata 1996). In gastrin-deficient mice, gastric acid secretion was impaired (Friis-Hansen 1998). Moreover, both parietal and ECL cell numbers were decreased, while mucous neck cells increased, suggesting that gastrin has a role in regulating cell differentiation in gastric mucosa (Koh 1997).

Pancreas

In the exocrine pancreas of dogs, some degree (~20 %) of CCK2R expression was identified, but the physiological role has not been addressed (Fourmy 1987). In the adult calf, CCK2R was predominantly expressed in the exocrine pancreas; however, low levels were monitored at the early stage of birth, suggesting the importance of CCK2R in proliferation rather than controlling cell proliferation in the pancreas (Le Meuth 1993). In the pig, CCK2R expression was predominantly found in the pancreas, and the high-dose treatment of CCK2R antagonist, YF476, blocked the enzyme secretion from the pancreas (Evilevitch 2004). However, experiments using other antagonists (L-365,260

or PD 135156) did not inhibit the pancreatic secretion in pigs (Lhoste 2002). Therefore, the role of CCK2R in pancreatic secretion is still questionable.

Also, CCK2R expressions were identified in the human pancreas by RT-PCRs and radio ligand bindings (Tang 1996; Ji 2001; Reubi 2003); however, the precise role of CCK2R in human pancreas is still unclear. There is evidence that gastrin treatment increases pancreatic blood flow, and it is inhibited by the treatment of CCK2R antagonist (Griesbacher 2006).

In the human endocrine pancreas, islet cells—especially glucagon-producing cells—were identified as major sites for expressing CCK2R by RT-PCR and immunohistochemistry. Those cells released glucagon by CCK2R agonist-stimulation, demonstrating the role of CCK2R in glucagon release in pancreatic islet cells (Saillan-Barreau 1999).

The first relevance of CCK2R in pancreas growth was addressed by observing pancreatic hypertrophy by chronic administration of synthetic gastrin (pentagastrin) to the rat (Mayston 1971). Additionally, increased pancreas weights were recorded in transgenic mice overexpressing CCK2Rs in pancreatic acinar cells (Clerc 2002), which supports the role of CCK2R in pancreatic growth.

Intestine

In normal human colonic mucosa, the expression of CCK2R is still a matter of debate. Generally, CCK2R appears to be undetectable in the normal intestine tissue by RT-PCR (Monstein 1996). There is a study that showed protein expression of CCK2R in normal human colon tissues, while adjacent colorectal cancer tissue exhibited both CCK2R and CCK2i4svR (Laverman 2008), but there was no determination of a precise function of CCK2R in human colon. However, there is some feasible evidence that

CCK2R has a physiological role in the intestine. For example, one study indicates that CCK2R is involved in the contraction of the ileum of guinea pig (Dal Forno 1992). Also, similar studies in either human and guinea pig distal colon supports the possibility that CCK2R mediates intestine motility (Fornai 2007; Fornai 2007).

Central Nervous System (CNS)

CCK and CCK2R are abundantly found in human brain tissue, such as the cerebral cortex, cerebellum, basal ganglia, and amygdala (Wang 2005). CCK2R has been known to control animal behavior such as anxiety (Wang 2005), satiety (Clerc 2007), notice of pain (Noble 1993), panic attack (Bradwejn 2001), and memory processes (Daugé 2001). However, the detailed mechanisms for regulating those functions have not been answered.

Kidney

There is no evidence that CCK2R is present in human kidney, but other studies in guinea pig and rat indicate the role of CCK2R in the kidney. In mammal kidney, the expression of CCK2R were confirmed by Northern blot and immunohistochemistry (de Weerth 1998; von Schrenck 2000). Also, gastrin treatment in proximal tubular cells (MCT cells) of mice showed increased thymidine incorporation, suggesting the role of CCK2R in cell proliferation in the kidney (de Weerth 1998). In rat kidney, gastrin stimulation resulted in interfererence with absorption of sodium and potassium (von Schrenck 2000).

Blood Cells

The presence of CCK2R in the human lymphoblastic Jurkat T cell line and normal human T lymphocytes was reported in several published papers (Dornand 1995; Cuq 1997; Oiry 1997). Both CCK2R and CCK2i4svR were indentified in human mononuclear cells, and gastrin exerted an antiproliferation effect (Schmitz 2001). However, the specific role of the receptor subtype has not been addressed.

Adrenal Gland

mRNA expression of CCK2R was found in human adrenal zona glomerulosa cells, and aldosterone secretion from adrenal gland is dependent on CCK2R activation via cyclic AMP pathway (Mazzocchi 2004).

I. III. CCK2R and its Clinical Relevance

Cancers

The expression of CCK2R has been reported in many human tissues, including the gastrointestinal tract (fundic mucosa, parietal cells, enterochromaffin-like [ECL] cells in stomach (Reubi 1997; Schmitz 2001), pancreatic islets (Reubi 2003), and colon (Schmitz 2001)), nervous system (cerebral cortex of brain (Lee 1993)), and immune system (peripheral white blood cells (Iwata 1996)). The main physiological role of CCK2R is believed to be mediation of the growth and repair of gastric mucosa. Therefore, the involvement in cell growth is naturally linked with several human gastrointestinal malignancies including gastric cancer, pancreatic adenocarcinoma, esophageal adenocarcinoma, colon cancer, and small cell lung cancer (Dufresne 2006).

Helicobacter pylori (HP) infection, which is believed to play a role in the development of gastric cancer, resulted in the increase in gastrin secretion from G cells. The gastrin stimulation promoted Hp growth in bacteria culture (Chowers 1999). The role of HP in the tumorigenesis of human gastric cancer is assumed to promote excessive cell growth via epidermal growth factor (EGF), cyclooxygenase-2 (Cox-2), and Bcl-2-dependent signaling pathways (Konturek 2006). The treatment of gastrin in the gastric cancer cell line AGS expressing CCK2R showed increased cell migration and invasion, which are crucial parameters for carcinogenesis (Wroblewski 2002).

In the majority of human pancreatic adenocarcinomas, the expression of both gastrin and CCK2R has been reported (Caplin 2000; Reubi 2003). However, the precise mechanism by which CCK2R contributes to pancreatic cancer development and growth is still unclear. Barrett's esophagus, which is considered a premalignant condition for esophageal cancer, expresses gastrin and CCK2R (Haigh 2003). ERK and Cox-2 activation in Barrett's esophagus resulted in cell proliferation (Abdalla 2004; Moore 2004). Human colorectal cancer, which was the second leading cause of cancer-related deaths in the United States in 2008 (Jemal 2008), are known to express CCK2Rs. This has been confirmed by radiolabeled gastrin binding (Upp 1989), RT-PCR (Biagini 1997), and Western blotting (Laverman 2008).

Other Diseases

As stated earlier, gastric ulcer has a close relationship with HP infection. There is the link between HP infection and increased gastrin from G cells (Levi 1989). Also, human pancreatitis tissue samples showed abundant CCK2R expression (Reubi 2003). However, whether CCK2R overexpression is related to pancreatitis has not been clarified since CCK2R expression is also found in normal pancreas.

II. CCK2R mediated Signaling Pathways

II. I. Activation of CCK2R

Depending on classical theory of GPCR activation, the binding of a specific agonist to the receptor results in the conformational change of GPCR from inactive to active state. The altered conformation allows the dissociation of heterotrimeric G proteins (Gαβγ) from receptors, resulting in the exchange of GDP to GTP on the Gα subunit. Then, the activated Gα subunit is dissociated from Gβγ dimer, and both released Gα subunit and Gβγ dimer are known to promote diverse cellular responses by stimulating GPCR-associated effectors including adenylyl and guanylyl cyclases, phospholipase C (PLC), phosphoinositide 3-kinases (PI3Ks), cGMP phosphodiesterases, cAMP, cGMP, inositol-1,4,5-triphosphate (IP3), 1,2,-diacylglycerol (DAG), and phosphatidylinositol-3,4,5-triphosphate (Hamm 1998). Among Gα subunits, CCK2R is known to couple with Gαq, which is insensitive for pertussis toxin (PTX) (Jagerschmidt 1995). Pommier et al. provided new evidence that CCK2R is coupled to two signaling pathways by coupling pertussis toxin (PTX)-sensitive and insensitive G proteins (Pommier 1999; Pommier 2003). These findings suggest that other PTX-sensitive Gα subusits, such as Gαi and Gαo, may be involved in CCK2R-mediated signaling pathways. However, CCK2R mainly activates the Gαq subunit and promotes intracellular signal transduction via PLC-dependent cascades (Paulssen 2000). The activated PLC induces the hydrolysis of membrane phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 induces release of Ca^{2+} from endoplasmic reticulum (ER) by activating ligand-gated Ca^{2+} channels, then increased $[Ca^{2+}]i$ affects ion channels on plasma membrane for Ca^{2+} influx, resulting in

rapid transient increase in $[Ca^{2+}]i$. The increased levels of $[Ca^{2+}]i$ and DAG are the necessary components for Protein Kinase C (PKC) activation.

PKC

Members of PKCs are the secondary-messenger kinases transducing plasma membrane-originated signals by phosphorylating downstream effectors. There are many known substrates for PKC-mediated phosphorylation. GPCRs are also the targets for PKC-mediated phosphoyrlation, and the amino-acid sequences for phosphorylation are serine or threonine residues on the third intracellular loop domain and/or C-terminal tail of GPCRs.

The family of PKC can be divided by three distinct subfamilies: classical (cPKC; α, βI, βII, γ), novel (nPKC; θ, δ, ε, η), and atypical (aPKC; ζ, λ/ι). Those sub-families have distinct NH_2 terminal regulatory domain for activation. The structure of PKCs consists of two main domains, which are the regulatory domain and the kinase domain. cPKCs have C1 and C2 regulatory domains. The C1 domain has the binding sites for DAG/phorbol 12-myristate 13-acetate (PMA) and phosphatidylserine (PS). The C2 domain has the Ca^{2+} binding site for their activation. nPKCs have similar C1 and C2 domains compared to cPKCs, but there is no Ca^{2+} binding site on their C2 regulatory domain, therefore they exhibit Ca^{2+}-independent activation for their regulation. aPKCs only have the C1 domain, which has the binding sites for phosphatidylinositol 4,5-bisphosphate (PIP2) and ceramide, and they are not regulated by DAG, PMA, or Ca^{2+} (Steinberg 2008). The PKC activation is initiated by the phosphorylation on the activation loop site by phosphoinositide dependent kinase 1 (PDK1). This initial phosphorylation results in a series of autophosphorylation of PKCs, which allows binding of cofactors on their regulatory domains. Further, it promotes conformational changes

to expel the inhibitory binding of the pseudosubstrate domain of PKCs from the substrate binding pocket leading to expose the substrate for PKC-mediated phosphorylation (Griner 2007).

PKCs have been investigated as target molecules for anticancer drug development after the finding that PMA-mediated activation of PKC promotes tumorigenesis (Castagna 1982; Nishizuka 1984). However, it is still unclear that the tumor-promoting effect by PMA treatment is caused by PKC activation or inhibition, because the chronic activation of PKCs by PMA treatment depletes PKC activity. PKCs are involved in cell proliferation, differentiation, apoptosis, and adhesion through activating Ras/Raf/MEK/ERK, NF-κB, caspase, p38 MAPK, or JNK pathways (Yang 2003). Both upregulation and downregulation of PKCs have been reported in many human cancers, however it is totally dependent on tissue types. For example, overexpression of PKC-α has been reported in human prostate and liver cancer (Koren 2004; Tsai 2005); whereas downregulation of PKC-α has been observed in human colon cancer (Kahl-Rainer 1994).

Both cPKC and nPKC are activated by gastrin stimulation. In rat colonic cells, gastrin treatment shows the translocation of PKC α and –β onto the plasma membrane, which is considered the indicator for PKC activation (Yassin 1995). The transfection of CCK2R into MKN-28, a human gastric cancer cell line, promotes PKC δ activation by gastrin treatment (Ogasa 2003). The activation of PKC α, -ε, -η is confirmed in AGS cells overexpressing CCK2R (Sturany 2002).

Phosphatidylinositol 3-Kinase

Among phosphatidylinositol (PI) 3-kinases, class I PI-3 kinase is composed of a 110 kDa catalytic domain and an 85 kDa regulatory domain, and it is activated by receptor tyrosine kinase (RTK) activity (Reedijk 1992). Gastrin stimulation is known to

activate class I PI-3 kinase (Kowalski-Chauvel 1996; Daulhac 1999; Ferrand 2004) in Chinese hamster ovary cells overexpressing CCK2R, rat intestinal epithelial cells, and rat exocrine pancreatic tumor cells. The molecular mechanism for PI3-kinase activation involves interaction of 85 kDa regulatory domain with phosphorylated insulin receptor substrate-1 (IRS-1) by Src kinase mediated by CCK2R (Daulhac 1999). The downstream effector of PI-3 kinase is AKT pathway, and PI-3 kinase/AKT pathway is involved in cell proliferation and anti-apoptosis (Harris 2004).

Focal Adhesion Kinase and Associated Proteins

p125-focal adhesion kinase (FAK) is activated by membrane receptors, such as integrins, RTKs, and GPCRs. The phosphorylation of p125-FAK forms p125-FAK/Src complex for phosphorylation of downstream effectors such as paxillin, talin, Shc, and p130Cas (Schlaepfer 2004). By CCK2R activation, the phosphorylation of p125-FAK was observed in mouse NIH3T3 cells (Taniguchi 1994), and also the phosphorylation of p125-FAK, paxillin, and p130Cas were monitored in Colo 320, a human colorectal cancer cell line, overexpressing CCK2R (Yu 2004). The phosphorylation of p130Cas was observed in rat AR42J cells by G17 stimulation, and also it was Src dependent (Daulhac 1999).

JAK/STAT

Janus kinases (JAKs) are non-receptor tyrosine kinases and have four isozymes: JAK1, JAK2, TYK2, and JAK3. The activation of JAKs leads to phosphorylate STATs, which are transcription factiors. Among JAK-related pathways, CCK2R activation leads to JAK2/STAT3 activation in AR42J cells and also transgenic mice expressing CCK2Rs (Ferrand 2005).

Extracellular Signal-regulated Kinase

The activation of PKC leads to activation of mitogen-activated protein kinases (MAPKs), which consist of the following serine/threonine kinases: ERK1,2, ERK3,4, ERK5, ERK7,8, *c-jun* NH2-terminal kinases (JNKs), and p38 MAPKs. The activation of MAPKs regulates cell proliferation, differentiation, apoptosis, and migration (Yang 2003; Dhillon 2007). Among them, the study of ERK1,2 activation via the PKC/Raf/MEK/ pathway has been well established regarding cell growth, differentiation, and apoptosis (Tsunoda 1988; Yassin 1999). ERK1,2 can be activated by RTKs, integrins, and GPCRs. These receptors are activated by various stimuli such as stress, growth factors, cytokines, and hormones. These cell-surface stimuli are delivered into intracellular space via the small G protein, RAS and/or Raf kinase for ERK1,2 activation. Activation of Raf kinase phosphorylates ERK kinase (MEK) 1,2, leading to phosphorylation of ERK1,2 (Dhillon 2007). The activation of ERK1,2 phosphorylates transcription factors such as Ets-like protein-1 (Elk-1), cellular-myelocytomatosis oncogene (c-Myc), c-Fos, and nuclear factor κB (NF-κB), which can affect the cell cycle in regulating cell proliferation and apoptosis (Boutros 2008).

In gastric epithelial (AGS) cells stably expressing CCK2R, G17 stimulation promoted cell migration via the PKC/ERK sinaling axis (Noble 2003). The inhibition of ERK activation with either a MEK inhibitor, PD98059, or a PKC inhibitor, GF109203X, inhibited G17 induced the proliferation of AR42J cells, which endogenously express CCK2Rs, indicating that the activation of PKC/MEK/ERK1,2 pathway via CCK2R is involved in cell proliferation (Stepan 1999). In NIH3T3 cells, the overexpression of CCK2R promotes cell growth and DNA synthesis via the MEK/ERK pathway when the cells are challenged with either CCK or gastrin (Taniguchi 1994). The rat pancreatic tumor cell line, AR42J, which is known to express CCK2R endogenously, activates

ERK1,2 with PKC and Src kinase-dependent manner (Daulhac 1999). The CCK2R-mediated ERK1,2 activation is also required for Ca^{2+} release, and the inhibition of ERK1,2 with either chemical inhibitors or dominant negative mutant for MEK results in reduced amplitude of Ca^{2+} release by gastrin stimulation (Olszewska-Pazdrak 2004).

Extracellular Signal-regulated Kinase and its Clinical Significance

Aberrant activations of ERK are found in several human diseases including cancers. Ras mutations, which lead aberrant ERK activation, are commonly found in human pancreatic, thyroid, colon, and non--small cell lung cancers (Roberts 2007).

Moreover, RAS mutations also are related to abnormal development. For example, K-RAS mutation exhibits constitutive ERK activation and it causes Noonan syndrome, which is characterized by short stature, facial dysmorphism, and heart malformation (Schubbert 2006). Also, the mutation on B-Raf causes Cardio-facio-cutaneous syndrome, which is characterized by distinct facial appearance and heart defects. It shows elevated B-Raf kinase activity by mutation and causes higher levels of ERK phosphorylation compared to wild type B-Raf (Rodriguez-Viciana 2006).

The difference in the strength and duration in ERK1,2 signaling can alter the gene-expression profile, which can result in cell growth and differentiation. For example, in PC12 cells epidermal growth factor (EGF) stimulation results in transient increases in ERK1,2 followed by cell proliferation; whereas fibroblast growth factor (NGF) stimulation promotes cell differentiation by sustained ERK1,2 activity (Nguyen 1993; Marshall 1995), suggesting that the failure of controlled ERK1,2 activity is related to abnormal cell growth resulting in cell malignancy.

In polycystic kidney disease patients, the loss of polycystin-1 or -2 in cilia on kidney epithelial cells causes alternation of the signaling pathway between ERK and

cyclic AMP leading to cell proliferation and loss of epithelial polarity (Yamaguchi 2004). In human islet cells from pancreas, the inhibition of ERK activation by interfering with Raf induced β-cell apoptosis, suggesting the relevance of ERK in human diabetes (Alejandro 2008).

III. GPCR Desensitization

The major role of GPCRs is transferring the signals on the plasma membrane into the cell interior to respond a variety of extracellular stimuli, such as hormones, neurotransmitters, chemokines, odorant, taste, and light. Approximately more than 800 genes have been identified as the genes expressing GPCRs. Because of the ability to modulate intracellular signaling, GPCRs have been primary targets for drug development (Pierce 2002; Luttrell 2008). The modulation of GPCR activity by agonist stimulation is finely tuned by many interacting proteins such as heterotrimeric G proteins, GPCR kinases (GRKs), second-messenger kinases, and/or other interacting signaling molecules. In general, agonist-induced GPCR activation leads to dissociation of G-proteins from receptors, and it allows receptor phosphorylation by protein kinases, followed by endosome-mediated receptor internalization from the plasma membrane into an intracellular space that isolates receptors physically from the plasma membrane to prevent further agonist stimulation. Internalized receptors undergo uncoupling of agonists and dephosphorylation of receptors, whereby receptors then can be either recycled onto the plasma membrane for further activation, otherwise receptors localize into lysosomes for degradation.

The continuous and repetitive agonist administration to GPCR often results in dampened responsiveness, which is defined as desensitization. In general, GPCR desensitization is very quickly achieved (seconds to minutes) when receptors were

exposed to agonist stimulation. Once agonists bind to their receptors, it promotes the conformational changes of receptors leading to the dissociation of G-proteins, which allows receptor phosphorylation and the association of β-arrestins, which inhibit physical interaction between receptors and G-proteins for high affinity to agonists.

As a regulatory step, the failure of balanced receptor desensitization and resensitization has been linked with various human diseases, such as night blindness by constitutive desensitization of rhodopsin receptors (Sullivan 1993), heart failure by increased expression of GRKs (Ungerer 1994), rheumatoid arthritis by CXC4 receptor (Nanki 2000), and nephrogenic diabetes by vasopressin 2 receptor (Barak 2001). This dissertation focuses on the mechanisms of CCK2R desensitization and the role of other regulators that affect receptor desensitization.

III.I. GRK-mediated GPCR Desensitization

In an inactive state, a GPCR is bound to heterotrimeric G proteins that consist of Gα, - β, -γ subunit on its 3rd intracellular domain or C-terminus domain. The binding of agonists to GPCRs promotes conformational changes that lead to the release of G proteins from the interacting domains on GPCRs. The released Gα subunit exchanges GDP to GTP on the guanine nucleotide binding site, then activated Gα subunit is dissociated from Gβγ complex (Pierce 2002). These changes recruit GRKs for receptor phosphorylation at serine or threonine residues within intracellular receptor domains, and it promotes the binding of β-arrestins, which are the agonist-dependent adaptor proteins to mediate receptor desensitization and internalization. β-arrestin binding onto receptor recruits other proteins needed for formation of clathrin-coated pits, and these complexes are internalized by endosomal endocytosis (Pierce 2001). The fate of internalized receptors could be determined by stability of the receptor-β-arrestin complex. For

receptor recycling onto the plasma membrane, dissociation of β-arrestin binding and dephosphorylation of receptors are considered crucial parameters for determining the internalized receptor's destiny. The receptors having low affinity for β-arrestins show fast resensitization by recycling onto plasma membrane, whereas the receptors showing high affinity can remain in the endosomes for extended periods of time. This presents slower receptor resensitization and can be the target for degradation at lysosomes for downregualtion (McDonald 2001).

III.II. Second Messenger Kinase-mediated GPCR Desensitization

As stated earlier, the second messenger kinases were initially believed to promote receptor phosphorylation and desensitization before GRKs were discovered. However, it is clear that the activity of PKA or PKC phosphorylates downstream effectors as well as some GPCRs for controlling receptor-mediated signaling. Since GRKs have been reported as primary kinases for the agonist-dependent phosphorylation of many GPCRs, the role of secondary messenger kinases in receptor phosphorylation and desensitization was thought as a supplementary mechanism for GRK-mediated phosphorylation. However, recent findings suggest that secondary kinases also have the primary role in regulating receptor activity. In P2Y1 purinergic receptors, PKC activity mediates receptor desensitization, but GRKs are not involved (Hardy 2005), and also PKC β activity is required for phosphorylation and desensitization in HEK293 cells overexpressing D3 dopamine receptors (Cho 2007). Moreover, metabotropic glutamate receptor 4 is not desensitized or internalized by agonist stimulation, but the activation of PKC promotes receptor desensitization and internalization. Interestingly, this receptor couples to Gαi, which cannot activate phospholipase C (PLC)-dependent PKCs that are activated by Gαq-coupled receptors (Mathiesen 2006). This finding suggests that

different GPCRs having different downstream effectors may interact with each other and affect desensitization by the mechanism of heterologous desensitization.

In addition to their individual role for GPCR desensitization, both GRKs and second-messenger kinases can recognize and phosphorylate identical receptors and interact with each other for coordinately desensitizing GPCRs in some cases. The phosphorylation of β2 adrenergic receptors inhibits Gαi coupling onto receptors by either PKA, PKC, or GRK2, suggesting those kinases desensitize receptors by distinct pathways (Pitcher 1992). In gastrin-releasing peptide receptor (GRPR), both GRK2 and PKC phosphorylate GRPR in vitro, and the activation of PKC by phorbol myristate acetate (PMA) treatment results in receptor phosphorylation *in vivo*. PMA-induced GRPR phosphorylation also inhibits both IP_3 generation and $[Ca^{2+}]i$ increase by agonist stimulation (Ally 2003). Interestingly, some studies suggest that GRKs and second-messenger kinases interact with each other for receptor desensitization. In *in vitro* experiments, GRK2 was phosphorylated by purified PKC, and phosphorylated GRK2 showed enhanced ability to phosphorylate rhodopsin receptors (Chuang 1995). PKC-α, -γ, -δ phosphorylated GRK2 (Krasel 2001). Also, GRK5 was phosphorylated by PKC activity in *in vitro* and *in vivo* experiments (Pronin 1997). Moreover, PKA is also known to mediate GRK phosphorylation and it affected on β2 adrenergic receptor desensitization (Cong 2001; Li 2006).

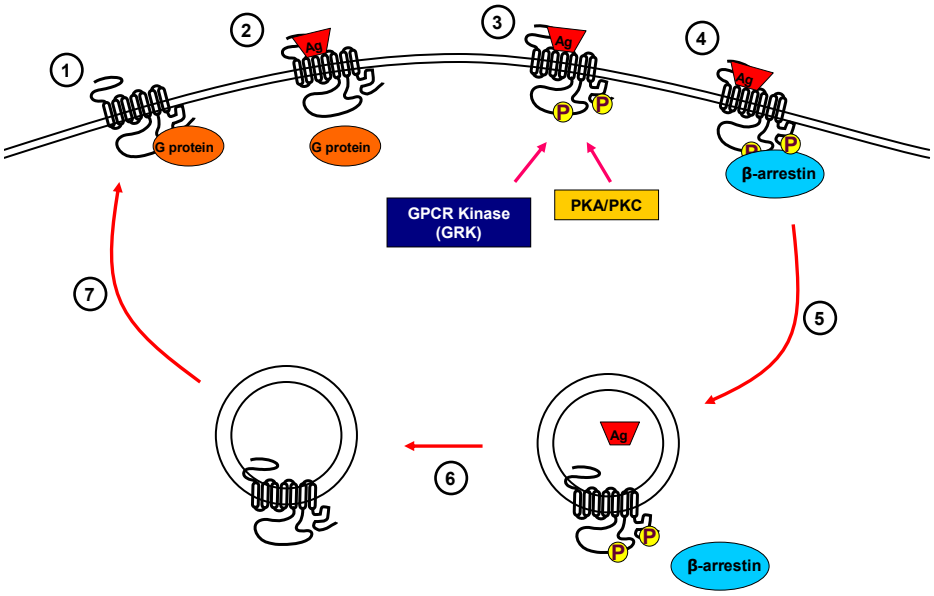

Figure 1.3: Illustration of GRK and/or second messenger kinases-mediated GPCR desensitization. (1) the agonist unoccupied GPCR associates with G protein in basal state, and (2) agonist (Ag) occupied GPCR loose affinity for G proteins, and releases. (3) GRK and/or second messenger kinases (PKA or PKC) phosphorylates 3rd intracellular loop domain and/or C-terminus tail of GPCR, and (4) it promotes β-arrestin binding, and desensitization, (5) leadding to GPCR internalization. (6) Internalized GPCR undergoes dissociation of agonist and dephosphorylation, and (7) dephosphorylated GPCR may be recycled or be targeted for degradation (downregulation of GPCR).

III.III. RKIP-mediated GPCR Desensitization and ERK Activation

Raf-1 kinase inhibitor protein (RKIP) is a 23-kDa protein originally found by yeast two hybrid screening in the search for interacting proteins for Raf-1 kinase domain. It is a member of phosphatidylethanolamine binding protein (PEBP) family (Yeung 1999). RKIP is known to bind to Raf-1, but it is not the kinase or substrate for Raf-1, rather it is an inhibitor for Raf-1 by interfering with Raf-1-mediated downstream phosphorylation, which leads to MEK/ERK activation. Therefore, interfering RKIP

activity results in ERK activation, and it is also suggests the role of RKIP in cancer progression since RKIP suppresses Raf-1-induced AP-1-mediated transcription and transformation (Yeung 2000). Moreover, the reduced levels of RKIP were reported in many human cancers, such as prostate (Fu 2003), breast (Hagan 2005), colorectal (Minoo 2007), and melanoma cancers (Park 2005).

However, RKIP-mediated ERK activation cannot be simply explained when GPCR and GPCR kinase 2 (GRK2) are added onto RKIP-mediated ERK activation because of the fact that GRK2-mediated receptor desensitization is also affected by RKIP activity (Lorenz 2003). The GRK family plays a major role in the phosphorylation of GPCRs, and this phosphorylation promotes receptor desensitization by recruiting β-arrestins. The RKIP activity is now known to regulate GRK2 activity for receptor phosphorylation and desensitization. As shown in Figure 1.4, RKIP suppresses Raf-1 activity leading to ERK activation in the basal state. However, when GPCRs and/or receptor tyrosine kinases are activated, PKC activity phosphorylates Serine 153 on RKIP. The phosphorylated RKIP (p-RKIP) looses the affinity for Raf-1 to activate the Raf-1/MEK/ERK signaling axis, and the dissociated p-RKIP prefers to bind GRK2 for inhibition to interfere with GPCR phosphorylation, leading to further inhibition of GPCR desensitization and internalization. This results in enhanced GPCR-mediated signaling (Lorenz 2003; Goel 2004; Huang 2007).

The most interesting finding from RKIP-mediated GPCR desensitization is that PKC is a pivotal modulator to integrate all receptor-mediated signaling pathways. In other words, PKC not only controls receptor-mediated ERK signaling, but it also regulates receptor activity by affecting receptor phosphorylation and desensitization. Taken together, those findings highly raise the role of RKIP in CCK2R desensitization, internalization, and receptor-mediated ERK activation, since PKC-mediated RKIP

phosphorylation interferes with GRK2 activity (Lorenz 2003). Furthermore, it is more interesting that RKIP is the substrate for PKC; *in vitro* kinase assay confirmed that the RKIP phosphorylation on Serine 153 is mediated by PKC-α, -βI, -βII, -γ, -δ and –ζ, and these are the members of cPKCs except PKC-δ, and -ζ (Corbit 2003; Lorenz 2003).

RKIP involvement in CCK2R desensitization and ERK signaling has not been studied yet. For this dissertation, I address the role of RKIP in CCK2R desensitization and ERK activation.

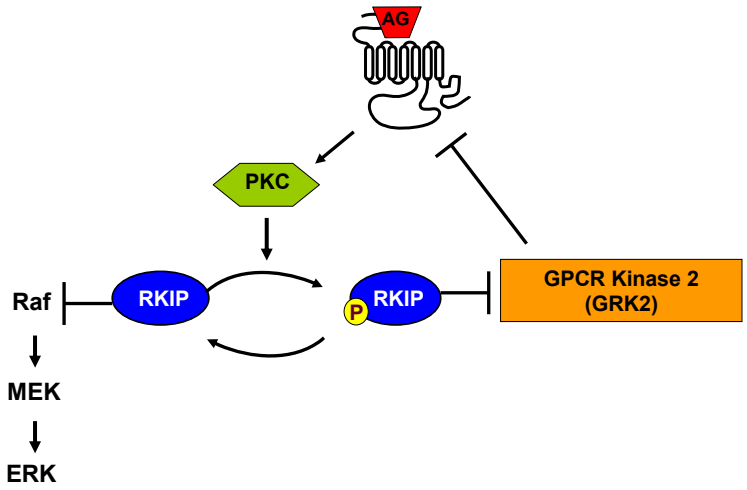

Figure 1.4: Illustration of RKIP-mediated ERK signaling and GPCR desensitization. In the basal state, RKIP physically blocks Raf-1-mediated ERK signaling by association. PKC activation by agonist-stimulated GPCRs resulted in phosphorylation at serine 153 on RKIP, which makes RKIP dissociation from Raf-1 to promote Raf-1/MEK/ERK signaling axis. Phosphorylated RKIP prefers to bind GRK2 for inhibiting GRK2-mediated GPCR phosphorylation and desensitization. Reduced receptor desensitization allows promote more ERK activation via PKC/MEK pathway.

IV. Specific Aims

As stated earlier, the main roles of CCK2R are gastric acid secretion, proliferation, and differentiation of gastric mucosa, and these various actions mediated by ERK1,2 activation. Therefore, it is critical to understand how CCK2R-mediated ERK1,2 activation is controlled and how receptor activity is finely modulated by receptor desensitization. The ultimate goal of my dissertation was to understand how CCK2R-mediated ERK1,2 activation is regulated and which modulators can conduct CCK2R desensitization. However, the mechanism of CCK2R desensitization is poorly understood in the relevance of receptor phosphorylation, the kinases involved, and the effects on receptor-mediated ERK activation. The growing body of evidence suggests that GRK-mediated GPCR desensitization is a universal mechanism for controlling receptor activity. Additionally, the finding that GRK activity can be controlled by RKIP activity via PKC-mediated phosphorylation in β2 adrenergic receptor highly suggests that RKIP may regulate CCK2R desensitization and ERK activation via the same mechanism. Therefore, the central hypothesis for my dissertation was RKIP mediates CCK2R desensitization and ERK activation by inhibition of GRK2 activity.

Through this dissertation, I focus on the function of RKIP in CCK2R desensitization and ERK activation. In Chapter 2, I address the function of RKIP in CCK2R desensitization. In Chapter 3, I address the function of RKIP in CCK2R-mediated ERK activation. The conclusions and future directions are addressed in Chapter 4.

V. The Cell Model for CCK2R

Trough this dissertation, I used a human embryonic kidney 293 (HEK293) cell line stably expressing CCK2R receptors as a cell model for measuring CCK2R

desensitization and ERK activation because there is no reliable human cell line endogenously expressing CCK2R. The HEK293 cell line originated from epithelial tissue, which is a major expressor for CCK2R. Also, the HEK293 cell line has historically been used to study GPCR signaling and desensitization because it is very easy to transfect and manipulate for various experimental purposes.

This cell line was established as follows: human CCK2R construct in pEGFP-N1 vector (BD Biosciences), which has an enhanced green fluorescent protein (EGFP) tag on its C-terminus, was stably transfected into HEK293 cells by using LipofectAMINE Plus Reagent (Invitrogen). The cells were designated as HEK-CCK2R. Briefly, 4.0×10^5 HEK293 cells were seeded onto 6-well plates one day before transfection; the following day, 2 μg of plasmids were transfected into the cells with ~80% confluence using LipofectAMINE Plus. After the transfection, the cells were initially selected under G418 (800 μg/ml) to enrich the positive transfectants, and the cells expressing EGFP-tagged receptor constructs were selected by FACSAria cell sorter in the University of Texas Medical Branch Flow Cytometry and Cell Sorting Core Facility. The receptor expression level of the cell line was measured by agonist binding using [^{125}I]-cholecystokinin octapeptide (1.2 pmol of receptor/mg of membrane protein). In maintaining the CCK2R-transfected HEK293 cell line, the cells were grown in Dulbelcco's Modified Eagle's Medium (DMEM, Cellgro® Mediatech), supplemented with 400 μg/ml G418 and 10% heat-inactivated fetal bovine serum (FBS, Cellgro® Mediatech) in a 5% CO^2 atmosphere at 37°C.

Chapter 2. CCK2R Desensitization

I. Introduction

For receptor desensitization, the following combined events are generally required: receptor phosphorylation, release of G proteins, and receptor internalization to intracellular compartments (Ferguson 2001). Receptor phosphorylation is a very rapid response (seconds) to agonist stimulation, and it leads to dissociation of G proteins from receptors. Bonovic *et al*. discovered that β2 adrenergic receptor kinase (now known as GRK2) phosphorylates receptors in an agonist-dependent manner (Benovic 1986). Second-messenger kinases (protein kinase A [PKA], or protein kinase C [PKC]) had been considered major kinases involved in receptor phosphorylation. However, accumulated evidence confirmed that most of GPCRs are the substrates for GRKs, and GRK-mediated receptor phosphorylation is now considered a more universal mechanism than second messenger kinase-mediated GPCR desensitization. As serine/threonine kinases, both GRKs and second-messenger kinases phosphorylate amino residues within the third intracellular loop domain and/or the C-terminus tail domain on GPCRs (Pitcher 1998).

Raf-1 kinase inhibitor protein (RKIP) was originally found as an inhibitory protein for Raf-1, a mediator for ERK signaling, by yeast two-hybrid screening. In its basal state, RKIP suppresses Raf-1 activity by formation of the RKIP/Raf-1 complex, which results in interfering with formation of the Raf-1/MEK/ERK complex for ERK activation. The phosphorylation of RKIP, which is an active state, dissociates from Raf-1 allowing the formation of an ERK-signaling complex (Yeung 1999). Moreover, in 2003 Lorenz et al. revealed that RKIP also modulates β2-adrenergic receptor desensitization by inhibiting GRK2 activity when PKC phosphorylates RKIP. In this

study, they showed agonist-induced PKC activation resulted in the phosphorylation of Serine 153 on RKIP, which lowered its high affinity for binding Raf-1 for inhibition. Then, phosphorylated RKIP was dissociated from Raf-1 and binds to GRK2 for physical inhibition to reduce GRK2-mediated receptor desensitization (Lorenz 2003). Since *in vitro* kinase assays confirmed that RKIP phosphorylation on Serine 153 is mediated by PKC-α, -βI, -βII, -γ, -δ and –ζ (Corbit 2003; Lorenz 2003), it is possible to consider that CCK2R-induced PKC activity may phosphorylate RKIP to inhibit CCK2R desensitization via the same mechanism. Therefore, the working hypothesis through this chapter was that PKC-dependent RKIP phosphorylation regulates CCK2R desensitization via inhibition of GRK2. To test this hypothesis, the function of PKC in CCK2R desensitization was addressed, and the function of RKIP in CCK2R desensitization was addressed. Then, the specific mechanism for RKIP-mediated CCK2R desensitization was addressed.

II. PKC mediated CCK2R desensitization

As mentioned previously, GPCR desensitization mediated by receptor phosphorylation, and the PKC family is one of the potential kinases involved in GPCR phosphorylation. The question addressed was whether PKC inhibition affects CCK2R desensitization. To figure out PKC involvement in CCK2R desensitization, the PKC activity was chemically introduced with PKC inhibitors. The strength of CCK2R activity was determined by measuring the change in intracellular calcium concentration $[Ca^{2+}]i$ with G17 stimulation, as an indicator for CCK2R activity. In CCK2R, Gq-mediated phospholipase C (PLC) activation leads to the hydrolysis of membrane for generating inositol-1,4,5-triphosphate (IP3), 1,2,-diacylglycerol (DAG). IP3 binds to ligand-gated Ca^{2+} channels on endoplasmic reticulum (ER), and it induces a rapid release

of Ca^{2+} from ER (Hamm 1998). To measure the receptor activity as an indicator for receptor desensitization, the change in $[Ca^{2+}]i$ by a series of G17 stimulations was monitored by employing the Ca^{2+} binding dye, Fura 2/AM. Then, Ca^{2+} concentration was calculated by the ratio of fluorescence emissions at both 340 nm and 380 nm.

Receptor Desensitization by Intracellular Ca^{2+} Imaging

Receptor desensitization was observed by using agonist-induced increases in $[Ca^{2+}]i$ as a measure of receptor activity. Gastrin (G17) induced the change in $[Ca^{2+}]i$ and was monitored by measuring the Ca^{2+} binding dye, Fura 2/AM (Invitrogen). HEK-CCK2R cells were grown onto 25-mm laminin-coated coverslips in DMEM, 10% FBS with 50% confluence, and washed with a physiological medium (25 mM HEPES pH 7.4, 125 mM NaCl, 5 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 6 mM glucose). The cells were incubated with 2 μM Fura2/AM in the same medium for 1 hour. To discriminate Ca^{2+} releasing from ER and Ca^{2+} channel-induced Ca^{2+} influx on the plasma membrane, Ca^{2+} influx was removed by using calcium-free physiological medium through the experiments. The cells were pretreated for 15 min with either vehicle (dimethyl sulfoxide, DMSO), 5 μM GF109203X (Calbiochem), which is a pan PKC inhibitor for downregulating both cPKCs (PKC- α, -βI, -βII, -γ) and nPKCs (PKC - θ, -δ, -ε, -η), or 5 μM Gö6976, (Calbiochem) for inhibiting cPKC activity only. The cells were then challenged by a series of 1-nM G17 stimulations to desensitize receptor activity, and the amplitude of increase in $[Ca^{2+}]i$ was measured by using a Nikon Diaphot inverted microscope and a CCD camera (Dage-MTI Inc.). At least 30 cells were analyzed per time point in each experiment, and each experiment was performed three times. For statistical analysis, the changes of peak height were compared by using an

one-way ANOVA, and Bonferroni post-tests were performed to compare values at each timepoint (***, $p < 0.001$).

Results and Discussion

As shown in Figure 2.1.A, with successive application of G17, the gradual decrease in magnitude of calcium response was observed in vehicle-treated cells, which indicates receptors were desensitized by agonist stimulation. The peaks of $[Ca^{2+}]i$ were gradually decreased with the series of G17 stimulations, indicating that CCK2R was desensitized to continuous G17 stimulation for a time course (Figure 2.1.D). In both PKC inhibitors treated, the overall amplitudes of $[Ca^{2+}]i$ were decreased compared to vehicle control; moreover, the peaks of $[Ca^{2+}]i$ were almost lost after a third G17 stimulation, suggesting that CCK2R became desensitized faster when PKC activity was inhibited (Figure 2.1.B and C). In particular, Gö6976-treated cells, in which cPKCs were inhibited, exhibited almost no responsiveness to the G17 stimulation after the second challenge (Figure 2.1.B). This finding implies that the inhibition of cPKC is enough to enhance CCK2R desensitization considering that GF109203X inhibits both PKC and nPKC families together. The peak-height of the curve for $[Ca^{2+}]i$ by G17 stimulation is represented in Figure 2.1.D. In this graph, both PKC inhibitor treatments show enhanced desensitization compared to vehicle-treated cells, and it also implies that the responsiveness of CCK2R to G17 is reduced when PKCs were inhibited in the cells. This finding suggests that PKC activity inhibits CCK2R desensitization by affecting receptor desensitization machineries, and cPKC activity has a role in controlling CCK2R desensitization. Furthermore, this data implies that cPKCs may act as antagonists for the inhibition of GRKs, which promote receptor desensitization by phosphorylating receptors.

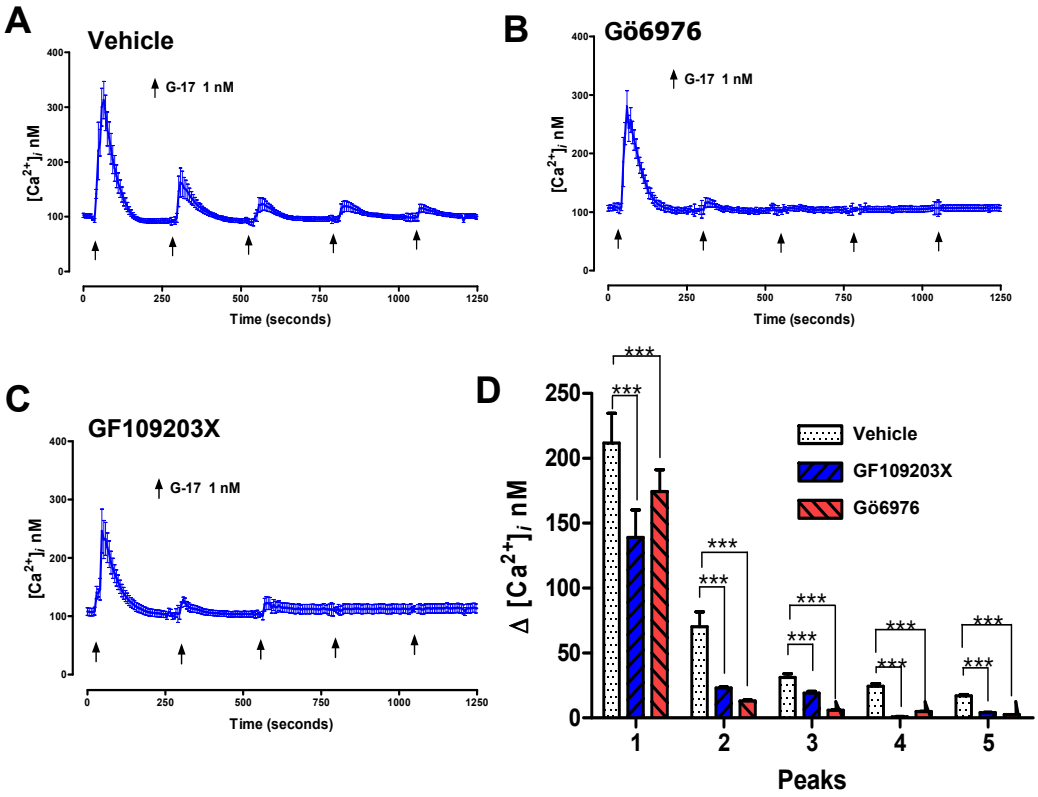

Figure 2.1: PKC-mediated, agonist-dependent CCK2R desensitization.
HEK-CCK2R cells were grown onto lamin-coated cover slips one day before Ca^{2+} imaging, and either vehicle, Gö6976, or GF109203X were pretreated for 15 min. The cells were challenged by a series of 1-nM G17 stimulations to desensitize receptor activity. **A**, **B**, and **C**, are the representative graphes for the changes of $[Ca^{2+}]i$ in vehicle, Gö6976, or GF109203X-treated cells respectively. The arrow indicates 1 nM G17 stimulation. **D**. The changes of peak-height of $[Ca^{2+}]i$ at G17 stimulation were plotted.

The Effects of PKC Inhibitors on Agonist Binding onto CCK2R

The previous data suggests the role of PKCs in CCK2R desensitization; however, if these PKC inhibitors lower agonist binding on CCK2R by affecting agonist affinity, the fast desensitization observed would be a side effect of both Gö6976 and GF109203X, not by inhibiting PKC isozymes. Therefore, it was critical to show that both chemical

inhibitors have minimal effects on agonist binding to justify the role of PKC in receptor desensitization.

To assess the effects of PKC inhibitors on agonist binding onto CCK2R, radio ligand binding experiments were performed using [^{125}I]-cholecystokinin octapeptide ([^{125}I]-CCK-8), which shows the same affinity as G17 because they share the last five C-terminal amino acids with amidated modification. For measuring specific binding, non-specific binding was defined by applying excessive amounts of unlabelled CCK-8 for competition. Also, to avoid artifact by agonist-induced receptor internalization, crude plasma membrane was used for the binding experiments.

Membrane Preparation

For membrane preparation, HEK-CCK2R cells were grown onto 2 of 150-mm dishes with 90% confluence, and the cells were washed with 20 ml of phosphate-buffered saline (PBS) and incubated with 10 ml of solution A (10 mM Hepes, pH 7.4, 1 mM EGTA, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 100 μM benzaminidine) for swelling cells for 20 min at 4°C. The cells were harvested and placed in an ice cold 50 ml Dounce homogenizer. The cells were gently homogenized by 20-22 strokes with the tight pestle, and 1 mM phenylmethylsulfonyl fluoride (PMSF) was added just before homogenizing. The post nuclear membrane fraction was separated from the nuclei and cell debris by centrifugation at 750 × *g* for 15 min at 4°C, and only the supernatant was carefully collected for another centrifugation at 75,000 × *g* for 45 min at 4°C. The pellet was resuspended with Solution A containing 12% sucrose, and the aliquots for this membrane-enriched fraction were quick frozen in liquid nitrogen and stored -80°C until radio ligand binding experiment was performed.

Radio Ligand Binding

Prepared HEK-CCK2R membrane was thawed on ice, and protein concentration was determined by Bradford protein assay. For the radio ligand binding experiment, 0.4 μg of membrane fraction was incubated with either vehicle (DMSO), 5 μM GF109203X, or 5 μM Gö6976 in binding solution (20 mM Hepes pH 7.4, 100 mM NaCl, 0.3% BSA, 3 mM $MgSO_4$, 1 mM EGTA) for 15 min, then 250 pM of [^{125}I]-CCK-8 (specific activity = 2200 Ci/mmol, PerkinElmer Life Sciences) was added for initiating binding at 30°C up to 90 minutes to reach the equilibrium. For non-specific binding, another set of binding reactant was prepared, and 1 μM of unlabelled CCK-8 was added for competing radio-labeled CCK-8. After the time course, the binding reaction was terminated by placing reactants into an ice bath and adding 4 ml of ice cold solution B (20 mM Tris pH 8.0, 100 mM NaCl, 25 mM $MgCl_2$) to the reaction, and radio ligand-receptor complexes were harvested by filtration over 25 mm circular GF/F (pore size 0.7 μm) glass fiber filters (Whatman). The filters were washed four times with 4 ml of ice cold solution B, then transferred to glass tubes for measuring the amount of radioactivity in a Cobra II gamma counter (Parkard Instrument Company). All reactions were run in triplicate for accuracy, and total binding was managed less than 8% of the total counts added to the assays. For measuring specific binding (SB), the averages of non-specific binding (NB) for each set of experiments were subtracted from the amounts of total binding. For analyzing binding data, the values of SB were plotted for a time course and analyzed using the one sit binding hyperbola equation ($Y = Bmax \times X / (Kd + X)$) by Prism 4.0 software (GraphPad Software).

Results and Discussion

As shown in Figure 2.2, the pretreatment with either vehicle, Gö6976, or GF109203X did not significantly affect radio ligand binding kinetics or the amount of ligand binding (Bmax) onto CCK2R, implying that both chemicals have negligible effects on agonist-receptor binding affinity. Therefore, I assumed that faster desensitization by chemical PKC inhibitors indeed resulted from the inhibition of PKC activities, not from interfering with G17 binding affinity on the receptors.

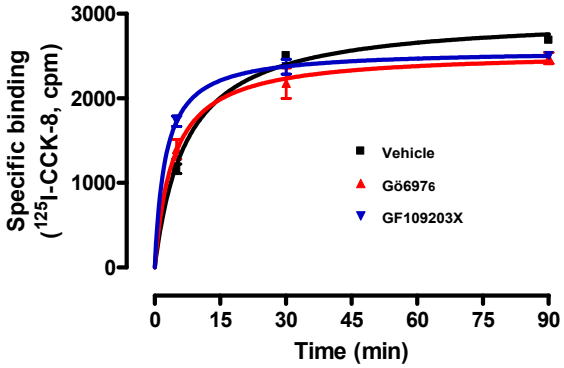

Figure 2.2: The effects of PKC inhibitors agonist binding onto CCK2R.
Radio-ligand binding experiments were performed using ^{125}I-CCK-8 with prepared crude membrane from HEK-CCK2R. The membrane was pretreated with either vehicle (black), Gö6976 (red), or GF109203X for 15 min before adding radio ligands. The upper graph shows non-linear progression curves with a time course by one site binding hyperbola. The numbers below are the values for the binding experiment.

PKC-α dependent CCK2R Desensitization

As shown in Figure 2.1, the inhibition of cPKC activity resulted in fast CCK2R desensitization. The next question was which cPKCs regulate CCK2R desensitization. The cPKC family is composed of PKC-α, β, γ isozymes. To avoid the side effects of chemical inhibitors, individual PKC isozymes were inhibited by employing a siRNA technique. As a first approach, the expression of endogenous PKC-α was downregulated by double-stranded siRNA for PKC-α (Santa Cruz Biotech) in HEK-

CCK2R cells, and the effect of this knockdown on CCK2R desensitization was addressed.

PKC-α siRNA Transfection and CCK2R Desensitization

One day before siRNA transfection, HEK-CCK2R cells were split onto 6-well plates in DMEM containing 10% FBS without antibiotics to reach ~50% confluence on the day of transfection. Either 400 pmole of non-target (Santa Cruz Biotech) or PKC-α siRNA (Santa Cruz Biotech) was diluted in 250 µl of OpTi-MEM (Invitrogen), and also each 5 µl of Lipofectamine™ 2000 (Invitrogen) was diluted in another 250 µl of OpTi-MEM. After 5 minutes of incubation at room temperature (RT), both the diluted siRNAs and the diluted Lipofectamine™ 2000 were combined and mixed gently together and incubated for 20 minutes at RT. Then, the siRNA-Lipofectamine™ 2000 complexes were added onto the cells. After 24 hours of transfection, the cells were plated onto laminin-coated cover slips for desensitization study. After 72 hours of transfection, CCK2R desensitization was monitored by challenging 0.1 nM G17 as previously described. The reduction level of PKC-α was determined by immunoblotting using an anti-PKC-α antibody (Santa Cruz Biotech). For statistical analysis of CCK2R desensitization, the changes of peak height by G17 stimulation were compared by using an one-way ANOVA between the non-target and PKC-α siRNA transfected cells, and Bonferroni post-tests were performed to compare values at each timepoint (***, $p < 0.001$).

Results and Discussion

Because chemical inhibition of cPKCs resulted in enhancing desensitization, the first question was whether the silencing PKC-α, which belongs to cPKC family, can

mimic enhancing receptor desensitization as shown by chemical inhibitions of cPKCs. After the transfection of either PKC-α siRNA or non-target siRNA into HEK-CCK2R cells, the receptor desensitization was monitored by Ca^{2+} imaging, as seen in Figure 2.3.B and C. In this experiment, PKC-α siRNA-treated cells were more sensitive to G17 stimulation and generated higher peaks than non-target siRNA control cells (Figure 2.3.C), even though PKC-α siRNA did not block the expression of PKC-α completely (Figure 2.3.A, upper panel). This suggests that PKC-α activity is required for normal CCK2R desensitization. In other words, PKC-α activity is not involved in inhibiting receptor desensitization, which was observed by inhibiting the cPKC family (PKC-α, -β, -γ) together. Recently, Bailey et al. reported that the inhibition of PKC-α by a chemical inhibitor resulted in attenuated desensitization of μ-opioid receptor, and it also was observed in PKC-α knockout mice (Bailey 2009). These reports coincide with the finding that PKC-α siRNA promoted attenuated CCK2R desensitization. One possible explanation for this is PKC-α enhances GRK2 activity by its phosphorylation. *In vitro* kinase assay indicated that GRK2 was phosphorylated by purified PKC-α, -γ, -δ, not by PKC-β, and also that Serine 29 on GRK2 is phosphorylated by PKC-α in HEK293 cells (Krasel 2001). Another possibility to consider is that PKC-α phosphorylates CCK2R directly for receptor desensitization. If PKC-α can phosphorylate either GRKs or CCK2R directly, it is possible that the knockdown of PKC-α results in attenuation of agonist-mediated receptor desensitization.

In this study, the knockdown of PKC-α did not promote faster desensitization, which was observed in previous experiments using chemical inhibition of the cPKC family together. Rather, the inhibition of PKC-α resulted in slow CCK2R desensitization compared to control, and this result was opposite of what was expected. Therefore, to find cPKC isozymes responsible for inhibiting CCK2R desensitization, the

remaining cPKC isozymes, such as PKC-β, and –γ, should be tested to prove their individual role in CCK2R desensitization.

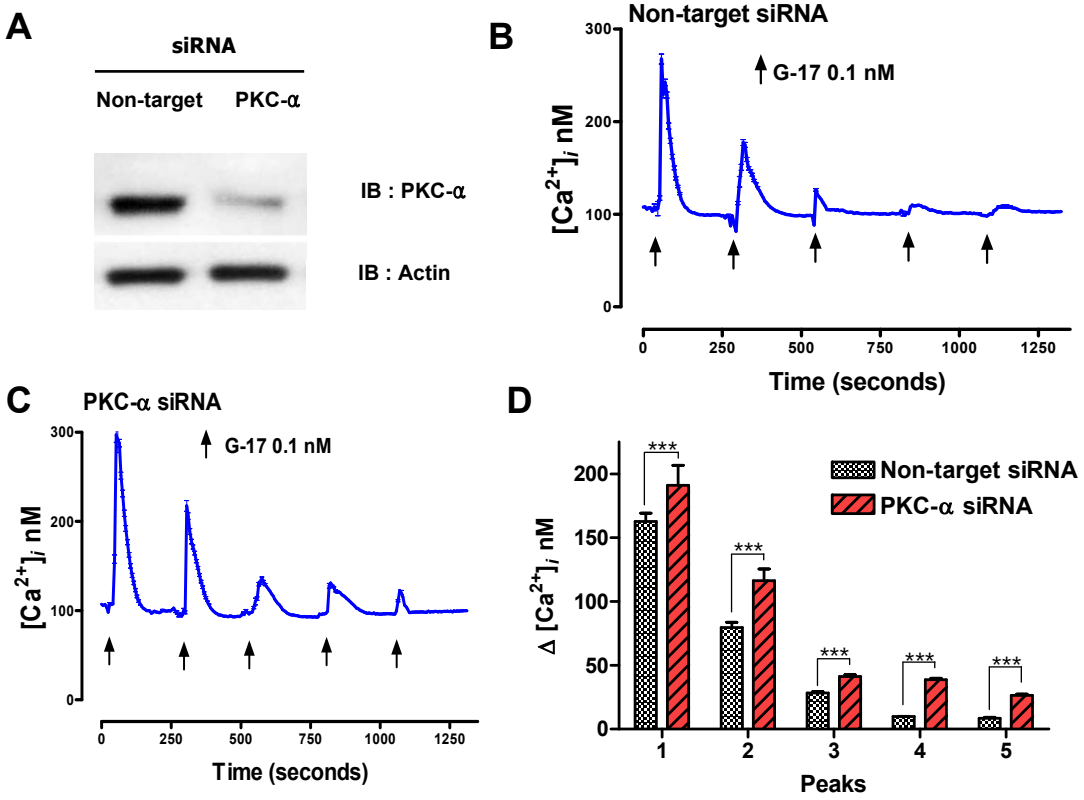

Figure 2.3: The role of PKC-α in CCK2R desensitization.
HEK-CCK2R cells were transfected with either non-target siRNAs (control) or PKC-α siRNAs. After 72 hours of siRNA transfection, CCK2R desensitization was monitored by Ca^{2+} imaging. **A**. To determine the knockdown of PKC-α by siRNA, an immunoblot was performed by using an antibody for PKC-α, and this blot was reproved with an anti-actin antibody to show equivalent amount of proteins were loaded onto each lane. **B**. The representative graph for the changes of $[Ca^{2+}]i$ in HEK-CCK2R transfected with non-target siRNA (control). The arrow indicates the series of G17 stimulation. **C**. The representative graph for the changes of $[Ca^{2+}]i$ in PKC-α siRNA transfected cells. **D**. The changes of peak-height of $[Ca^{2+}]i$ at G17 stimulation were plotted.

PKC-β dependent CCK2R Desensitization

To further screen cPKCs involved in CCK2R desensitization, the role of PKC-β in CCK2R desensitization was addressed. In the cPKC family, only PKC-β is not known to phosphorylate GRK2, which mediates agonist-dependent GPCR

phosphorylation. Therefore, it was reasonable to consider that PKC-β has an inhibitory role in receptor desensitization. To test this hypothesis, the role of PKC-β in CCK2R desensitization was assessed by inhibiting endogenous expression of PKC-β by siRNA transfection.

PKC-β siRNA Transfection and CCK2R Desensitization

PKC-β has two isoforms by alternative splicing. PKC-βII is a splice variant for PKC-βI, and both share the majority of the sequence together. Since PKC-βII has some distinctive additional amino acid residues compared to PKC-βI, PKC-β siRNA (Santa Cruz Biotech), which is specific for common sequence to both isozymes, was employed for knockdown.

One day before siRNA transfection, HEK-CCK2R cells were split onto 6-well plates in DMEM, 10% FBS without antibiotics to reach ~50% confluence on the day of transfection. Either 200 pmole of non-target (Santa Cruz Biotech) or PKC-β siRNA (Santa Cruz Biotech) was diluted in 250 µl of OpTi-MEM (Invitrogen). Each 6 µl of Lipofectamine^{TM} 2000 (Invitrogen) was diluted in another 250 µl of OpTi-MEM. After 5 minutes of incubation at RT, both the diluted siRNAs and the diluted Lipofectamine^{TM} 2000 were combined and mixed gently together and incubated for 30 minutes at RT. Then, the siRNA-Lipofectamine^{TM} 2000 complexes were added onto the cells. After 24 hours of transfection, the cells were plated onto laminin-coated cover slips for desensitization study. After 72 hours of transfection, CCK2R desensitization was monitored by challenging 0.1 nM G17 as previously described. The reduction level of PKC-β was determined by immunoblotting using an anti-PKC-βII antibody (Santa Cruz Biotech), which recognize PKC-βI and PKC-βII together. For statistical analysis of CCK2R desensitization, the changes of peak height by G17 stimulation were compared

by using an one-way ANOVA between the non-target and PKC-β siRNA transfected cells, and Bonferroni post-tests were performed to compare values at each time point (***, $p < 0.001$; *, $P < 0.05$).

Results and Discussion

The desensitization experiments using PKC-β siRNA indicated that PKC-β siRNA-treated cells were more sensitive to G17 and generated higher peaks of $[Ca^{2+}]i$ by G17 stimulation than non-target siRNA control cells (Figure 2.4.B and C). This result implies that PKC-β activity is required for normal CCK2R desensitization, and PKC-β activity is not interfering with agonist-dependent CCK2R desensitization. As seen in Figure 2.1, cPKC inhibition by Gö6976 resulted in enhanced G17-induced CCK2R desensitization. However, the individual knockdown of either PKC-α or –β did not mimic the treatment of Gö6976, rather both PKCs appeared to be required for G17-mediated CCK2R desensitization. The remaining candidate for cPKC-mediated inhibition of CCK2R desensitization is PKC-γ, however this possibility appears to be unlikely considering a report that PKC-γ can phosphorylate GRK2 *in vitro* (Krasel 2001). Because PKC-mediated GRK2 phosphorylation eventually activates GRK2 to phosphorylate agonist-occupied receptors, it should desensitize receptors. However, it is still worth trying to knockdown PKC-γ for addressing cPKC involvement in inhibition of G17-induced CCK2R desensitization, because the aforementioned experiments were performed by *in vitro* kinase assay using purified PKC-γ and GRK2, which does not simply mean that PKC-γ activity promotes GRK2 phosphorylation and induces more CCK2R desensitization in HEK293 cells. It is also possible that the PKC inhibitions with PKC-specific chemical reagents are not specific to target only PKC isozymes. There are some possible side effects of GF109203X and Gö6976. Other kinases or

proteins involved in CCK2R desensitization might be affected by them. Moreover, it is also possible that individual knockdowns of cPKCs by specific siRNAs may not be the same as the chemical-induced knockdown of cPKCs together.

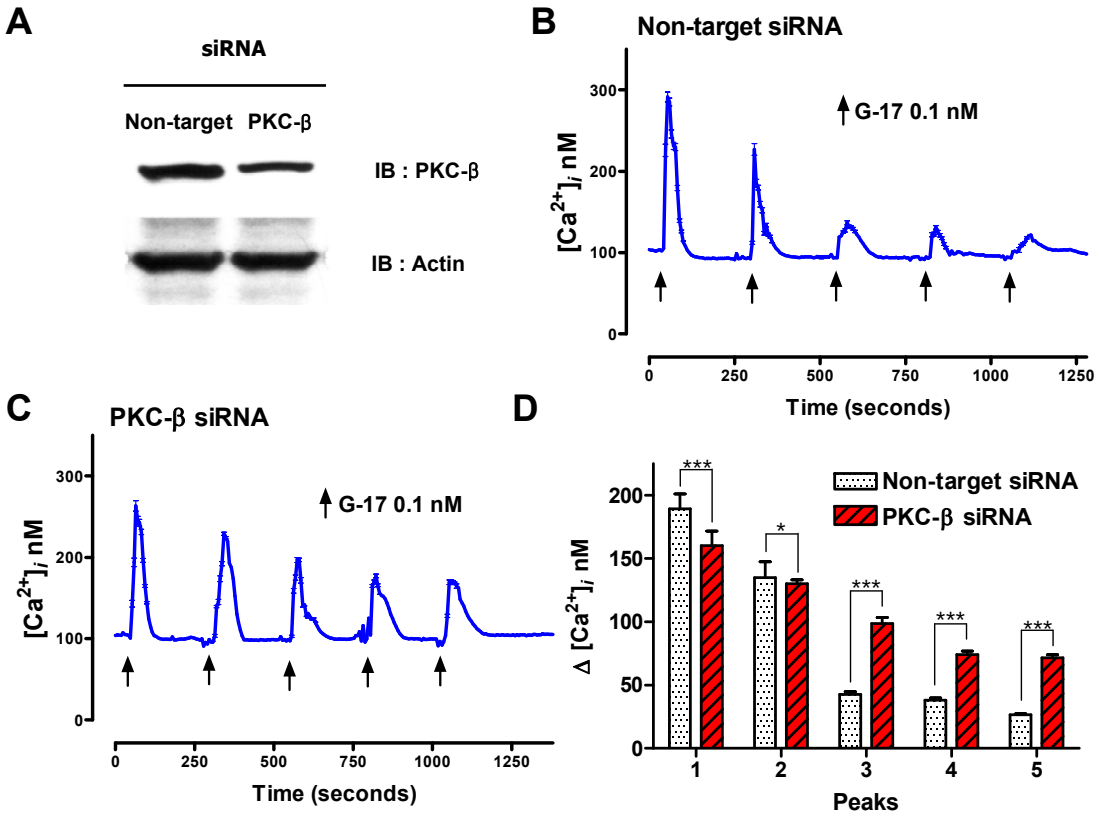

Figure 2.4: The role of PKC-β in CCK2R desensitization.
HEK-CCK2R cells were transfected with either non-target siRNAs (control) or PKC-β siRNAs. After 72 hours of siRNA transfection, CCK2R desensitization was monitored by Ca^{2+} imaging. **A**. To determine the knockdown of The changes of $[Ca^{2+}]i$ in HEK-CCK2R transfected with non-target siRNA (control). by siRNA, an immunoblot was performed by using an antibody for PKC-βII, and this blot was reproved with an anti-actin antibody to show equivalent amount of proteins were loaded onto each lane. **B**. The representative graph showing the changes of $[Ca^{2+}]i$ in HEK-CCK2R transfected with non-target siRNA (control). The arrow indicates the series of G17 stimulation. **C**. The representative graph showing the changes of $[Ca^{2+}]i$ in PKC-β siRNA transfected cells. **D**. The changes of peak-height of $[Ca^{2+}]i$ at G17 stimulation were plotted.

PKC-δ dependent CCK2R Desensitization

PKC-δ is one of the nPKC isozymes, and it is known to be activated by G17-mediated CCK2R (Ogasa 2003). Therefore, it is possible that G17-mediated PKC-δ activation may act as a negative regulator for CCK2R activation, because activated PKC-δ may desensitize receptors via phosphorylation. However, in previous data, the treatment with GF109203X, which blocks both cPKCs and nPKCs together, indicated enhanced CCK2R desensitization even though inhibition of cPKCs by Gö6976 was enough to show enhanced CCK2R desensitization. To address the role of PKC-δ in CCK2R desensitization, the effect of PKC-δ knockdown by siRNA on CCK2R desensitization was measured by Ca^{2+} imaging.

PKC-δ siRNA Transfection and CCK2R Desensitization

One day before siRNA transfection, HEK-CCK2R cells were split onto 6-well plates in DMEM, 10% FBS without antibiotics to reach ~50% confluence on the day of transfection. Either of 200 pmole of non-target (Santa Cruz Biotech) or PKC-δ siRNA (Santa Cruz Biotech) was diluted in 250 µl of OpTi-MEM (Invitrogen). Each 6 µl of Lipofectamine™ 2000 (Invitrogen) was diluted in another 250 µl of OpTi-MEM. After 5 minutes of incubation at room temperature (RT), both the diluted siRNAs and the diluted Lipofectamine™ 2000 were combined and mixed gently together and incubated for 30 minutes at RT. Then, the siRNA-Lipofectamine™ 2000 complexes were added onto the cells. After 24 hours of transfection, the cells were plated onto laminin-coated cover slips for desensitization study. After 72 hours of transfection, CCK2R desensitization was monitored by challenging 0.1 nM G17 as previously described. The reduction level of PKC-δ was determined by immunoblotting using an anti-PKC-δ antibody (Santa Cruz Biotech), and this blot was reprobed with an anti-actin antibody for

loading control. For statistical analysis of CCK2R desensitization, the changes of peak height by G17 stimulation were compared by using an one-way ANOVA between the non-target and PKC-δ siRNA transfected cells, and Bonferroni post-tests were performed to compare values at each time point (***, $p < 0.001$).

Results and Discussion

The desensitization experiments using PKC-δ siRNA showed that PKC-δ siRNA-treated cells were much slower in G17-induced desensitization compared to non-target siRNA control. As seen in Figure 2.7, the knockdown of PKC-δ indicated much higher and distinctive peaks of $[Ca^{2+}]i$ by a series of G17 stimulations than non-target siRNA control cells (Figure 2.5.A and B). As with previous data using either PKC-α, or PKC-β siRNA (Figures 2.3 and 2.4), PKC-δ activity also appears to be required for normal CCK2R desensitization.

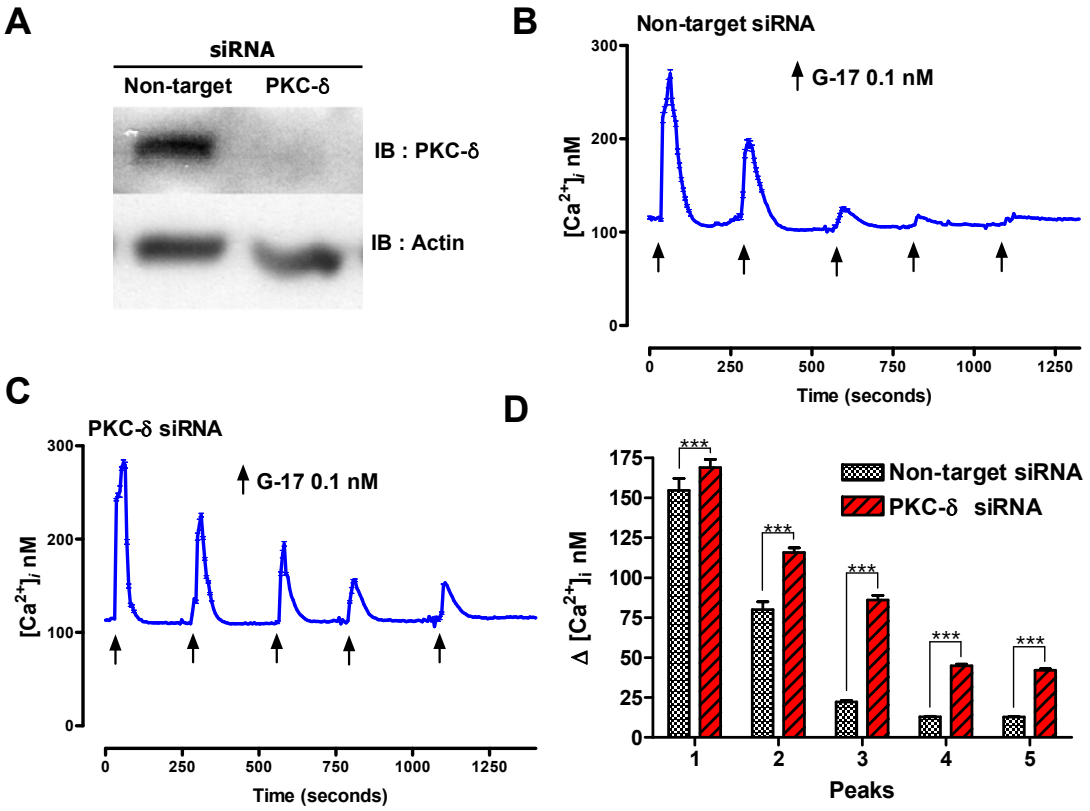

Figure 2.5: The role of PKC-δ in CCK2R desensitization.
HEK-CCK2R cells were transfected with either non-target siRNAs (control) or PKC-δ siRNAs. After 72 hours of siRNA transfection, CCK2R desensitization was monitored by Ca^{2+} imaging. **A**. The PKC-δ levels of reduction by either non-target or PKC-δ siRNA were determined by immunoblot (upper panel), and the levels of actin were measured as loading controls (bottom panel). **B**. The representative graph showing the changes of $[Ca^{2+}]i$ in HEK-CCK2R transfected with non-target siRNA (control). **C**.The arrow indicates the series of G17 stimulation. The representative graph showing the changes of $[Ca^{2+}]i$ in PKC-δ siRNA transfected cells. **D**. The changes of peak-height of $[Ca^{2+}]i$ at G17 stimulation were plotted.

III. GRK2-dependent CCK2R Desensitization

The inhibition of PKC promoted enhanced CCK2R desensitization in the previous data was very interesting because PKC-dependent RKIP activity is known to interfere with GPCR desensitization by inhibiting GRK2. GRK2-mediated GPCR desensitization is well established in many other receptors, however the role of GRK2 in CCK2R desensitization has not been addressed. If GRK2 acts as a modulator for CCK2R desensitization, RKIP may interfere with GRK2 activity via PKC-dependent association. Therefore, the first question was whether GRK2 is involved in CCK2R desensitization. To address the question, the role of GRK2 in CCK2R desensitization was determined by inhibiting GRK2 using siRNA techniques.

GRK2 siRNA Transfection and Measuring CCK2R Desensitization

One day before siRNA transfection, HEK-CCK2R cells were split onto 6-well plates in DMEM, 10% FBS without antibiotics to reach ~50% confluence on the day of transfection. Either of 200 pmole of non-target (Santa Cruz Biotech) or GRK2 siRNA (Santa Cruz Biotech) was diluted in 250 µl of OpTi-MEM (Invitrogen), and also each 5 µl of Lipofectamine^{TM} 2000 (Invitrogen) was diluted in another 250 µl of OpTi-MEM. After 5 minutes of incubation at RT, both the diluted siRNAs and the diluted Lipofectamine^{TM} 2000 were combined and mixed gently together and incubated for 20 minutes at RT, then the siRNA- Lipofectamine^{TM} 2000 complexes were added onto the cells. After 24 hours of transfection, the cells were plated onto laminin-coated cover slips for desensitization study. After 72 hours of transfection, CCK2R desensitization was monitored by challenging 1 nM G17 as previously described, and the level of GRK2 knockdown was assessed by immunoblotting using an anti-GRK2 antibody (Santa Cruz Biotech). For statistical analysis of CCK2R desensitization, the changes of peak height

by G17 stimulation were compared by using an one-way ANOVA between the non-target and GRK2 siRNA transfected cells, and Bonferroni post-tests were performed to compare values at each time point (***, $p < 0.001$).

Results and Discussion

After 72 hours of transfection with either GRK2 siRNA or non-target siRNA, the receptor desensitization was monitored by Ca^{2+} imaging (Figure 2.6.B and C). Compared to the non-target control group, the GRK2 inhibition by siRNA exhibited more distinct Ca^{2+} peaks with a series of G17 stimulations. This observation implies that CCK2R is more sensitive to G17 stimulation when the level of GRK2 was inhibited by siRNA, indicating the role of GRK2 in CCK2R desensitization. It is reasonable, therefore, to think that GRK2 activity mediates CCK2R phosphorylation leading to receptor desensitization. It was not surprising to know that GRK2 regulates CCK2R desensitization because GRK-mediated GPCR desensitization is a well established mechanism for GPCR regulation. However, GRK2 involvement in CCK2R desensitization was very interesting, because it increases the possibility that RKIP regulates GRK2 activity for CCK2R desensitization. As stated earlier, several published articles indicated the role of RKIP in regulating GPCR desensitization by controlling GRK2 activity for receptor phosphorylation. PKC has also been shown to phosphorylate RKIP, which leads to exchange interacting partner molecules from Raf-1 to GRK2. If the same mechanism applies to CCK2R desensitization, it can explain previous data that shows inhibition of cPKCs resulted in enhanced CCK2R desensitization because RKIP-mediated GRK2 inhibition would be impaired if PKC activity was inhibited. Therefore, it was critical to clarify the involvement RKIP in CCK2R desensitization. To answer the question, the effect of RKIP on CCK2R

desensitization was monitored by transfecting RKIP siRNA into HEK-CCK2R cells. Moreover, the role of p-RKIP in CCK2R desensitization was elucidated by using a phosphorylation mutant for RKIP.

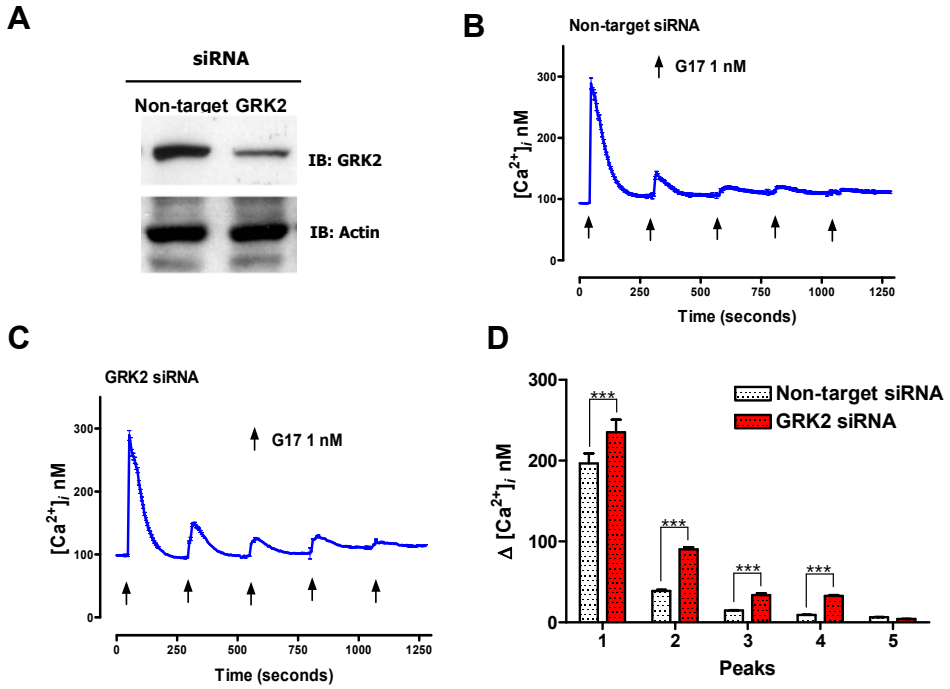

Figure 2.6: The role of GRK2 in CCK2R desensitization.
HEK-CCK2R cells were transfected with either non-target siRNA (control) or GRK2 siRNA, then agonist-induced CCK2R desensitization was monitored by challenging 1 nM G17. **A**. The level of GRK2 knockdown was assessed by immunoblotting using an anti-GRK2 antibody (upper panel), and the levels of actin were measured as loading controls (bottom panel). **B**. The representative graph showing the changes of $[Ca^{2+}]i$ in HEK-CCK2R transfected with non-target siRNA (control). The arrow indicates the series of G17 stimulation. **C**. The representative graph showing the changes of $[Ca^{2+}]i$ in GRK2 siRNA-transfected cells. **D**. The changes of peak-height of $[Ca^{2+}]i$ at G17 stimulation were plotted.

IV. RKIP-mediated CCK2R Desensitization

The involvement of both PKC and GRK2 in CCK2R desensitization in the previous data strongly raised the potential role of RKIP in CCK2R desensitization. The working hypothesis was that RKIP negatively regulates CCK2R desensitization. To

address the question, the endogenous RKIP was silenced by transfecting RKIP siRNA (Santa Cruz Biotech) into HEK-CCK2R cells. The question addressed was whether the inhibition of RKIP facilitates G17-induced CCK2R desensitization. Since activated RKIP (p-RKIP) interferes with GRK2 activity for receptor desensitization, the inhibition of RKIP would result in enhanced CCK2R desensitization.

RKIP siRNA Transfection and Measuring CCK2R Desensitization

For siRNA transfections, HEK-CCK2R cells were plated onto a 6-well plate in 2.0 ml of DMEM 10% FBS without any antibiotic to reach 50% confluence on the following day. Either 200 pmole of non-target (Santa Cruz Biotech) or RKIP siRNA (Santa Cruz Biotech) was diluted in 250 µl of OpTi-MEM (Invitrogen), and also each 5 µl of Lipofectamine^{TM} 2000 (Invitrogen) was diluted in another 250 µl of OpTi-MEM. After 5 min of incubation at RT, both the diluted siRNAs and the diluted Lipofectamine^{TM} 2000 were combined and mixed gently together and incubated for 20 min at RT. Then, the siRNA-Lipofectamine^{TM} 2000 complexes were added onto the cells. After 24 hours of transfection, the cells were split onto laminin-coated cover slips, and incubated another 48 hours for CCK2R desensitization assay (for a total of 72 hours of siRNA transfection).

The CCK2R desensitization was measured by Ca^{2+} imaging as a measure of receptor activity, similar to the procedure previously described. The levels of knockdown by RKIP siRNAs were confirmed by immunoblots using an anti-RKIP antibody (Milipore) with 1:1000 dilution in TBST.

For statistical analysis of CCK2R desensitization, the changes of peak height by G17 stimulation were compared by using an one-way ANOVA between the non-target

and RKIP siRNA transfected cells, and Bonferroni post-tests were performed to compare values at each time point (***, $p < 0.001$; *, $P < 0.05$).

Results and Discussion

After 72 hours of the transfection of either of RKIP siRNA or non-target siRNA for the control, the receptor desensitization was monitored by Ca^{2+} imaging (Figure 2.7.B and C). Compared to the non-target siRNA control group, silencing RKIP by siRNA exhibited reduced Ca^{2+} response to a series of G17 stimulations, as well as a loss of peaks after the second stimulation. This observation indicates that reduced RKIP levels were not sufficient to interfere with CCK2R desensitization, suggesting that RKIP acts as an inhibitory protein for desensitizational machinery for CCK2R. Since previous GRK2 data indicates that CCK2R desensitization is mediated by GRK2 activity, it would be natural to link RKIP and GRK2 for regulating CCK2R desensitization. Therefore, it is also possible to assume that RKIP may suppress GRK2-mediated CCK2R desensitization via inhibiting GRK2 activity by physical association. Since the reduced total RKIP pool by siRNA would be not enough to inhibit GRK2 by binding, the uninterfered GRK2 activity should phosphorylate and desensitize CCK2R much faster than non-target siRNA-treated cells.

Based on the literature, RKIP is phosphorylated at serine 153 by PKC activity, and phosphorylated RKIP (p-RKIP) interacts with GRK2 for its inhibition. Therefore, it is necessary to confirm whether p-RKIP interferes with CCK2R desensitization.

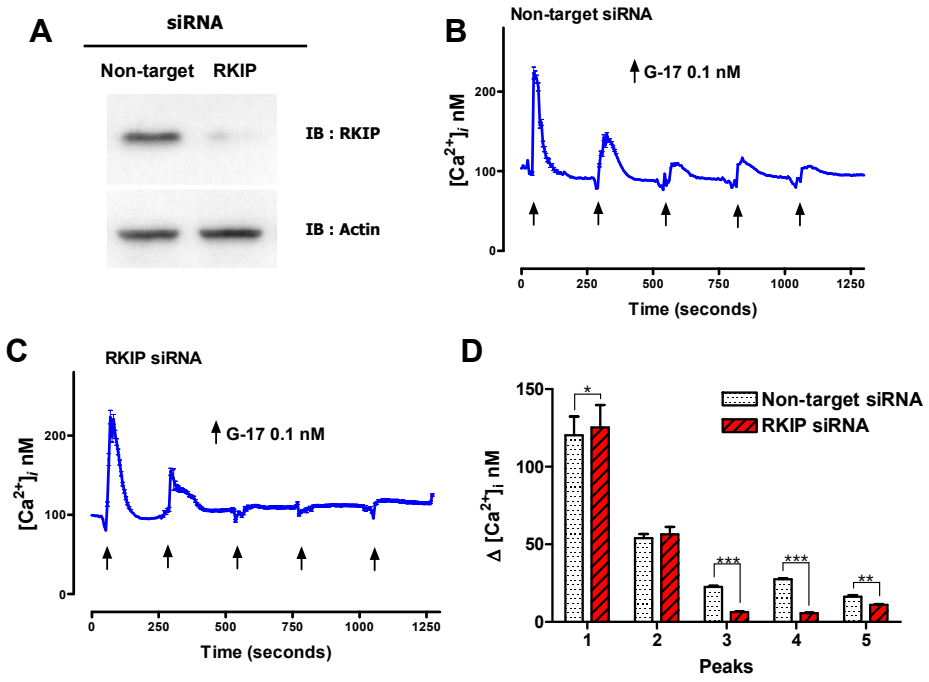

Figure 2.7: The role of RKIP in CCK2R desensitization.
By siRNA transfection, RKIPs were knocked down in HEK-CCK2R cells, then agonist-induced CCK2R desensitization was monitored by challenging 0.1 nM G17. **A**. Immunoblots showing the level of RKIP knockdown (top panel) by anti-RKIP and the loading control (bottom panel) by anti-actin antibodies. **B**. The representative graph showing the changes of $[Ca^{2+}]i$ in HEK-CCK2R transfected with non-target siRNA (control). The arrow indicates the series of G17 stimulations. **C**. The representative graph showing the changes of $[Ca^{2+}]i$ in RKIP siRNA-transfected cells. **D**. The changes of peak-height of $[Ca^{2+}]i$ at G17 stimulation were plotted.

Serine 153 on RKIP-mediated Attenuation of CCK2R Desensitization

As mentioned in the introduction, RKIP activity was controlled by PKC-mediated phosphorylation at Serine 153 (S153) on RKIP. The phosphorylation at S153 on RKIP results in switching its interacting partner from Raf-1 to GRK2, and the interaction between phosphorylated RKIP and GRK2 inhibits the ability of GRK2 for receptor desensitization (Lorenz 2003). Even though the role of RKIP on CCK2R desensitization was shown in the previous experiment by knockdown RKIP expression, it

did not give any specific information about how RKIP regulates CCK2R desensitization. Therefore, through this experiment, the role of the phosphorylation S153 on RKIP in CCK2R desensitization was addressed by introducing the phosphorylation-deficient mutant for RKIP. The phosphorylation-deficient mutant for RKIP was generated by changing S153 to alanine (S153A) on RKIP by site-directed mutagenesis based on a wild-type RKIP construct tagged with flag at the C-terminus (RKIP-flag), which was kindly provided by Dr. Walter Kolch at the University of Glasgow. By transfecting either S153A-RKIP, WT-RKIP, or vector (pcDNA 3.1+) construct into HEK-CCK2R cells, the role of S153 on RKIP in CCK2R desensitization was addressed.

The Site-directed Mutagenesis for the Phosphorylation-deficient Mutant for RKIP

To disrupt potential PKC phosphorylation sites at S153, serine at 153 on RKP was replaced with Ala (S153A) using QuikChange® II XL Site-Directed Mutagenesis Kit (Stratagene).

For mutating serine to alanine, two complementary primers (5'–GGCAAATTCAAGGTGGCGGCCTTCCGTAAAAAGTATG-3', 5'-CATACTTTTTACGGAAGGCCGCCACCTTGAATTTGCC-3') were designed based on the recommendation by the QuikChange® II XL Site-Directed Mutagenesis Kit, and chemically synthesized and purified by Sigma Aldrich. The polymerase chain reaction (PCR) for generating RKIP mutant was performed as follows. For PCR reaction, the following reagents were added: each 125 ng of prepared oligonucleotide primers, 10 ng of parental WT-RKIP constructs as templates, 5 μl of 10X reaction buffer, 1 μl of dNTP mix, 3 μl of QuikSolution, 1 μl of *PfuUltra* HF DNA polymerase (2.5 U/μl) and ddH_2O to 50 μl of final volume. All reagents were provided by Stratagene except the two primers and WT-RKIP construct.

Table 2.1 Cycling Parameters for PCR Reaction for S153-RKIP

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	7 minutes
3	1	68°C	7 minutes

***Dpn* I digestion for removing parental constructs**

After 18 cycles of amplification, the parental WT-RKIP constructs were specifically digested and removed by *Dpn* I endonuclease, which digest methylated and hemimethylated parental DNA. For this reaction, 10 U of *Dpn* I was added to the amplification reaction and gently mixed with a pipette tip followed by spin down for 1 min. The reactant was incubated for 1 hour at 37°C to digest the parental construct remaining in the PCR reaction.

Transformation

The prepared mutant constructs were transformed into XL10-Gold® Ultracompetent Cells (Stratagene) for bacterial colonies for S153A-RKIP constructs. Briefly, the aliquot having 45 μl of XL10-Gold® Ultracompetent Cells was thawed on ice, 2 μl of β-mercaptoethanol was added to the cells, and the cells were swirled every 2 minutes for 10 minutes on ice. 2 μl of *Dpn* I-treated DNA construct for RKIP mutant was added to the competent cells and incubated on ice for 30 minutes. The transformation was performed by heat shock at 42°C for 30 seconds followed by incubation on ice for 2 minutes. Preheated (37°C) 0.5 ml of SOC broth was added to the cells, and the cells were incubated at 37°C for 1 hour with vigorous shaking at 250

rpm. For colony selection, the cells were grown onto LB plates having ampicilin, and the colonies from the LB plates were grown for plasmid isolation.

DNA Sequencing for S153A-RKIP

To confirm the exchange amino acid from serine 153 to alanine by mutagenesis, DNA sequencing was performed in the protein chemistry core laboratory at UTMB using 18 mer sequencing oligonucleotides (5'-GGCCGCTAAAGTGTGACG-3'). The confirmed phosphorylation deficient mutant for RKIP was designated as S153A-RKIP, and used following overexpression experiment for measuring the effects on CCK2R desensitization.

Overexpression of S153A-RKIP, WT-RKIP or vector construct in HEK-CCK2R

For transient transfection of either S153A-RKIP, WT-RKIP, or vector construct (pcDNA3.1+), HEK-CCK2R cells were grown onto a 6-well plate in 2.0 ml of DMEM containing 10% FBS without any antibiotic to reach 90% confluence on the day of transfection. Each 4.0 µg of either S153A-RKIP, WT-RKIP or vector construct (pcDNA3.1+) was diluted in 250 µl of OpTi-MEM (Invitrogen), and also each 10 µl of Lipofectamine^{TM} 2000 (Invitrogen) was diluted in another 250 µl of OpTi-MEM. After 5 min of incubation at RT, the diluted plasmids and the dilution of Lipofectamine^{TM} 2000 were combined and mixed gently together. The reaction was maintained for 20 min at RT to form plasmid-lipofectamin complexes; then the plasmid-Lipofectamine^{TM} 2000 complexes were added onto the cells. After 24 hours of transfection, the cells were split onto laminin-coated cover slips and incubated another 48 hours for CCK2R desensitization assay (for 72 hours of transfection). The levels of RKIP were confirmed by immunoblots using an anti-RKIP antibody (Milipore). For statistical analysis of

CCK2R desensitization, the changes of peak height by G17 stimulation were compared by using an one-way ANOVA, and Bonferroni post-tests were performed to compare values at each time point (***, $p < 0.001$; *, $P < 0.05$).

Results and Discussion

After 72 hours of transfection, the effect of the phosphorylation of S153 on RKIP in CCK2R desensitization was assessed by Ca^{2+} imaging as previously described. As shown in Figure 2.8, the transient transfection of S153A-RKIP, which is the phosphorylation deficient mutant for RKIP, exhibited markedly reduced Ca^{2+} responses by a series of G17 stimulations compared to vector- or WT-RKIP-transfected cells. This result exactly mimics the earlier observation using RKIP siRNA for RKIP knockdown. The immunoblotting for measuring RKIP expression indicated that there were overexpressed and endogenous RKIP in both RKIP overexpressed cells (Figure 2.8.A). The small differences of overexpressed RKIPs account for the flag tag on the RKIP constructs. Even though the weak Ca^{2+} peaks were still observed in S153A-RKIP transfected cells during the series of G17 stimulations, possibly the endogenous expression of RKIP (Figure 2.8.A) was responsible for the weak response to G17 stimulation. Moreover, overexpressing WT-RKIP showed more distinct Ca^{2+} response compared to vector control or S153A-RKIP, suggesting that RKIP acts as an inhibitory protein for CCK2R desensitization. This observation shows strong evidence that the phosphorylation of RKIP on S153 inhibits CCK2R desensitization and also raises the possibility that RKIP exerts influence on CCK2R desensitization by inhibiting GRK2 activity via physical association of phosphorylated RKIP.

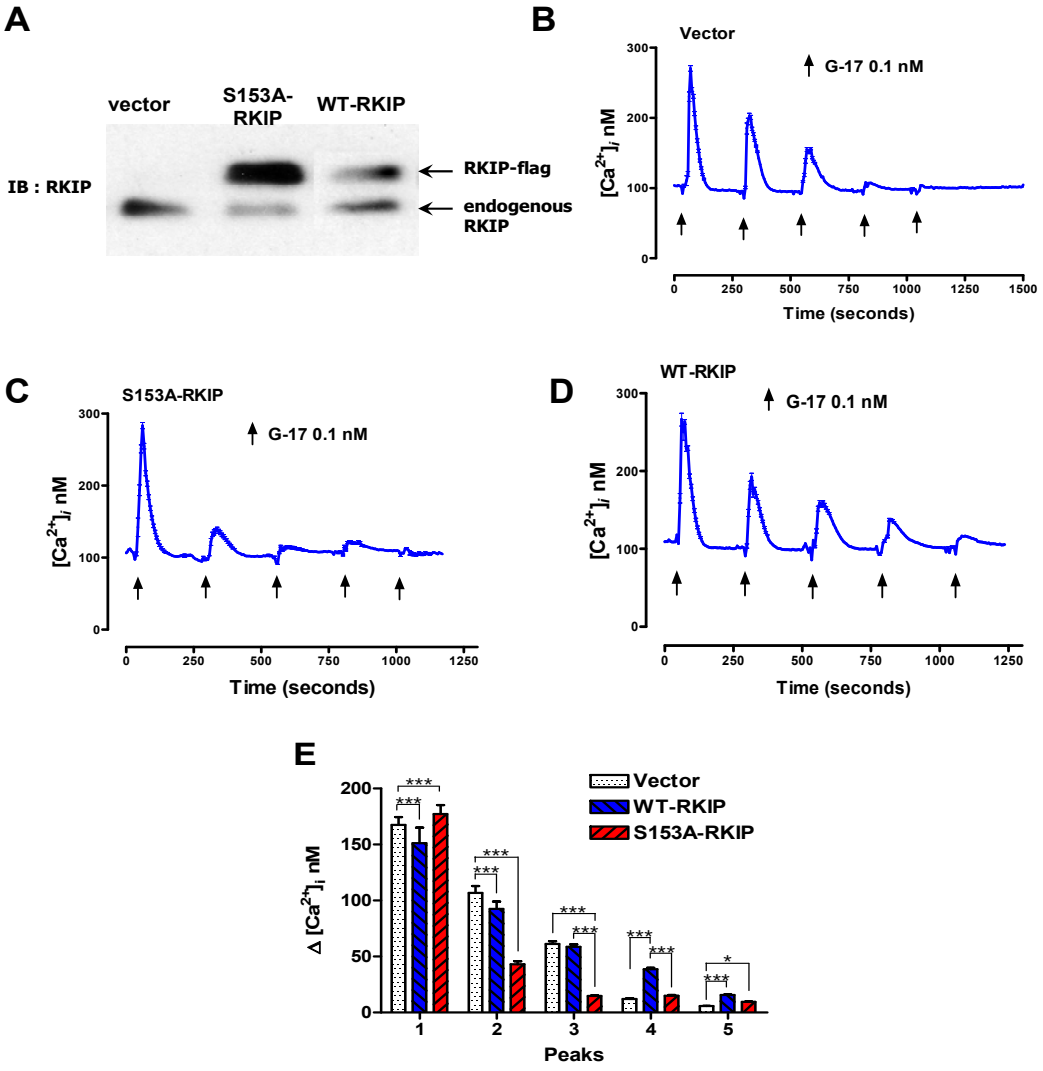

Figure 2.8: The role of RKIP in CCK2R desensitization by using an RKIP mutant. HEK-CCK2R cells were transfected with either vector (pcDNA 3.1+), wild type (WT), or S153A-RKIP, then agonist-induced CCK2R desensitizations were monitored by challenging 0.1 nM G17. **A**. Immunoblots showing the levels of RKIP after the transfection of either vector, S153A-RKIP, or WT-RKIP. **B**. The representative graph showing the changes of $[Ca^{2+}]i$ in vector-transfected cells. The arrow indicates the series of G17 stimulations. **C**. The representative graph showing the changes of $[Ca^{2+}]i$ in S153A-RKIP-transfected cells. **D**. The representative graph showing the changes of $[Ca^{2+}]i$ in WT-RKIP-transfected cells. **E**. The changes of peak-height of $[Ca^{2+}]i$ at G17 stimulation were plotted.

V. Summary of CCK2R Desensitization

Table 2.2 Summary of CCK2R desensitization

	Treatment	$t_{1/2}$ Maximum Ca^{2+} response value (min) ($p < 0.05$)	CCK2R desensitization compared to control
PKC inhibitors	Vehecle control	2.21 ± 0.17	
	GF109203X	1.50 ± 0.19	Increase
	Gö6976	0.98 ± 0.12	Increase
PKC-α inhibition	Non-target siRNA	3.81 ± 0.18	
	PKC-α siRNA	4.26 ± 0.48	Decrease
PKC-β inhibition	Non-target siRNA	5.57 ± 0.94	
	PKC-β siRNA	8.54 ± 1.64	Decrease
PKC-δ inhibition	Non-target siRNA	3.67 ± 0.26	
	PKC-δ siRNA	7.64 ± 0.83	Decrease
GRK2 inhibition	Non-target siRNA	1.62 ± 0.07	
	GRK2 siRNA	2.85 ± 0.17	Decrease
RKIP inhibition	Non-target siRNA	2.92 ± 0.31	
	RKIP	2.62 ± 0.23	Increase
RKIP overexpression	Vector control	6.99 ± 0.82	
	S153A-RKIP	1.75 ± 0.06	Increase
	WT-RKIP	8.61 ± 0.96	Decrease

As seen in Figure 2.1, PKC inhibitor-treated cells showed a rapid desensitization compared to vehicle control. The cPKC subfamily was considered as responsible PKC isozymes, since cPKC inhibition by Gö6976 was enough to enhance CCK2R desensitization. Therefore, PKC-α and -β, which are the members of cPKCs, were selectively knocked downed by siRNA to discriminate specific isozymes involved in this. However, surprisingly, non-e of each inhibition did not mimic a rapid desensitization, which was observed in PKC inhibitor-treated experiments. Rather, the silencing of PKC-α or -β promoted an attenuated CCK2R desensitization. This finding indicates that at least each knockdown of PKC-α or -β interferes with normal CCK2R desensitization, and probably agonist-induced receptor phosphorylation or other desensitization mechanism is inhibited when PKC-α or -β is inhibited. Therefore, it raises the possibility that the inhibition of PKC-γ, the remaining untested cPKC isozyme,

may lead to a rapid CCK2R desensitization as PKC-inhibitor treatments. Otherwise, Gö6976 treatment may induce unexpected side effects involved in receptor desensitization. Also, it may be possible that one single knockdown of cPKCs is not enough for mimicking the effects of Gö6976.

The role of PKC-δ, a member of nPKC, in CCK2R also was addressed by using siRNA, however it also showed an attenuated CCK2R desensitization observed in the inhibition of PKC-α and –β. Interestingly, all of the independent silencing for either PKC-α, -β, or -δ showed the same attenuated CCK2R desensitization. It suggests that different PKC isozymes act in a redundant function for receptor desensitization, and these redundant roles cannot be compensated by other PKC isozymes when one of PKC isozymes is depleted, considering that the inhibition of either PKC-α, -β, or -δ resulted in an attenuated CCK2R desensitization.

As shown in Figure 2.6, GRK2 involvement in CCK2R desensitization was confirmed by silencing GRK2 with siRNA. The next question was to prove RKIP involvement in receptor desensitization, since RKIP could be a modulator for controlling GRK2 activity for CCK2R desensitization. As shown in Figure 2.7, the inhibition of RKIP by siRNA showed an enhanced CCK2R desensitization, indicating that RKIP acts as an inhibitory protein for receptor desensitization. Therefore, both findings strongly support the hypothesis that RKIP inhibits CCK2R desensitization by inhibiting GRK2 activity. The next question was to address the role of PKC-mediated phosphorylation at Serine 153 (S153) on RKIP in CCK2R desensitization. As seen in Figure 2.8, the prevention of the phosphorylation at S153 by using the phosphorylation-deficient mutant did not interfere with CCK2R desensitization, rather it reduced Ca^{2+} response to G17 stimulation, suggesting CCK2R desensitization is inhibited when the phosphorylation of S153 was impaired. Since the phosphorylation at S153 on RKIP was known to be

mediated PKC activity based on published data, it implies that PKC-mediated phosphorylation at S153 on RKIP inhibits CCK2R desensitization, and it also raised the possibility that the phosphorylaiton of RKIP inhibits GRK2 activity required for receptor desensitization.

VI. cPKC-independent CCK2R Phosphorylation

GPCR phosphorylation is a key event for receptor desensitization and internalization. PKA, PKC, and GRK are the known kinases involved in receptor phosphorylation (Pitcher 1998). The second-messenger kinases, PKA and PKC, are known to phosphorylate receptor indiscriminately with GRK mediation. The agonist occupation is not required for second kinase-mediated GPCR phosphorylation, whereas GRK-mediated receptor phosphorylation requires agonist-occupied receptors (Ferguson 2007; Luttrell 2008).

In some cases, both GRKs and second-messenger kinases phosphorylate identical receptors. For example, β2 adrenergic receptors are phosphorylated and desensitized by GRK2 and PKA, and PKA-mediated receptor phosphorylation acts as the switch that converts Gαs-mediated cAMP activation into Gαi-mediated ERK activation (Daaka 1997). Moreover, in many cases, GPCR phosphorylation involves multiple phosphorylation sites by multiple kinases, and some prime phosphorylation triggers sequential phosphorylation events resulting in receptor desensitization. The phosphorylation study using HEK293 cells overexpressing δ opioid receptors indicated that the C-terminal tail of the receptor has two phosphorylation sites, Threonine 358 and Serine 363. Further, the phosphorylation of Serine 363 is required for the phosphorylation of Threonine 358, and this phosphorylation leads to receptor internalization (Kouhen 2000).

The mechanism for CCK2R phosphorylation has been poorly investigated. Very recently, Langer *et al.* provided specific phosphor amino residues, Serine 434 and Threonine 439 on the C-terminal tail of CCK2R by CCK stimulation, and showed that these phosphor residues interact with regulators of G protein signaling 2 (RGS2) proteins for generation of inositol phosphate. However, they did not provide which protein kinases were involved in receptor phosphorylation (Langer 2009). However, this finding provides a very interesting speculation because RGS2 is phosphorylated by PKC activity. *In vitro* kinase assays confirmed that purified PKC-α, -β, or -γ isozyme is capable of phosphorylating RGS2, and RGS2 phosphorylation is also shown in Cos7 cells (monkey kidney fibroblast cells) treated with PMA, an activator for cPKCs and nPKCs (Cunningham 2001).

The previous desensitization data using Gö6976, a PKC inhibitor for cPKCs, indicates that inhibition of cPKC did not impair CCK2R desensitization, rather it resulted in enhanced CCK2R desensitization. This finding raises the possibility that cPKC isozymes may not be involved in receptor phosphorylation, which is known to be the prior step for receptor desensitization. To test this hypothesis, the cPKC involvement in CCK2R phosphorylation was tested by performing receptor *in vivo* phosphorylation assays using Gö6976 to block cPKCs.

In vivo Receptor Phosphorylation

The cells were grown onto 25-cm^2 T-flasks with 80–90% confluence. Prior to labeling, the medium was replaced with phosphate-free DMEM supplemented with 25 mM HEPES for 1 hour at 37°C. Then, the cells were labeled with 200 μci/mL [^{32}P] ortho-phosphate in the same medium for 1 hour at 37°C for labeling intracellular proteins. For inhibiting PKCs, either vehicle (DMSO) or 5 μM Gö6976 was pretreated

for 15 min, and the cells were challenged with or without 100 nM G17 for 20 min followed by two washings with PBS. The cells were then solubilized with 1 ml of ice cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1% Non-idet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM Na3VO4, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 100 μM benzaminidine 1 mM PMSF) for 30 min at 4°C. For isolating proteins, the cell lysates were spun down at 10,000 × g, and the protein concentrations were measured. For immunoprecipitating CCK2Rs, each 1 mg of total cell lysate was incubated with 2.0 μg of anti-CCK2R antibodies (Santa Cruz Biotech), and 40 μl Protein A/G PLUS-Agarose (Santa Cruz Biotech) on a constant rotator at 4°C overnight. The bead-antibody-receptor complexes were pelleted at 1,000 × g for 5 min, and the supernatants were carefully removed. The beads were washed four times with 1 mL of ice cold RIPA buffer. After the final wash, the pellets were resuspended in 40 μl of 1 X Laemmli sample buffer, supplemented with 20 mM DTT, and incubated for 30 minutes at 37°C. The prepared protein samples were separated onto 8% SDS-polyacrylamide gel, and transferred to a polyvinylidene fluoride (PVDF, Millipore) membrane for autoradiography. For the loading control, the precipitated receptor levels were assessed by making an immunoblot using an anti-EGFP antibody (Invitrogen), which recognizes EGFP on the C-terminus of receptors. After autoradiography and immunoblotting, the levels of CCK2R phosphorylation and total precipitated receptors were measured by densitometry using Multi Gauge software (Fujifilm). Statistical comparisons of the levels of CCK2R phosphorylation were performed by Student T-Test.

Results and Discussion

In vivo receptor phosphorylation data indicated that CCK2Rs were phosphorylated with G17-dependent in both DMSO- (vehicle) and Gö6976-treated groups, and the inhibition of cPKCs by Gö6976 did not inhibit agonist-induced receptor phosphorylation as well as the basal receptor phosphorylation (agonist-independent) (Figure 2.9). This data implies that cPKC activity is not required for basal- and agonist-induced CCK2R phosphorylation. Also, considering the finding that inhibition of cPKC did not result in reduced CCK2R phosphorylation, it is possible to assume that cPKCs do not potentiate the activity of GRKs, which are primary kinases for GPCR phosphorylation. In previous receptor desensitization data, the inhibition of cPKCs indicated enhanced CCK2R desensitization, which might mean that inhibition of cPKCs promotes more CCK2R phosphorylation. There were some increases in the levels of receptor phosphorylation when the cells were treated with Gö6976 with or without G17 stimulation compared to vehicle control. However, the increase in receptor phosphorylation by Gö6976 treatment was not statistically significant at 95% level ($p < 0.20$) compared to vehicle-treated cells. Those experiments were performed four times to analyze data statistically, but the differences between vehicle and Gö6976 were insufficient to produce statistical difference. For more precise analysis, more phosphorylation experiments still needed to be done.

This finding suggests that GRKs has a role in receptor phosphorylation, and CCK2R is desensitized via GRK-mediated receptor phosphorylation. Also, it is possible that the primary role of cPKCs is basically inhibitory for GRK-mediated receptor phosphorylation and desensitization. Because of the previous observation that the inhibition of cPKC activity facilitated G17-mediated CCK2R desensitization in Figure 2.1, the role of cPKC might be in controlling GRK activity leading to receptor

desensitization. Interestingly, as mentioned in the previous introduction, RKIP regulates GRK2 activity by PKC-mediated RKIP phosphorylation (Lorenz 2003); therefore, it is possible that CCK2R desensitization may be regulated by a RKIP-dependent mechanism. Furthermore, there is evidence that the phosphorylated residue, serine 153 on RKIP, is the substrate for cPKC isozymes (Corbit 2003). It further raises the possibility that cPKC-mediated RKIP phosphorylation inhibits GRK2 activity to phosphorylate CCK2R leading to receptor desensitization.

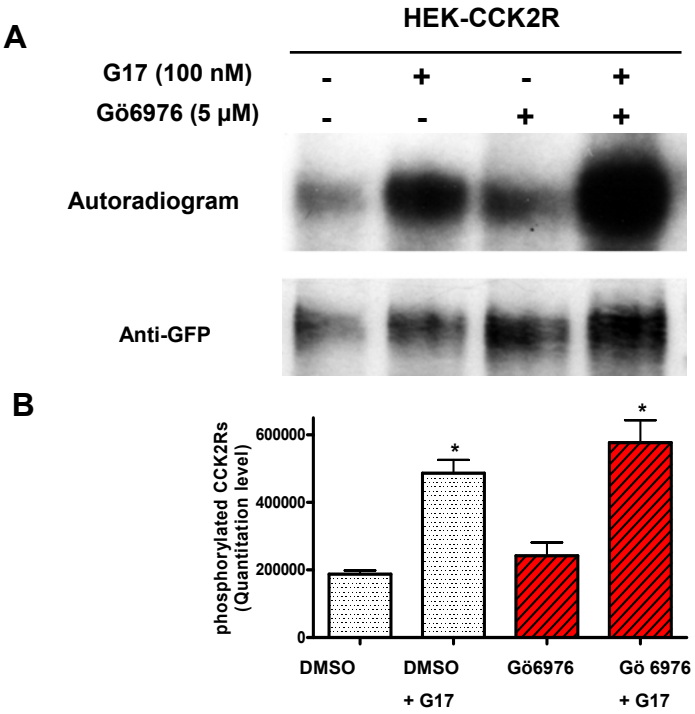

Figure 2.9: PKC-independent CCK2R phosphorylation.
HEK-CCK2R cells were metabolically labeled with [^{32}P] ortho-phosphate, and challenged with 100 nM G17 for 20 min with or without pretreatment of 5 μM Gö6976, then CCK2Rs were immunoprecipitated with anti-CCK2R antibodies. **A**. Top panel shows phosphorylated CCK2R by autoradiogram. Bottom panel shows the amount of precipitated receptors by an immunoblot with anti-EGFP antibodies. **B**. The phosphorylation experiments were run four times, and the phosphorylated receptor levels were measured and normalized with precipitated receptor levels by densitometry using Multi Gauge V. 2.02 (Fujifilm). Statistical comparisons of the levels of CCK2R phosphorylation were performed by Student T-Test (*, $p < 0.05$, untreated [basal] versus G17 stimulated)

VII. cPKC-mediated CCK2R Internalization

Agonist-induced GPCR activation promotes GPCR internalization accompanied by receptor phosphorylation and desensitization. GPCR internalization can be explained as a way of GPCR downregulation. GPCRs on the plasma membrane undergo agonist-promoted receptor internalization by endocytosis, which is the way of physical isolation from the plasma membrane for downregulation. As seen in desensitization data, receptors become resistant for agonist stimulation within a relatively short period of time (seconds to minutes). However, the internalization regulates receptor activity more slowly (minutes to hours) because internalized receptors can be classified into various endosomal vesicles, which determine receptor destiny to be replaced on the plasma membrane for another stimulation (resensitization) or be destroyed in the lysosomes (downregulation of receptor activity).

GPCR internalization is also known to regulate ERK activation via β-arrestins. β-arrestin-mediated receptor internalization recruits signaling effectors such as Raf-1, MEK, and ERK to form signaling complexes for ERK activation (DeFea 2000; Luttrell 2001). Reversely, ERK activity also regulates GPCR internalization via phosphorylating Serine 412 on β-arrestin-1, and the phosphorylated β-arrestin-1 is unable to localized onto plasma membrane for the formation of receptor/β-arrestin-1 complex, and it leads to inhibition of receptor internalization (Lin 1999).

In the previous chapter, the inhibition of cPKCs resulted in enhancing CCK2R desensitization, which may also lead to facilitate receptor internalization. The fast desensitization simply can be interpreted as a rapid receptor phosphorylation that promotes fast receptor internalization via β-arrestin-mediated endocytosis. However, it is also possible to assume that the increased ERK activity may inhibit agonist-induced CCK2R internalization, considering the fact that the cPKC inhibition by Gö6976

exhibited increase in ERK activation as seen in Chapter 3. In this case, the increased ERK may phosphorylate more β-arrestin-1, resulting in interference with β-arrestin-1-mediated receptor internalization.

Through this experiment, the involvement of cPKC in CCK2R internalization was addressed. To answer the question, the agonist-induced CCK2R internalization was monitored under Gö6976 treatment, which is the condition for ERK activation. For measuring agonist-induced CK2R internalization, radiolabeled ligand binding experiments were performed using [^{125}I]-CCK-8.

Internalization of [^{125}I]-CCK-8

HEK-CCK2R cells were grown onto laminin-coated 48-well plates with 60% confluence, After washing with PBS, the cells were incubated in 100 μl of binding buffer (DMEM, 25 mM HEPES, 0.1% bovine serum albumin) with either of 5 μM Gö6976 or DMSO (vehicle) for 15 min, and the cells were placed into a 4°C water bath, and 0.25 nM of [^{125}I]-CCK-8 (specific activity = 2200 Ci/mmol, PerkinElmer Life Sciences) were added for 2 hours at 4°C. To initiate receptor internalization, the temperature was increased to 37°C, and internalization was continued for 1 hour. To terminate reactions, the cells were washed with PBS once, and the remaining radioligands on plasma membranes were removed with acidic wash solution (0.2 N acetic acid and 0.5 M NaCl) twice. The cells were solubilized with 100 μl of 1 N NaOH, and radio activities were measured by a Cobra II gamma counter (Parkard Instrument Company). All reactions were run in triplicate for accuracy, and total bindings were managed less than 10% of the total counts added to the assays. For non-specific binding, another set of binding reactant was performed using 1 μM of unlabelled CCK-8 for competing radiolabeled CCK-8. For measuring specific binding (SB), the averages of non-specific

binding (NB) for each set of experiments were subtracted from the amounts of the total binding. Statistical comparisons of the levels of CCK2R internalization were performed by Student T-Test at 60 min time points (***, $p < 0.001$)

Results and Discussion

The inhibition of cPKC by Gö6976 treatment exhibited significant reduction (74% decrease compared to average of DMSO treated) in [^{125}I]-CCK-8 internalization at 60 min, compared to the DMSO-treated group. This indicates that more than 70% of internalization was inhibited when cPKCs were knocked down by Gö6976 treatment (Figure 2.10). As shown in previous phosphorylation assays, Gö6976 treatment did not interfere with agonist-induced CCK2R phosphorylation. However, this data suggests that cPKC activity is required for agonist-induced CCK2R internalization. This finding was very interesting because, in general, receptor phosphorylation promotes receptor internalization. Therefore, this data implies that cPKC activity is required for agonist-mediated CCK2R internalization, however it affects receptor endocytosis, but this is not regulated by CCK2R phosphorylation. There are two possible theories for an explanation. First, it may be possible to presume that cPKC activity affects β-arrestin binding onto receptors. As mentioned in the introduction, the receptor internalization has a close relationship with ERK activation in the relevance of the phosphorylation of β-arrestin. ERK-mediated β-arrestin phosphorylation interferes with agonist-induced receptor internalization (Lin 1999), therefore it is possible to presume that increased ERK activation by Gö6976 impairs CCK2 internalization via ERK-mediated β-arrestin phosphorylation. Second, another possibility is that cPKC activity is required for formation of endosomal vesicles involved in receptor endocytosis. Recent studies suggest a novel role of PKC in regulating endosomal trafficking. Prolonged activation

of classical PKCs results in accumulation of pericentrion, which is considered as endocytic vesicles near the Golgi complex. This pericentrion is colocalized with Rab11, which is the marker for recycling endosome (Becker 2003); and also cPKC activity is needed for clathrin-dependent and -independent (caveolin-mediated) endocytosis (Idkowiak-Baldys 2006).

However, the finding that cPKC is required for CCK2R internalization does not explain how RKIP phosphorylation by cPKCs can interfere with GRK2 activity for receptor desensitization. If cPKCs are the isozymes for RKIP phosphorylation, inhibition of cPKCs would result in enhanced receptor internalization. Rather, this finding explains why individual knockdowns of cPKCs (PKC-α, or -β) did not exhibit enhanced CCK2R desensitization, which was observed in cPKC inhibition with Gö6976 treatment. To address more precisely the role of PKC in CCK2R internalization, the individual knockdowns of PKC isozymes would be required. If the inhibition of one of the PKC isozymes could result in facilitation of agonist-induced CCK2R internalization, it would raise the possibility that the PKC isozyme also promotes RKIP phosphorylation and regulates GRK activity to modulate CCK2R desensitization.

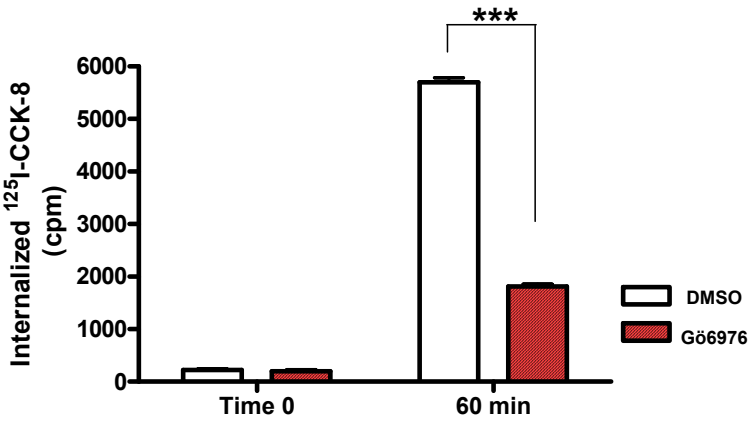

Figure 2.10: cPKC-dependent CCK2R internalization.
HEK-CCK2R cells were grown onto laminin-coated plates and pretreated with DMSO (vehicle control) or 5 μM Gö6976 on ice. The internalizations of [^{125}I]-CCK-8 were measured by incubating the cells for 60 min at 37°C. The amount of internalized [^{125}I]-CCK-8 was determined by Cobra II gamma counter (Parkard Instrument Company) after removing residual radioligands on the plasma membrane with 2 X washing with acidic solution. Statistical comparisons of the levels of CCK2R internalization were performed by Student T-Test at 60 min time points (***, $p < 0.001$)

VIII. Agonist-dependent GRK2 Association onto CCK2R

The GRK2-mediated CCK2R desensitization and cPKC-independent CCK2R phosphorylation strongly imply that GRK2 phosphorylates CCK2R in an agonist-dependent manner. The addressed question was whether PKCs affect GRK2 activity for regulating CCK2R desensitization. Based on the desensitization experiments using two different PKC inhibitors (Gö6976 or GF109203X), the inhibition of cPKCs by PKC inhibitors showed enhanced CCK2R desensitization to the series of G17 stimulations, and

it strongly implies that cPKC activity may inhibit the ability of GRK2 in phosphorylating CCK2R. If cPKCs are inhibited by chemical PKC inhibitors, it may promote more physical binding of GRK2 onto CCK2Rs. To test this hypothesis, co-immunoprecipitation experiments were performed using an anti-CCK2R antibody.

Co-immunoprecipitation of GRK2 and CCK2R

HEK-CCK2R cells were grown onto 25-cm^2 T-flasks with 80–90% confluence. For inhibiting PKCs, either vehicle (DMSO), 5 μM GF109203X, or 5 μM Gö6976, was pretreated for 15 min, and the cells were challenged with or without 100 nM G17 for 5 min followed by PBS washing. To fix and stabilize the receptor complexes, the cells were incubated with dithiobis succinimidyl propionate (DSP, Thermo Fisher Scientific) for 20 min at RT. DSP is a membrane-permeable crosslinker, and it reacts rapidly with amines in lysine of proteins, then forms stable amine bonds. After crosslinking with DSP, the cells were washed twice with PBS and solubilized with 1 ml of ice cold radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1% Non-idet P-40, 0.5% deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM Na3VO4, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 100 μM benzaminidine 1 mM PMSF) for 30 min at 4°C. For isolating proteins, the cell lysates were spun down at 10,000 × g, and the protein concentrations were measured. For immunoprecipitating CCK2Rs, each 1 mg of total cell lysate was incubated with 2.0 μg of goat polyclonal anti-CCK2R antibody (Santa Cruz Biotech) for 1 hour. For the negative control, the same amount of normal goat IgG (Santa Cruz Biotech) was added instead of anti-CCK2R antibody. Then, 40 μl Protein A/G PLUS-Agarose (Santa Cruz Biotech) was added to the cell lysate and antibody mix and incubated on a constant rotator at 4°C overnight. The bead-antibody-receptor complexes were pelleted at 1,000

× g for 5 min, and the supernatants were carefully removed. The beads were washed four times with 1 ml of ice cold RIPA buffer. After the final wash, the pellets were resuspended in 40 µl of 1 X Laemmli sample buffer supplemented with 20 mM DTT, and incubated for 30 min at 37°C. The prepared protein samples were separated onto 8% SDS-polyacrylamide gel, and transferred to a polyvinylidene fluoride (PVDF, Millipore) membrane, followed by blocking with 5% skim milk in TBST for 1 hour. For immunoblotting for GRK2, mouse monoclonal anti-GRK2 antibody (Santa Cruz Biotech) was diluted as 1:1000 in TBST. For the loading control, the precipitated receptor levels were assessed by making an immunoblot using an anti-EGFP antibody (Invitrogen), since a receptor construct has an EGFP tag on its C-terminus tail. After making immunoblots, the levels of GRK2 and the total precipitated receptors were measured by densitometry using Multi Gauge software (Fujifilm). Statistical comparisons of the levels of GRK2 association with CCK2R were performed by Student T-Test.

Results and Discussion

By co-immunoprecipitation of GRK2 with anti-CCK2R antibodies, the association of GRK2 onto CCK2Rs was monitored as seen in Figure 2.11. First, it is clear that GRK2 binding onto receptors was totally dependent on G17 stimulation in all groups. PKC inhibition by either GF109203X or Gö6976 also indicated agonist-dependent GRK2 association. As shown in Figure 2.11.B, the intensity of GRK2 was determined by densitometry and normalized by the amount of receptors precipitated after three independent experiments. Without G17 stimulation (basal state), either GF109203X- or Gö6976-treated cells showed greater increases in GRK2 association (98% and 116% increases, respectively) compared to vehicle control on average. Also, G17-stimulated cells showed augmentation in GRK2 association when PKC inhibitors

were treated (GF109203X: 23%, and Gö6976: 55% increase compared to vehicle control with G17 stimulation). However, statistically these are not significant differences by Student's t-test at 95% level ($p = 0.052$). Therefore, the initial hypothesis that the inhibition of PKC promotes more GRK2 association onto CCK2R is not justified by the data. However, co-immunoprecipitation experiments measuring the amount of GRK2 association onto CCK2R, did not mean that the associated GRK2 has the kinase activity involved in CCK2R phosphorylation, which leads to receptor desensitization. Since GRK2 association onto CCK2R was very specific to G17 stimulation, most of the associated GRK2 may be in an activated state (phosphorylated GRK2) for receptor phosphorylation. However, this experiment could not discriminate only activated (phosphorylated) GRK2 from total GRK2 levels. Also, one of the possible explanations may be there were insufficient levels of endogenous GRK2 expression compared to CCK2R because HEK-CCK2R cells are a stable cell line that overexpress CCK2R by stable transfection. Therefore, if the majority of CCK2R interacts with GRK2 in an agonist-dependent manner, probably more GRK2 association would be unlikely even though PKC inhibition created a more favorable situation for promoting more interactions between GRK2 and CCK2R.

Taken together, the conclusion of this experiment is that the association of GRK2 onto CCK2R was markedly dependent on G17 stimulation, and the inhibition of PKC by chemical inhibitors did not interfere with GRK2 association onto CCK2R. However, this finding does not undermine the previous observation that suggests the role of PKC in CCK2R desensitization, considering that at least PKC inhibitions by chemical inhibitors did not exhibit a decrease in GRK2 association.

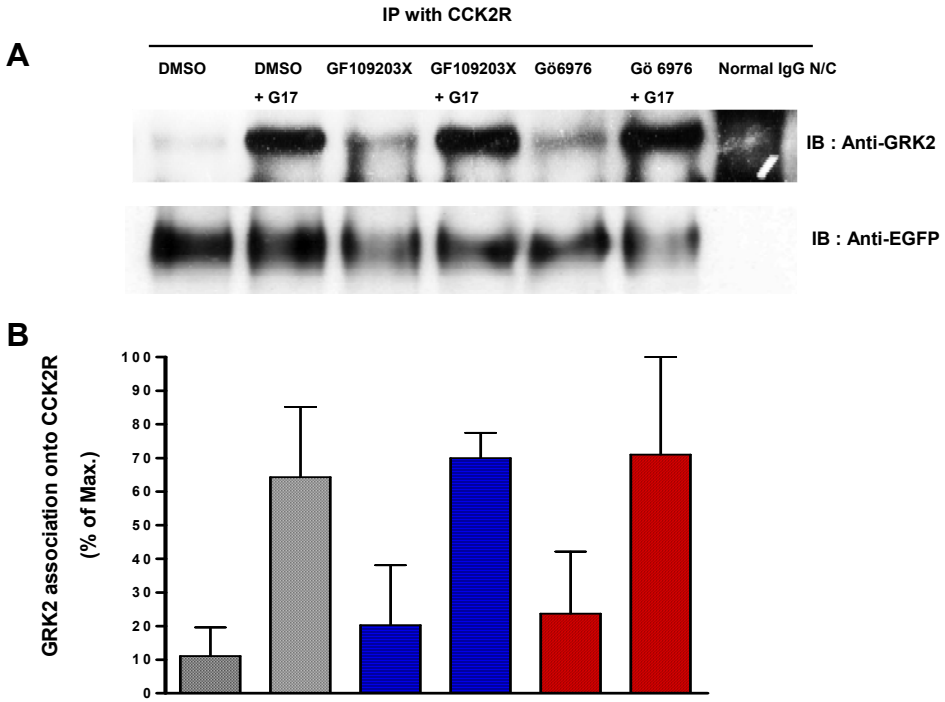

Figure 2.11: The effects of PKC inhibitors on GRK2 association onto CCK2R.
HEK-CCK2R cells were pretreated either DMSO (vehicle), 5 μM GF109203X, or 5 μM Gö6976 for 15 min, then the cells were challenged with or without 100 nM G17 for 10 min followed by cross-linking by DSP treatment for 20 min at RT. After isolation of total proteins, 1 mg of total cell lysates were used for immunoprecipitating with anti-CCK2R antibodies, then the immunoblottings were performed for GRK2 and EGFP. **A**. Top panel shows the levels of co-immunoprecipitated GRK2 by immunoblot. Bottom panel shows amount of precipitated receptors by an immunoblot with anti-EGFP antibodies. **B**. The experiments were done in triplicate, and the amount of GRK2 was calculated over the receptor levels measured by densitometry using Multi Gauge V. 2.02 (Fujifilm).

IX. RKIP Association onto GRK2 via PKC Activation

The previous experiments indicated that RKIP interfered with agonist-induced CCK2R desensitization, and it was confirmed by either downregulating or overexpressing RKIPs in HEK-CCK2R cells. Moreover, it was also identified that the phosphorylation of S153 on RKIP was critical for regulating CCK2R desensitization. As introduced previously, the phosphorylation of RKIP on S153 is dependent on PKC

activity (Lorenz 2003). Through this experiment, I tested the hypothesis that RKIP associates with GRK2 in a PKC-dependent manner. To detect interaction between RKIP and GRK2, I performed transient transfection of both constructs into HEK-CCK2R cells, since endogenous RKIP was not immunoprecipitated by RKIP antibodies. This difficulty was also mentioned in a previous published report (Hagan 2006). To co-immunoprecipitate the RKIP and GRK2 complex, the overexpressed RKIP was immunoprecipitated by using flag tag on its C-terminus.

Transient Transfection of RKIP and GRK2 into HEK-CCK2R

For transient transfection of wild type RKIP and GRK2, the following constructs were provided by other investigators. RKIP-flag in pcDNA 3.1 (+), as described in the previous experiment, and GRK2 in pcDNA 3 were kindly provided by Dr. Jeffrey Benovic at Thomas Jefferson University.

For transfection of both constructs, HEK-CCK2R cells were grown onto a 100-mm culture dish in 15 ml of DMEM containing 10% FBS without any antibiotic to reach 90% confluence on the day of transfection. Both 2.0 μg of RKIP-flag and 6.0 μg of GRK2 plasmids were diluted in 1.5 ml of OpTi-MEM (Invitrogen). Also, 40 μl of Lipofectamine^{TM} 2000 (Invitrogen) was diluted in another 1.5 ml of OpTi-MEM. After 5 min of incubation at RT, the diluted plasmids and the dilution of Lipofectamine^{TM} 2000 were combined and mixed gently together. The reaction was maintained for 20 min at RT to form plasmid-lipofectamin complexes. Then, the plasmid-Lipofectamine^{TM} 2000 complexes were added onto the cells. After 24 hours of transfection, the cells were split into 6 of 25 cm^2 T-flasks, and incubated another 48 hours before treatments with PKC effectors (1 μM PMA, 5 μM Gö6976, or DMSO [vehicle]).

Co-immunoprecipitation of GRK2 with RKIP

After 72 hours of transient transfection, the cells were placed in 25-cm^2 T-flasks with 80–90% confluence. For pretreatment with PKC effectors, either vehicle (DMSO), 5 μM Gö6976, or 1 μM PMA was used for pretreatment for 15 min, then the cells were treated with or without 100 nM G17 for 5 min for stimulation. The cells were washed twice with PBS and harvested with 1 ml of ice cold lysis buffer containing 20 mM HEPES, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.1% NP‐40, 10 mM NaF, 1 mM Na3VO4, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 mM PMSF, followed by sonication (3 × 5 seconds). The cell lysates were spun down at 10,000 × g for 15 min, and the total protein concentrations were measured. For co-immunoprecipitating RKIP-flag and GRK2 complexes, each 500 μg of total cell lysate was incubated with 20 μl of ANTI-FLAG® M2 Affinity Gel (Sigma-Aldrich) for at least 2 hours. For the negative control, the same amount of ANTI-FLAG® M2 Affinity Gel was incubated with lysis buffer. The beads were pelleted by spin down, the supernatants were carefully removed, and the beads were washed four times with 1 mL of ice cold PBS. After the final wash, the pellets were resuspended in 20 μl of 1 X Laemmli sample buffer supplemented with 20 mM DTT and boiled for 4 min. The prepared samples were separated onto 4–12% Bis-Tris gel (Invitrogen), and transferred to a polyvinylidene fluoride (PVDF, Millipore) membrane and blocked with 5% skim milk in TBST for 1 hour. For immunoblotting for GRK2, rabbit anti-GRK2 antibody (Santa Cruz Biotech) was diluted at 1:1000 in TBST. The precipitated receptor levels were assessed by making an immunoblot using a rabbit anti-RKIP antibody (Santa Cruz Biotech). The experiments were performed three times for accuracy, and the levels of GRK2 and the total precipitated RKIPs were measured by densitometry using Multi

Gauge software (Fujifilm). Statistical comparisons of the levels of GRK2 association with RKIP were performed by Student T-Test at 60 min time points (*, $p < 0.05$)

Results and Discussion

As seen in Figure 2.12, co-immunoprecipitation of GRK2 with RKIP indicated that only PMA treatment alone showed significant increase in GRK2 association onto RKIP, suggesting that PMA-sensitive PKC isozymes promote RKIP association with GRK2. However, the cPKC inhibition by Gö6976 did not show any effect on the association between RKIP and GRK2, suggesting that cPKCs are not the kinases for phosphorylating RKIP for GRK2 association. Moreover, G17 treatment did not enhance the association, suggesting that CCK2R-induced PKC isozymes are not involved in RKIP phosphorylation. Rather, G17 treatment with PMA exhibited reduced GRK2 levels compared to PMA alone. The observation that the inhibition of cPKC did not inhibit RKIP and GRK2 interaction indicates that cPKC is not responsible for phosphorylating RKIP for the association with GRK2. Probably other PKC isozymes activated by unknown mechanisms have the role for RKIP phosphorylation in HEK-CCK2R cells. However, the marked increase of GRK2 association observed in the PMA-treatment group indicates the activation of PKCs promotes the physical interaction between RKIP and GRK2, which inhibits GRK2-mediated GPCR desensitization.

Even though several reports indicate that G17 stimulation promotes PKC activation, G17 treatment did not exhibit any increase in the association of RKIP and GRK2, suggesting that the activation of PKC isozymes by CCK2R has no direct relationship with RKIP. In fact, the activation of CCK2R is known to potentiate activity of PKC-α, -β, -δ, -ε, and -η (Yassin 1995; Sturany 2002; Ogasa 2003). However, there is no evidence which PKC isozymes are activated by G17 stimulation in HEK-CCK2R

cells. PMA stimulation is known to activate cPKC (PKC- α, -β,-γ) and the nPKC (PKC- θ, -δ, -ε, -η) family, because PMA binds the C1 domain of both PKC families for activation. Based on *in vitro* kinase assays, RKIP is phosphorylated by cPKC (PKC- α, -β, -γ), PKC-ζ (atypical PKC), and PKC-δ (nPKC) families (Corbit 2003; Lorenz 2003).

To show more direct involvement of PKC in the RKIP and GRK2 association, the additional PKC knockdown by GF109203X would be helpful, because RKIP is also known to be phosphorylated by PKC-δ, a member of nPKCs. Additionally, it would be clear if endogenous RKIP and GRK2 are co-immunoprecipitated in HEK-CCK2R cells. However, as mentioned earlier, RKIP immunoprecipitation is still a technically difficult procedure. Alternatively, it might be helpful if much less basal association of RKIP and GRK2 would be observed by using smaller amounts of both RKIP-flag and GRK2 constructs in transient transfection. In that case, one could discriminate the effects of G17 and Gö6976 on the association of RKIP and GRK2 from the less basal level of association.

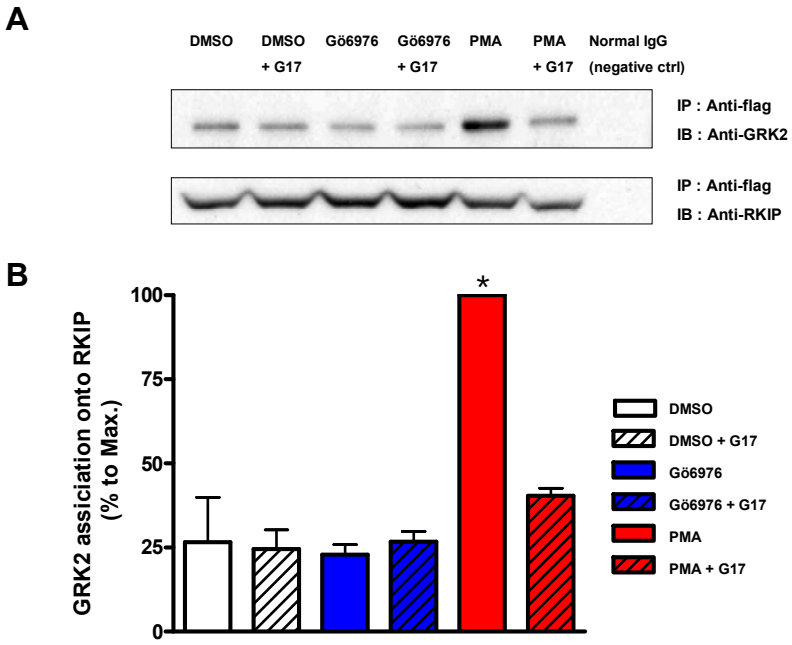

Figure 2.12: PKC effectors on GRK2 association onto RKIP.
HEK-CCK2R cells were pretreated either DMSO (vehicle), 5 μM Gö6976, or 1 μM PMA for 15 min, then the cells were challenged with or without 100 nM G17 for 5 min. After isolation of total proteins, each of 500 μg of total cell lysates was used for immunoprecipitating with anti-flag antibodies, then the immunoblottings were performed for GRK2 and RKIP. **A**. Top panel shows the levels of co-immunoprecipitated GRK2 by immunoblot. Bottom panel shows the amount of precipitated RKIP by an immunoblot with anti-RKIP antibodies. **B**. The experiments were run in triplicate, and the amount of GRK2 was calculated over the levels of precipitated RKIP by densitometry. Statistical comparisons of the levels of p-ERK were performed by Student T Test at each of the time points (*, $p < 0.05$).

X. Summary of Chapter 2.

In this chapter, the role of PKC in CCK2R desensitization was first addressed. By using chemical PKC inhibitors, a rapid CCK2R desensitization was observed, however the knockdown PKC isozymes (either PKC-α, -β, or -δ) by siRNAs conversely showed an attenuated CCK2R desensitization. Therefore, to identify the PKC isozymes

involved in the inhibition of CCK2R desensitization, more desensitization experiments using multiple siRNAs for PKC isozymes are required.

The role of GRK2 in CCK2R desensitization was confirmed by using siRNA. Since the inhibition of GRK2 resulted in an attenuated CCK2R desensitization, it suggests that GRK2 may phosphorylate CCK2R in an agonist-dependent manner. Also, this hypothesis is supported by the finding that strong association of GRK2 with CCK2R, only when the cells were challenged with G17. Based on this finding, RKIP involvement in CCK2R desensitization was also addressed. The data indicate that RKIP activity inhibits CCK2R desensitization, suggesting that it may inhibit GRK2 activity involved in receptor desensitization. Finally, the role of PKC-mediated phosphorylation at Serine 153 on RKIP was addressed by employing a phosphorylation-deficient mutant for RKIP. The data indicate that this phosphorylation is required for inhibition of CCK2R desensitization. Also, co-immunoprecipitation experiments confirmed that the association between RKIP and GRK2 requires PMA-sensitive PKC activity, but CCK2R-induced PKC activity does not appear to be involved in RKIP association onto GRK2. Taken together, these findings prove that RKIP acts as inhibitory modulator for CCK2R desensitization, and the mechanism for this inhibition involves physical inhibition of GRK2 with phosphorylated RKIP. Also, it strongly suggests that PMA-sensitive PKCs, but not CCK2R-induced PKCs, are required for RKIP phosphorylation required for GRK2 association.

Chapter 3. CCK2R-mediated ERK Activation

I. Introduction

The altered conformation by agonist binding allows the dissociation of heterotrimeric G proteins (Gαβγ) from receptors. Among Gα subunits, CCK2R is known to couple with Gαq/11 for activating signaling pathways via PLC-dependent cascades (Paulssen 2000). The activated PLC produces inositol 1,4,5-triphosphate (IP3) and 1,2-diacylglycerol (DAG). Then, released IP3 promotes release of Ca^{2+} from endoplasmic reticulum (ER), which increases in $[Ca^{2+}]i$ by Ca^{2+} influx via opening calcium channels on the plasma membrane. The increased levels of $[Ca^{2+}]i$ and DAG are the necessary components for Protein Kinase C (PKC) activation. The PKC isozymes activated by CCK2R are PKC-α, -β, -δ, -ε, and -η (Yassin 1995; Sturany 2002; Ogasa 2003).

Agonist-induced CCK2R activation promotes ERK1/2 (ERK) activation via the PKC/Raf/MEK signaling axis. Administration of G17 or CCK indicated Raf-1 and ERK activation (Seufferlein 1995). In a CCK2R-transfected cell line, the inhibition of Raf-1 resulted in complete loss of ERK activation, whereas Ras mutant exerted no effects on ERK activation, indicating that ERK activation is dependent of Raf rather than Ras (Hocker 2004). Also, PD98059, an inhibitor for MEK, completely inhibited gastrin-induced ERK activation (Cramer 2008) in AGS-B gastric cancer cells stably expressing CCK2R.

The studies for RKIP indicate that RKIP is involved in the Raf/MEK/ERK signaling axis and basically acts as an inhibitory modulator for ERK activation (Hagan 2006). The inhibitions of RKIP by antisense RKIP RNA or RKIP antibodies indicated activation of MEK-1/ERK, whereas the overexpression of RKIP blocked ERK signaling

(Yeung 1999). In the basal state, RKIP interferes with Raf-mediated phosphorylation of MEK by forming a complex with Raf, therefore MEK cannot activate ERK (Yeung 2000). This concept of RKIP-mediated ERK activation is also applied to GPCR-mediated ERK activation. Based on the findings of Lorenz *et al*. in 2003, PKC-mediated RKIP phosphorylation on serine 153 acts as a switch for changing the RKIP interacting partner from Raf to GRK2, resulting in the inhibition of GRK2-mediated GPCR desensitization (Lorenz 2003). Interestingly, PKC-mediated RKIP phosphorylation also acts as a signal enhancer for GPCR-mediated ERK activation. Since phosphorylated RKIP (p-RKIP) interferes with GRK2-mediated receptor desensitization and internalization, the slower receptor desensitization by p-RKIP allows it to generate more signal molecules resulting in enhanced ERK activation. In fact, HEK293 cells expressing β2 adrenergic receptors showed a marked increase in ERK activation and decrease in agonist-induced receptor internalization when RKIPs were overexpressed (Lorenz 2003).

Previous findings indicated that the inhibition of either PKC or RKIP led to enhanced CCK2R desensitization and GRK2 inhibition resulting in attenuated receptor desensitization in Chapter 2. These observations suggest that PKC-mediated phosphorylation of RKIP inhibit GRK2-mediated CCK2R desensitization. Therefore, it strongly implies that ERK activation may be regulated by PKC-mediated RKIP phosphorylation in HEK-CCK2R cells. To test this hypothesis, first the effects of PKC knockdown on ERK activation were determined, and the PKC isozyme responsible for ERK activation was identified. Finally, the effects of RKIP on ERK activation were addressed in this chapter.

II. PKC-mediated CCK2R-induced ERK Activation

As stated earlier, agonist-induced CCK2R activates ERK via the PKC/Raf/MEK signaling axis, and several PKC isozymes (PKC-α, -β, -δ, -ε, and –η) are known to be activated by CCK2R. However, the PKC isozymes involved in CCK2R-mediated ERK activation have not been addressed. To determine the effects of PKC on ERK activation, chemical PKC inhibitors were used as follows: Gö6976 for inhibition of cPKCs, GF109203X for inhibition of both cPKCs and nPKCs, and Rottlerin for PKC-δ. The effects of each PKC inhibitor on G17-induced ERK activation were monitored by immunoblotting with phosphorylated ERK (p-ERK) antibodies.

PKC knockdown by chemical inhibitors and immunoblotting for p-ERK

HEK-CCK2R cells were grown onto 24-well plates, and the cells were pretreated with either DMSO (vehicle), 5 μM GF109203X, 5 μM Gö6976, or 10 μM Rottlerin for 15 min before 10 nM G17 stimulation over a time course (from 0 to 60 min). The cells were washed with PBS twice and incubated with 200 μl of lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, 1% Non-idet P-40, 0.5% sodium deoxycholate, 0.1 % sodium dodecyl sulfate (SDS), 10 mM NaF, 1 mM Na_3VO_4, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 100 μM benzaminidine 1 mM PMSF) for 20 min on ice, followed by centrifugation at 10,000 × g. Total cell lysates were obtained from the supernatants, and the protein concentration was determined by Bradford assay. Then, each 25 μg of total cell lysates was mixed with 2X Laemmli sample buffer and boiled for 3 min; then the prepared protein samples were separated onto 10% polyacrylamide gel. The separated proteins were transferred onto a polyvinylidene fluoride (PVDF, millipore) membrane, and blocked with 5% skim milk in Tris buffered saline tween 20 (TBST, 50 mM Tris-HCl pH. 7.4, 135 mM NaCl, 0.1%

tween 20) for 1 hour. For detection of phosphorylated ERK1/2 (p-ERK), mouse monoclonal anti-phospho-ERK (p-ERK, Santa Cruz Biotech) antibody was probed with 1:3000 dilution in TBST for 1 hour or overnight, and the membrane was washed with TBST for 15 min followed by 3 X 5 min of washing with TBST. Horseradish peroxidase (HRP) conjuagated secondary antibodies were used for detection of primary antibodies, and chemiluminescent detection was performed using ECL Plus™ Western Blotting Reagent Pack (GE Health Care Life Sciences). After the first blot, the membrane was stripped using Restore Western Blot Stripping Buffer for anti-actin (Santa Cruz Biotech) blot for loading control, and re-blocked with 5% skim milk in TBST. For detection of actin, 1:6000 dilution of goat polyclonal anti-actin antibody was used. Each blot was quantified by densitometry and normalized by actin controls. Statistical comparison of the p-ERK activation was performed by using a two-way ANOVA between G17-stimulated cells in the absence and presence of inhibitors (†, $p < 0.001$), and Bonferroni post-tests were performed to compare values at each time point (**, $p < 0.01$; *, $p < 0.05$).

Results and Discussion

The effects of PKC inhibition by chemical PKC inhibitors on ERK activation were measured by immunobloting with p-ERK antibodies as shown in Figure 3.1. In Figure 3.1.A, the inhibition of cPKCs by Gö6976 treatment did not inhibit ERK activation compared to DMSO control (vehicle), rather this exhibited enhanced ERK activation with a time course in HEK-CCK2R cells (†, $p < 0.001$). However, the inhibition of both cPKCs and nPKCs by GF109203X treatment resulted in inhibition of ERK activation, especially in early time points (before 30 min of G17 stimulation) compared to vehicle-treated cells. This finding implies that GF109203X sensitive PKC

isozymes (novel PKC isozymes: -δ, -ε, -η, -θ,) are responsible for receptor-mediated ERK activation, considering that both Gö6976 and GF109203X block cPKC isozymes; and only GF109203X, which also inhibits nPKC isozymes, showed reduced ERK activation over a time course for G17 stimulation. However, it was very interesting to consider why Gö6976-treated cells showed more ERK activation than any other group, especially in the later time points. In previous desensitization data, both PKC inhibitors represented enhanced receptor desensitization compared to the control group, and it implies receptor activity was reduced by both PKC inhibitor treatments. Therefore, it is reasonable to presume that inhibition of PKC would result in reduced levels of ERK activation. However, considering another finding that inhibition of cPKC inhibited CCK2R internalization, it is possible to assume that enhanced receptor desensitization by cPKC inhibition does not involve receptor internalization, which is considered as a negative regulator for receptor-mediated ERK activation (Lin 1999). Therefore, if this assumption is right, it is possible that the inhibition of CCK2R internalization could allow more ERK activation, and even the receptors are rapidly desensitized when cPKCs are inhibited.

To screen which nPKC isozymes are involved in receptor-mediated ERK activation, HEK-CCK2R cells were pretreated with 10 μM Rottlerin, which is known as a specific inhibitor for PKC–δ. As seen in Figure 2.2.B, the inhibition of PKC-δ by Rottlerin treatment almost blocked G17-induced ERK activation (from 0 to 30 min) compared to the DMSO control. Considering both GF109203X and Rottlerin inhibit PKC-δ and these PKC inhibitor-treated cells showed diminished ERK activation especially in early time points (before 10 min of G17 stimulation), PKC-δ might be a potential isozyme, which mediates G17-induced ERK activation via CCK2R. However, there is growing controversy over the specificity of Rottlerin, because Rottlerin is also

known to block PKC-α, -β, -γ, and other kinases with slightly lower affinity (3- to 5-fold lower than PKC-δ). Furthermore, it lowers intracellular adenosine triphosphate (ATP) pool, which is required for activation of most of PKCs (Soltoff 2007). For that reason, small interfering RNAs (siRNAs) for inhibiting specific PKC isozymes were introduced to narrow down the target PKCs involved in receptor-mediated ERK activation.

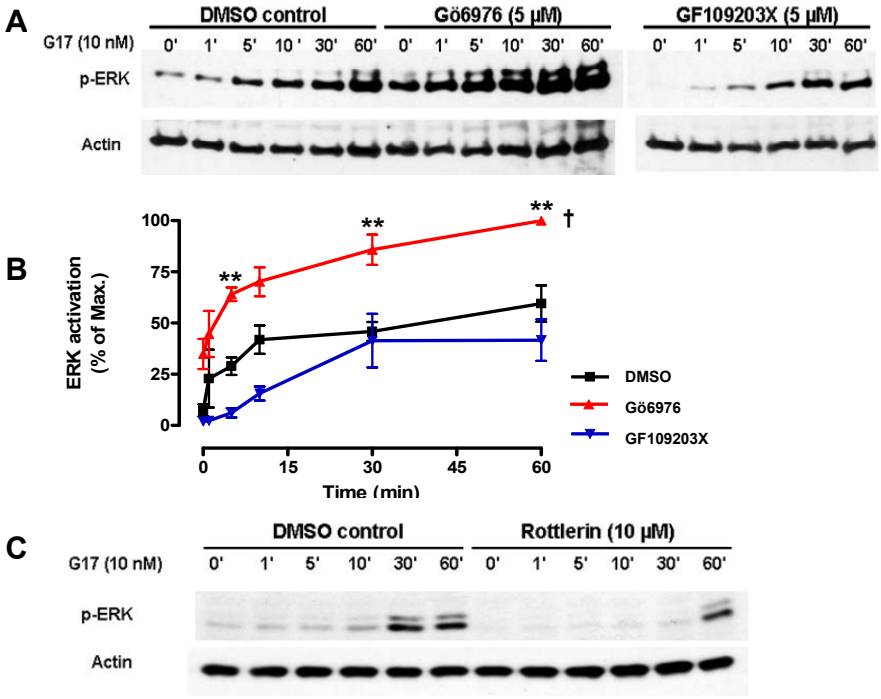

Figure 3.1: PKC mediated ERK activation.
HEK-CCK2R cells were pretreated with either DMSO (vehicle) or PKC inhibitors, and challenged with 10 nM G17 for ERK activation. **A**. the cells were pretreated with either DMSO (vehicle), 5 μM Gö6976 or 5 μM GF109203X for 15 min before 10 nM G17 stimulation over a time course (0, 1, 5, 10, 30, and 60 min) at 37°C, then the equal amount of total proteins were analyzed by Western blot for p-ERK (upper panel), and the blot was reprobed for actin (bottom panel). **B**. ERK activation kinetics of either DMSO (vehicle), Gö6976, or GF109203X-treated HEK-CCK2R cells. The experiments were run in triplicate, and the levels of p-ERK were measured by densitometry. Total levels of p-ERK were normalized by the levels of actin control, and the relative amounts (%) to the maximal p-ERK were plotted for the graph. Statistical comparison of the p-ERK activation was performed by using a two-way ANOVA between G17-stimulated cells in the absence and presence of inhibitors (†, $p < 0.001$), and Bonferroni post-tests were performed to compare values at each of the time (**, $p < 0.01$; *, $p < 0.05$). **C**. The cells were pretreated with either DMSO (vehicle), or 10 μM Rottlerin, and the levels of p-ERK (upper panel) and actin (bottom panel) were detected.

PKC-δ-dependent CCK2R-induced ERK Activation

In the previous data, either GF109203X- or Rottlerin-treated HEK-CCK2R cells indicated diminished ERK activation especially early in the time course. Since PKC-δ is sensitive for both chemical inhibitors, this suggested that PKC-δ may control CCK2R-mediated ERK activation. To answer the role of PKC-δ in ERK activation, the endogenous PKC-δ was knocked down by siRNA transfection. The siRNA for PKC-δ was synthesized and obtained from Santa Cruz Biotech, and either specific siRNA for PKC-δ or non-target siRNA control was transfected into HEK-CCK2R cells using Lipofectamine^{TM} 2000 as previously described. After 24 hours of transfection, the cells were split onto 24-well plates. The effects of siRNAs on ERK activation were monitored at 72 hours of transfection by challenging the cells with 10 nM G17 for a time course (0, 1, 5, 10, and 30 min). Immunoblottings were performed for either p-ERK, PKC-δ, or actin as previously described. Statistical comparison of the p-ERK activation was performed by using a two-way ANOVA between G17-stimulated cells in the non--target and PKC-δ siRNA (†, $p < 0.05$), and Bonferroni post-tests were performed to compare values at each of the times (**, $p < 0.01$; ***, $p < 0.001$).

Results and Discussion

As shown in Figure 3.2, the CCK2R-mediated ERK activation was diminished when PKC-δ was inhibited by siRNA transfection, compared to non--target siRNA-transfected cells. In PKC-δ siRNA-transfected cells, the ERK activation was markedly reduced (Figure 3.2.A, right panel), compared to non--target siRNA-transfected cells (Figure 3.2.A, left panel) (†, $p < 0.05$). This finding coincides with previous data that either GF109203X- or Rottlerin-treated HEK-CCK2R cells showed diminished ERK activation. Considering both chemical PKC inhibitors are targeting PKC-δ, this finding

indicates that PKC-δ, a member of calcium-independent novel PKC family, is a modulator for CCK2R-mediated ERK activation. Even though there is no direct evidence that G17 activates PKC-δ via CCK2R in HEK-CCK2R cells, there is reliable evidence that PKC-δ is activated by G17 stimulation in the human gastric cancer cell line, MKN-28, transfected with CCK2R (Ogasa 2003). Therefore, if G17-mediated CCK2R activation promotes PKC-δ activation, ERK activation might be mediated via the CCK2R/PKCδ/MEK signaling axis. However, there is still a possibility that other nPKC isozymes (such as PKC-θ, -ε, -η) regulate CCK2R-induced ERK activation. For further screening potential of PKC isozymes involved in ERK activation, inhibition of other PKC isozyme are required.

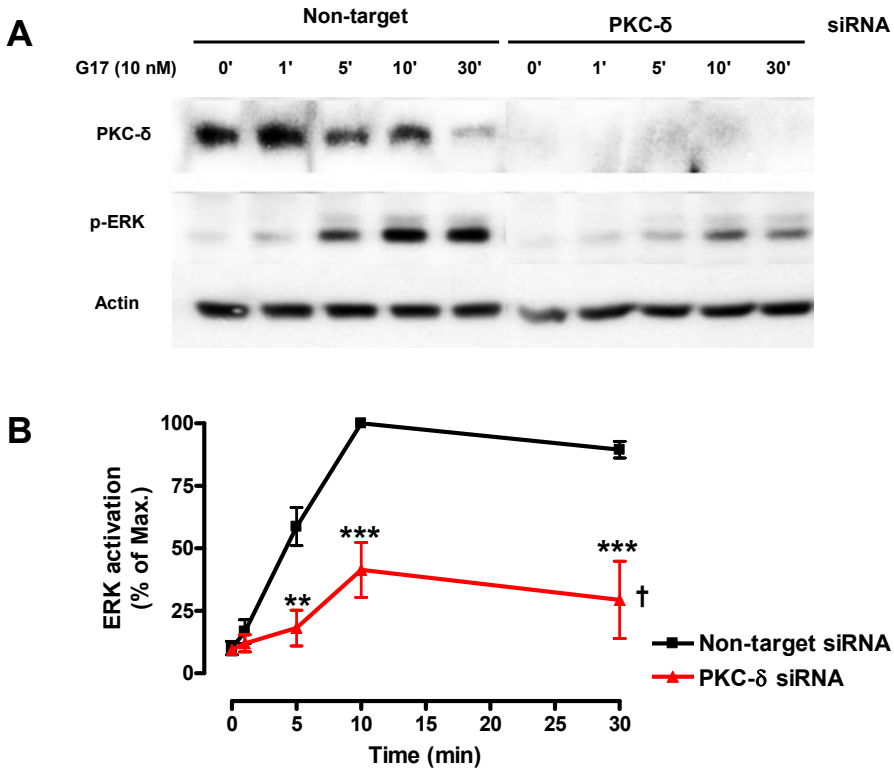

Figure 3.2: PKC-δ-mediated ERK activation.
HEK-CCK2R cells were transfected with either non--target siRNA (control) or PKC-δ siRNA for 72 hours. These cells were challenged with 10 nM G17 for ERK activation for a time course (0, 1, 5, 10, and 30 min), and immunoblottings were performed for p-ERK, PKC-δ, and actin. **A**. Representative immunoblots for PKC-δ (upper panel), p-ERK (middle panel), and actin (bottom panel). **B**. ERK activation kinetics of non--target or PKC-δ siRNA transfected HEK-CCK2R cells. The experiments were run in triplicate, and the levels of p-ERK were measured by densitometry. Total levels of p-ERK were normalized by the levels of actin control, and the relative amounts (%) to the maximal p-ERK were plotted for the graph. Statistical comparison of the p-ERK activation was performed by using a two-way ANOVA between G17-stimulated cells in the non--target and PKC-δ siRNA (†, $p < 0.05$), and Bonferroni post-tests were performed to compare values at each of the times (**, $p < 0.01$; ***, $p < 0.001$).

III. The role of GRK2 in CCK2R-mediated ERK Activation

As seen in the previous chapter, GRK2 is involved in G17-induced CCK2R desensitization (Figure 2.9). The role of GRK2 in receptor desensitization also suggests that GRK2-mediated CCK2R desensitization may inhibit ERK activation, since receptor desensitization would result in loss of the responsiveness to agonist stimulation. Therefore, if GRK2 is knocked down, it would exhibit a greater increase in ERK activation. To test this hypothesis, the role of GRK2 in ERK activation was addressed by inhibiting endogenous GRK2 by siRNA transfection.

After 72 hours of GRK2 siRNA transfection as previously described, the cells were challenged with 10 nM G17 for a time course (0, 1, 5, 10, 30, and 60 min). Each 25 μg of total cell lysates was resolved onto 10% polyacrylamide gel, transferred onto PVDF membrane, and blocked with 5% skim milk in TBST. For immunoblottings of p-ERK and actin, the same procedures were performed as described earlier. To measure the reduced levels of GRK2 by siRNA transfection, mouse polyclonal anti-GRK2 antibody (Santa Cruz Biotech) was diluted as 1:1000 in TBST. Statistical comparison of the p-ERK activation was performed by using a two-way ANOVA between G17-stimulated cells in the non--target and GRK2 siRNA (†, $p < 0.05$), and Bonferroni post-tests were performed to compare values at each of the times (*, $p < 0.05$).

Results and Discussion

The data indicated that GRK inhibition by siRNA promoted a greater increase in ERK activation over a time course, compared to the non--target siRNA control group (Figure 3.3) (†, $p < 0.05$). The data showed a significant difference in G17 stimulation in the time point at 30 min (*, $p < 0.05$), suggesting that the inhibition of GRK2 exerted inhibition of CCK2R desensitization. As seen earlier, the inhibition of GRK2 resulted in

attenuated CCK2R desensitization (Figure 2.9); therefore, this finding explains how receptor desensitization affects receptor-mediated signaling pathways. Presumably, the attenuated CCK2R desensitization, which is caused by depleted GRK2 by siRNA, would produce more IP3 and DAG for activating the PKC-mediated signaling axis for ERK activation. The elevation of ERK activation by GRK2 siRNA was also reported in HEK293 cells expressing angiotensin II receptors, showing GRK2 as a major protein kinase involved in receptor phosphorylation (Kim 2005). Moreover, GRK2 involvement in ERK activation also suggests the possible role of RKIP in CCK2R-mediated ERK activation, since RKIP is known to inhibit GRK activity. Therefore, it was very interesting whether RKIP affects ERK activation. As the Raf-1 kinase inhibitor protein, RKIP is known to inhibit ERK activation by inhibiting Raf-1 kinase activity involved in phosphorylation of MEK, which is the upstream effector for ERK. Therefore, the role of RKIP had been focused on the interaction with Raf-1, before Lorenz et al. found that the PKC-mediated phosphorylation of RKIP interacted with GRK2 for inhibiting GPCR desensitization, which also resulted in potentiated ERK activation (Lorenz 2003). For that reason, the role of RKIP in CCK2R-mediated ERK activation was assessed as follows.

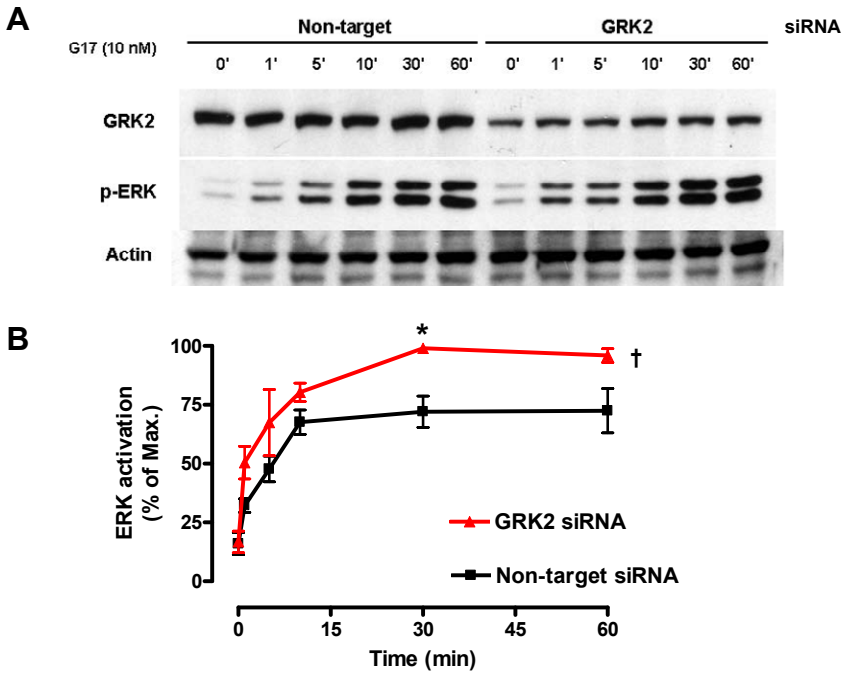

Figure 3.3: The role of GRK2 in CCK2R-mediated ERK activation.
HEK-CCK2R cells were transfected with either non--target siRNA (control) or GRK2 siRNA for 72 hours. These cells were challenged with 10 nM G17 for ERK activation for a time course (0, 1, 5, 10, 30, and 60 min), and immunoblottings were performed for p-ERK, GRK2, and actin. **A**. Representative immunoblots for GRK2 (upper panel), p-ERK (middle panel), and actin (bottom panel). **B**. ERK activation kinetics of non-target or GRK2 siRNA transfected HEK-CCK2R cells. The experiments were run in triplicate, and the levels of p-ERK were measured by densitometry. Total levels of p-ERK were normalized by the levels of actin control, and the relative amounts (%) to the maximal p-ERK were plotted for the graph. Statistical comparison of the p-ERK activation was performed by using a two-way ANOVA between G17-stimulated cells in the non-target and GRK2 siRNA (†, $p < 0.05$), and Bonferroni post-tests were performed to compare values at each of the times (*, $p < 0.05$).

IV. The Role of RKIP in CCK2R-mediated ERK Activation

As described in the previous chapter, the inhibition of RKIP showed enhanced G17-induced CCK2R desensitization, however the inhibition of GRK2 exhibited attenuated receptor desensitization. These observations strongly suggest a role of RKIP

in regulating ERK activity in HEK-CCK2R cells. In the previous experiment, the inhibition of GRK2 by siRNA showed a greater increase in ERK activation, compared to the non-target siRNA-transfected cells. Considering the opposite effect on CCK2R desensitization compared to GRK2, it is possible to expect that the inhibition of RKIP would weaken ERK activation. To test this hypothesis, the role of RKIP in ERK activation was addressed by inhibiting endogenous RKIP by siRNA transfection.

After 72 hours of RKIP siRNA transfection as previously described, the cells were challenged with 10 nM G17 for a time course (0, 1, 5, 10, and 30 min). Each 25 μg of total cell lysates was resolved onto 12% polyacrylamide gel, transferred onto PVDF membrane, and blocked with 5% skim milk in TBST. For immunoblottings of p-ERK and actin, the same procedures were performed as described earlier. The levels of knockdown by RKIP siRNAs also were confirmed by immunoblots using an anti-RKIP antibody with 1:1000 dilution in TBST. The experiments were run in triplicate, and the levels of p-ERK were measured by densitometry. Total levels of p-ERK were normalized by the levels of actin control, and the relative amounts (%) to the maximal p-ERK were plotted for the graph. Statistical comparison of the p-ERK activation was performed by using a two-way ANOVA between G17-stimulated cells in the non-target and RKIP siRNA (†, $p < 0.05$), and Bonferroni post-tests were performed to compare values at each of the times (***, $p < 0.001$).

Results and Discussion

As seen in Figure 3.4, the effects of RKIP knockdown by siRNA on CCK2R-mediated ERK activation were monitored by immunoblotting for p-ERK. The data indicated that the inhibition of RKIP showed markedly reduced ERK activation compared to the non-target siRNA-transfected group (†, $p < 0.05$). This result was

expected since RKIP knockdown showed enhanced CCK2R desensitization. Presumably, the depleted RKIP levels were insufficient to interfere with GRK2 activity. Therefore, this suggests the possibility that more GRK2 activity can induce rapid CCK2R desensitization to the agonist stimulation.

Theoretically, unphosphorylated RKIP in the basal state inhibits ERK activation by association onto Raf-1. This association disrupts the interaction between Raf-1 and MEK, which is required for ERK activation. Therefore, if RKIP is knocked down by siRNA, more ERK activation in the basal state might be expected. However, the data indicated that ERK activation did not result in a significant increase in the basal state data. This discrepancy might be explained as follows. It may be possible to assume that some degree of the constitutive RKIP phosphoylation by either the basal CCK2R and/or PKC activities could be able to interfere with GRK2-mediated receptor desensitization resulting in more basal level of ERK activation. Therefore, RKIP inhibition would exhibit less ERK activation compared to the control group in the basal state. Because HEK-CCK2R cells were grown in the media supplemented with 10% FBS, it may be possible that the phosphorylated RKIPs are upregulated. This change in the balance between unphosphorylated and phosphorylated RKIPs may result in ERK activation. The phosphorylation of RKIP is dependent on PKC activity, which is induced by an increase in signaling molecules such as calcium, DAG, PMA, and IP3. Moreover, epidermal growth factor (EGF) receptor (EGFR), a member of receptor tyrosine kinases, is also known to activate PKCs resulting in RKIP phosphorylation (Corbit 2003). This finding also raises the possibility that EGFR and CCK2R synergistically induce more phosphorylated RKIPs by transactivation of EGF by CCK2R activation in the presence of serum.

Taken together, this data indicates that inhibition of RKIP by siRNA reduced ERK activation over a time course. Considering the role of RKIP in inhibition of GRK2, this finding also coincides with the previous observation that ERK activation was increased by the inhibition of GRK2.

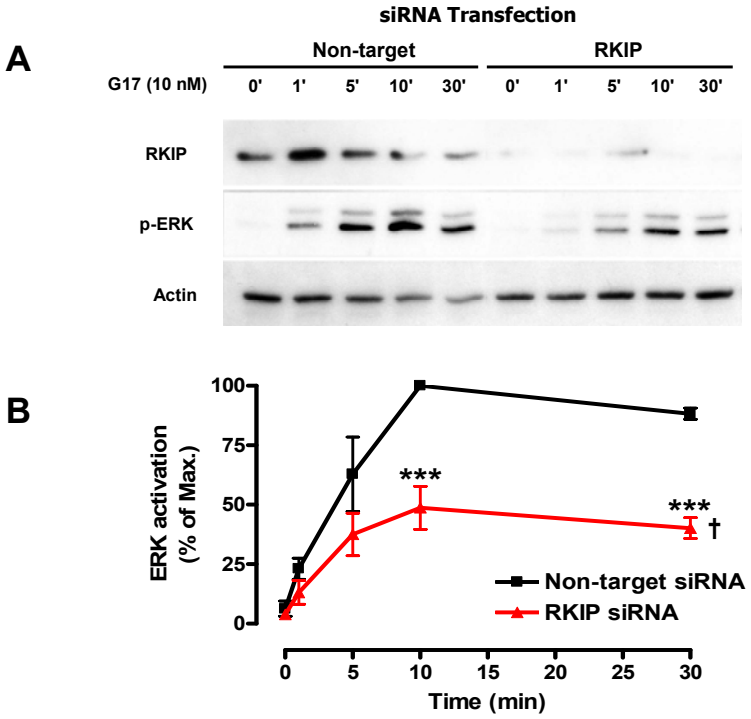

Figure 3.4: RKIP-mediated ERK activation.
HEK-CCK2R cells were transfected with either non-target siRNA (control) or RKIP siRNA for 72 hours. These cells were challenged with 10 nM G17 for ERK activation for a time course (0, 1, 5, 10, and 30 min), and immunoblottings were performed for p-ERK, RKIP, and actin. **A**. Representative immunoblots for RKIP (upper panel), p-ERK (middle panel), and actin (bottom panel). **B**. ERK activation kinetics of non-target or RKIP siRNA-transfected HEK-CCK2R cells. The experiments were run in triplicate, and the levels of p-ERK were measured by densitometry. Total levels of p-ERK were normalized by the levels of actin control, and the relative amounts (%) to the maximal p-ERK were plotted for the graph. Statistical comparison of the p-ERK activation was performed by using a two-way ANOVA between G17-stimulated cells in the non-target and RKIP siRNA (†, $p < 0.05$), and Bonferroni post-tests were performed to compare values at each of the times (***, $p < 0.001$).

The Role of S153 on RKIP in CCK2R-mediated ERK Activation

In previous experiments, I have shown that RKIP inhibition by siRNA reduced CCK2R-mediated ERK activation. Since RKIP is phosphorylated on S153 by PKC activity, the next question was whether the phosphorylation at S153 on RKIP affects ERK activation. The phosphorylation at S153 on RKIP results in inhibition of receptor desensitization by interfering with GRK2, and the ERK activation was increased by overexpressing wild type RKIP (Lorenz 2003). Also, I have shown that the phosphorylation at S153 on RKIP inhibited CCK2R desensitization (Figure 2.11). Therefore, it was also interesting to consider whether this phosphorylation can potentiate ERK activation. Through this experiment, the role of the phosphorylated S153 on RKIP in CCK2R-mediated ERK activation was addressed by transfecting either S153A-RKIP (the phosphorylation-deficient mutant for RKIP) or WT-RKIP.

Either S153A-RKIP, WT-RKIP, or vector construct (pcDNA3.1+) was transiently transfected into HEK-CCK2R cells as previously described. The following day, the cells were divided onto 24-well plates. After a total of 48 hours of transfection, the cells were challenged with 10 nM G17 for a time course (0, 1, 5, and 10 min). Each 35 μg of total cell lysates was resolved onto 12% polyacrylamide gel, transferred onto PVDF membrane, and blocked with 5% skim milk in TBST. For immunoblottings of p-ERK and actin, the same procedures were performed as described earlier. To confirm the expressions of RKIP constructs, an immunoblot for RKIP was performed by using an anti-RKIP antibody with 1:1000 dilution in TBST. The experiments were run in duplicate, and the levels of p-ERK were measured by densitometry. Total levels of p-ERK were normalized by the levels of actin control, and the relative amounts (%) to the maximal p-ERK were plotted in the graph. Statistical comparison of the p-ERK activation was performed by using a two-way ANOVA between G17-stimulated cells in

the vector, S153A-RKIP, and WT-RKIP (†, $p < 0.05$), and Bonferroni post-tests were performed to compare values at each of the times (*, $p < 0.001$).

Results and Discussion

By immunoblottig for p-ERK, the effects of transient transfection of either S153A-RKIP, WT-RKIP, or vector construct (pcDNA3.1+) on CCK2R-mediated ERK activation was addressed (Figure 3.5). Compared to the vector- or S153A-RKIP-transfected cells, the cells transfected with WT-RKIP showed significant increase in ERK activation at the 5-min time point (***, $p < 0.001$). This finding indicates that increased levels of RKIP positively potentiated ERK activation and strongly supports the previous data using RKIP siRNA. Considering previous CCK2R desensitization data, it is possible to assume that the RKIIP-mediated inhibition of CCK2R desensitization promoted enhanced ERK activation. However, S153A-RKIP transfected cells did not show any significant difference compared to vector-transfected cells. It also did not mimic the effects of RKIP siRNA, which showed attenuated ERK activation. Since S153A-RKIP is the phosphorylation-deficient mutant, it might be unable to compete or inhibit endogenous RKIP for regulating GRK2.

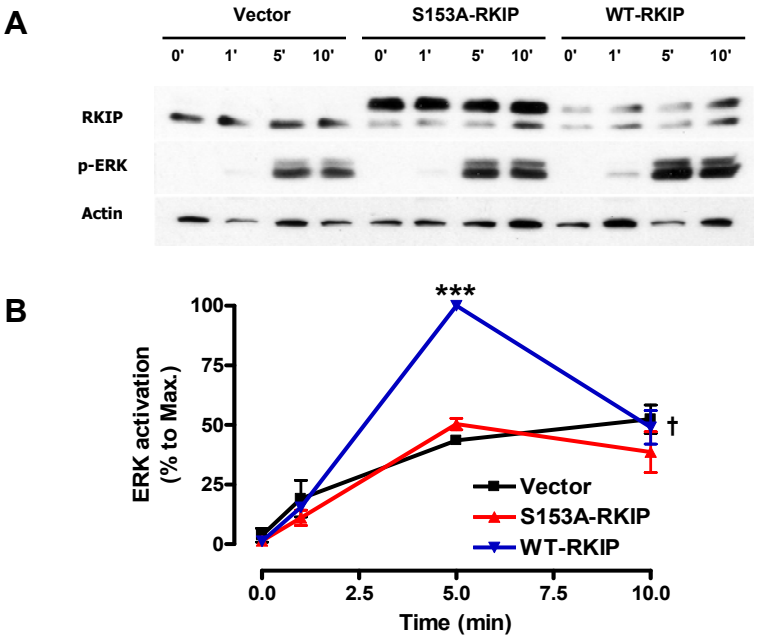

Figure 3.5: The effects of transient transfection of RKIP constructs in ERK activation. HEK-CCK2R cells were transfected with either pcDNA3.1 (vector control), S153A-RKIP, or WT-RKIP constructs for 72 hours. These cells were challenged with 10 nM G17 for ERK activation for a time course (0, 1, 5, and 10 min), and immunoblottings were performed for p-ERK, RKIP, and actin. **A**. Representative immunoblots for p-ERK (upper panel), RKIP (middle panel), and actin (bottom panel). **B**. ERK activation kinetics of non-target- or RKIP siRNA-transfected HEK-CCK2R cells. The experiments were run in duplicate, and the levels of p-ERK were measured by densitometry. Total levels of p-ERK were normalized by the levels of actin control, and the relative amounts (%) to the maximal p-ERK were plotted in the graph. Statistical comparison of the p-ERK activation was performed using a two-way ANOVA between G17-stimulated cells in the vector, S153A-RKIP, and WT-RKIP (†, $p < 0.05$); and Bonferroni post-tests were performed to compare values at each of the times (***, $p < 0.001$).

V. Summary of Chapter 3.

Table 3.1 CCK2R-induced ERK activation

Targets	Treatment	ERK Activation
PKC	Inhibition of cPKC and nPKC by GF109203X	Decrease
	Inhibition of cPKC by Gö6976	Increase
	Inhibition of PKC-δ	Decrease
GRK2	Inhibition of GRK2 by siRNA	Increase
RKIP	Inhibition of RKIP by siRNA	Decrease
	Overexpression of WT-RKIP	Increase
	Overexpression of S153-RKIP	No effect

In this chapter, I tested the hypothesis that CCK2R-induced ERK activation is regulated by PKC-mediated RKIP phosphorylation. To test the hypothesis, the first approach was to identify PKC isozymes involved in CCK2R-induced ERK activation. As seen in Figure 3.1, the inhibition of both cPKCs and nPKCs by GF109203X indicated diminished ERK activation compared to vehicle control group, while inhibition of cPKC by Gö6976 resulted in augmentation of ERK activation. This finding suggested that nPKCs are responsible in the PKC/MEK/ERK signaling axis activated by CCK2R. PKC isozymes activated by CCK2R have been known to be PKC-α, -β, -δ, -ε, and -η (Yassin 1995; Sturany 2002; Ogasa 2003), and PKC-δ, -ε, and –η are members of nPKC. Therefore, the effect of PKC-δ inhibition on ERK activation was tested as a first approach to screen nPKCs, and it showed diminished ERK activation by either Rottlerin or PKC-δ siRNA treatments. This observation indicates that PKC-δ is the one of PKC

isozymes involved in CCK2R-induced ERK activation, however PKC-δ does not appear to phosphorylate RKIP via CCK2R activity considering previous findings that inhibition of PKC-δ by siRNA did not enhance CCK2R desensitization. Therefore, I assume that other nPKCs may be involved in CCK2R-mediated RKIP phosphorylation, and this phosphorylation might act as an inhibitor for GRK2-mediated CCK2R desensitization. Otherwise, the phosphorylation of RKIP may rely on other PKC activities rather than CCK2R-mediated PKC activity. Also, the augmentation of ERK activation by the inhibition of cPKCs by Gö6976 suggests that cPKC may not be involved in RKIP phosphorylation. If cPKCs are involved in RKIP phosphorylation, the inhibition of cPKCs should exhibit reduced ERK activation because the inhibition of PKCs responsible for RKIP phosphorylation would not inhibit GRK2 activity for receptor desensitization.

In previous desensitization data, the inhibition of GRK2 showed reduced CCK2R desensitization, therefore the role of GRK2-mediated CCK2R desensitization in ERK activation was also addressed. The GRK2 inhibition by siRNA indicated increased CCK2R-mediated ERK activation, indicating that receptor desensitization mediated by GRK2 negatively regulates ERK activation by reducing receptor activity.

Finally, the involvement of RKIP in CCK2R-mediated ERK activation was addressed by either inhibition of RKIP by siRNA or overexpression of wild type RKIP. The data indicated that RKIP inhibition promoted reduced ERK activation, whereas RKIP overexpression exhibited potentiating ERK activation. Moreover, overexpression of S153A-RKIP, which is a PKC-mediated phosphorylation deficient mutant, did not increase ERK activation compared to the vector control group. Therefore, these findings strongly suggest that PKC-mediated RKIP phosphorylation enhances ERK activation by inhibition of GRK2.

Chapter 4. Conclusions and Future Directions

PKC-mediated CCK2R Desensitization

Agonist-induced CCK2R desensitization was monitored by Ca^{2+} imaging using different PKC inhibitors and specific siRNA for PKC isozymes. The inhibition of PKC by Gö6976 (inhibition for cPKCs) and GF109203X (inhibition for cPKCs and nPKCs) showed a rapid desensitization compared to the vehicle control group. This finding suggested that the inhibition of cPKCs was enough to facilitate CCK2R desensitization. Therefore, I assumed that cPKCs have inhibitory effects on receptor desensitization, and cPKC-mediated inhibition of CCK2R desensitization might involve RKIP phosphorylation for GRK2 inhibition. However, the inhibition of either PKC-α or -β, the members of cPKC family, resulted in attenuated CCK2R desensitization (Figures 2.5 and 2.6). It implies that both PKC-α and -β do not inhibit receptor desensitization, rather these PKCs are required for enhanced receptor desensitization. The remaining target for cPKC is PKC-γ. Therefore, the effects of PKC-γ in CCK2R desensitization should be tested for deducing the final conclusion for cPKC-mediated CCK2R desensitization. Also, it is possible that one single knockdown of cPKC isozymes maybe not enough to mimic a rapid desensitization as cPKC inhibitor. Therefore, if PKC-γ knockdown does not induce a rapid CCK2R desensitization, the combined knockdown of cPKCs by siRNAs will be tested for CCK2R desensitization to address the role of cPKCs in CCK2R desensitization. Receptor desensitization should affect receptor-induced ERK activation; therefore, if cPKCs inhibit receptor desensitization, the inhibition of cPKCs should result in decrease in ERK activation. However, in Chapter 3, the inhibition of cPKCs by Gö6976 promoted an increase in ERK activation compared

to vehicle control. This finding may also suggest that cPKC does not interfere with CCK2R desensitization. However, the relationship between functional receptor desensitization and ERK activation is not always simplified as described, since there are evidence that receptor internalization promoted by receptor desensitization is also known to induce ERK activation. For example, the inhibition of internalization by knockdown of β-arrestin resulted in attenuated ERK activation in β2-adrenergic receptor (Daaka 1998).

For searching PKC isozymes involved in receptor desensitization, PKC-δ, a member of nPKCs, was selected for inhibition by siRNA, and it also showed attenuated CCK2R desensitization, suggesting that PKC-δ is not the PKC isozyme responsible for RKIP phosphorylation.

Taken together, the data suggests that the CCK2R desensitization is inhibited by the activity of cPKCs. However, this role of cPKCs only was confirmed when all cPKCs were inhibited together by Gö6976, and each knockdown of either PKC-α, or -β did not mimic the effects of either Gö6976 or GF109203X treatment. Therefore, it is also possible that chemical PKC inhibitors may have some side effects for other kinase-related proteins that affect CCK2R desensitization. However, radioligand binding data indicated that at least both chemical PKC inhibitors did not affect agonist binding onto CCK2R receptors. Therefore, it is still unclear which PKC isozymes mediate inhibition of CCK2R desensitization by RKIP-mediated inhibition of GRK2. Based on other literature, PKC-α, -βI, -βII, -γ, -δ and –ζ, have been identified as the protein kinases involved in the phosphorylation at Serine 153 on RKIP (Corbit 2003; Lorenz 2003). However, those findings do not mean that the identified PKCs are capable of phosphorylating RKIP *in vivo,* because those experiments were done by *in vitro* kinase

assays using purified PKCs. Also, there is still a possibility that other PKCs and/or other protein kinases are involved in RKIP phosphorylation.

PKC-mediated CCK2R-induced ERK Activation

The effects of PKC inhibition on CCK2R-mediated ERK activation were also assessed by performing immunoblotings for p-ERK. The cPKC inhibition by Gö6976 showed enhanced CCK2R-mediated ERK activation compared to the vehicle control group, suggesting that cPKCs do not modulate the PKC/MEK/ERK axis directly. Rather, cPKC activity may inhibit this signaling axis for ERK activation. Furthermore, the inhibition of both cPKC and nPKC by GF109203X treatment diminished CCK2R-mediated ERK activation, suggesting the role of nPKCs in ERK activation. Also, the inhibition of PKC-δ with Rottlerin exhibited a loss of CCK2R-mediated ERK activation, and this finding was additionally confirmed by the inhibition of PKC-δ by siRNA. Taken together, PKC-δ is the modulator for CCK2R-induced ERK activation, but PKC-δ does not appear to mediate RKIP phosphorylation, considering the fact that inhibition of PKC-δ did not result in enhanced CCK2R desensitization, which was expected if PKC-δ mediates inhibition of CCK2R desensitization by phosphorylating RKIP. Therefore, through PKC-δ experiments, it is possible to conclude that CCK2R mediates ERK activation via PKC-δ activation. However, receptor-mediated PKC-δ activation does not involve RKIP phosphorylation, which leads to inhibition of GRK2-mediated CCK2R desensitization.

PKC-mediated CCK2R Desensitization and ERK Activation

By inhibiting cPKCs, I have shown three distinct findings in CCK2R: facilitation of CCK2R desensitization, enhancement of ERK activation, and inhibition of CCK2R

internalization. Considering the observation that the inhibition of cPKC did not affect CCK2R phosphorylation, it is possible to consider that impaired CCK2R internalization results from the defect in endocytosis machinery, not in receptor phosphorylation. If cPKC is required for receptor internalization, but also inhibits GRK activity, this could explain how cPKC can regulate both receptor desensitization and internalization. In fact, cPKC is known to mediate endosomal trafficking and also inhibits GRK2 activity via phosphorylating RKIP. Further, the fact that the inhibition of cPKCs resulted in impaired CCK2R internalization strongly suggests the possibility that increased ERK activity is correlated with impaired receptor internalization. This hypothesis is also supported by previous data for GRK2, which showed that GRK2 inhibition by siRNA resulted in slow CCK2R desensitization and enhanced ERK activation. This data implies that if GRK2-mediated receptor phosphorylation is impaired, it potentiates ERK signaling by accumulated receptors onto the plasma membrane for further activation.

In general, GPCR phosphorylation promotes receptor internalization, but growing evidence suggests that receptor phosphorylation alone is not enough to induce receptor internalization, rather it would be the correct expression that the phosphorylated receptors can recruit the components for receptor endocytosis. The key molecules for triggering endocytosis are β-arrestins, and the β-arrestin binding is generally dependent on GRK-mediated GPCR phosphorylation. There is no reliable evidence that PKA- or PKC-mediated receptor phosphorylation recruits β-arrestin binding onto receptors, even PKA or PKC phosphorylates identical receptors as GRK does (Tobin 2008). Therefore, it would be more interesting if I could address the role of β-arrestin in PKC-mediated CCK2R internalization.

Recently, Dr. Lefkowitz's group reported that the parathyroid hormone receptor (PHR)-mediated ERK activation pathway can be dissected by two distinct pathways: G

protein- and β-arrestin-dependent pathways. They confirmed that the G protein-dependent pathway activates p-ERK in early phase, while the β-arrestin-dependent pathway regulates ERK activity in late phase of agonist stimulation (Gesty-Palmer 2006). This finding also explains the role of receptor internalization in ERK activation, considering the fact that β-arrestins are the key regulators for agonist-stimulated GPCR internalization. Conversely, the activation of ERK interferes with GPCR internalization by phosphorylating β-arrestin (Lin 1999). Therefore, if the inhibition of cPKCs promotes more ERK activation through an unknown mechanism, it also may interfere with agonist-induced receptor internalization. I assumed that PKC-dependent RKIP mediated inhibition of GRK2 is the mechanism for enhancing ERK and inhibiting CCK2R internalization.

RKIP-mediated CCK2R Desensitization

The phosphorylation of RKIP by PKC is known to inhibit GRK2-mediated receptor desensitization, and it also raises the possibility that RKIP may act as a regulator between PKC and GRK2 for CCK2R desensitization. In fact, RKIP inhibition by siRNA indicated enhanced agonist-induced CCK2R desensitization, and the overexpression of WT-RKIP showed slower desensitization. This finding indicates the role of RKIP in CCK2R desensitization. Also, the overexpression of S153A-RKIP, the phosphorylation-deficient mutant, promoted enhanced CCK2R desensitization, indicating the importance of PKC-mediated RKIP phosphorylation.

RKIP-mediated CCK2R-induced ERK Activation

The role of RKIP in ERK activation also was addressed by transfecting either RKIP siRNA or RKIIP constructs. The data indicated that RKIP was required for

CCK2R-mediated ERK activation, suggesting the possible role for inhibiting GRK2. Because GRK2 was required for CCK2R desensitization, and inhibition of GRK2 resulted in enhanced ERK activation, RKIP-mediated GRK2 inhibition would result in potentiation of ERK activation. This hypothesis is supported by previous data that ERK activation was intensified when RKIP was overexpressed, whereas overexpression of S153A-RKIP did not increase ERK activation in HEK-CCK2R cells.

RKIP-mediated CCK2R Desensitization and ERK Activation

The mechanism of how RKIP regulates CCK2R desensitization and ERK activation was investigated. First, the effects of PKC inhibitors in GRK2 association onto CCK2R were addressed by co-immunoprecipitation. If PKC-dependent RKIP phosphorylation inhibits GRK activity, the increased levels of GRK2 association can be expected. However, the data indicated that the increases of GRK2 association onto CCK2R by inhibition of PKCs were not enough to make significant differences compared to the vehicle control. However, it very nearly gave a significant difference between vehicle- and Gö6976-treated groups when the cells were challenged with G17 ($p = 0.052$). Therefore, there is still a possibility that cPKC-mediated RKIP phosphorylation is required for GRK2 association. However, more experiments need to be done.

By overexpressing RKIP and GRK2, the role of PKC effectors in the association of RKIP and GRK2 also was addressed. The data indicated that PMA stimulation for 5 min was enough to promote strong association between RKIP and GRK2. However, cPKC inhibition with Gö6976 did not inhibit the association. Moreover, G17 stimulation did not potentiate this association, suggesting that G17-mediated CCK2R activation did not activate the PKC isozyme involved in RKIP phosphorylation. Therefore, it is possible to postulate that RKIP phosphorylation is mediated by other

PKCs activated by PMA. However, it might be possible that the expression levels of both constructs were too high to generate specific interaction mediated by PKC mediated RKIP phosphorylation. Therefore, it might be helpful if appropriate amounts of both constructs were transfected into HEK-CCK2R cells for discriminating the effects of G17 and/or PKC inhibitors in RKIP and GRK2 binding. In spite of this consideration, the data strongly raises the possibility that RKIP phosphorylation is mediated by other signaling pathways resulting in PKC activation. One of the possible candidates would be the receptor tyrosine kinase (RTK) family since published reports indicate that EGF stimulation is very efficient for phosphorylating RKIPs. Therefore, it will provide valuable information if the effects of other stimulants for RTKs in RKIP association with GRK2. I assume that EGF stimulation would promote strong association of RKIP on GRK2, since there is a report that EGF stimulation resulted in RKIP association onto GRK2 (Corbit 2003), and also EGF receptors have synergic effects for potentiating CCK2R-mediated signaling (Olszewska-Pazdrak 2004).

Because phosphorylated RKIP is known to inhibit GPCR internalization via inhibiting GRK2, it is also interesting if inhibition of RKIP by siRNA facilitates agonist-induced CCK2R internalization. Also, it will address the more specific role of RKIP, showing the effects of the overexpression of either S153A-RKIP or WT-RKIP in CCK2R internalization. Because RKIP regulates CCK2R desensitization and ERK activation, I expect that overexpression of WT-RKIP would interfere with agonist-induced CCK2R internalization.

The Model of RKIP-mediated CCK2R Desensitization and ERK Activation

In this dissertation, I have shown that RKIP has a role in regulating CCK2R desensitization and ERK activation. As shown in Figure 5.1, RKIP phosphorylation is

dependent on PKC activity, but there is no evidence that CCK2R-mediated PKC activity phosphorylates RKIP. Therefore, I assume that PMA-sensitive PKC activation other than CCK2R-induced PKCs should have a role in RKIP phosphorylation. Presumably, RTK-mediated PKC activation is one of the mechanisms for activating RKIP-involved PKCs. PMA-sensitive PKC-mediated RKIP phosphorylation at S153 promotes dissociation of RKIP from Raf, and it associates with GRK2. This inhibitory association leads to GRK2-mediated CCK2R desensitization. Therefore, G17-challenged CCK2R can deliver more signals from the plasma membrane into the intracellular space, and more PKC activity including PKC-δ results in more ERK activation. Therefore, it can be concluded that RKIP enhances CCK2R-induced ERK activation by inhibiting CCK2R desensitization.

Conclusively, RIKIP positively regulates ERK activation by inhibiting CCK2R desensitization via physical association with GRK2 for its inhibition. Therefore, the significance of my research can be summarized as follows. CCK2R involves maintaining gastrointestinal cells by regulation of cell proliferation and differentiation; also CCK2R activates the ERK-signaling pathway, which is considered an important regulator for cell proliferation, differentiation, apoptosis, and survival. Through this dissertation I proved RKIP's involvement in CCK2R desensitization and ERK activation. The function of RKIP for maintaining balanced CCK2R activation should be considered as a vital role in maintaining normal cell conditions in gastrointestinal tissues. Therefore, the failure of this balanced RKIP-regulated, CCK2R-induced ERK activation may result in cell malignancy and ultimately lead to the development of human cancers.

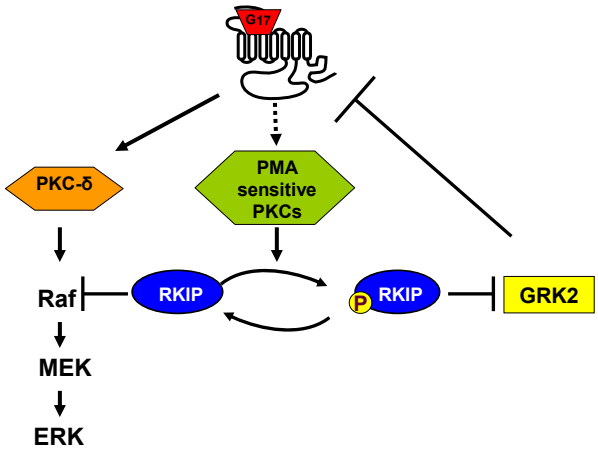

Figure 4.1: Illustration of RKIP-mediated CCK2R desensitization and ERK activation. In the basal state, RKIP physically blocks Raf-1-mediated ERK signaling by association. PMA sensitive-PKC activation promotes the phosphorylation of RKIP, which makes RKIP dissociation from Raf-1 to promote Raf-1/MEK/ERK signaling axis. The phosphorylated RKIP prefers to bind GRK2 for inhibiting GRK2-mediated GPCR phosphorylation and desensitization. RKIP-mediated inhibition of CCK2R desensitization allows it to generate more signaling molecules for activating PKCs including PKC-δ for potentiation of the Raf/MEK signaling axis resulting in ERK activation.

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VITA

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Jeseong Park was born in Seoul, Korea on July 19th, 1972, to the proud parents Hae-Seok Park and Myoung-Seock Kong. Jeseong attended Dankook University in Korea , where he received a Bachelor of Sciences in Biological Science in 1998. He continued graduate studies at Dankook University for his master degree. While majoring zoology for his Master's degree under the tutelage of Dr. Gun-sik Tae, Jeseong conducted research related to the cloning of anti-bacterial genes in insect. In 2000, he started a professional work as a technician at Chung-Ang University in Korea, until he entered Graduate School of Biomedical Sciences at the University of Texas Medical Branch in 2002 for Ph. D degree. He has since worked in the laboratory of Dr. Mark Hellmich at UTMB studying cholecystokinin-2 receptor-mediated signaling and the mechanism of receptor desensitization.

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PUBLICATIONS

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SUMMARY OF DISSERTATION

Raf-1 kinase inhibitor protein (RKIP) is initially known as a suppressor for Raf-1-mediated ERK activation. Moreover, recent findings indicate that RKIP also has a role in G-protein-coupled receptor (GPCR) desensitization. Protein kinase C (PKC)-mediated phosphorylation at Serine 153 (S153) on RKIP switches RKIP association from Raf-1 to GPCR kinase-2 (GRK2) for inhibiting GRK2-mediated G-protein-coupled receptor (GPCR) desensitization. As a member of the GPCR superfamily, Cholecystokinin-2 receptor (CCK2R) is a physiological receptor for gastrin (G17) and activates extracellular signal-regulated kinase (ERK) via the PKC activity. The inhibition of classical PKCs (cPKC, PKC-α,-β, and -γ) by Gö6976 indicated the augment in ERK activation compared to vehicle control, suggesting cPKC's involvement in CCK2R desensitization. CCK2R-mediated ERK activation was significantly decreased when PKC-δ was selectively silenced by siRNAs, indicating that PKC-δ, a member of the novel PKC family, mediates CCK2R-induced ERK activation. Furthermore, the data for CCK2R desensitization showed that inhibited cPKC activity by Gö6976 facilitated CCK2R desensitization. However, the silencing for PKC-α,-β, or –δ by siRNAs indicated that each knockdown of PKC isozymes attenuated CCK2R desensitization. The PKC involvement in CCK2R desensitization and ERK activation also suggested a potential role of RKIP in regulation of CCK2R activity. By either silencing or overexpressing RKIPs, I proved that RKIP acts as a suppressor for CCK2R desensitization, and the phosphorylation at S153 on RKIP plays a crucial role for inhibiting desensitization. The RKIP-mediated inhibition of CCK2R desensitization also resulted in augmentation of receptor-induced ERK activation, and this finding indicates that RKIP acts as a modulator for CCK2R-mediated signaling. The mechanism for RKIP-mediated receptor desensitization was investigated by co-immunoprecipitation of GRK2 with RKIP. The data indicated that RKIP strongly associated onto GRK2 when PKC was activated by phorbol 12-myristate 13-acetate (PMA) treatment, but either G17 stimulation or Gö6976 did not affect on the association. It suggests that PMA-sensitive PKC isozymes are responsible for RKIP phosphorylation;

however, CCK2R-mediated PKC isozymes are not involved in RKIP phosphorylation directly, rather PKC activation by other cellular mechanisms mediate RKIP phosphorylation resulting in GRK2 association. Therefore, I conclude that RKIP mediates CCK2R desensitization and ERK activation through PKC activation.

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