IOWA STATE UNIVERSITY
Digital Repository

Retrospective Theses and Dissertations

Iowa State University Capstones, Theses and Dissertations

2005

Phospholipid regulation by somatostatin in insulin-secreting [beta]-cells

Justin Adam Grodnitzky
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

 Part of the Pharmacology Commons

Recommended Citation
Grodnitzky, Justin Adam, "Phospholipid regulation by somatostatin in insulin-secreting [beta]-cells " (2005). *Retrospective Theses and Dissertations*. 1861.
https://lib.dr.iastate.edu/rtd/1861

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

Phospholipid regulation by somatostatin in insulin-secreting β–cells

by

Justin Adam Grodnitzky

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Co-majors: Physiology (Pharmacology); Toxicology

Program of Study Committee:
Walter H. Hsu, Co-major Professor
Anumantha Kanthasamy, Co-major Professor
Janice Buss
Joel Coats
Tim Day

Iowa State University

Ames, Iowa

2005

UMI Number: 3184616

INFORMATION TO USERS

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleed-through, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

UMI®

UMI Microform 3184616

Copyright 2005 by ProQuest Information and Learning Company.

All rights reserved. This microform edition is protected against unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Justin Adam Grodnitzky

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

Co-major Professor

Signature was redacted for privacy.

For the Co-major Program

Signature was redacted for privacy.

For the Co-major Program

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

1,4,5-trisphosphate (IP_3)

ADP-ribosylation factor (ARF)

Adrenocorticotropin (ATCH)

Arf nucleotide-binding-site opener (ARNO)

Arginine vasopressin (AVP)

Diacylglycerol (DAG)

Endoplasmic reticulum (ER)

G-protein coupled receptor (GPCR)

G-protein inward rectifing K^+ channels (GIRK)

Guanine nucleotide exchange factor (GEF)

G-protein receptor kinase (GRK)

Intracellular calcium concentration ($[Ca^{2+}]_i$)

phosphatidic acid (PA)

phosphatidylcholine (PC)

phospholipase A (PLA)

Phospholipase D (PLD)

Phospholipase C (PLC)

phosphorylate phosphosinsitol 4-phosphosate (PIP)

phosphoinsitol 4,5-bisphosphate (PIP_2)

pleckstrin homology (PH)

protein kinase A (PKA)

protein kinase C (PKC)

somatostatin (SS)

voltage-dependent Ca^{2+} channel (VDCC)

LIST OF FIGURES

CHAPTER 1.

CHAPTER 2.

LIST OF TABLES

CHAPTER 3.

CHAPTER 1: INTRODUCTION

Dissertation Organization

This dissertation is organized into four chapters. Chapter 1 is a general introduction with background information about each of the other chapters. The second chapter "**Somatostatin increases phospholipase D activity and PIP2 synthesis in clonal β-cells HIT-T15**" has been published in the journal of *Molecular Pharmacology*. Chapter 3 "Somatostatin Receptors Signals through EFA6-Arf6 to Activate PLD in Clonal β-Cells, HIT-T15" will be submitted to the *Journal of Biological Chemistry*. Chapter 4 consists of a general conclusion and direction for future research.

Introduction

The focus of this dissertation is to determine the signaling cascade responsible for the paradoxical increase in intracellular calcium concentrations ($[Ca^{2+}]_i$) when somatostatin (SS) and arginine vasopressin (AVP) treatments are added to HIT-T15 cells. In this dissertation, I will demonstrate the ability of SS to activate the small G-protein Arf6 through EFA6, a guanine nucleotide exchange factor. I will further demonstrate that Arf6 stimulates phospholipase D1 (PLD1) to hydrolyse phosphatidylcholine (PC) to phosphatidic acid (PA). PA will stimulate PIP-5 kinase to phosphorylate phosphosinsitol 4-phosphosate (PIP) to phosphoinsitol 4,5-bisphosphate (PIP_2) (Fig. 1). This increase in PIP_2 can act as additional substrate for phospholipase C (PLC), which produces its downstream effect in increasing $[Ca^{2+}]_i$. The details of this mechanism and the components involved will be elucidated in the introduction. HIT-T15 cells are a hamster tumor cell-line of pancreatic β-cells. These cells, like all pancreatic β-cells are known for their ability to secrete the hormone insulin. Insulin is

essential for regulating metabolic processes, most notably the storage of glucose and lipids in the body[1]. Abnormal secretion or response to insulin can lead to the disease state known as diabetes mellitus. Diabetes is classified by high blood glucose levels and abnormal lipid levels in patients[1]. Attenuation of this epidemic disease can only occur with a better understanding of the normal physiological and cellular signaling responsible for insulin secretion and its regulation. SS is important for the regulation of insulin secretion from pancreas β-cells. In order to fully appreciate SS's novel signaling mechanism discussed in this dissertation introduction, background information will provide into all signaling components and signaling paradigm that are involved in this mechanism.

G-Protein Coupled Receptors:

Many endogenous hormones communicate with pancreatic β-cells to both stimulate and inhibit insulin secretion. The two hormones this dissertation will focus on are SS and AVP. AVP is known to stimulate insulin secretion[2], while SS is a classic inhibitory hormone[1]. These hormones interact with seven-transmembrane domain receptors. These receptors have an extracellular N-terminus and an intracellular C-terminus[3]. These receptors are coupled to heterotrimeric G-proteins, and are generally referred to as G-protein coupled receptors (GPCRs). There are six families of GPCR receptors with each family sharing at least 20% homology in amino acid in the transmembrane region[3]. Class A is known as the rhodopsin-like receptors and consists of such receptors as rhodopsin, adrenergic, chemokine, opioids, SS, melatonin receptors, etc[3]. Class B consists of such receptors as calcitonin, glucagons, latrotoxin, secretin receptors, etc[3]. Class C consists of metabotropic glutamate, GABA-B, calcium-sensing receptors, etc[3]. Class D consists of STE2 pheromone receptors[3]. Class E consists of STE3 pheromone receptors[3].

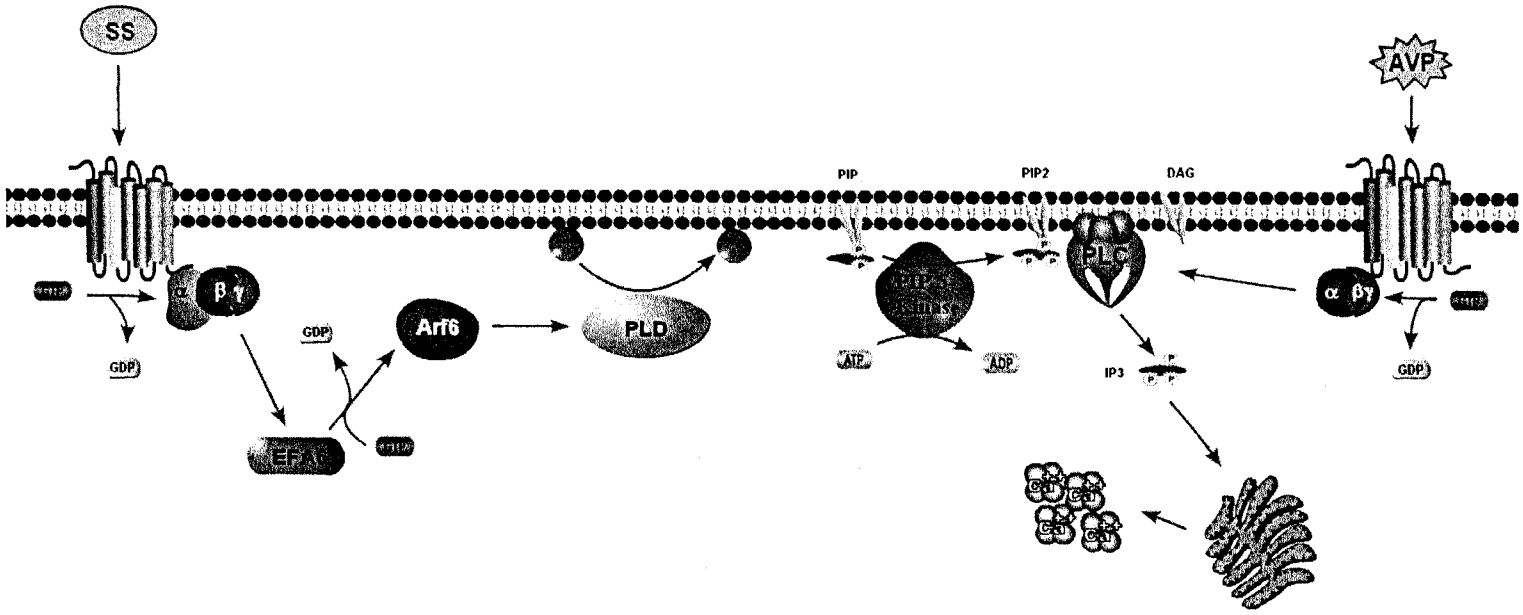

Figure 1. Proposed model of SS-induced increase in PIP_2 and cross-talk with AVP. SS receptors activation releases the bg-dimer and directly activates EFA6, a GEF for Arf6. This causes Arf6 to release GDP and to bind GTP. The active Arf6 will then bind to and activate PLD. PLD will convert phosphatidylcholine (PC) into phosphatidic acid (PA). PA, a known activator of PIP 5-kinase will activate this enzyme synthesizing more PIP_2, providing extra substrate for preactivated PLC-β by AVP. This increase in DAG and IP_3 levels and $[Ca^{2+}]_i$ leads to insulin release.

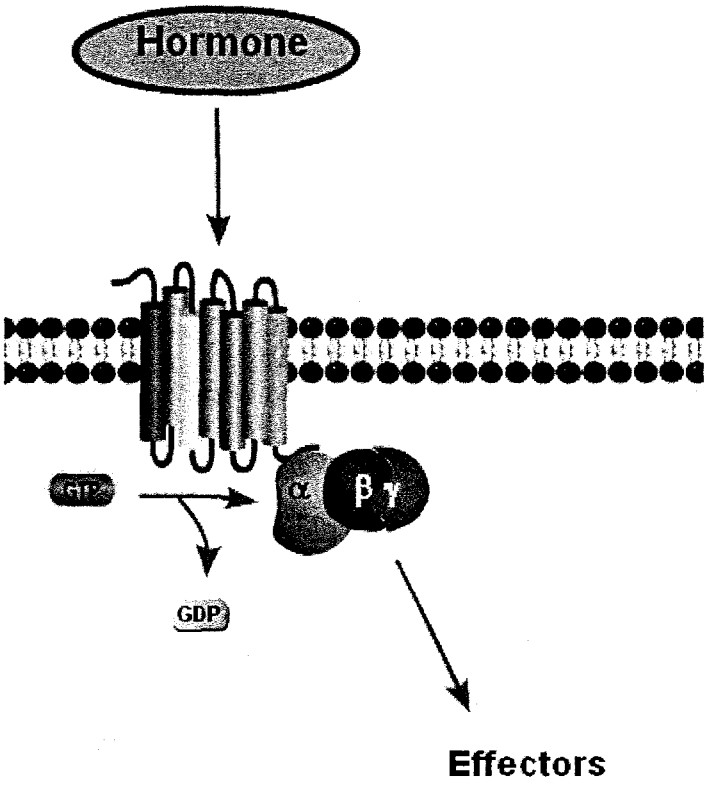

Figure 2. General model of G-protein coupled receptor activation.

Class F consists of cAMP receptors[3]. Heterotrimeric G-proteins consist of three subunits, α, β, and γ. The α subunit possesses GTPase activity[4]. While the α-subunit is in its inactive GDP-bound state, the βγ-dimer and the α-subunit are coupled together and are attached to a GPCR. When a ligand binds to a GPCR, it changes the α-subunit conformation to allow GDP to leave, replacing it with GTP. This reaction converts the α-subunit into the active form. In the GTP-bound state, the α-subunit displaces itself from the plasma membrane and the βγ-dimer. These subunits now are able to exert their effects on respective signaling molecules (Fig. 2). The intrinsic GTPase activity of the α-subunit hydrolyzes GTP back to GDP, which inactivates the α-subunit and resets the system. GPCRs regulate many physiological processes and play a very important role in the etiology and treatment of many pathologic states. Each receptor couples to a distinct α-subunit. The various α-subunits interact with specific effectors, which leads to the

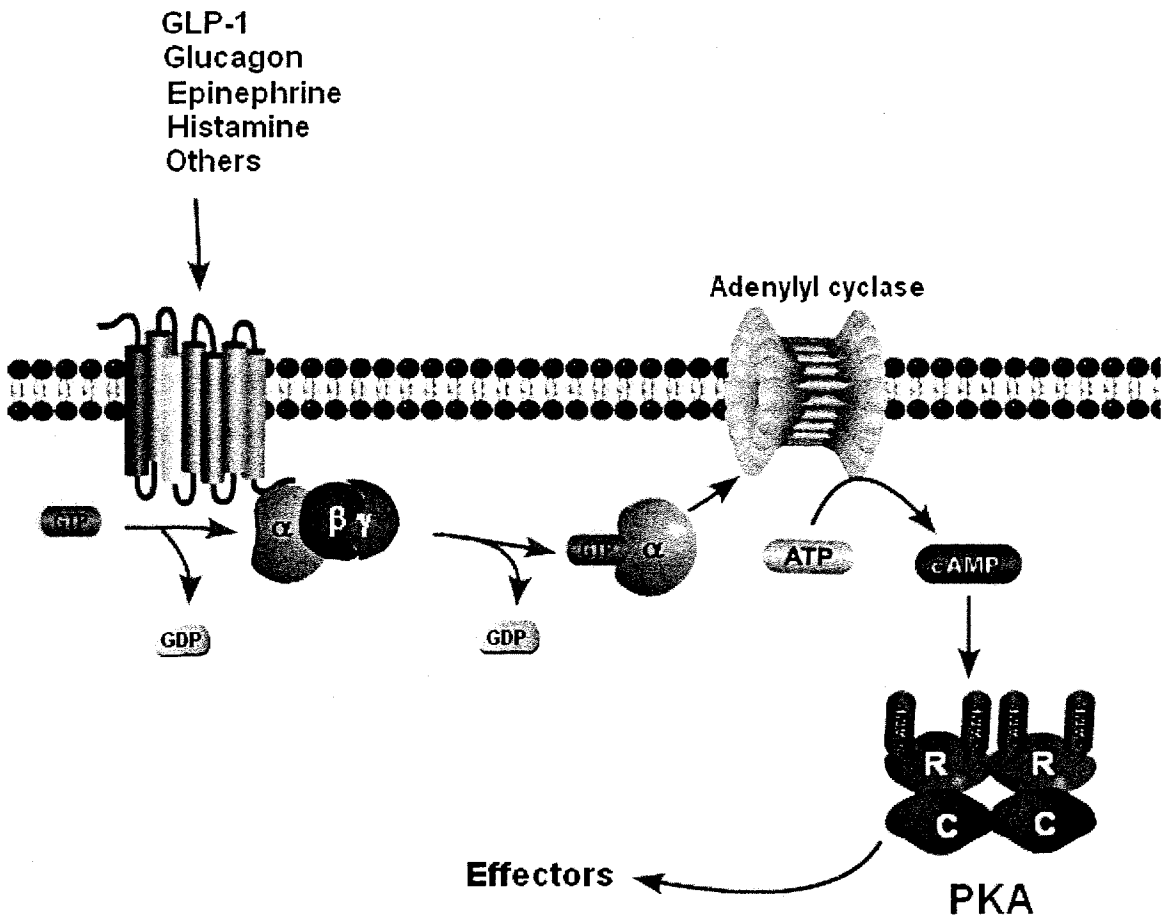

Figure 3. Hormone operated activation of $G\alpha_s$ and adenylyl cyclase pathway. Hormones such as those listed, bind to the receptor, changing the structural conformation of both the receptor and the α-subunits. These structural changes release GDP from the α-subunit, allowing it to bind to GTP. This active form of α_s dissociates from the membrane, allowing it to activate adenylyl cyclase. Adenylyl cyclase converts ATP to cAMP. PKA is then activated by cAMP to phosphorylate its effector.

initiation of the diverse signaling cascade associated with GPCR activation (Fig. 2).The classic α-subunits involved in GPCR signaling are $G\alpha_s$, $G\alpha_q$, and $G\alpha_{i/o}$[4]. These subunits signal through distinct effectors. For example, $G\alpha_s$ is known for its ability to stimulate adenylyl cyclase[3]. Many hormone receptors couple through $G\alpha_s$. β-adrenergic, adenosine A_1, glucagon, AVP-V_2, histamine H_2, glucagon-like peptide, and vasoactive intestinal

polypeptide receptors are a few examples of $G\alpha_s$ coupled-receptors[5]. Active $G\alpha_s$ stimulates adenylyl cyclase causing an increase in cAMP production. cAMP then binds to the regulatory subunits of protein kinase A (PKA), a Ser/Thr-specific kinase. The binding of cAMP to the regulatory subunits of PKA allows the two catalytic subunits of the enzyme to dissociate from the regulatory subunits and interact with downstream effectors (Fig. 3). Traditionally, the stimulation of PKA causes an excitatory cellular response.

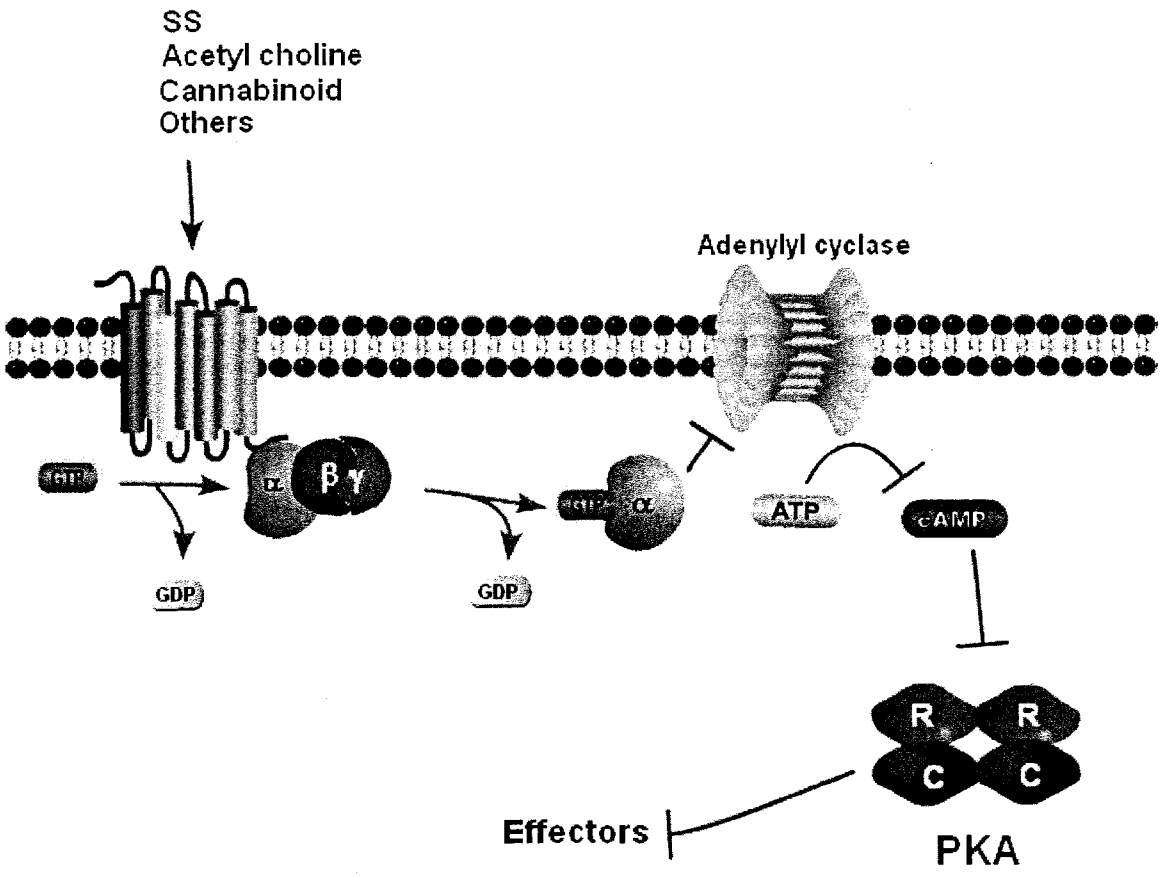

Figure 4. Hormone operated activation of $G\alpha_i$ and inhibition of adenylyl cyclase pathway. Hormones, such as those listed, bind to the receptor, changing the structural conformation of both the receptor and the α-subunits. These structural changes release GDP from the α-subunit, allowing it to bind to GTP. This active form of αi dissociates from the membrane allowing it to inhibit adenylyl cyclase. Adenylyl cyclase is then unable to converts ATP to cAMP, which lowers PKA activity.

The antithesis of $G\alpha_s$ is $G\alpha_{i/o}$. These α-subunits are known to inhibit adenylyl cyclase activity, decreasing levels of cAMP[3]. The lower cAMP levels in cells attenuate PKA activity, producing the opposite cellular response to $G\alpha_s$ (Fig. 4). The attenuation of PKA produced

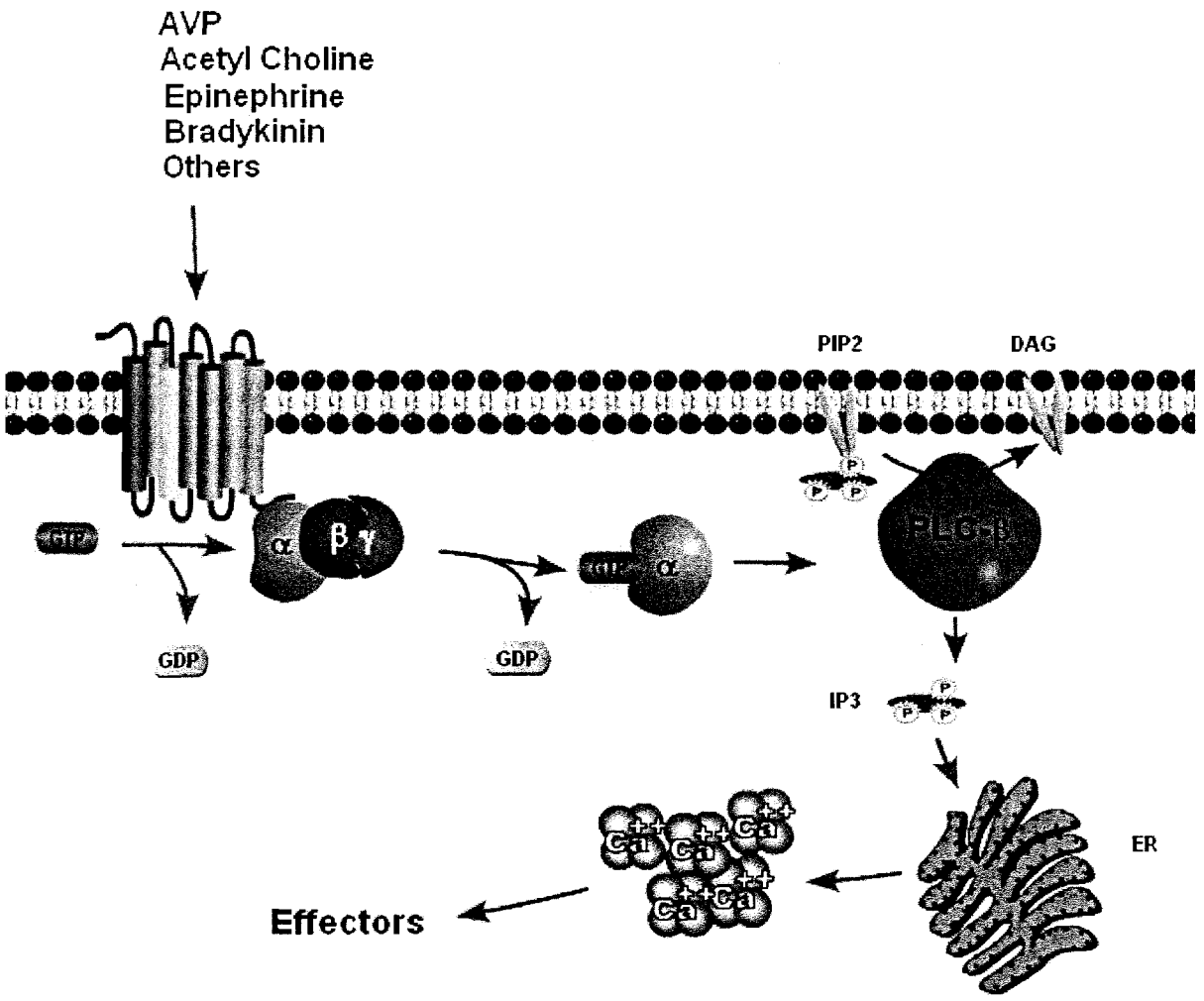

Figure 5. Hormone-operated activation of $G\alpha_q$ and phospholipase C pathway. Hormones, such as those listed, bind to the receptor, changing the structural conformation of both the receptor and the α-subunits. These structural changes release GDP from the α-subunit, allowing it to bind to GTP. This active form of αq dissociates from the membrane, allowing it to bind and activate PLC, converting PIP_2 to IP_3 and DAG.

by $G\alpha_{i/o}$ leads to the inhibitory effect associated with many of the hormones whose receptors couple to $G\alpha_{i/o}$[4]. SS, M_2 and M_4 muscarinic, cannabinoid, and α_2-adrenergic receptors are examples of GPCRs that exert their activity through its coupling to $G\alpha_{i/o}$[4].

$G\alpha_q$ subunits exert their effect on a completely different downstream effector than $G\alpha_s$ and $G\alpha_{i/o}$ subunits. $G\alpha_q$ binds and activates PLC[4]. Although there are four PLC isoforms: PLC-β, PLC-δ, PLC-γ, and PLC-ε, $G\alpha_q$ only activates PLC-β by binding to the G-box domain located on the C-terminus of PLC-β[6]. This enzyme specifically hydrolyzes the membrane phospholipids, PIP_2. PLC cleaves PIP_2 into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 can bind to its receptors on the endoplasmic reticulum (ER), which releases Ca^{2+} from this organelle (Fig. 6). The increase in $[Ca^{2+}]_i$ initiates exocytosis and activates numerous Ca^{2+}-sensitive proteins. DAG, the other product of the PLC reaction, is well known for its ability to activate novel and conventional forms of protein kinase C (PKC)[6]. This $G\alpha_q$ / PLC mediated pathway is activated by many endogenous ligands including hormones and neurotransmitters. The activation of PKC affects cell proliferation and other cell reactions.

The heterotrimeric G-protein α-subunit was classically thought to be the predominant mechanism for receptor-mediated activation of intracellular signaling. The βγ-dimer coupled to the α-subunits, however; it is now appreciated as an important mediator for cell signaling. This dissertation focuses on the βγ-dimer that couples to SS receptors. βγ-dimer is more promiscuous than α-subunit in activating downstream effectors. The β-subunit exists in a β-propeller structure, WD40 repeats. βγ-dimers are known to interact with many signaling proteins such as G-protein receptor kinase (GRK) II and III, adenylyl cyclase, Rho guanine-

nucleotide exchange factor (GEF), PLC, phospholipase A (PLA), G-protein inward rectifying K^+ channels (GIRK), and voltage-dependent Ca^{2+} channel (VDCC)[7].

As indicate above, βγ-dimer can exert its effect on many different effectors, and each plays an essential role in normal cell processing. For example, receptor desensitization is an essential part of cell signaling and this process is critically regulated by βγ-dimer. βγ-dimers released from activated receptor binds to the PH-domain of GRKs, leading to the phosphorylation of activated receptors. The phosphorylated receptors are then bound by arrestin, leading to their homologus desensitization (Fig. 6).

Another important regulator of cell signaling is adenylyl cyclase. This enyzme plays an important role in hormone secretion and gene regulation. Adenylyl cyclase is known to be both stimulated and inhibited by βγ-dimers. βγ-dimer can stimulate adenylyl cyclase isoforms II and IV activity, while inhibiting adenylyl cyclase I activity. Rho-GEF activation by βγ-dimer leading to actin skeleton rearragement is another example of the significance of βγ-dimer in cellular signaling. PLC activation is essential for hormone mediate exocytosis and gene regulation is also regulated by βγ-dimer. Isoforms of PLC-β demonstrate a wide degree of activty towards the βγ-dimer. By binding to the PH-domain of PLC-β, βγ-dimer can stimulate PLC-β 3 and 4, while PLC-β 2 and 1 seem less affected by βγ-dimer activation[7].

There are 6 known β-subunits; 5 distinct isoforms and 1 splice variant of isoform 5[8]. At least 12 different isoforms of the γ-subunit exist in mammalian cells. βγ-dimer can mediate many effectors as indicated above, but the reason not all GPCRs can regulate all of the diverse range of effectors is in part due to Gβγ-isoform specificity for certain distinct

downstream effectors[9, 10, 11]. For example, VDCC are closed upon βγ-dimer release from activated SS and M_4 muscarinic receptors. However, although the SS receptor effect is mediated by $G\beta_1$, while M_4 receptor's effect is mediated by $G\beta_3$[10]. Defined βγ-isoforms can also cause a specific enhancement of GRKII translocation and activation. The binding of GRKII to β-adrenergic and rhodopsin receptors and the subsequent phosphorylation of these receptors is highly dependent on which β-isoform is present. For rhodopsin receptors, the ability of $G\beta_2\gamma_2$ to increase GRKII binding affinity and phosphorylation rate is greater than that of $G\beta_1\gamma_2$[7]. For β-adrenergic receptors, the converse is true. $G\beta_1\gamma_2$ increases GRKII binding affinity and phosphorylation rate more so than $G\beta_2\gamma_2$[7]. In addition, any substitution with a different Gγ-subunit inhibits the ability of GRKII to bind and phosphorylate both rhodopsin and β-adrenergic receptors[7]. These results indicate certain distinct βγ-dimer isoforms can preferentially activate downstream effectors.

Arginine Vasopressin and Somatostatin:

SS and to a lesser extent AVP will be the two GPCR-mediating hormones examined in this dissertation due to their modulation of cell signaling in pancreatic β-cell. AVP is also known as antidiuretic hormone (ADH) and has three receptors, AVP_{1a}, AVP_{1b}, and AVP_2[12]. All these receptors are GPCRs. V_{1a} and V_{1b} act through a PLC dependent pathway, while V_2 signals through $G\alpha_s$ to activate adenylyl cyclase[11]. This hormone is traditionally found in the posterior pituitary gland, but is also present in the perivascular compartments of the pancreas[13,14]. This nonapeptide hormone exerts a number of physiological effects in mammalian systems. AVP plays a major role in regulating body fluid volume, osmolarity and contributes to the maintenance of blood pressure because of the antidiuretic properties[11].

AVP received its name because of its potent vasoconstricting propreties. V_{1a} receptor mediates most of the vasoconstricting properties associated with AVP, while enhancing adrenocorticotropin (ATCH) release is mediated by V_{1b} receptors[12]. In addition, AVP has been shown to increase insulin release[15].

Somatostatin (SS) is a peptide hormone secreted from δ-cells of pancreatic islets, the central and peripheral nervous systems, and gastrointestinal mucosa[16]. In the central and peripheral nervous systems, SS acts as a neurotransmitter to inhibit the release of other neurotransmitters and hormones such as growth hormone, thyrotropin-releasing hormone, thyroid-stimulating hormone, corticotropin-releasing hormone, adrenocorticotropic hormone, acetylcholine, norepinephrine and dopamine[17]. SS also can inhibit endocrine secretions of the gastrointestinal tract and pancreas, such as gastrin, vasoactive intestinal peptide, gastric inhibitory peptide, insulin, glucagon and pancreatic polypeptide[15, 16]. SS is released in two sizes; a 14-amino acid peptide and its prohormone N-terminal extend form, a 28-amino acid peptide[16]. Somatostatin receptor (SSTR), a $G_{i/o}$-coupled receptor, has 5 subtypes - SSTR1–5[18,19]. These receptors all couple to $G\alpha_i$ subunit, and transduce their physiological signal through many effectors ranging from adenylyl cyclase[20], K^+ channel[21], tyrosine phosphatase[22], phospholipase C-β[23], VDCC[24], Na^+-H^+ antiporter[25], and MAP kinase[26]. In this dissertation I will focus on phospholipase D as a novel effector for SS receptor.

PLD activity:

Phospholipase D (PLD) is known for its enzymatic properties to hydrolyze PC to PA. The conversion of PC to PA regulates many cellular processes such as endocytosis, exocytosis, cell proliferation, and cell migration[27,28,29,30,31]. There are two mammalian PLD isoforms,

PLD1 and PLD2. These isoforms differ in their subcellular location and regulation. PLD1 is located in the cytosol, Golgi body, nucleus, and plasma membrane, whereas PLD2 seems to be largely found in the plasma membrane[32]. Each isoform is capable of existing as a splice variant, leading to PLD1a, PLD1b, PLD2a, and PLD2b[33,34]. There are many potential regulators of PLD, but only a few regulators can stimulate both isoforms. The membrane phospholipids, PIP_2, and PKC are both known to stimulate both isoforms of PLD[35,36]. The family of small G-proteins, ADP-Ribosylation Factor (Arf) and Rho are potent stimulators of PLD1, but are unable to stimulate the endogenous forms of PLD2[26,37]. PLD activity can also be stimulated by various GPCRs. A plethora of hormones have been shown to stimulate PLD activity through signaling their GPCRs. Angiotensin II[38], bradykinin[39], carbachol[40], lysophosphatidic acid[41], gonadotropin releasing hormone[42], AVP[43], endothelin[44], thyroid-stimulating hormone[45], prostaglandin $F_{2\alpha}$[46] are examples of the prevalent nature of hormones stimulating PLD. The mechanisms by which these hormones regulate PLD activity are very diverse. One mechanism by which PLD may be stimulated is through PLC-dependent signaling pathway. PLC catalyzes the conversion of PIP_2 to IP_3 and DAG. IP_3 mobilizes Ca^{2+} from ER stores. This increase in $[Ca^{2+}]_i$ is known to activate conventional isoforms of PKC[47]. DAG, the other product of PLC reaction, can also stimulate PKC through its interaction with the C1-domain of both conventional and novel PKC isoforms[47]. Both conventional and novel PKC isoforms are known to enhance the activity of PLD. Another mechanism for receptor-mediated stimulation of PLD is through the activation of small G-proteins. The signaling mechanisms responsible for small G-proteins regulation of PLD are diverse, but two small G-proteins have been firmly established to regulate PLD activity through GPCRs. The small G-proteins Rho and Arf can mediate GPCR activation of PLD. Stimulation of Rho by activation

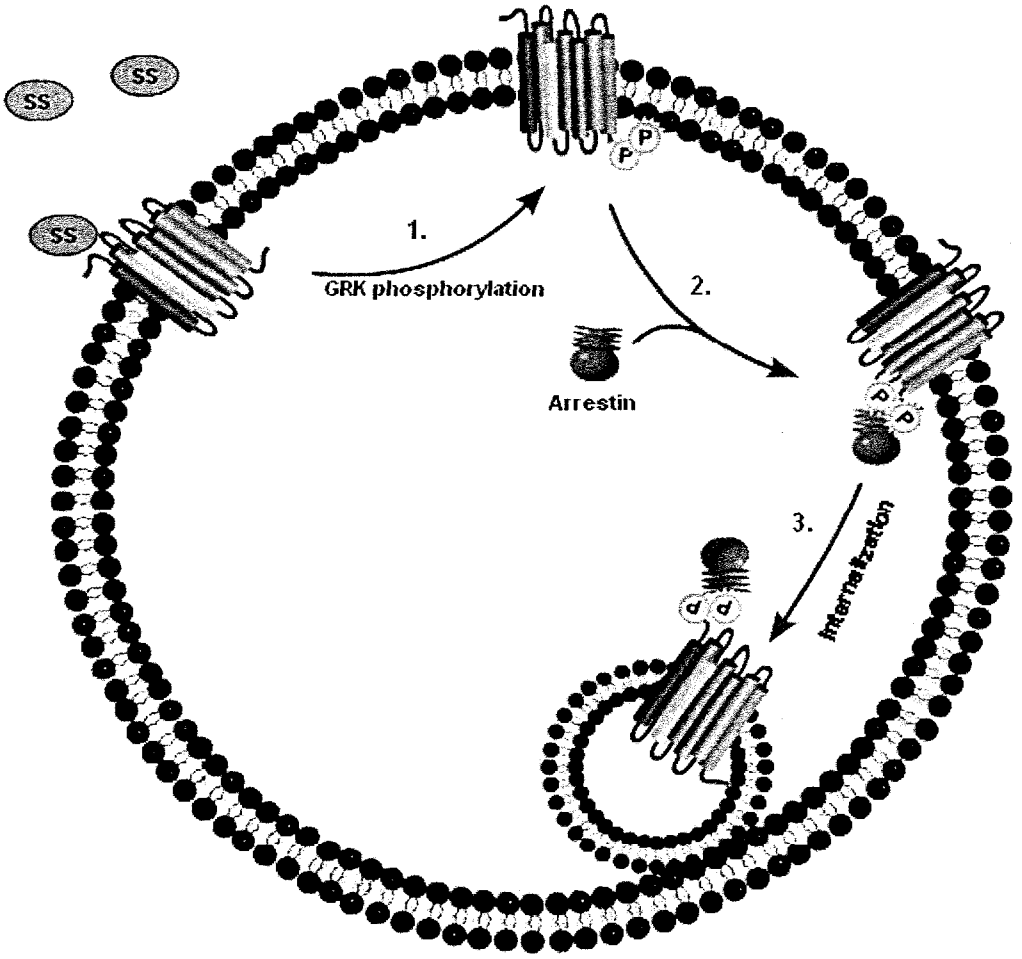

Figure 6. Role of β-arrestin in model of GPCR desensitization. (1) SS receptors are phosphorylated by GRKs on their carboxyl-terminal tails. (2) Arrestin translocates and binds the SS-occupied GRK-phosphorylated receptors, causing receptor desensitized. (3) Arrestin triggers SS receptors for internalize via a clathrin-coated dependent endocytosis mechanism.

of GPCRs is attributed to the associated βγ-dimer binding to the PH-domain of a guanine nucleotide exchange factor for Rho[48] and its direct interaction with $G\alpha_{12/13}$-subunit[49, 50]. These interactions cause GDP to dissociate from Rho, which allows Rho to bind GTP, switching it to its active form. There have been several reports of GPCR activation occurring due to the ability of Arf proteins to directly interact with activated GPCRs. This signal

cascade has been demonstrated in 5-hydroxy tryptamine-2a receptors[51], gonadotropin-releasing hormone receptors[52] and M_3 muscarinic receptors[39]. These studies show the involvement of Arf1, Arf3, and Arf6(see below) in the GPCR-mediated activation of PLD. This signaling cascade is dependent on Arf proteins interacting with the NxxPY motif located on the third intracellular loop of these receptors. NPxxY motif is a common motif found in 94% of Class A family GPCRs[53] and is found in SSTR2 receptor, the SS receptor present in HIT-T15 cells[54]. These studies also demonstrate that the activation of a GPCR can stimulate the conversion of Arf-GDP to its active Arf-GTP bound state, Arf-GTP can then bind to the NxxPY region of the receptor. Another well documented model for GPCR-mediated activation of Arf6 involves the stimulation of β-adrenergic receptors and other GPCRs. Stimulation of these receptors leads to the dissociation of βγ-dimer and subsequent activation of GRK. GRK then phosphorylates the receptors, recruiting β-arrestin to the receptor and leading to receptor desensitization[55, 56, 57]. Upon β-arrestin binding, Arf nucleotide-binding-site opener (ARNO), an Arf GEF, scaffolds with the desensitized receptor complex, activating Arf6. These previous examples are evidence of how Arfs can be involved in modulating signaling cascades initiated upon hormone receptor binding. In this dissertation, I report that SS can activate PLD through an Arf6 dependent pathway, causing a subsequent increase in PIP_2 concentration[58].

Small G-Proteins Arf:

Arf is a member of the Ras GTPase superfamily, which consists of 20-kDa guanine nucleotide-binding proteins. Arfs are inactive when they are bound to GDP, but become active when GTP displaces GDP from its binding site. The activated Arfs are known to bind to various proteins, including PLD[56], PIP 5-kinase[59], coatomers (proteins involved in vesicles

trafficking between the Golgi and ER)[60, 61], Arfaptin (protein that coordinates the action of Arf and Rho proteins)[62] and G-protein βγ-subunits[63].

There are 3 classes of Arfs found in mammals. Class I consists of Arf1, Arf2, and Arf3, which play a critical role in the transport from ER to Golgi complex and intra-Golgi transport. Arf4 and Arf5 make up class II whose function is not well understood. Arf 6 makes up class III, and has been implicated in many plasma membrane events[64]. Many of Arf's effects stem from their modulation of the membrane-lipid composition. Arfs are known to activate PLD and increase PIP_2 synthesis. In HL60 cells, 45% of the PIP_2 increase was attributed to Arf1-induced activation of PLD, while the other 55% was attributed to Arf1's direct activation of PIP 5-kinase[65]. This result is representative of numerous studies indicating the significances of Arfs in PIP_2 regulation through both a direct activation of PIP 5-kinase and an indirect activation of PIP 5-kinase through the interaction with PLD[64, 65].

In this dissertation, Arf6 has been implicated as an important mediator of SS regulation of PLD1 within HIT-T15 cells. Arf6 plays an important role in membrane trafficking, endocytosis, exocytosis, and actin cytoskeleton rearrangement[66]. Arf6 ability to regulate cortical actin cytoskeleton has a profound effect on cell migration[67], wound healing[68] and phagocytosis[69]. Arf6 has also known to regulate cell polarity. In hippocampal neurons, Arf6 regulates dendritic branching. In addition to Arf6's effects contributed to its ability to regulate actin cytoskeleton, Arf6 also regulates plasma membrane receptors such as β_2-adrenergic[70] and luteinizing hormone receptors[71].

Cells	Gq-coupled receptors	Gi/o-coupled receptors	References
HIT-T15 β-cells	AVP_1 receptors	Somatostatin 2 receptors	81
SH-SY5Y Cells	M_3 muscarinic receptors	δ-opioid receptors	82
FRTL-5 Thyroid cells	α_1 -adrenergic receptors	Adenosine receptors	83
NG 108-15 cells	P_2 -Purinergic and Bradykinin receptors	Opioid receptors	84
CHO cells	P_2 -Purinergic receptors	Neuropeptide Y_1, adenosine A_1 and $5\text{-}HT_{1B}$ receptors	85
DDT1 MF-2 Smooth muscle cells	Bradykinin receptors	Adenosine A_1 Receptors	86

Table 1. Coincidental increase in $[Ca^{2+}]_i$ due to cross-talk between G_q- and $G_{i/o}$-coupled receptors in various cell types.

GEF regulation of Arf activity:

Activation of Arfs via GDP-GTP exchange is modulated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs)[72]. GEFs promote the GTP-bound active state of Arfs, whereas GAPs enhance the intrinsic GTPase activity of Arfs, thereby promoting the GDP-bound inactive state. GEFs of Arfs can be placed into two categories: high molecular weight GEFs and low molecular weight GEFs. High molecular weight GEFs are divided into two subfamilies Gea/Gnom/GBF family and Sec7/BIG family all with a molecular weight >100kDa. Low molecular weight GEFs are divided into two subfamilies: Arf nucleotide-binding site opener (ARNO)/cytohesin/ general receptor for

phosphoinositides (GRP) family and the EFA6 family[71,73]. Brefeldin A, a fungal toxin, inhibits the activity of high molecular weight GEFs, but does not inhibit the activity of low molecular weight GEFs[71,72]. This dissertation indicates Arf6 is an important mediator in the SS signaling pathway. EFA6, GEF100 and ARNO are the only known GEFs for Arf6. All of these are low molecular weight brefeldin A-insensitive GEFs. EFA6 is a 71-kDa protein that catalyzes the exchange of GDP from the Arf inactive state to its GTP-bound active state will be shown to play a role in SS-induced increase in PLD activity. The Sec 7 domain of EFA6 and all other GEFs of Arfs enhance nucleotide exchange rate in Arfs[71]. Tandem to the Sec 7 domain of the low molecular weight GEFs is a PH domain. The EFA6 family consists of four members, EFA6 A, B, C and D[74]. EFA6, also known as EFA6A, is the most extensively studied, and has been found predominately in the brains, but also in the colon, small intestine, and ovaries[74]. EFA6B is ubiquitously expressed in mammal tissues and has demonstrated Arf6 GEF activity in-vitro[74]. EFA6C and EFA6D have been identified as GEFs of Arf6 because of sequence homology and the presence of a tandem Sec 7 domain and PH domain[72]. Neither of these proteins has been analyzed to determine if they possess true GEF activity.

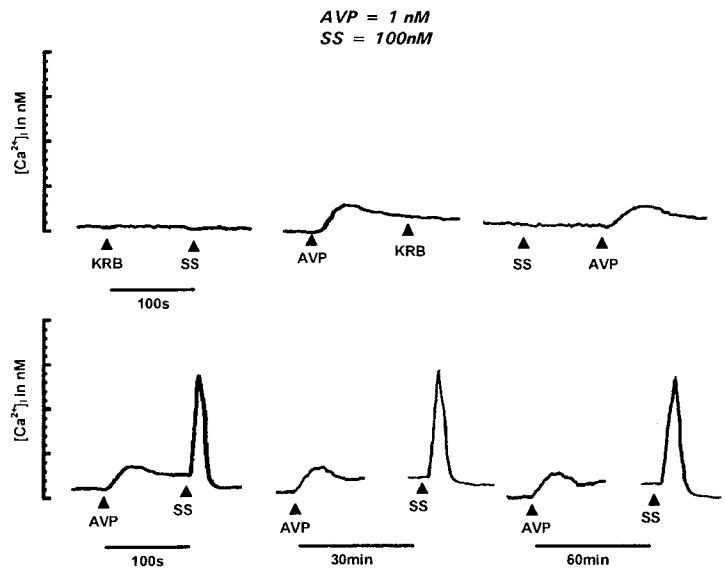

Figure 7. Effect of SS and/or AVP on $[Ca^{2+}]_i$ in HIT-T15 cells, which shows that SS in the presence of AVP increased $[Ca^{2+}]_i$ (lower panel). These results suggest that SS increases PIP_2, which serves as the substrate for AVP-activated PLC. Upper panel: No increase in $[Ca^{2+}]_i$ was observed after 100 nM of SS treatment alone was applied (left-hand panel). The typical increase in $[Ca^{2+}]_i$ by 1 nM AVP was observed (middle panel). Pretreatment with SS did not induce a further increase in $[Ca^{2+}]_i$ by AVP (right-hand panel). Traces are representative of 4 experiments. Each line represents the result from 1.5 million .cells. (From *Biochem. J.* (2002) *364*: 33-39.)

Crosstalk between $G\alpha_i$ and $G\alpha_q$: Signaling via GPCRs can lead to many cellular responses, ranging from regulation of hormone release to stimulation of gene transcription. Crosstalk between two GPCRs can lead to paradoxical cellular responses in many systems[75]. Signaling between G_q-coupled receptors and $G_{i/o}$-coupled receptors has been shown to produce a synergistic increase in $[Ca^{2+}]_i$ in numerous cell types[76, 77, 78]. Normally, activation of G_q-coupled receptors increases $[Ca^{2+}]_i$ via the PLC pathway, whereas activation of $G_{i/o}$-coupled receptors inhibits adenylyl cyclase[79, 80]. However, when these receptors are activated concurrently, they produce a synergistic increase in $[Ca^{2+}]_i$ via enhancement of PLC-β activity[81,82,83,84,85,86]. Various cell types have demonstrated the ability to perform this coincidental signal between numerous G_q- and $G_{i/o}$-coupled receptors (Table 1). This enhancement by $G_{i/o}$-coupled receptors is in part mediated by $G_{i/o}$-βγ dimer[87, 88]. We previously reported that in the presence of AVP, SS increased $[Ca^{2+}]_i$ and evoked a paradoxical, transient release of insulin from HIT-T15 cells[81](Fig. 7). Pretreatment with SS for 100 s does not enhance AVP-induced increase in

$[Ca^{2+}]_i$. The focus of this dissertation is to determine the mechanism behind the paradoxical increase in $[Ca^{2+}]_i$. Others have found similar results using SS as a $G_{i/o}$-coupled receptor agonist. In SH-SY5Y cells, SS increases $[Ca^{2+}]_i$ after pretreatment with carbachol, a cholinergic agonist, which signals via G_q [89]. In another β-cell line RINm5F, cyclopentyladenosine, a $G_{i/o}$-coupled receptor agonist, does not increase $[Ca^{2+}]_i$ when administered alone, but increases $[Ca^{2+}]_i$ after pretreatment with carbachol[90]. The crosstalk effects of SS with AVP in HIT-T15 cells are mediated by $G_{i/o}$, the PLC pathway and Ca^{2+} release from ER[81]. The increase in $[Ca^{2+}]_i$ by SS is attributable to a crosstalk between G_q and $G_{i/o}$[81]. To date, studies examining crosstalk signals between G_q- and $G_{i/o}$-coupled receptors have been performed through quantification of inositol phosphates and/or $[Ca^{2+}]_i$ [87, 88, 89]. In addition, antibodies raised against PLC-β isozymes[91] or PLC inhibitors such as U-73122[81] have also been used to investigate the crosstalk mechanism of $G_{i/o}$-coupled SS receptors. The results from some of these studies suggest that the βγ dimer of $G_{i/o}$ directly activates PLC-β [86,90]. However, in our previous study we found treatment with SS alone, even at a high concentration of 1 μM, failed to increase $[Ca^{2+}]_i$ or insulin release[81]. Therefore, we set out to demonstrate in this dissertation that SS increases PIP_2 synthesis in β-cells, thereby increasing the substrate for preactivated PLC-β by AVP. Although others have suggested that enhancement of G_q signals by $G_{i/o}$ is through activation of PLC-β[87] or interaction with a step after PLC activation[88], none of them have attributed the effect of $G_{i/o}$ to a step before PLC activation, except that Schmidt et al.[92, 93] suggested that $G_{i/o}$ mediates an increase in PIP_2 levels. In this dissertation SS treatment decreased PIP levels but increased PIP_2 levels, suggesting that SS may increase PIP_2 synthesis. Thus, we have suggested a novel mechanism

for cross-talk between G_q, and $G_{i/o}$. That SS can increase PIP_2 synthesis, which in turn provides extra substrate for preactivated PLC-β by AVP to generate high levels of IP_3. Without a preactivated PLC-β, SS failed to increase IP_3 levels (Fig. 3) and $[Ca^{2+}]_i$[81]. In addition to the ability of SS to increase $[Ca^{2+}]_i$, SS also increases PIP_2 levels within these cells. The ability of SS to increase PIP_2 in β-cells gives rise to the exciting possibility that SS may contribute in cell signaling through the diverse action of PIP_2 independent of the PLC pathway.

The role of PIP_2 in cellular regulation:

PIP_2 mediates various cell signaling processes in two distinct ways as an enzyme substrate and as an anchoring signaling lipid. PIP_2 is converted into IP_3 and phosphatidylinositol 3,4,5-trisphospate (PIP_3) and these enzymatic products that mediate many cellular signaling processes. Phosphatidylinositol 3-kinase phosphorylates PIP_2 to form PIP_3. PIP_3 is a membrane lipid, which serves as a binding site for various proteins and mediates many downstream effects. The biochemistry of PIP_3 has been reviewed in great detail elsewhere[94]. The most well-known use of PIP_2 is as a substrate for PLC. PLC cleaves PIP_2 into IP_3 and diacylglycerol. The other way PIP_2 mediates cellular process is as an anchoring signaling lipid. PIP_2 is able to translocate cytosolic proteins to distinct membrane regions of the cell, providing region-specific microdomains on which signal transduction can occur[95, 96]. Many proteins contain specific phosphatidylinositide-binding domains. Five of these domains have been characterized, i.e., epsin amino-terminal homology (ENTH) domain, Fab1, YOTB, Vac1, and EEA1 (FYVE) domain, band 4.1, ezrin, radixin, and moesin (FERM) domain, pleckstrin homology (PH) domain and a phox homology (PX) domain[97]. The PH domain is a phosphatidylinositide-binding domain, which is the eleventh most common domain found in

humans[97]. Because of the vast number of proteins that contain phosphatidylinositide-binding domains, many proteins have the potential to utilize PIP_2 as a membrane-anchoring lipid. These interactions can initiate a cascade of downstream reactions. For example, PIP_2 membrane anchoring property is responsible for many cellular processes such as clathrin-mediated endocytosis[98,99], actin rearrangement[100, 101], vesicle docking [102], and membrane ruffling[103]. While full potential of PIP_2's effects on cellular function has yet to be discovered, it is clear that PIP_2 plays a vital role in many cellular processes. Understanding regulation of PIP_2 synthesis within the cell will give a clear picture of the signaling pathways that utilize PIP_2 as a signaling molecule.

Regulation of PIP_2 is tightly controlled by various kinases as well as phosphatases. In the classic synthesis pathway, PIP 5-kinase type 1 phosphorylates phosphatidylinositol (PtdIns)[104] towards the 5' terminus to form PtdIns(4,5)P_2, representing the major route for PIP_2 synthesis[95]. The conversion of PtdIns(5)P to form PtdIns(4,5)P_2 through PIP 4-kinase, also known as PIP 5-kinase type 2, is the alternative route for PIP_2 synthesis and is not as well understood as type 1 kinase acyivity. PIP 5-kinase type 1, the predominant enzyme for PIP_2 synthesis, has 3 isoforms, α, β, γ [105]. PIP 5-kinase type 1 is activated by several input signals that include PLD and small G-proteins such as Rho, Rac, and Arf[106,107,108.] Because of the ability SS to increase PIP_2, regulators of PIP_2 synthesis were investigated in this disseration.

This dissertation will demonstrate the ability of the $\beta\gamma$-dimer coupled to SS receptor signal through EFA6, a guanine nuclear exchange factor, to activate the small G-protein Arf6. We further demonstrated that Arf6 will activate PLD1 to hydrolyze PC to phosphatidic acid (PA). PA will stimulate PIP-5 Kinase to PIP to PIP_2 (Fig. 1). We believe this novel

mechanism of cross-talk and new effectors for SS provide exciting insights to β-cell signaling.

References:

[1]. Gangong W. F. Endocrine functions of the pancreas & regulation of carbohydrate metabolism. In review of medical physiology, 20thed. Appleton and Lange, Norwalk. 2001, pp 322-343.

[2] Dunning B. E., Moltz J. H., Fawcett C. P. (1984) Modulation of insulin and glucagon secretion from the perfused rat pancreas by the neurohypophysial hormones and by desamino-D-arginine vasopressin (DDAVP). *Peptides*, **5**: 871-875.

[3] Flower D. R., (1999) Modelling G-protein-coupled receptors of drug design. *Biochimica et Biophysica Acta.,* **1422**: 207-234.

[4] Krauss G. G-protein coupled signal transmission pathways. In Biochemistry of Signal Transduction and Regulation.Wiley-VCH, Weinheim (Federal Republic of Germany), 2000, pp173-211.

[5] Spiegel A. M., Jones T. L. Z, Simonds W. F., Weinstein L. S. G Protein –Coupled Receptors. In G-Proteins, CRC press, 1994, 6-17.

[6] Rhee S. G., (2001) Regulation of Phosphoinositide-specific phospholipase C. Annu. *Rev. Biochem.*, **70**: 281-312.

[7] Clapham D. E., Neer E. J. (1997) G protein βγ subunit. *Annu. Rev. Pharmacol. Toxicol.*, **37**: 167-203.

[8] Muller S., Hekman M., Lohse M. J. (1997) Specific enhancement of β-adrenergic receptor kinase activity by defined G-protein β and γ subunits. *Proc. Natl. Sci. U.S.A.*, **90**: 10439-10443.

[9] Camps M., Hou C., Sidiropoulos D., Stock J. B., Jakobs K. H., Giershik P. (1992) Stimulation of phospholipase C by guanine-nucleotide-binding protein βγ subunits. *Eur. J. Biochem.* **206**: 821-832.

[10] Tang W., Gilman A. G. (1991) Type-specific regulation of adenylyl cyclase by G protein βγ subunits. *Science,* **254**: 1500-1503.

[11] Herlitze S., Garcia D. E., Mackie K., Hille B., Scheuer T., Catterall W. A. (1996) Modulation of Ca^{2+} channels by G-protein βγ subunits. *Nature,* **380**: 258-262.

[12] Birnbaumer M. (2000) Vasopressin Receptors. *T.E.M.,* **11**: 406-410.

[13] Amico J. A., Finn F. M. Haldar J. (1988) Oxytocin and vasopressin are present in human and rat pancreas. *Am. J. Med. Sci.,* **296**: 303–307.

[14] Mechaly I., Macari F., Laliberte M. F., Lautier C., Serrano J. J., Cros G., Grigorescu F. (1999) Identification by RT-PCR and immunolocalization of arginine vasopressin in rat pancreas. *Diabetes Metab.,* **25**: 498-501.

[15] Dunning B.E., Moltz J.H., Fawcett C. P. (1984) Modulation of insulin and glucagon secretion from the perfused rat pancreas by the neurohypophysial hormones and by desamino-D-arginine vasopressin (DDAVP). *Peptides*, **5**: 871-875.

[16] Gangong W. F. Endocrine functions of the pancreas & regulation of carbohydrate metabolism. In review of medical physiology, 20thed. Appleton and Lange, Norwalk. 2001, pp 322-343.

[17] Patel Y. C. (1999) Somatostatin and its receptor family. *Front Neuroendocrinol,* **20**: 157-98.

[18] Reisine T., Bell G. I. (1995) Molecular biology of somatostatin receptors. *Endocr. Rev.,* **16**: 427-42.

[19] Yamada Y., Post S. R., Wang K., Tager H. S., Bell G. I., Seino S. (1995) Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract and kidney of human. *Proc Natl. Acad. Sci., USA* **89**: 251-255.

[20] Hadcock J., Strnad J., Eppler C. (1994) Rat somatostatin receptor type 1 couples to G proteins and inhibition of cyclic AMP accumulation. *Mol. Pharmacol.,* **45**: 410-416.

[21] Karschin A. (1995) Molecular single-cell analysis identifies somatostatin tpe 1 (sst1) receptors to block inwardly rectifying K+ channels in rat brain oligodendrocytes. *Neuroreport.,* **7**: 121-124.

[22] Buscail L., Delesque N., Esteve J. P., Saint-Laurent N., Prats H., Clerc P., Robberecht P., Bell G. I., Liebow C., Schally A., Vaysse N., Susini C. (1994) Stimulation of tyrosine phosphatase and inhibition of cell proliferation by somatostatin analogues: mediation by human somatostatin receptor subtypes SSTR1 and SSTR2. *PNAS,* **91**: 2315-2319.

[23] Akbar M., Okajima F., Tomura H., Majid M. A., Yamada Y., Sino S., Kondo Y. (1994) Phospholipase C activation and Ca^{2+} mobilization by cloned human somatostatin receptor subtypes 1-5 in transfected COS-7 cells. *FEBS letters,* **348**: 192-196.

[24] Roosterman D., Glassmeier G., Baumeister H., Scherubl H., Meyerhof W. A. (1998) A somatostatin receptor 1 selective ligand inhibits Ca2+ currents in rat insulinoma 104639 cells. *FEBS Letters,* **425**: 137-140.

[25] Hou C., Gilbert R., Barber D. (1994) Subtype-specific signaling mechanisms of somatostatin receptors SSTR1 and SSTR2. *J. Biol. Chem.,* **269**: 10357-10362.

[26] Florio T., Yao H., Carey K., Dillon T., Stork P. (1999) Somatostatin activation of mitogen-activated protein protein kinase via somatostatin receptor 1 (SSTR1). *Mol. Endocrinology.,* **13**: 24-37.

[27] Exton J. H. (1997) New developments in phospholipase D. *J. Biol. Chem.,* **272**: 15579-15582.

[28] Stutchfield J., Cockcroft S. (1993) Correlation between secretion and phospholipase D activation in differentiated HL60 cells. *Biochem. J.,* **293**: 649-655.

[29] Xie M., Jacobs L. S., Dubyak G. R. (1991) Regulation of phospholipase D and primary granule secretion by P2-purinergic and chemotactic peptide-receptor agonists is induced during granulocytic differentiation of HL-60 cells. *J. Clin. Invest.,* **88**: 45-54.

[30] Foster D. A., Lizhong X. (2003) Phospholipase D in cell proliferation and cancer. *Molecular Cancer Research.*, **1**: 789-800.

[31] Cui Y., English D. K., Siddiqui R. A., Heranyiova M., Garcia J. G. (1997) Activation of endothelial cell phospholipase D by migrating neutrophils. *J. Investig. Med.*, **45:** 388-93.

[32] Liscovitch M., Czarny M., Fiucci G., Lavie Y.,Tang X. (1999) Localization and possible functions of phospholipase D isozymes. *Biochim. Biophys. Acta.*, **1439**: 245-263.

[33] Hammond S. M., Jenco J. M., Nakashima S., Cadwaller K., GU Q. M., Cook S., Nozawa Y., Prestwich G. D, Froman M. A., & Morris A. J. (1997) Characterization of two alternately spliced forms of phospholipase D1. *J. Biol. Chem.*, **272**: 3860-3868.

[34] Steed P. M., Clark K. L., Boyar W. C., Lasala D. J. (1998) Characterization of human PLD2 and analysis of PLD isoform splice variants. *FASEB J.*, **12**: 1309-1317.

[35] Hodgkin M. N., Masson M. R., Powner D. J., Saqib K. M., Ponting C. P., Wakelam M. J. O. (2000) Phospholipase D regulation and localization is dependent upon a phosphatidylinositol 4,5-bisphosphate-specific PH domain. *Curr. Biol.*, **10**: 43-46.

[36] Divecha N., Roefs M., Halstead J. R., D'Andrea S., Fernandez-Borga M., Oomen L., Saqib K. M., Wakelam M. L. O, D'Santos C. (2000) Interaction of the type 1a PIPkinase with

phospholipase D: a role for the local generation of phosphatidylinositol 4,5-bisphosphate in the regulation of PLD2 activity. *EMBO J.,* **19**: 5440-5449.

[37] Lopez I., Arnold R. S., Lambeth J. D. (1998) Cloning and initial characterization of a human phospholipase D2 (hPLD2). *J. Biol. Chem.,* **273**: 12846-12852.

[38] Bollag W. B., Jung E. M., Calle R. A. (2002) Mechanism of angiotensin II-induced phospholipase D activation in bovine adrenal glomerulosa cells. *Mol. Cell. Endocrinol.*, **192**: 7-16.

[39] Lee S. D., Lee B. D., Kim Y., Suh P. G., Ryu S. H. (2000) Bradykinin activates phospholipase D2 via protein kinase C delta in PC12 cells. *Neurosci. Lett.,* **294**: 130-132.

[40] Mitchell R., Robertson D. N., Holland P. J., Collins D., Lutz E. M., Johnson M. S. (2003) ADP-ribosylation factor-dependent phospholipase D activation by the M_3 muscarinic receptor. *J. Biol. Chem.,* **278**: 33818-33830.

[41] Ha K. S., Yeo E. J., Exton J. H. (1994) Lysophosphatidic acid activation of phosphatidylcholine-hydrolysing phospholipase D and actin polymerization by a pertussis toxin-sensitive mechanism. *Biochem. J.,* **303**: 55-59.

[42] Liscovitch M., Amsterdam A. (1989) Gonadotropin-releasing hormone activates phospholipase D in ovarian granulose cells. Possible role in signal transduction. *J. Biol. Chem.*, **264**: 11762-11767.

[43] Miwa M., Kozawa O., Suzuki A., Watanabe Y., Shinoda J., Oiso Y. (1995) Vasopressin activates phospholipase D through pertussis toxin-insensitive GTP-binding protein in aortic smooth muscle cells: function of Ca2+/calmodulin. *Biochem. Cell. Biol.*, **73**: 191-199.

[44] Fahimi-Vahid M., Gosau N., Michalek C., Han L., Jakobs K. H., Schmidt M., Roberts N., Avkiran M.,Wieland T. (2002) Distinct signaling pathways mediate cardiomyocyte phospholipase D stimulation by endothelin-1 and thrombin. *J. Mol. Cell. Cardiol.*, **34**: 441-453.

[45] Gupta S., Gomez-Munoz A., Matowe W. C., Brindley D. N., Ginsberg J. (1995) Thyroid-stimulating hormone activates phospholipase D in FRTL-5 thyroid cells via stimulation of protein kinase C. *Endocrinology.*, **136**: 3794-3799.

[46] Kato Y., Banno Y., Dohjima T., Kato N., Watanabe K., Tatematsu N., Nozawa Y. (1997) Involvement of Rho family proteins in prostaglandins F2 alpha-induced phospholipase D activation in the osteoblast-like cell line MC3T3-E1. *Prostaglandins.*, **54**: 475-492.

[47] Nishizuka Y. (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science, 258: 607-614.

[48] Niu J, Profirovic J, Pan H, Vaiskunaite R, Voyno-Yasenetskaya T. G. (2003) Protein betagamma subunits stimulate p114RhoGEF, a guanine nucleotide exchange factor for RhoA and Rac1: regulation of cell shape and reactive oxygen species production. *Circ. Res.*, **93**: 848-56.

[49] Kozasa T., Jiang X., Hart M. J., Sternweis P. M., Singer W. D., Gilman A. G., Bollag G., Sternweis P. C. (1998) p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. *Science, 280*: 2109–2111.

[50] Hart M. J., Jiang X., Kozasa T., Roscoe W., Singer W. D., Gilman A. G., Sternweis P. C., Bollag G. (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by $G\alpha_{13}$. *Science,* **280**:2112–14.

[51] Robertson D. N., Johnson M. S., Moggach L. O., Holland P. J., Lutz E. M., Mitchell R. (2003) Selective interaction of ARF1 with the carboxy-terminal tail domain of the 5-HT2A receptor. *Mol. Pharmacol.,* **64**:1239-1250.

[52] Mitchell R., McCulloch D., Lutz E., Johnson M., MacKenzie C., Fennell M., Fink G., Zhou W., Sealfon S.C. (1998) Rhodopsin-family receptors associated with small G-proteins to activate phospholipase D. Nature, **392**: 411-414.

[53] Oliveira L., Paiva A. C. M., Vriend G. (1999) A low resolution for the interaction of G proteins with G protein-coupled receptors. *Protein Engineering,* **12**: 1087-1095.

[54] Cheng H., Yibchok-anun S., Coy D. H., Hsu W. H. (2002) SSTR2 mediates the somatostatin-induced increase in intracellular Ca(2+) concentration and insulin secretion in the presence of arginine vasopressin in clonal beta-cell HIT-T15. *Life Sci.,* **71**: 927-936.

[55] Claing A., Chen W., Miller W. E., Vitale N., Moss J., Premont R. T., Lefkowitz R. J. (2001) β-Arrestin-mediated ADP-ribosylation factor 6 activation and β2-adrenergic receptor endocytosis. *J. Biol. Chem.* **276**: 42509-42513.

[56] Mukherjee S., Gurevich V. V, Jones J. C. R., Casanova J. E., Frank S. R., Maizels E. T., Bader M. F., Kahn R. A., Palczewski K., Aktories K., Hunzicker-Dunn M. (2000) The ADP ribosylation factor nucleotide exchange factor ARNO promotes β-arrestin release necessary for luteinizing hormone/choriogonadotropin receptor. *Proc. Natl. Acad. Sci. U.S.A.,* **97:** 5901-5906.

[57] Andresen B. T, Jackson E. K, Romero G. G. (2001) Angiotensin II signaling to phospholipase D in renal microvascular smooth muscle cells in SHR. *Hypertension*, **37**: 635-639.

[58] Cheng H., Grodnitzky J. A., Yibchok-Anun S., Ding J., Hsu W. H. (2005) Somatostatin increases phospholipase D activity and PIP2 synthesis in clonal β-cells HIT-T15. *Mol. Pharmacol.,* Epub ahead of print.

[59] Randazzo P. A. (1997) Functional interaction of ADP-ribosylation factor 1 with phosphatidylinositol 4,5-bisphosphate. *J. Biol. Chem.,* **272:** 7688-7692.

[60] Donaldson J. G., Jackson C. L. (2000) Regulators and effectors of the ARF GTPases. *Curr. Opin. Cell Biol.* **12**: 475-482.

[61] Zhao L., Helms J. B., Brugger B., Harter C., Martoglio B., Graf R., Brunnen J., Wieland F. T. (1997) Direct and GTP-dependent interaction of ADP ribosylation factor 1 with coatomer subunit β. *Proc. Natl. Acad. Sci. U.S.A.,* **94:** 4418-4423.

[62] Kanoh H., Williger B. T., Exton J. H. (1997) Arfaptin 1, a putative cytosolic target protein of ADP-ribosylation factor, is recruited to Golgi membranes. *J. Biol. Chem.* **272:** 5421-29.

[63] Franco M., Paris S., Chabre M. (1995) The small G-protein ARF1GDP binds to the Gtβγ

subunit of transducin, but not to Gtα GDP-Gtβγ. *FEBS Lett.*, **362**:286-290.

[64] Moss J., Vaughan M. (1998) Molecules in the ARF orbit. *J. Biol. Chem.*, **273**: 21431-21434.

[65] Skippen A., Jones D. H., Morgan C. P., Li M., Cockcroft S. (2001) Mechanism of ADP ribosylation factor-stimulated phosphatidylinositol 4,5-bisphosphate synthesis in HL60 cells. *J. Biol. Chem.*, **277**: 5823-5831.

[66] Donaldson J. G. (2003) Multiple roles for Arf6: Sorting, Structuring and at plasma membrane. *J. Biol. Chem.*, **278**: 41573-41576.

[67] Palacios F., Price L., Schweitzer J., Collard J. G., D'Souza-Schorey C. (2001) An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. *EMBO J.*, **20**: 4973-4986.

[68] Santy L. C., Casanova J. E. (2001) Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J Cell Biol.*, **154**: 599-610.

[69] Niedergang F., Colucci-Guyon E., Dubois T., Raposo G., Chavrier P. (2003) ADP ribosylation factor 6 is activated and controls membrane delivery during phagocytosis in macrophages. *J. Cell Biol.,* **161**: 1143-1150.

[70] Claing A., Chen W., Miller W. E., Vitale N., Moss J., Premont R. T., Lefkowitz R. J. (2001) beta-Arrestin-mediated ADP-ribosylation factor 6 activation and beta 2-adrenergic receptor endocytosis. *J. Biol. Chem.,* **276**: 42509-42513.

[71] Salvador L. M., Mukherjee S., Kahn R. A., Lamm M. L., Fazleabas A. T., Maizels E. T., Bader M. F., Hamm H., Rasenick M. M., Casanova J. E., Hunzicker-Dunn M. (2001) Activation of the luteinizing hormone/choriogonadotropin hormone receptor promotes ADP ribosylation factor 6 activation in porcine ovarian follicular membranes. *J. Biol. Chem.,* **276**: 33773-33781.

[72] Donaldson J. G., Jackson C. L. (2000) Regulators and effectors of the ARF GTPases. *Curr. Opin. Cell Biol.*, **12**: 475-482.

[73] Jackson C. L., Casanova J. E. (2000) Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors. *Trends Cell Biol.*, **10**: 60-67.

[74] Derrien V., Coullaul C., Franco M., Martineau S., Montcouttier P., Houlgatte R., and Chavrier P. (2002) A conserved C-terminal domain of EFA6-family ARF6-guanine

nucleotide exchange factors induces lengthening of microvilli-like membrane protusions. *J. Cell Sci.*, **115**: 2867-79.

[75] Selbie L. A., Hill S. J., (1998) G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. *TiPs*, **19**: 87-93.

[76] Muller S. and Lohse M. J. (1995) The role of G-protein βγ subunits in signal transduction. *Biochem. Soc. Trans.*, **23**:141-148.

[77] Quitterer U. and Lohse M. J. (1999) Crosstalk between $G\alpha_i$- and $G\alpha_q$-coupled receptors is mediated by Gβγ exchange. *Proc. Natl. Acad. Sci. U.S.A.*, **96**:10626-10631.

[78] Yeo A., Samways D. S., Fowler C. E., Gunn-Moore F., and Henderson G. (2001) Coincident signalling between the Gi/Go-coupled δ-opioid receptor and the Gq-coupled m3 muscarinic receptor at the level of intracellular free calcium in SH-SY5Y cells. *J. Neurochem.*, **76**:1688-1700.

[79] Patel Y. C., Greenwood M. T., Warszynska A., Panetta R., and Srikant C. B. (1994) All five cloned human somatostatin receptors (hSSTR1-5) are functionally coupled to adenylyl cyclase. *Biochem. Biophys. Res. Commun.*, **198**:605-612.

[80] Rhee S. G. (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu. Rev. Biochem.*, **70**:281-312.

[81] Cheng H., Yibchok-anun S., Park S. C., and Hsu W. H. (2002) Somatostatin-induced paradoxical increase in intracellular Ca^{2+} concentration and insulin release in the presence of arginine vasopressin in clonal HIT-T15 β-cells. *Biochem. J.*, **364**:33-39.

[82] Yeo A., Samways D. S., Fowler C. E., Gunn-Moore F., Henderson G. (2001) Coincident signalling between the Gi/Go-coupled delta-opioid receptor and the Gq-coupled m3 muscarinic receptor at the level of intracellular free calcium in SH-SY5Y cells. *J. Neurochem.*, **76**: 1688-1700.

[83] Okajima F., Sato K., Sho K., Kondo Y. (1989) Stimulation of adenosine receptor enhances alpha 1-adrenergic receptor-mediated activation of phospholipase C and Ca2+ mobilization in a pertussis toxin-sensitive manner in FRTL-5 thyroid cells. FEBS Lett., **248**: 145-149.

[84] Okajima F., Tomura H., Kondo Y. (1993) Enkephalin activates the phospholipase C/Ca2+ system through cross-talk between opioid receptors and P2-purinergic or bradykinin receptors in NG 108-15 cells. A permissive role for pertussis toxin-sensitive G-proteins. *Biochem. J.*, **290**: 241-247.

[85] Megson A. C., Dickenson J. M., Townsend-Nicholson A., Hill S. J. (1995) Synergy between the inositol phosphate responses to transfected human adenosine A1-receptors and constitutive P2-purinoceptors in CHO-K1 cells. *Br. J. Pharmacol.,* **115:** 1415-24.

[86] Gerwins P., Fredholm B. B. (1992) Stimulation of adenosine A1 receptors and bradykinin receptors, which act via different G proteins, synergistically raises inositol 1,4,5-trisphosphate and intracellular free calcium in DDT1 MF-2 smooth muscle cells. *Proc. Natl. Acad. Sci. U.S.A.,* **89**: 7330-7334.

[87] Selbie L. A., King N. V., Dickenson J. M., and Hill S. J. (1997) Role of G-protein βγ subunits in the augmentation of P_2Y_2 (P_{2U}) receptor-stimulated responses by neuropeptide Y Y_1 $G_{i/o}$-coupled receptors. *Biochem. J.,* **328:**153-158.

[88] Chan J. S., Lee J. W., Ho M. K., and Wong Y. H. (2000) Preactivation permits subsequent stimulation of phospholipase C by G_i-coupled receptors. *Mol. Pharmacol.,* **57**: 700-708.

[89] Connor M., Yeo A., and Henderson G. (1997) Neuropeptide Y Y_2 receptor and somatostatin sst_2 receptor coupling to mobilization of intracellular calcium in SH-SY5Y human neuroblastoma cells. *Br. J. Pharmacol.,* **120**: 455-463.

[90] Biden T. J., and Browne C. L. (1993) Cross-talk between muscarinic- and adenosine-receptor signalling in the regulation of cytosolic free Ca^{2+} and insulin secretion. *Biochem. J.*, **293**: 721-728.

[91] Murthy K. S., Coy D. H., and Makhlouf G. M. (1996) Somatostatin receptor-mediated signaling in smooth muscle. Activation of phospholipase C-β3 by $G_{\beta\gamma}$ and inhibition of adenylyl cyclase by $G_{\alpha i1}$ and $G_{\alpha o}$. *J. Biol. Chem.*, **271:** 23458-23463.

[92] Schmidt M., Nehls C., Rumenapp U., and Jakobs K. H. (1996) m3 Muscarinic receptor-induced and G_i-mediated heterologous potentiation of phospholipase C stimulation: role of phosphoinositide synthesis. *Mol. Pharmacol.*, **50:**1038-1046.

[93] Schmidt M., Lohmann B., Hammer K., Haupenthal S., Nehls M. V., and Jakobs K. H. (1998) Gi- and protein kinase C-mediated heterologous potentiation of phospholipase C signaling by G protein-coupled receptors. *Mol. Pharmacol.*, **53:**1139-1148.

[94] Stephens L. R., Jackson T. R., Hawkins P. T. (1993) Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-triphosphate: a new intracellular signaling system? *Biochim. Biophy. Acta.*, **1179**: 27-75.

[95] Botelho R. J., Teruel M., Dierckman R., Anderson R., Wells A., York J. D., Meyer T., Grinstein S. (2000) Localized biphasic changes in phosphatidylinositol-4,5-bisphosphate at sites of phagocytosis. *J. Cell Biol.,* **151**: 1353-1368.

[96] Haugh J. M., Codazzi F., Teruel M., Meyer T. J. (2000) Spatial sensing in fibroblasts mediated by 3' phosphoinositides. *J. Cell Biol.,* **151**: 1269-1280.

[97] Cullen P. J., Cozier C. E., Banting G., Mellor H. (2001) Modular phosphoinositide-binding domains--their role in signalling and membrane trafficking. *Curr. Biol.,* **11**: R882-893.

[98] Gillooly D. J., Stenmark H. (2001) Cell Biology. A lipid oils the endocytosis machine. *Science,* **291**: 993-994.

[99] Marin T. F. (2001) $PI(4,5)P_2$ regulation of surface membrane traffic. *Curr. Opin Cell Biol.,* **13**: 493-499.

[100] Raucher D., Stauffer T., Chen W., Shen K., Guo S. (2000) Phosphatidylinositol 4,5-bisphosphate functions as a second messenger that regulate cytoskeleton-plasma membrane adhesion. *Cell,* **100**: 221-228.

[101] Tolias K. F., Hartwig J. H., Ishihara H., Shibasaki Y., Cantley L. C., Carpenter C. L. (2000) Type Iα phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr. Biol.,* **10**: 153-156.

[102] Brown F. D., Rozelle A. L., Yin H. L., Balla T., Donaldson J. G. (2001) Phosphatidylinositol 4,5-bishphosphate and Arf6-regulated membrane traffic. *J. Cell Biol.,* **154:** 1007-1017.

[103] Honda A., Nogami M., Yokozeki T., Yamazaki M., Nakamura H., Watanabe H., Kawamoto K., Nakayama K., Morris A. J., Frohman M. A., Kanaho Y. (1999) Phosphatidylinositol 4-phosphate 5-kinase α is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell,* **99:** 521-532.

[104] Loijens J. C., Boronenkov I. V., Parker G. J., Anderson R. A. (1996) The phosphatidylinositol 4-phosphate 5-kinase family. *Adv. Enzyme Regul.,* **36**: 115-140.

[105] Ishihara H., Shibasaki Y., Kizuki N., Wada T., Yazaki Y., Asano T., Oka Y. (1998) Type I phosphatidylinositol-4-phosphate 5-kinases. Cloning of the third isoform and deletion/substitution analysis of members of this novel lipid kinase family. *J. Biol. Chem.,* **273**: 8741-8748 .

[106] Tolias K. F., Hartwig J. H., Ishihara H., Shibasaki Y., Cantley L. C., Carpenter C. L. (2000) Type Iα phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr. Biol.*, **10**: 153-156.

[107] Oude Weernink P. A., Schulte P., Guo Y., Wetzel J., Amano M., Kaibuchi K., Haverland S., Voss M., Schmidt M., Mayr G. W., Jakobs K. H. (2000) Stimulation of phosphatidylinositol-4-phosphate 5-kinase by Rho kinase. *J. Biol. Chem.*, **275:** 10168-10174.

[108] Jones D. H., Morris J. B., Morgan C. P., Kondo H., Irvine R. F., Cockcroft S. (2000) Type I phosphatidylinositol 4-phosphate 5-kinase directly interacts with ADP-ribosylation factor 1 and is responsible for phosphatidylinositol 4,5-bisphosphate synthesis in the Golgi compartment. *J. Biol. Chem.*, **275:** 13962-13966.

CHAPTER 2: SOMATOSTATIN INCREASES PHOSPHOLIPASE D ACTIVITY AND PIP_2 SYNTHESIS IN CLONAL β-CELLS HIT-T15

Henrique Cheng, Justin A. Grodnitzky, Sirintorn Yibchok-anun, Jing Ding, and Walter H. Hsu

Molecular Pharmacology 67: 2162-2172, 2005

Department of Biomedical Sciences, Iowa State University, Ames, Iowa

J. A. Grodnitzky and H. Cheng contributed equally to the present work

Running title: Somatostatin increases PLD & PIP_2 in β-Cells

Correspondence Author: Dr. Walter H. Hsu, Department of Biomedical Sciences, Iowa State University, Ames, IA 50011-1250. Phone: 515-294-6864. FAX: 515-294-2315. E-mail: whsu@iastate.edu

The number of text pages: 29

The number of figures: 10

The number of references: 40

The number of words in the Abstract: 249

The number of words in the Introduction: 543

The number of words in the Discussion: 1433

ABBREVIATIONS AVP, arginine vasopressin; ct-βARK, C-terminus of the β-adrenergic receptor kinase; DAG, diacylglycerol; ER, endoplasmic reticulum; fura-2AM, fura-2 acetoxymethyl ester; GPCR, G protein-coupled receptor; IP_3, inositol 1,4,5-trisphosphate; KRB, Krebs-Ringer bicarbonate buffer; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; PLD, phospholipase D; PA, phosphatidic acid; PBt, phosphatidylbutanol; PC, phosphatidylcholine; PTX, pertussis toxin; RFU, relative fluorescent unit; zLYCK, carbobenzyloxy-leucine-tyrosine-chloromethylketone.

ABSTRACT

In the presence of arginine vasopressin (AVP), somatostatin increases $[Ca^{2+}]_i$, leading to a transient increase in insulin release from clonal β-cells HIT-T15 via $G_{i/o}$ and phospholipase C (PLC) pathway (Cheng et al., 2002a). The present study was to elucidate the mechanisms underlying somatostatin-induced $[Ca^{2+}]_i$ increase in the presence of AVP. We found the effect of somatostatin was mediated by βγ subunits, but not by α subunit of $G_{i/o}$. Since somatostatin alone failed to increase $[Ca^{2+}]_i$, we hypothesized that somatostatin increases PIP_2 synthesis, providing extra substrate for preactivated PLC-β to generate IP_3. Somatostatin alone did not increase IP_3 levels, but AVP + somatostatin did. Somatostatin increased PIP_2 levels, but decreased PIP levels. We further hypothesized that PLD mediates somatostatin-induced changes in PIP_2 levels. Both the phospholipase D (PLD) inhibitors and antibody vs. PLD1 antagonized AVP-somatostatin-induced increases in $[Ca^{2+}]_i$. PLD inhibitor also antagonized somatostatin-induced increase in PIP_2 levels. In addition, somatostatin increased PLD activity. These results suggested that activation of somatostatin receptors that are coupled to the βγ dimer of $G_{i/o}$ leads to PLD1 activation, thus promoting

receptors that are coupled to the βγ dimer of $G_{i/o}$ leads to PLD1 activation, thus promoting the synthesis of phosphatidic acid. Since phosphatidic acid activates PIP-5 kinase, this evokes an increase in PIP_2 synthesis. The PIP_2 generated by somatostatin administration increases substrate for preactivated PLC-β, which hydrolyzes PIP_2 to form IP_3, leading to an increase in $[Ca^{2+}]_i$. The regulation of PIP_2 synthesis by $G_{i/o}$-coupled receptors via PLD activation represents a novel signaling mechanism for somatostatin and a novel concept in the crosstalk between G_q- and $G_{i/o}$-coupled receptors in β-cells.

INTRODUCTION

Signaling via the large family of G protein-coupled receptors (GPCRs) can lead to many cellular responses, ranging from regulation of hormone release to stimulation of gene transcription. Crosstalk between two GPCRs can lead to paradoxical cellular responses. Signaling between G_q-coupled receptors and $G_{i/o}$-coupled receptors produces a synergistic increase in $[Ca^{2+}]_i$ in numerous cell types (Muller and Lohse, 1995; Quitterer and Lohse, 1999; Yeo et al., 2001). PLC is known to hydrolyze phosphatidylinositol 4,5-bisphosphate (PIP_2) into the second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 triggers Ca^{2+} release from the endoplasmic reticulum (ER), whereas DAG activates protein kinase C (Berridge et al., 1984; Nishizuka, 1984). Normally, activation of Gq-coupled receptors increases $[Ca^{2+}]_i$ via the PLC pathway, whereas activation of $G_{i/o}$-coupled receptors inhibits adenylyl cyclase (Patel et al., 1994; Rhee, 2001). However, when these receptors are stimulated concurrently, they produce a synergistic increase in $[Ca^{2+}]_i$ via enhancement of PLC-β activity (Muller and Lohse, 1995; Quitterer and Lohse, 1999; Yeo et al., 2001). This enhancement by Gi/o-coupled receptors is mediated by $G_{i/o}$-βγ dimer (Selbie et al., 1997; Quitterer & Lohse, 1999; Chan et al., 2000).

Recently, we reported that in the presence of AVP, somatostatin increased $[Ca^{2+}]_i$ and evoked a paradoxical, transient insulin release from HIT-T15 cells (Cheng et al., 2002a). Pretreatment with somatostatin for 100 s does not enhance AVP-induced increase in $[Ca^{2+}]_i$ (Cheng et al., 2002a). Others have found similar results using somatostatin as a $G_{i/o}$-coupled receptor agonist. For example, in SH-SY5Y cells, somatostatin increases $[Ca^{2+}]_i$ after pretreatment with carbachol, a cholinergic agonist, which signals via G_q (Connor et al., 1997). In another β-cell line RINm5F, cyclopentyladenosine, a $G_{i/o}$-coupled receptor agonist, does not increase $[Ca^{2+}]_i$ when administered alone, but increases $[Ca^{2+}]_i$ after pretreatment with carbachol (Biden and Browne, 1993). The crosstalk effects of somatostatin with AVP in HIT-T15 cells are mediated by $G_{i/o}$, the PLC pathway and Ca^{2+} release from ER (Cheng et al., 2002a). The increase in $[Ca^{2+}]_i$ by somatostatin is attributable to a crosstalk between G_q and $G_{i/o}$ (Cheng et al., 2002a). To date, most of the studies examining crosstalk signals between G_q- and $G_{i/o}$-coupled receptors have been performed through quantification of inositol phosphates and/or $[Ca^{2+}]_i$ (Selbie et al., 1997; Chan et al., 2000; Yeo et al., 2001). In addition, antibodies vs. PLC-β isozymes (Murthy et al., 1996) or PLC inhibitors such as U-73122 (Cheng et al., 2002a) have also been used to investigate the crosstalk mechanism of $G_{i/o}$-coupled somatostatin receptors. The results from some of these studies suggest that the βγ dimer of $G_{i/o}$ directly activates PLC-β (Murthy et al., 1996; Chan et al., 2000). However, in our previous study, treatment with somatostatin alone, even at a high concentration of 1 μM, failed to increase $[Ca^{2+}]_i$ or insulin release (Cheng et al., 2002a). We, therefore, hypothesized that somatostatin increases PIP_2 synthesis in β-cells, thereby increasing the substrate for preactivated PLC-β by AVP. In addition, we hypothesized that somatostatin

activates PLD to increase the synthesis of phosphatidic acid, which in turn activates PIP-5 kinase to increase PIP_2 synthesis.

Our present findings indicate that somatostatin alone cannot increase IP_3 production. In addition, somatostatin-induced increase in IP_3 and $[Ca^{2+}]_i$ in the presence of AVP is mediated through the Gβγ-dimer of $G_{i/o}$, an increase in PLD activity, and a subsequent increase in PIP_2 synthesis.

MATERIALS AND METHODS

Materials. All reagents were purchased from Sigma Chemical (St. Louis, MO), except that fura-2 acetoxymethyl ester (fura-2AM) was from Molecular Probes (Eugene, OR), and rabbit polyclonal antibodies vs. $G\alpha_{i1}/G\alpha_{i2}$ and $G\alpha_{i3}/G\alpha_o$, and Gβ were from Biomol (Plymouth Meeting, PA), myo-[2-^{3}H]inositol and γ-^{32}P-ATP were from PerkinElmer Life Sciences (Boston, MA).

Cell Culture and Transfection. HIT-T15 cells were maintained in RPMI 1640 with 10 % FBS and aerated with 5 % CO_2-95 % air at 37°C. All experiments were performed using cells from passages 80-90. For Over-expression of the C-terminus of the β-adrenergic receptor kinase (ctβARK), HIT-T15 cells were transfected with pcDNAIIIB T8βark plasmid (donated by Dr. Silvio Gutkind, NIH), using Lipofectamine™ (Invitrogen, Carlsbad, CA) and according to the manufacturer's protocol.

Measurement of $[Ca^{2+}]_i$ in Single Cells. $[Ca^{2+}]_i$ was measured as previously described (ZhuGe and Hsu, 1995). Cells were loaded with 2 μM fura-2AM in Krebs-Ringer bicarbonate buffer (KRB) for 30 min at 37°C. Measurement was performed in custom-made 35-mm culture dishes or a 20 mm X 20 mm perifusion chamber on the stage of an inverted

fluorescence microscope (Carl Zeiss). Fluorescence images were obtained (λ_{ex}=340 and 380 nm; λ_{em}=510 ± 20 nm), background subtracted, and divided on a pixel-by-pixel basis to generate spatially resolved maps of the $[Ca^{2+}]_i$. The emitted signals were digitalized, recorded and processed using Attofluor Digital Fluorescence Imaging System (Atto Instruments, Rockville, MD). The $[Ca^{2+}]_i$ was calculated according to a previously published method (Grynkiewicz et al., 1985). Calibration was performed according the procedure provided by Attofluor, using fura-2 penta K^+ as a standard.

Measurement of $[Ca^{2+}]_i$ in Transfected Cells. T8βARK-transfected HIT-T15 cells were seeded into black-walled clear-base poly-D-lysine-coated 96-well plates (Costar, Corning) at a density of 10^5 cells per well in RPMI 1640, supplemented as described above, and cultured overnight. The cells were then incubated with FLIPR Calcium 3™ Assay Kit (Molecular Devices, Sunnyvale, CA) at 37°C for 60 min. After incubation, the plates were inserted into a fluorometric imaging plate reader (FLEXstation®II, Molecular Devices), and the fluorescence at λ_{ex}=488 nm, and λ_{em}=515–535 nm was used to monitor changes in $[Ca^{2+}]_i$. Data were expressed as percent increase of relative fluorescent units (RFU) / baseline RFU using Softmax Pro™.

Microinjection Protocol. Single cells were grown for 2 days on glass coverslips of custom-made 35-mm culture dishes. Thereafter, cells were loaded with fura-2AM, mounted on the stage of an inverted microscope. For microinjection of antibodies, two cells from each dish were injected with intracellular buffer and rabbit antibodies respectively, using a disposable glass pipette (VWR Scientific, West Chester, PA) held by a Narishige MW-3 micromanipulator. Pipettes were made by a PE-2 Micropipette puller (Narishige Scientific Instrument, Tokyo, Japan). All antibodies were diluted at 1:100 with intracellular buffer

solution containing (in mM): K_2HPO_4 27; NaH_2PO_4 8; KH_2PO_4 26; pH 7.3. Injection pressure was controlled by a pressure injection system (Picospritzer II, General Valve, Fairfield, NJ). A 30-min incubation period was allowed between the antibody injection and $[Ca^{2+}]_i$ measurement. For microinjection of PIP_2, one cell from each dish was injected with Na^+ salt of PIP_2, which had been dissolved and further diluted in intracellular buffer at the respective PIP_2 concentration or buffer alone as the control. Microinjection of PIP_2, PIP, and PA was performed at 100 s following the perfusion with AVP into the culture dish. At the end of each experiment, the cell was depolarized with 10 mM KCl to test membrane integrity.

Determination of PIP and PIP_2. PIP and PIP_2 levels were measured using thin layer chromatography as previously described (Norris and Majerus, 1994). HIT-T15 cells were labeled with 200 μCi/ml of γ-^{32}P-ATP in phosphate-free KRB for 60 min and washed twice with centrifugation at 300 x *g* for 2 min. For experiments, cells were resuspended at a density of 15 x 10^6 cells/ml/treatment. The reactions were terminated by addition of 1 ml ice cold 1 N HCl. Phospholipids were separated by a chloroform:methanol (1:1) mixture. The lower phase was dried under a stream of nitrogen, resuspended in 200-500 μl chloroform:methanol (1:1) mixture, and spotted on silica gel plates. PIP and PIP_2 were identified by co-migration with unlabeled standards, which were visualized by iodine staining and radiograph. One-cm blocks of the corresponding lanes for the samples were subsequently scraped and radioactivity was quantified by liquid scintillation counting.

Determination of IP_3. Measurement of inositol phosphates followed modified procedures from a published report (Hoque et al., 2001). Cells were labeled with 20 μCi/ml of myo-[2-

^{3}H]inositol at 37°C for 90 min by which time the incorporation of ^{3}H into inositol lipids had reached a plateau (Hoque et al., 2001).

Cells were washed twice in phosphate-free KRB and centrifuged at 300 x *g* for 2 min. For experiments, cells were resuspended at a density of 20 x 10^{6} cells/ml/treatment. The reactions were terminated by addition of 0.5 ml of ice cold 10% trichloroacetic acid and samples centrifuged at 3,000 x *g* for 20 min at 4°C. The supernatants were passed through a 200-400 mesh Dowex AG1-X 8 in formate form column (Bio-Rad Laboratories, Hercules, CA). Inositol phosphates were eluted by stepwise addition of 0.2, 0.5, and 1 M ammonium formate, which yielded IP_1, IP_2, and IP_3, respectively. Radioactivity associated with IP_3 from each sample was quantified by liquid scintillation counting.

Determination of PLD Activity. PLD assay was performed using a previously described method (O'Launaigh et al., 2002). Briefly, HIT-T15 cells were grown in 24-well plates overnight in RPMI 1640 medium with 10% FBS. The medium was then discarded and 500 µl of 3 µCi/ml of [^{3}H]myristic acid was added to each well for 60 min. Cells were then washed twice with KRB and 400 µl of KRB was added to each well. Treatments were applied 15 s after the addition of 0.5% 1-butanol. The reactions were terminated 30 s after treatment had been administered. Phosphatidylcholine (PC) and phosphatidylbutanol (PBt) were identified by migration with unlabeled standards, which were visualized by iodine staining and radiograph. One-cm blocks of the corresponding lanes for the samples were subsequently scraped and radioactivity was quantified by liquid scintillation counting.

Intracellular Delivery of antibodies vs. PLD. This technique was performed using BioPORTER™ protein delivery reagent (Gene Therapy Systems, San Diego, CA). Undiluted PLD antibodies, a gift from Dr. Sylvain Bourgoin of the University of Laval,

Canada, were delivered into HIT-T15 cells following manufacturer's protocol. Briefly, HIT-T15 cells were plated on a glass coverslip of custom-made 35-mm culture dish. Antibody vs. PLD (2 μg) (or normal rabbit plasma as control) was mixed with 1.5 μl of the protein delivery reagent and 150 μl of FBS-free RPMI medium and added to each well. The cells were incubated at 37°C for 4 h. The culture medium was then removed and replaced by KRB. Thereafter, cells were loaded with fura-2AM and determination of single cell $[Ca^{2+}]_i$ was performed as mentioned above.

Western Blot Analysis of PLD. PLD protein levels were determined by Western blot analysis. Ten μg of whole cell protein of HIT-T15 was separated by reduced SDS-PAGE (10%). Protein was transferred to PVDF membranes in transfer buffer (35 mM Tris, 190 mM glycine, 20% methanol). The PVDF membranes were blocked with 5% non-fat dry milk in PBS for 1 h at room temperature. The primary PLD antibodies were diluted 1:30 in wash buffer (0.01% Tween 20 in PBS) and incubated with the PVDF membranes for 1 h at room temperature. The blots were washed 3 times for 10 min each with wash buffer. The secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, Pierce, Rockford, IL) was diluted 1:2,000 in wash buffer and incubated with the PVDF membranes for 1 h at room temperature. The blots were then washed and developed using diaminobenzidine.

Data Analyses. All values are presented as mean ± SE. Results were analyzed using ANOVA and individual mean comparisons were made using the Least Significant Difference test. The significance level was set at $p < 0.05$.

RESULTS

Mediation by Gβγ of somatostatin-Induced Increase in $[Ca^{2+}]_i$ in the Presence of AVP.

The $G_{i/o}$-βγ dimer has been demonstrated to be the essential subunits of the G protein, which elicit this synergistic increase in $[Ca^{2+}]_i$ in the presence of G_q activation (Selbie et al., 1997; Quitterer and Lohse, 1999; Chan et al., 2000). In order to determine if our cell system is also dependent on $G_{i/o}$-βγ dimer, we microinjected antibodies vs. $G_{i/o}$ subunits into single cells in the presence of 1 nM AVP and then treated the cells with somatostatin (100 nM) to induce an increase in $[Ca^{2+}]_i$. Antibody (1:100) vs. Gβ reduced the response to somatostatin by 89 % ($p < 0.05$). In contrast, antibodies vs.$G\alpha_{i1}/G\alpha_{i2}$ or $G\alpha_{i3}/G\alpha_o$ (1:100) did not significantly change the response to somatostatin (Fig. 1). To further demonstrate the involvement of $G_{i/o}$-βγ dimer, ct-βARK was expressed in HIT-T-15 cells. Expressing ct-βARK has been shown to sequester Gβγ, thereby inhibiting its ability to stimulate downstream mediators (Inglese et al., 1992; Koch et al., 1994). Protein expression of the myc-tagged ct-βARK was determined by immunocytochemistry using anti-myc monoclonal antibody with 80-90% of HIT T-15 cells expressing the myc epitope (data not shown). When ct-βARK was expressed in HIT-T15 cells, it dramatically reduced somatostatin (100 nM)-induced increase in $[Ca^{2+}]_i$ in the presence of AVP (1 nM). In mock transfected HIT-T15 cells, somatostatin in the presence of AVP caused a 81% ± 14% increase in RFU/baseline RFU, while cells that expressed ct-βARK only elicited 14 % ± 9% increase in RFU/ baseline RFU when stimulated by AVP-somatostatin. All cells responded to 10 mM KCl by increasing $[Ca^{2+}]_i$ (data not shown). These results suggested that $G_{i/o}$-βγ dimer is essential for the somatostatin-induced increase in $[Ca^{2+}]_i$ in the presence of AVP.

Somatostatin- and AVP-Induced Increase in IP_3 Levels. Somatostatin alone was unable to elicit any increase in $[Ca^{2+}]_i$ in HIT-T15 cells (Cheng et al., 2002a). We then hypothesized that somatostatin does not directly activate PLC-β. We first determined the time course in which somatostatin + AVP produced the highest IP_3 levels. In the presence of AVP (1 nM), somatostatin (100 nM) increased IP_3 levels after 10-12 s of administration (Fig. 2A, $p < 0.05$). We then utilized this time frame to compare IP_3 levels among 4 treatment groups, terminating all reactions at 12 s post-somatostatin administration. Somatostatin (100 nM) alone failed to increase IP_3 levels. AVP (1 nM) alone induced a small, but significant increase in IP_3 levels (Fig. 2B). In cells pretreated with AVP (1 nM) for 100 s, followed by somatostatin (100 nM), IP_3 levels were the highest among 4 treatment groups. These results suggested that somatostatin alone cannot activate PLC-β in HIT-T15 cells, since it did not increase IP_3 levels. AVP (1 nM) alone increased IP_3 levels in these cells, and concurrent administration of AVP and somatostatin caused a synergistic increase in IP_3 levels.

Somatostatin-Induced Increase in PIP_2 and Decrease in PIP Levels. Since somatostatin alone was unable to increase IP_3 levels in HIT-T15 cells, we hypothesized that somatostatin increases PIP_2 synthesis, which in turn provides more substrate for pre-activated PLC-β by AVP. If this hypothesis is correct, injection of PIP_2 into single cells in the presence of 1 nM AVP should increase $[Ca^{2+}]_i$ in a similar manner to somatostatin administration. In the control group, microinjection of intracellular buffer into single cells did not increase $[Ca^{2+}]_i$ in the presence of AVP (Fig. 3). In the presence of AVP, microinjection of PIP_2 (5 - 50 amol) increased $[Ca^{2+}]_i$ in a concentration-dependent manner. In the absence of AVP, PIP_2 at the highest concentration studied (50 amol) failed to increase $[Ca^{2+}]_i$ (data not shown). Also, in the presence of AVP (1 nM), microinjection of PIP (50 amol) was unable to increase

$[Ca^{2+}]_i$ (data not shown). These results are consistent with our hypothesis that somatostatin increases PIP_2 levels, providing more substrate for preactivated PLC-β to synthesize IP_3 and increase $[Ca^{2+}]_i$. Again, all cells responded to 10 mM KCl by increasing $[Ca^{2+}]_i$ (data not shown).

We determined if AVP-somatostatin increased PIP_2 levels and the time course of this increase. In the presence of 1 nM AVP, 100 nM somatostatin increased PIP_2 at 8 s post-somatostatin administration ($p < 0.05$) (Fig. 4A). In addition, there was a decrease in PIP levels, the precursor for PIP_2, at 8 s post-somatostatin administration ($p < 0.05$). We then utilized this time frame to compared PIP_2/PIP levels among 4 treatment groups.

We determined if somatostatin increases PIP_2 and decreases PIP levels in the presence and absence of AVP. Somatostatin (100 nM) increased PIP_2 and decreased PIP levels in the presence and absence of 1 nM AVP ($p < 0.05$), compared to basal controls (Fig. 4B & 4C). AVP alone failed to alter PIP_2 or PIP levels. Since somatostatin with or without AVP decreased PIP levels but increased PIP_2 levels, these findings suggested that somatostatin increase the synthesis of PIP_2 from PIP or decrease the breakdown of PIP_2 to PIP.

Somatostatin-Induced Increase in PLD Activity. PIP_2 synthesis is catalyzed predominantly by phosphatidylinositol 4-phosphate (PIP) 5-kinase (Hawkins et al., 1992), which is activated by several input signals including PLD (Jenkins et al., 1994). PLD converts PC into phosphatidic acid (PA), which is known to activate PIP 5-kinase. We hypothesized that somatostatin stimulates PLD to increase PA formation, thereby activating PIP 5-kinase to increase PIP_2 synthesis. Somatostatin (100 nM) increased PLD activity by two-fold over the control group ($p < 0.05$, Fig. 5A). In contrast, 100 nM AVP did not increase PLD activity (Fig. 5A). Pretreatment of HIT-T15 cells with pertussis toxin (PTX,

100 ng/ml) for 14-18 h abolished somatostatin (100 nM)-induced activation of PLD ($p<0.05$, Fig. 5B), suggesting a $G_{i/o}$-coupled receptor-mediated response. HIT-T15 cells that expressed ct-βARK domain was able to abolish somatostatin (100 nM)-induced activation of PLD ($p<0.05$, Fig. 5C). In order to demonstrate that activation of PLD can lead to an increase in PIP_2 levels, which can be utilized by PLC as a substrate to promote IP_3 production and a subsequent increase in $[Ca^{2+}]_i$, we microinjected PA into HIT-T15 cells. Microinjection of PA (10 amol) in the absence of AVP (1 nM) produced no changes in $[Ca^{2+}]_i$; however, in the presence of AVP, it increased $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$: PA: 2.1 ± 0.8 nM; AVP + PA: 39.1 ± 8.8 nM, n = 10 cells, $p<0.05$). To determine if somatostatin's ability to increase PLD activity has an impact on PIP_2 levels and its subsequent increase in $[Ca^{2+}]_i$, we used 1-butanol, a PLD inhibitor, to determine its effects on somatostatin-induced increase in PIP_2 levels. Pretreatment of cells with 1-butanol (0.5%) abolished somatostatin-induced increase in PIP_2 levels ($p<0.05$. Fig. 6), but pretreatment with 2-butanol (0.5%), an inactive constitutional isomer of 1-butanol, did not (Fig. 6). To further demonstrate the involvement of PLD in AVP+somatostatin-induced increase in $[Ca^{2+}]_i$, cells were pretreated with PLD inhibitor 1-butanol. 1-butanol (0.5%) abolished AVP+somatostatin-induced increase in $[Ca^{2+}]_i$, but 2-butanol (0.5%), did not (Fig. 7). Pretreatment with another PLD inhibitor carbobenzyloxy-leucine-tyrosine-chloromethylketone (zLYCK, 10 μM) (Kessels et al., 1991) also abolished AVP+somatostatin-induced increase in $[Ca^{2+}]_i$ (Fig. 8). A high concentration of AVP (100 nM) caused an increase in both the control and zLYCK-pretreated cells (Fig. 8), suggesting that zLYCK does not inhibit G_q-PLC signaling pathway. To further classify PLD involvement in this pathway, we used antibodies vs. PLD1 and PLD2, respectively, to determine which PLD isoform was responsible for the paradoxical increase in $[Ca^{2+}]_i$ caused

by AVP-somatostatin. Only PLD1 was detected in HIT-T15 cells using Western blot analysis (Fig. 9, inset). Antibody vs. PLD1 inhibited somatostatin-induced $[Ca^{2+}]_i$ increase in the presence of AVP, while antibody vs. PLD2 did not (Fig. 9). These results suggested that PLD1 mediates somatostatin-induced increases in PIP_2.

DISCUSSION

Previously, we reported in clonal β-cells HIT-T15 that somatostatin increased $[Ca^{2+}]_i$ and transiently stimulated insulin release in the presence of AVP (Cheng et al., 2002a). These effects of somatostatin in HIT-T15 cell are due to activation of sstr2 (Cheng et al., 2002b), and are attributable to a crosstalk between $G_{i/o}$ and G_q (Cheng et al., 2002a). Crosstalks between G_q- and $G_{i/o}$-coupled receptors have been reported in other systems. For example, activation of $G_{i/o}$-coupled adenosine A_1-receptors enhances the stimulation of PLC-β by G_q-coupled receptors such as α_1-adrenergic, bradykinin, histamine H_1, and muscarinic receptors (Selbie and Hill, 1998). For such a crosstalk, activation of $G_{i/o}$ alone usually has no effect, but it enhances G_q-mediated increases in PLC-β activity, particularly when G_q is activated before $G_{i/o}$ (Muller and Lohse, 1995; Connor et al., 1997; Quitterer and Lohse, 1999; Yeo et al., 2001). Activation of $G_{i/o}$-coupled adenosine A_1-receptors, α_2-adrenoceptors in COS cells (Quitterer and Lohse, 1999) or δ- or κ-opioid receptors in SH-SY5Y cells (Yeo et al., 2001) enhances inositol phosphate formation generated by activation of G_q-coupled receptors. In CHO cells, neuropeptide Y, a $G_{i/o}$-coupled receptor agonist, enhances inositol phosphate formation generated by ATP, a G_q-coupled receptor agonist (Selbie et al., 1997). Somatostatin also increases $[Ca^{2+}]_i$ after activation of the G_q-coupled muscarinic receptors in SH-SY5Y cells (Connor et al., 1997).

To date, studies on the crosstalk between G_q and $G_{i/o}$ indicate that $G\beta\gamma$ of $G_{i/o}$ is responsible for the enhancement of G_q-generated signals (Selbie et al., 1997; Quitterer and Lohse, 1999; Chan et al., 2000). We demonstrated that antibody vs. Gβ nearly abolished somatostatin-induced increase in $[Ca^{2+}]_i$, whereas antibodies vs. $G\alpha_{i1}/G\alpha_{i2}$ and $G\alpha_{i3}/G\alpha_o$ failed to do so. In addition, over-expressing ct-βARK, which binds avidly to $G\beta\gamma$ (Inglese et al., 1992; Koch et al., 1994), greatly reduced somatostatin-induced increases in $[Ca^{2+}]_i$. Our findings are consistent with what are in the literature (Selbie et al., 1997; Quitterer and Lohse, 1999; Chan et al., 2000), and further suggest that in β-cells, the increase in $[Ca^{2+}]_i$ by somatostatin is mediated through $G\beta\gamma$.

Several studies have shown that $G\beta\gamma$ of $G_{i/o}$ can activate a number of enzymes, including PLC-β (Blake et al, 2001), PLA_2 (Kim et al., 1989), mitogen-activated protein kinase (Koch et al., 1994), Raf-1 (Pumiglia et al., 1995), βARK (Goldman et al., 1997), phosphatidylinositol 3-kinase (Lopez-Ilasaca et al., 1998) and adenylyl cyclase (Myung and Garrison, 2000). In intestinal smooth muscle cells, somatostatin alone activates PLC-β_3, thereby increasing IP_3 levels, $[Ca^{2+}]_i$ and contractions through the $G\beta\gamma$ of $G_{i/o}$ (Murthy et al., 1996).

Although others have suggested that enhancement of G_q signals by $G_{i/o}$ is through activation of PLC-β (Chan et al., 2000) or interaction with a step after PLC activation (Yeo et al., 2001), none of them have attributed the effect of $G_{i/o}$ to a step before PLC activation, except that Schmidt et al. (1996 and 1998) suggested that $G_{i/o}$ mediates an increase in PIP_2 levels. In the present study with HIT-T15 cells, after somatostatin treatment, PIP levels decreased but PIP_2 levels increased, suggesting that somatostatin may increase PIP_2

synthesis. Thus, we have suggested for the first time that somatostatin can increase PIP_2 synthesis, which in turn provides extra substrate for preactivated PLC-β by AVP to generate high levels of IP_3. Without a preactivated PLC-β, somatostatin failed to increase IP_3 levels (Fig. 3) and $[Ca^{2+}]_i$ (Cheng et al., 2002a). However, we cannot rule out the possibility that the increase in PIP_2 and decrease in PIP levels might have been due to a decrease in phosphatase activity. In addition, since the IP_3 assay that we used in the present study could not differentiate inositol 1,3,4-trisphosphate from 1,4,5-trisphosphate, some of the IP_3 observed in the present study could be attributable to inositol 1,3,4-trisphosphate. Nevertheless, our results are consistent with the findings using cyclopentyladenosine, an adenosine A_1-receptor agonist, in RINm5F cells, another β-cell line (Biden and Browne, 1993). In RINm5F cells, activation of adenosine A_1-receptors alone fails to increase IP_3 levels and $[Ca^{2+}]_i$, but increases them after activation of M_3 receptors (Biden and Browne, 1993). Our findings are further supported by those of the administration of PIP_2 into the cells, in which PIP_2 alone failed to increase $[Ca^{2+}]_i$, but PIP_2 in the presence of a small concentration of AVP (1 nM) increased $[Ca^{2+}]_i$. Because PIP_2 and PIP failed to increase $[Ca^{2+}]_i$ in the absence of AVP (1 nM), we believe that these phospholipids are unable to elicit any $[Ca^{2+}]_i$ response inHIT-T15 cells. The failure of microinjected PIP (50 amol) to induce changes in $[Ca^{2+}]_i$ in the presence of AVP (1 nM) demonstrated that the microinjection of PIP_2 in the presences of AVP, which caused an increase in $[Ca^{2+}]_i$, was due to PIP_2's usage as a substrate for low-grade PLC activation and not an artifact of the system. In HEK 293 cells with stable expression of M_3 receptors, carbachol increases PIP_2 levels for at least 30 min (Schmidt et al., 1996). The M_3 receptor-mediated increase in PIP_2 levels is attributable to G_i-protein coupling, since this effect was inhibited by PTX pretreatment. In HEK 293 cells,

activation of plasma membrane purinergic receptors and lysophosphatidic acid receptors, respectively, also increases PIP_2 levels for ≥40 min (Schmidt et al., 1998). These findings are somewhat different from those of ours, since somatostatin-induced increase in PIP_2 levels in HIT-T15 cells lasted <12 s, but the effect of $G_{i/o}$-coupled receptor agonists in HEK 293 cells lasted ≥40 min. It is not clear why these two systems are so much different in terms of the duration of the agonist-induced PIP_2 levels. However, in the study with HEK 293 cells, for preactivation of PLC, Schmidt et al. used G_q-coupled receptor agonists, which markedly lowered PIP_2 levels. Upon removal of the agonists, the PIP_2 levels increased again, which were above the initial control levels (Personal communications from Dr. K. H. Jakobs).

In the present study, we demonstrated for the first time that somatostatin can increase PLD activity. This effect of somatostatin was PTX-sensitive, and was blocked by expression of ct-βARK, suggesting that Gi/o-βγ dimer mediates this effect of somatostatin. We further hypothesized that somatostatin-induced increase in PLD activity mediates the increase in PIP_2 formation, since PLD is the enzyme that catalyzes the formation of PA, which may in turn activates PIP 5-kinase to increase PIP_2 synthesis (Hawkins et al., 1992). In the present study, we demonstrated that microinjection of PA increased $[Ca^{2+}]_i$, which has been used as an indicator for IP_3 increase. In addition, PLD inhibitors, 1-butanol and zLYCK, and antibody vs. PLD1 all blocked somatostatin-induced $[Ca^{2+}]_i$ increase in the presence of AVP. 1-Butanol further abolished somatostatin-induced PIP_2 increase. Therefore, the present findings strongly supported the notion that somatostatin increases PA synthesis, which in turn activates PIP-5 kinase, the enzyme catalyzing the formation of PIP_2.

In this system somatostatin was able to increase PIP_2 levels, providing extra substrate for PLC-β. On the other hand, PIP_2 can regulate a wide range of cellular processes, including

exocytosis (Cremona and De Camilli, 2001), clathrin-mediated endocytosis (Gillooly and Stenmark, 2001), actin rearrangement (Tolias et al., 2000), vesicle docking (Brown et al., 2001), opening of G-protein-gated inwardly rectifying K^+ channels (Zhang et al., 1999), K_{ATP} channels (Baukrowitz et al., 1998), membrane ruffling and trafficking (Honda et al., 1999). Because of the diverse role of PIP_2 in cellular processes, it is uncertain what impact somatostatin-induced increase in PLD activity and its subsequent increase in PIP_2 levels have on normal physiology of β-cells.

In the present study, when AVP was needed to cause a low-grade PLC activation, we used 1 nM AVP in most of the experiments. This concentration of AVP usually caused an increase in $[Ca^{2+}]_i$ of 0-50 nM, which was consistent with our previous study (Cheng et al., 2002a). However, in the experiment where butanols were studied (Fig. 7), AVP at 1 nM increased $[Ca^{2+}]_i$ by ~90 nM. In this particular experiment, probably due to different FBS used in cell culture, the cells became more responsive to AVP than usual. As a result of the hyperresponse to AVP, somatostatin-induced $[Ca^{2+}]_i$ increase was attenuated. This phenomenon was also seen in our previous study (Cheng et al., 2002a).

In summary, we have demonstrated a novel signaling mechanism for somatostatin. The activation of somatostatin receptors, which are coupled to $G_{i/o}$, leads to an increase in PIP_2 synthesis through Gβγ activation of PLD. The PIP_2 generated by somatostatin administration provides extra substrate for preactivated PLC-β, which hydrolyzes PIP_2, thereby increasing IP_3 levels, $[Ca^{2+}]_i$ and a transient release of insulin from HIT-T15 cells (Fig. 10). This is the first report regarding somatostatin-induced increase in PLD activity and PIP_2 synthesis by activation of $G_{i/o}$-coupled receptors.

ACKNOWLEDGMENTS

We greatly appreciate the generous donation of pcDNAIIIB T8βARK plasmid by Dr. Silvio Gutkind of NICDR, NIH and antibodies vs. PLD1 and PLD 2 by Dr. Sylvain Bourgoin of the University of Laval, Canada. We would also like to acknowledge Dr. Guangxing Bai for contribution with western blot analysis.

REFERENCES

Baukrowitz T, Schulte U, Oliver D, Herlitze S, Krauter T, Tucker SJ, Ruppersberg JP, and Fakler, B (1998) PIP_2 and PIP as determinants for ATP inhibition of K_{ATP} channels. *Science* **282:**1141-1144.

Berridge MJ, Heslop JP, Irvine RF, and Brown KD (1984) Inositol trisphosphate formation and calcium mobilization in Swiss 3T3 cells in response to platelet-derived growth factor. *Biochem J* **222**:195-201.

Biden TJ, and Browne CL (1993) Cross-talk between muscarinic- and adenosine-receptor signalling in the regulation of cytosolic free Ca^{2+} and insulin secretion. *Biochem J* **293**:721-728.

Blake BL, Wing MR, Zhou JY, Lei Q, Hillmann JR., Behe CI, Morris RA, Harden TK, Bayliss DA, Miller RJ, and Siderovski DP (2001) Gβ association and effector interaction selectivities of the divergent Gγ subunit $G\gamma_{13}$. *J Biol Chem* **276**:49267-49274.

Brown FD, Rozelle AL, Yin HL, Balla T, and Donaldson JG (2001) Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J Cell Biol* **154:**1007-1017.

Chan JS, Lee JW, Ho MK, and Wong YH (2000) Preactivation permits subsequent stimulation of phospholipase C by G_i-coupled receptors. *Mol Pharmacol* **57**:700-708.

Cheng H, Yibchok-anun S, Park S-C, and Hsu WH (2002a) Somatostatin-induced paradoxical increase in intracellular Ca^{2+} concentration and insulin release in the presence of arginine vasopressin in clonal HIT-T15 β-cells. *Biochem J* **364**:33-39.

Cheng H, Yibchok-anun S, Coy DH, and Hsu WH (2002b) SSTR2 mediates the somatostatin-induced increase in intracellular Ca^{2+} concentration and insulin secretion in the presence of arginine vasopressin in clonal β-cells HIT-T15. *Life Sci* **71**:927-936.

Connor M, Yeo A, and Henderson G (1997) Neuropeptide Y Y_2 receptor and somatostatin sst_2 receptor coupling to mobilization of intracellular calcium in SH-SY5Y human neuroblastoma cells. *Br J Pharmacol* **120**:455-463.

Cremona O and De Camilli P (2001) Phosphoinositides in membrane traffic at the synapse. *J Cell Sci* **114:**1041-1052.

Gillooly DJ and Stenmark H (2001) Cell biology. A lipid oils endocytosis machine. *Science* **291:**993-994.

Goldman PS, DeMaggio AJ, Hoekstra MF, and Goodman RH (1997) The β-adrenergic receptor kinase interacts with the amino terminus of the G protein β subunit. *Biochem Biophys Res Commun* **240:**425-429.

Grynkiewicz G, Poenie M, and Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* **260:**3440-3450.

Hawkins PT, Jackson TR, and Stephens LR (1992) Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase. *Nature* **358:**157-159.

Honda A, Nogami M, Yokozeki T, Yamazaki M, Nakamura H, Watanabe H, Kawamoto K, Nakayama K, Morris AJ, Frohman, MA, et al. (1999) Phosphatidylinositol 4-phosphate 5-

kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell* **99:**521-532.

Hoque KM, Pal A, Nair GB, Chattopadhyay S, and Chakrabarti MK (2001) Evidence of calcium influx across the plasma membrane depends upon the initial rise of cytosolic calcium with activation of IP_3 in rat enterocytes by heat-stable enterotoxin of *Vibrio cholerae* non-O1. *FEMS Microbiol Lett* **196:**45-50.

Inglese J, Koch WJ, Caron MG, and Lefkowitz RJ (1992) Isooprenylation in regulation of signal transduction by G-protein-coupled receptor kinases. *Nature* **359:**147-149.

Jenkins GH, Fisette PL, and Anderson RA (1994) Type I phosphatidylinositol 4-phosphate 5-kinase isoforms are specifically stimulated by phosphatidic acid. *J Biol Chem* **269:**11547-11554.

Kessels GCR, Gervaix A, Lew PFD, and Verhoeven AJ (1991) The chymotrypsin inhibitor carbobenzyloxy-leucine-tyrosine-chloromethylketone interferes with phospholipase D activation induced by formyl-methionyl-leucyl-phenylalanine in human neutrophils. *J Biol Chem* **266:**15870-15875.

Kim D, Lewis DL, Graziadei L, Neer EJ, Bar-Sagi D, and Clapham DE (1989) G-protein βγ-subunits activate the cardiac muscarinic K^+-channel via phospholipase A_2. *Nature* **337:**557-560.

Koch WJ, Hawes BE, Allen LF, and Lefkowitz RJ (1994) Direct evidence that Gi-coupled receptor stimulation of mitogen-activated protein kinase is mediated by Gβγ activation of p21ras. *Proc Natl Acad Sci USA* **91:**12706-12710.

Lopez-Ilasaca M, Gutkind JS, and Wetzker R (1998) Effects of FK506-binding protein 12 and FK506 on autophosphorylation of epidermal growth factor receptor. *J Biol Chem* **273:**2505-2508.

Muller S and Lohse MJ (1995) The role of G-protein βγ subunits in signal transduction. *Biochem Soc Trans* **23:**141-148

Murthy KS, Coy DH, and Makhlouf GM (1996) Somatostatin receptor-mediated signaling in smooth muscle. Activation of phospholipase C-β3 by $G_{\beta\gamma}$ and inhibition of adenylyl cyclase by $G_{\alpha i1}$ and $G_{\alpha o}$. *J Biol Chem* **271:**23458-23463.

Myung CS and Garrison JC (2000) Role of C-terminal domains of the G protein β subunit in the activation of effectors. *Proc Natl Acad Sci USA* **97:**9311-9316.

Nishizuka Y (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature* **308:**693-698.

Norris FA and Majerus PW (1994) Hydrolysis of phosphatidylinositol 3,4-bisphosphate by inositol polyphosphate 4-phosphatase isolated by affinity elution chromatography. *J Biol Chem* **269:**8716-8720.

O'Launaigh N, Pardo R, FensomeA, Allen-Baume V, Jones D, Holt MR, and Cockcroft S (2002) Continual production of phosphatidic acid by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells. *Mol Biol Cell* **13:**3730-3746.

Patel YC, Greenwood MT, Warszynska A, Panetta R, and Srikant CB (1994) All five cloned human somatostatin receptors (hSSTR1-5) are functionally coupled to adenylyl cyclase. *Biochem Biophys Res Commun* **198:**605-612.

Pumiglia KM, LeVine H, Haske T, Habib T, Jove R, and Decker SJ (1995) A direct interaction between G-protein βγ subunits and the Raf-1 protein kinase. *J Biol Chem* **270:**14251-14254.

Quitterer U and Lohse MJ (1999) Crosstalk between $G\alpha_i$- and $G\alpha_q$-coupled receptors is mediated by Gβγ exchange. *Proc Natl Acad Sci USA* **96:**10626-10631.

Rhee SG (2001) Regulation of phosphoinositide-specific phospholipase C. *Annu Rev Biochem* **70:**281-312.

Schmidt M, Nehls C, Rumenapp U, and Jakobs KH (1996) m3 Muscarinic receptor-induced and G_i-mediated heterologous potentiation of phospholipase C stimulation: role of phosphoinositide synthesis. *Mol Pharmacol* **50:**1038-1046.

Schmidt M, Lohmann B, Hammer K, Haupenthal S, Nehls MV, and Jakobs KH (1998) Gi- and protein kinase C-mediated heterologous potentiation of phospholipase C signaling by G protein-coupled receptors. *Mol Pharmacol* **53:**1139-1148.

Selbie LA, King NV, Dickenson JM, and Hill SJ (1997) Role of G-protein βγ subunits in the augmentation of P_2Y_2 (P_{2U}) receptor-stimulated responses by neuropeptide Y Y_1 $G_{i/o}$-coupled receptors. *Biochem J* **328:**153-158.

Selbie LA and Hill SJ (1998) G protein-coupled-receptor cross-talk: the fine-tuning of multiple receptor-signalling pathways. *Trends Pharmacol Sci* **19:**87-93.

Tolias KF, Hartwig JH, Ishihara H, Shibasaki Y, Cantley LC, and Carpenter CL (2000) Type Iα phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr Biol* **10:**153-156.

Yeo A, Samways DS, Fowler CE, Gunn-Moore F, and Henderson G (2001) Coincident signalling between the Gi/Go-coupled δ-opioid receptor and the Gq-coupled m3 muscarinic receptor at the level of intracellular free calcium in SH-SY5Y cells. *J Neurochem* **76:**1688-1700.

Zhang H, He C, Yan X, Mirshahi T, and Logothetis DE (1999) Activation of inwardly rectifying K^+ channels by distinct PtdIns(4,5)P2 interactions. *Nature Cell Biol* **1:**183-188.

ZhuGe R and Hsu WH (1995) Evidence for a caffeine- and ryanodine-sensitive Ca^{2+} store in porcine myometrial cells. *J Pharmacol Exp Ther* **275:**1077-1083.

FOOTNOTES

Address correspondence to: Dr. Walter H. Hsu, Department of Biomedical Sciences, Iowa State University, Ames, IA 50011-1250. Phone: 515-294-6864. FAX: 515-294-2315. E-mail: whsu@iastate.edu

LEGENDS FOR FIGURES

Fig. 1. Effect of antibodies vs. $G_{i/o}$ subunits on somatostatin (SS)-induced increase in $[Ca^{2+}]_i$ in the presence of AVP in HIT-T15 cells. 1A) Antibodies vs. Gβ, $G\alpha_{i1}/G\alpha_{i2}$ and $G\alpha_{i3}/G\alpha_o$, respectively, were diluted 1:100 and microinjected into single cells followed by a 30-min incubation period and Ca^{2+} image analysis. 100 nM somatostatin was given 100 s following administration of AVP (1 nM). Baseline $[Ca^{2+}]_i$ were approximately 100 nM. Values are the mean ± SE; n=10 cells/treatment from 3 independent cell preparations. *p<0.05 compared to somatostatin controls. 1B) Representative calcium trace of microinjected antibodies vs. Gβ, in the presence of AVP, inhibited SS induced increase in $[Ca^{2+}]_i$. 1C-D) Representative calcium traces of microinjected antibodies vs. $G\alpha_{i1}/G\alpha_{i2}$ and $G\alpha_{i3}/G\alpha_o$, respectively, in the presence of AVP, did not inhibited SS induced increase in $[Ca^{2+}]_i$.

Fig. 2. Somatostatin(SS)-induced increase in IP_3 levels in the presence and absence of AVP in HIT-T15 cells. A) Time course of AVP-somatostatin induced increase in IP_3 levels, as determined by ion-exchange chromatography. Somatostatin (100 nM) was given 100 s after AVP (1 nM) and experiments terminated at the respective time; *p<0.05 compared to 0 s (n=3). B) Determination of IP_3 levels among 4 treatment groups. Somatostatin (100 nM) was applied 100 s after AVP (1 nM); experiment was terminated at 12 s of somatostatin treatment; *p<0.05 compared to basal controls. Values are the mean ± SE (n=4 independent cell preparations).

Fig. 3. Effect of PIP_2 microinjection on $[Ca^{2+}]_i$ in the presence of AVP in HIT-T15 cells. 3A) Administration of PIP_2 (1.5-50 amol) into single cells increased $[Ca^{2+}]_i$ in a dose-

dependent manner after 100 s of AVP (1 nM). Values are the mean ± SE; n=3 independent cell preparations. (data not shown).3B) Representative calcium trace of HIT T-15 cells microinjected with intracellular buffer after the addition AVP not increase $[Ca^{2+}]_i$. 3C) Representative calcium trace of HIT-T15 cells microinjected with PIP_2 alone at 50 amol did not increase $[Ca^{2+}]_i$. 3D) Representative calcium trace of HIT-T15 cells microinjected with 1.5 amol of PIP_2 after the addition of AVP did not increase $[Ca^{2+}]_i$. 3E-G) Representative calcium traces of microinjected PIP_2 (5 amol, 15 amol, 50 amol, respectively) did increase $[Ca^{2+}]_i$, while in the presence of AVP in a dose dependent manner.

Fig. 4. Somatostatin(SS)-induced changes in PIP and PIP_2 levels in HIT-T15 cells. PIP and PIP_2 levels were determined by thin layer chromatography. Values are the mean ± SE. A) Time course for changes in PIP and PIP_2 levels by somatostatin in the presence of AVP. Somatostatin (100 nM) was administered 100 s after AVP (1 nM); experiment was terminated at the respective time. n=3 independent cell preparations. *$p<0.05$ compared to 0 s. B) and C). Somatostatin-induced changes in PIP and PIP_2 levels in the presence and absence of AVP. PIP_2 (B) and PIP (C) levels were determined after exposure to different treatments. Somatostatin (100 nM) was applied 100 s after AVP (1 nM) and experiments terminated at 8 s post-somatostatin administration. n=4 independent cell preparations. *$p<0.05$ compared to basal controls.

Fig. 5. Somatostatin(SS)-induced increase in PLD activity in HIT-T15 cells. Experiments were terminated 30 s after somatostatin or AVP had been administered. A) Effect of somatostatin and AVP. B) Effect of pertussis toxin (PTX). HIT-T15 cells were pretreated

overnight with PTX (100 ng/ml) prior to treatments. C) Effect of ct-βARK expression on somatostatin-induced increase on PLD activity. HIT-T15 cells were transfected 24 h prior to PLD assay. Values are the mean ± SE (n=4 independent cell preparations). *$p < 0.05$ compared to controls. PBt = phosphatidylbutanol, PC=phosphatidylcholine.

Fig. 6. Effect of 1-butanol and 2-butanol on somatostatin(SS)-induced increase in PIP_2 levels. HIT-T15 cells were pretreated with 0.5% 1-butanol or 2-butanol 5 min prior to somatostatin treatment. Experiments were terminated at 8 s post-somatostatin administration. Values are the mean ± SE; n=4 independent cell preparations. *$p<0.05$ compared to basal controls.

Fig. 7. Effect of 1-butanol and 2-butanol on somatostatin(SS)-induced (100 nM) increase in $[Ca^{2+}]_i$ in the presence of AVP (1 nM). HIT-T15 cells were pretreated with 1-butanol or 2-butanol (0.5%) 5 min prior to treatments. Cells treated with 1-butanol or 2-butanol were exposed to AVP (1 nM) for 150 s before the addition of somatostatin. Each line depicts the mean from 8-14 cells. The lines are representative of 4 independent experiments.

Fig. 8. Effect of zLYCK on somatostatin(SS)-induced (100 nM) increase in $[Ca^{2+}]_i$ in the presence of AVP (1 nM). HIT-T15 cells were pretreated with zLYCK (10 μM) 60 min prior to treatments. Control and zLYCK-treated cells were exposed to AVP (1 nM) for 120 s before the addition of somatostatin. After the 60-s co-exposure to AVP (1 nM) and somatostatin, cells were perifused with KRB for 120 s before the addition of 100 nM of

AVP. Each line depicts the mean from 5-8 cells. The lines are representative of 4 independent experiments.

Fig. 9. PLD immunoblot and effect of PLD antibodies on somatostatin(SS)-induced increase in $[Ca^{2+}]_i$ in the presence of AVP. Inset, 10 μg of the whole cell protein was run on SDS-PAGE, transferred to the PVDF membrane, and blotted with PLD antibodies. For $[Ca^{2+}]_i$ determination, HIT-T15 cells were pretreated with PLD antibodies using the BioPORTER® protein delivery system 4 h prior to Fura-2AM loading. Cells were exposed to AVP (1 nM) for 120 s before the addition of somatostatin (100 nM). AVP (100 nM) was added 120 s after somatostatin treatment. Each line depicts the mean from 5-8 cells. The lines are representative of 4 independent experiments.

Fig. 10. Summary of the crosstalk between AVP receptor (V_{1b}) and somatostatin receptor (sstr2) in stimulation of insulin release from β-cells. Activation of the $G_{i/o}$-coupled receptor by somatostatin increases PIP_2 synthesis from PIP through Gβγ. Gβγ activates PLD to increase the synthesis of phosphatidic acid (PA) from phosphatidylcholine (PC). PA activates PIP 5-kinase to increase synthesis of PIP_2, providing extra substrate for preactivated PLC-β by AVP. This increases in DAG and IP_3 levels and $[Ca^{2+}]_i$ lead to insulin release. ER: endoplasmic reticulum.

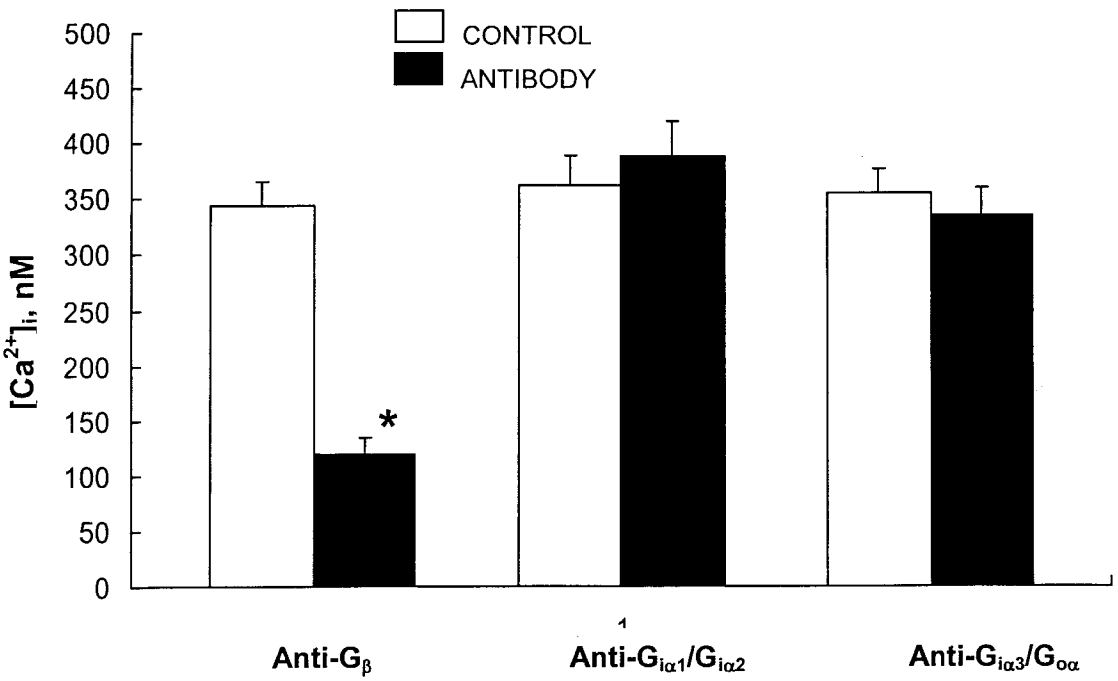
CONTROL
ANTIBODY
500
450
400
350
300
250
200
150
100
50
0
$[Ca^{2+}]_i$, nM
*
Anti-G_β
Anti-$G_{i\alpha1}/G_{i\alpha2}$
Anti-$G_{i\alpha3}/G_{o\alpha}$

Fig. 1A.

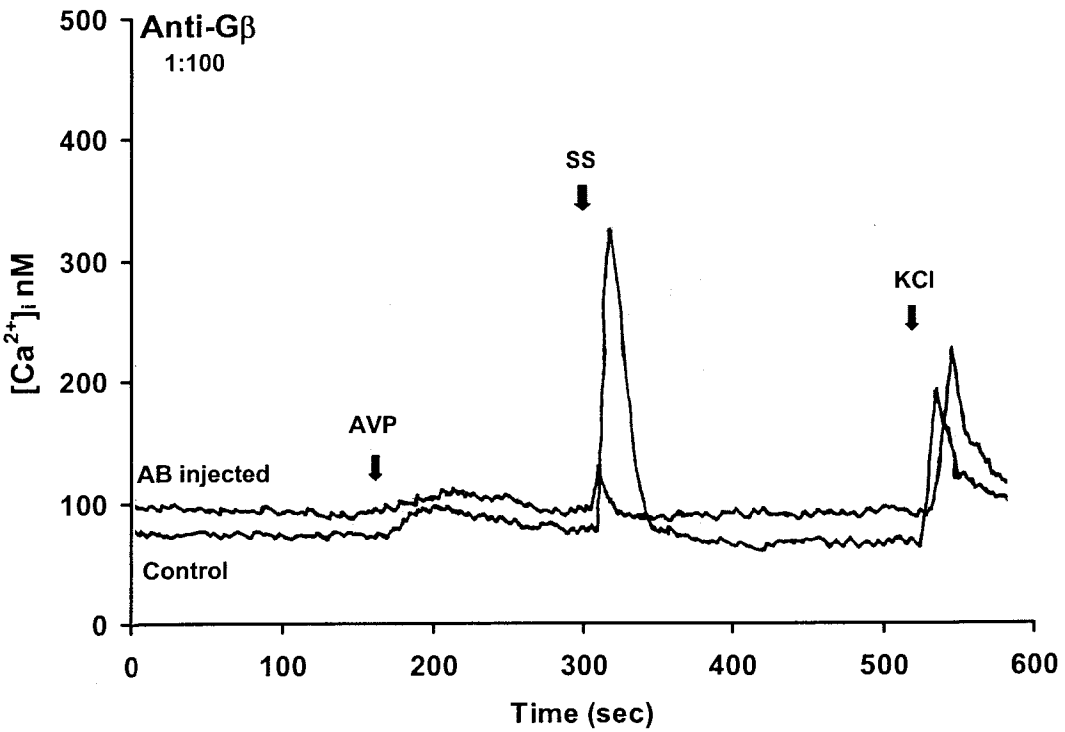
Anti-Gβ
1:100
SS
KCl
AVP
AB injected
Control
[Ca2+]i nM
Time (sec)
0
100
200
300
400
500
600

Fig. 1B

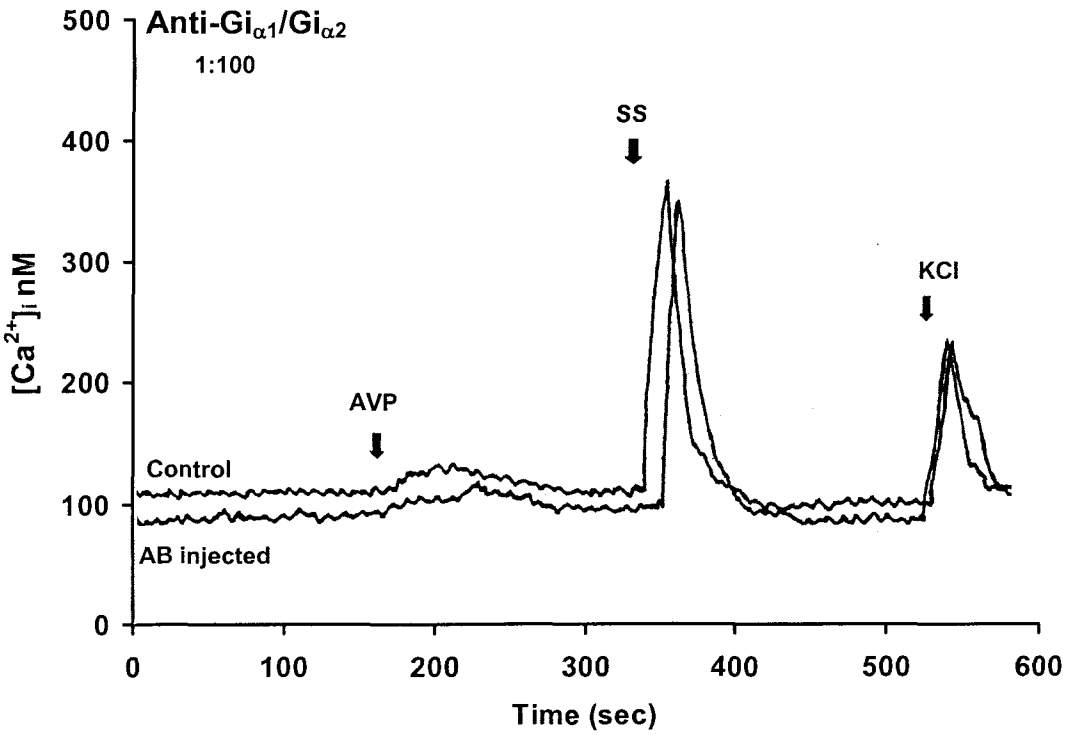
Anti-Gi$_{\alpha1}$/Gi$_{\alpha2}$
1:100
SS
KCl
AVP
Control
AB injected
[Ca^{2+}]$_i$ nM
Time (sec)
0
100
200
300
400
500
600

Fig. 1C

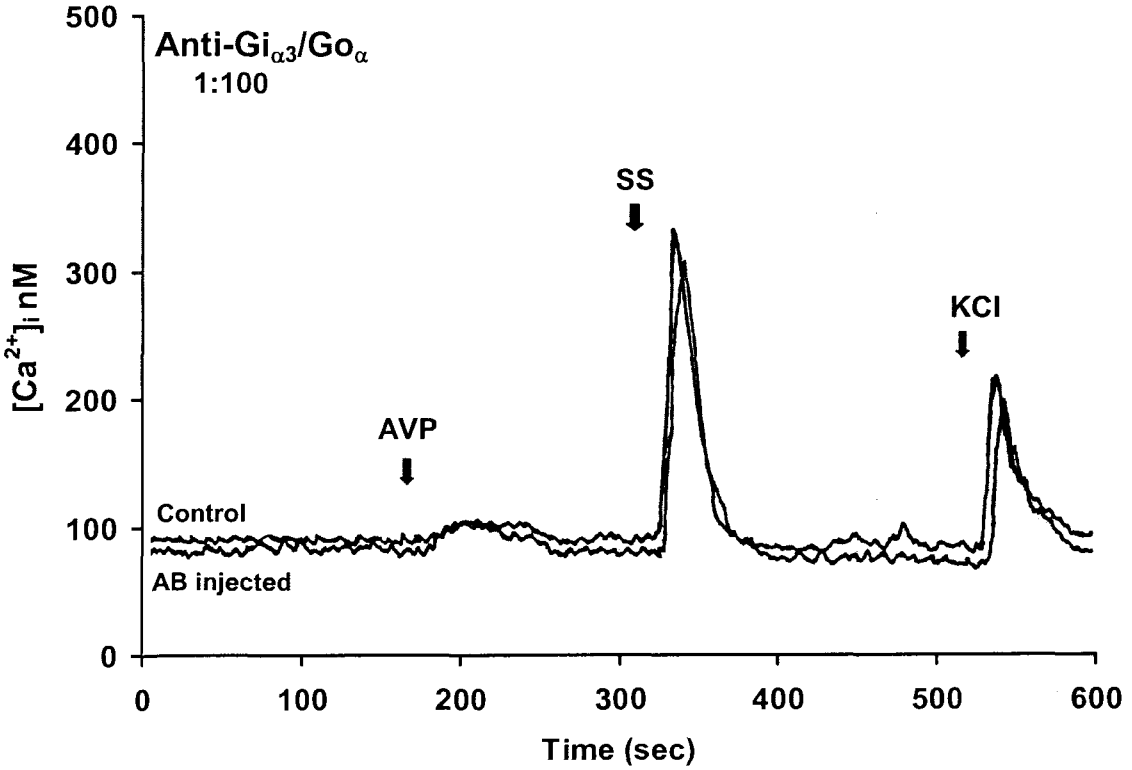
Anti-Gi$_{\alpha 3}$/Go$_{\alpha}$
1:100
SS
KCl
AVP
Control
AB injected
[Ca^{2+}]$_i$ nM
Time (sec)
0
100
200
300
400
500
600

Fig. 1D

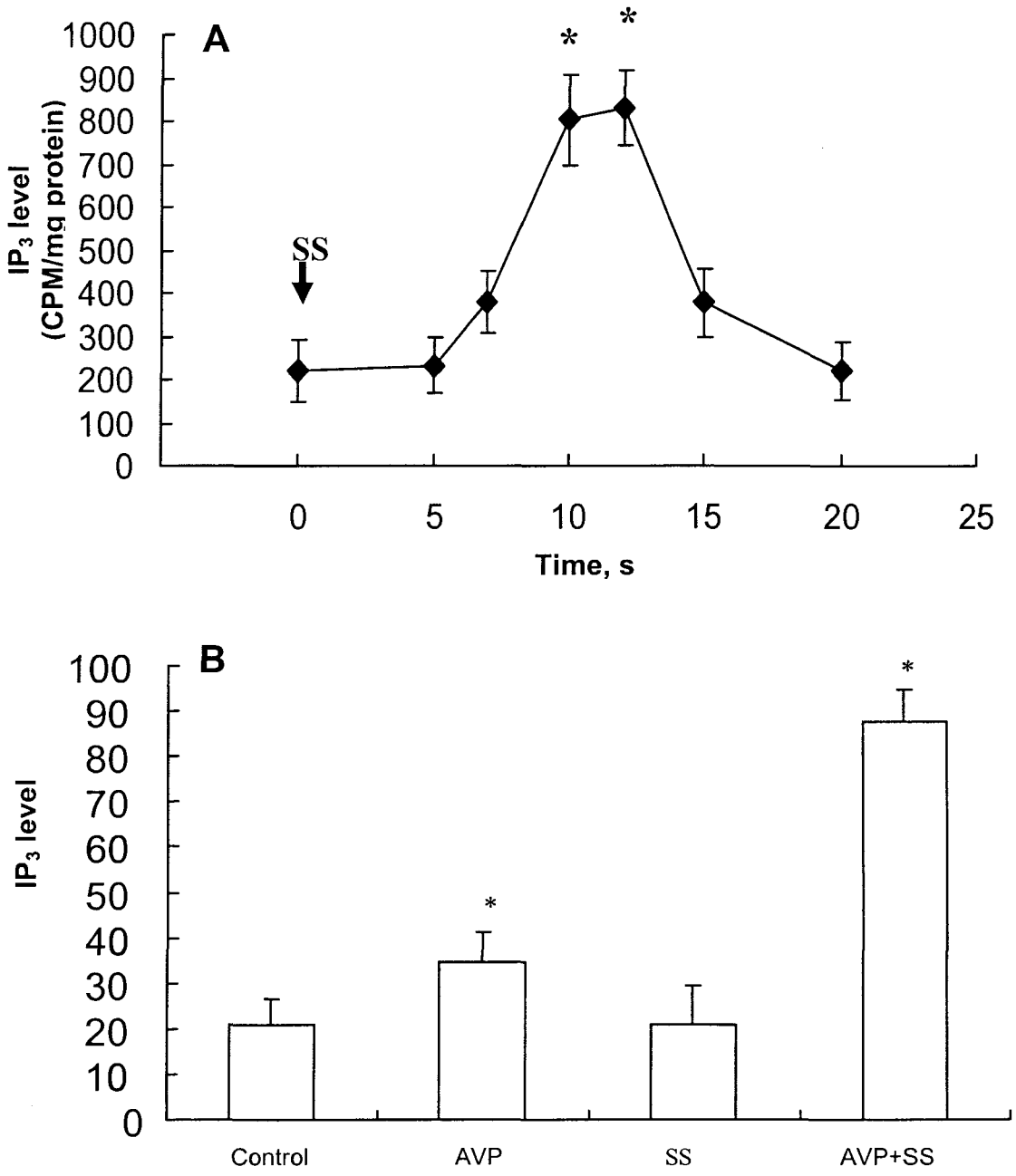
A
1000
900
800
700
600
500
400
300
200
100
0
IP3 level
(CPM/mg protein)
SS
*
*
0
5
10
15
20
25
Time, s
B
100
90
80
70
60
50
40
30
20
10
0
IP3 level
*
*
Control
AVP
SS
AVP+SS

Fig. 2

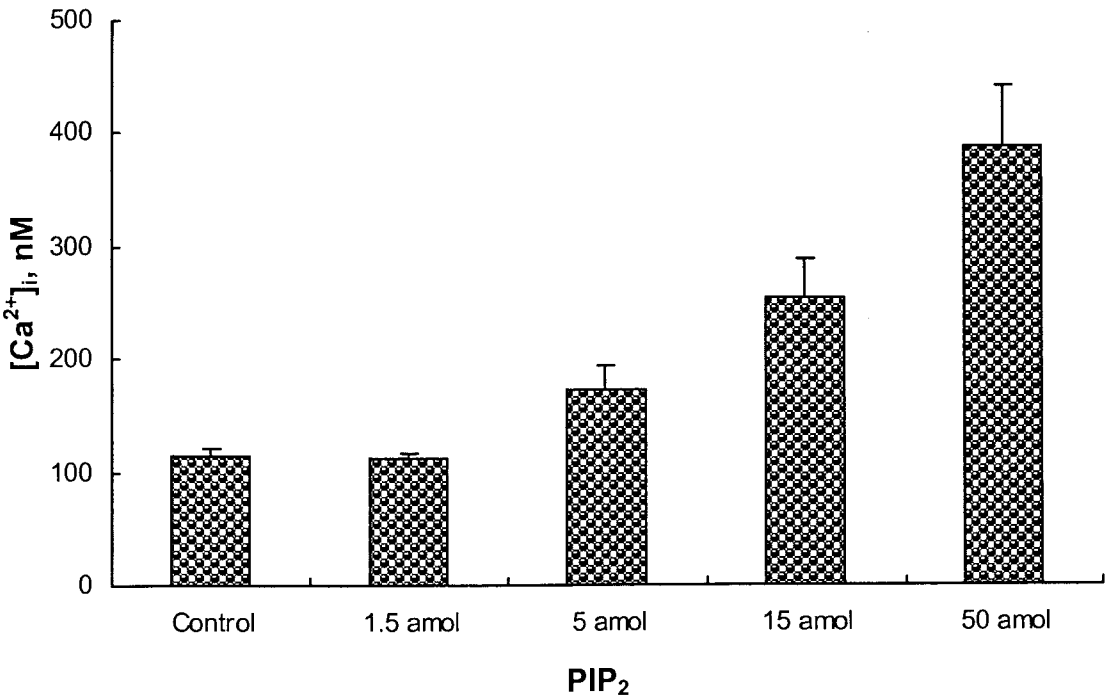
500
400
300
200
100
0
[Ca2+]i, nM
Control
1.5 amol
5 amol
15 amol
50 amol
PIP2

Fig. 3A.

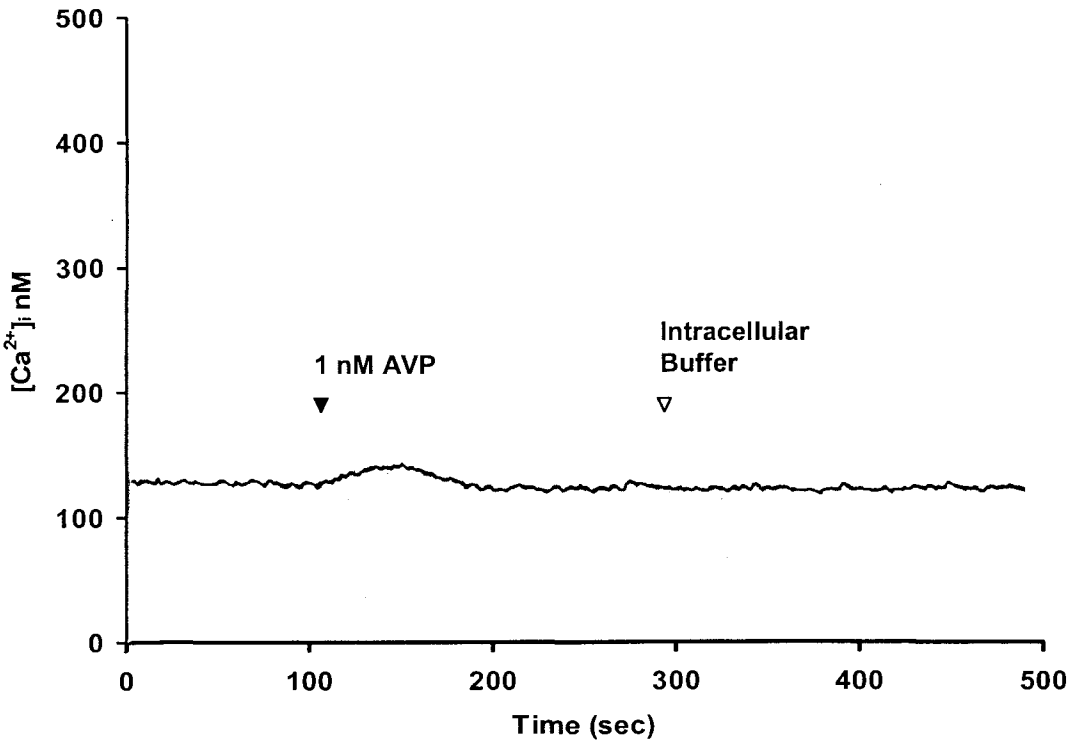
500
400
300
200
100
0
[Ca2+]i nM
1 nM AVP
Intracellular
Buffer
0
100
200
300
400
500
Time (sec)

Fig. 3B

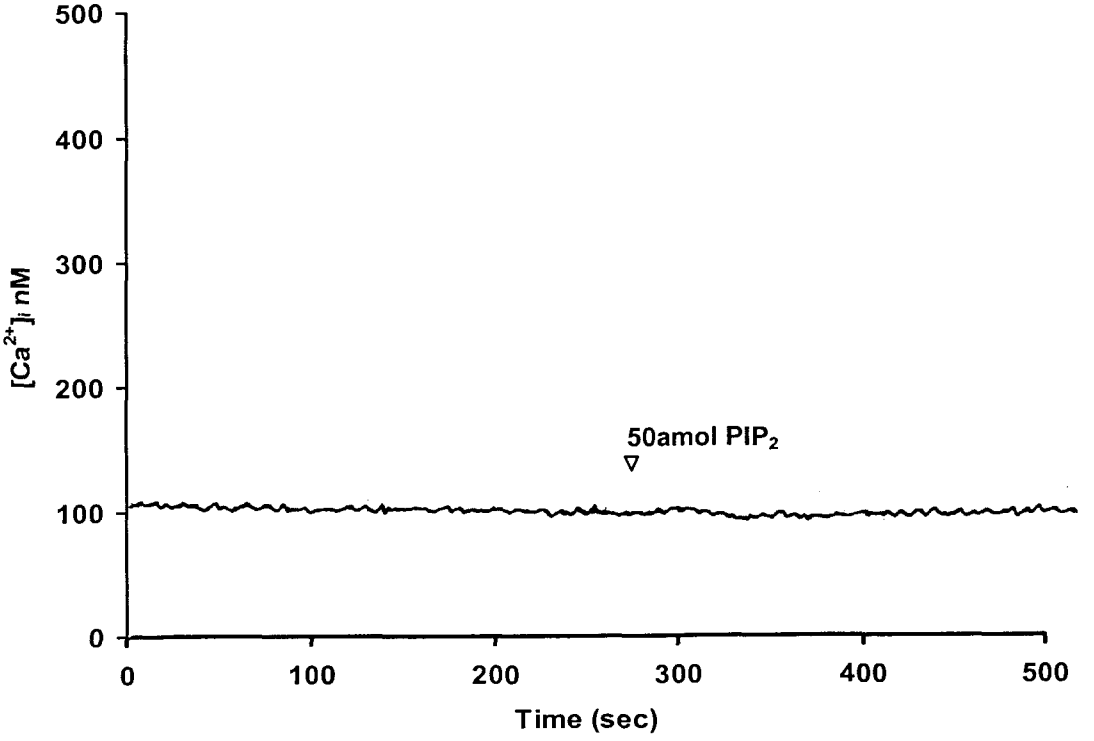
500
400
300
200
100
0
[Ca2+]i nM
50amol PIP2
0
100
200
300
400
500
Time (sec)

Fig. 3C

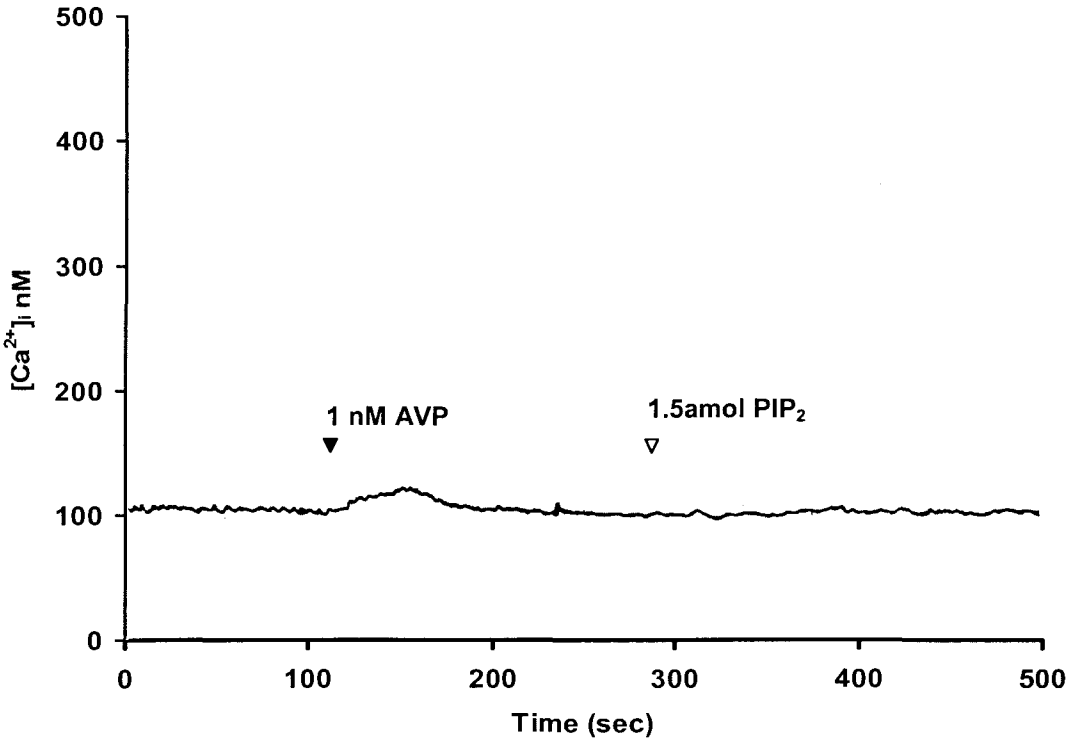
500
400
300
200
100
0
[Ca2+]i nM
1 nM AVP
1.5amol PIP2
0
100
200
300
400
500
Time (sec)

Fig. 3D

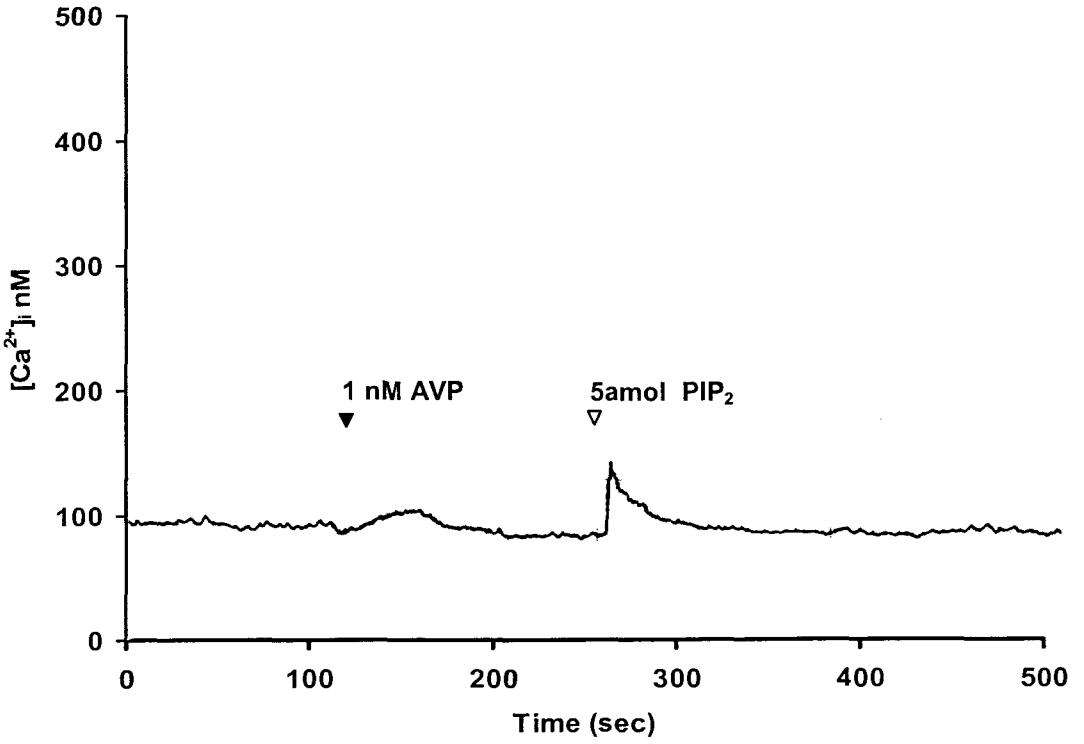
500
400
300
200
100
0
[Ca2+]i nM
1 nM AVP
5amol PIP2
0
100
200
300
400
500
Time (sec)

Fig. 3E

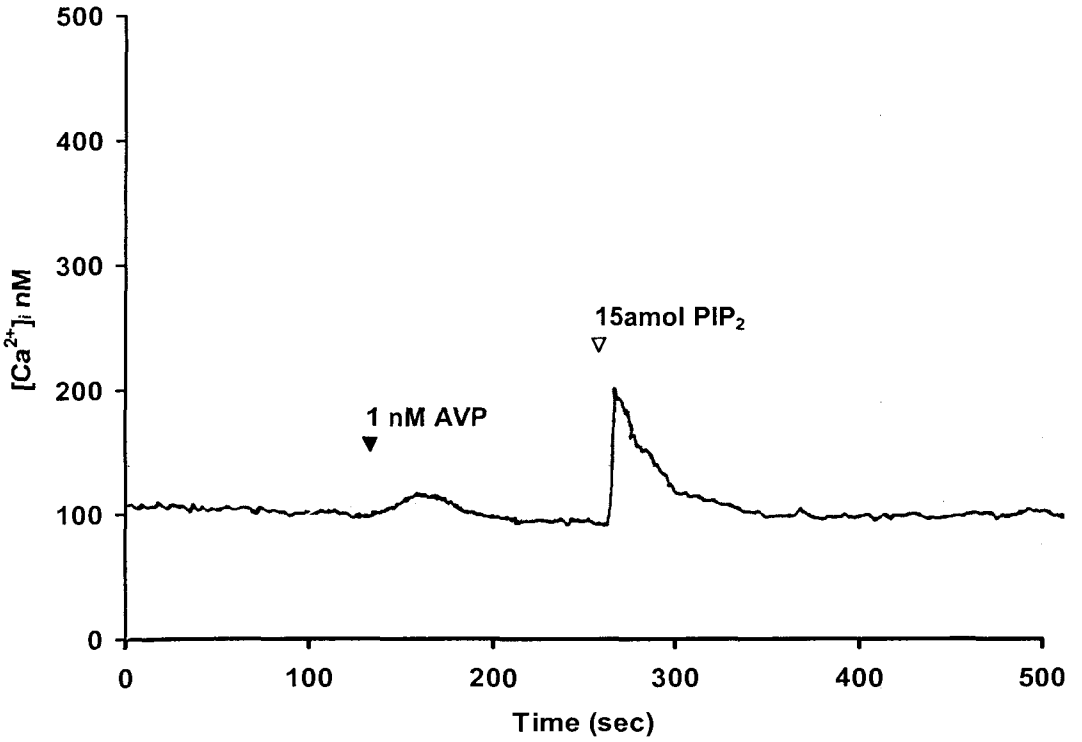
500
400
300
200
100
0
$[Ca^{2+}]_i$ nM
15amol PIP_2
1 nM AVP
0
100
200
300
400
500
Time (sec)

Fig. 3F

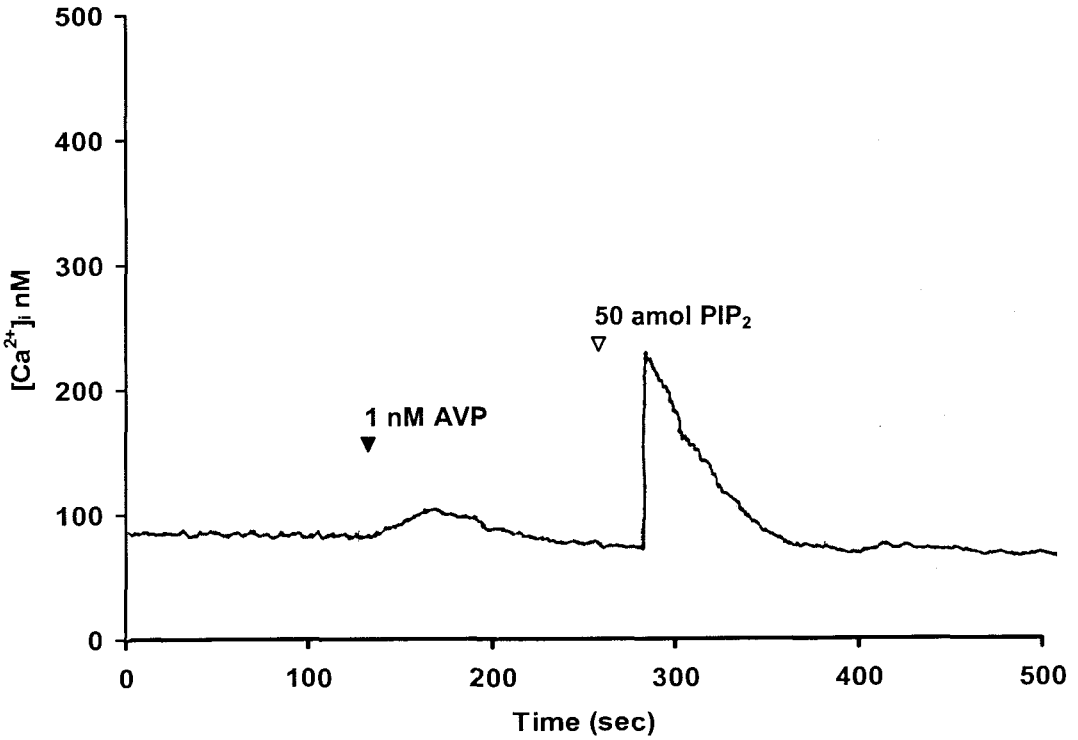
500
400
300
200
100
0
[Ca2+]i nM
50 amol PIP2
1 nM AVP
0
100
200
300
400
500
Time (sec)

Fig. 3G

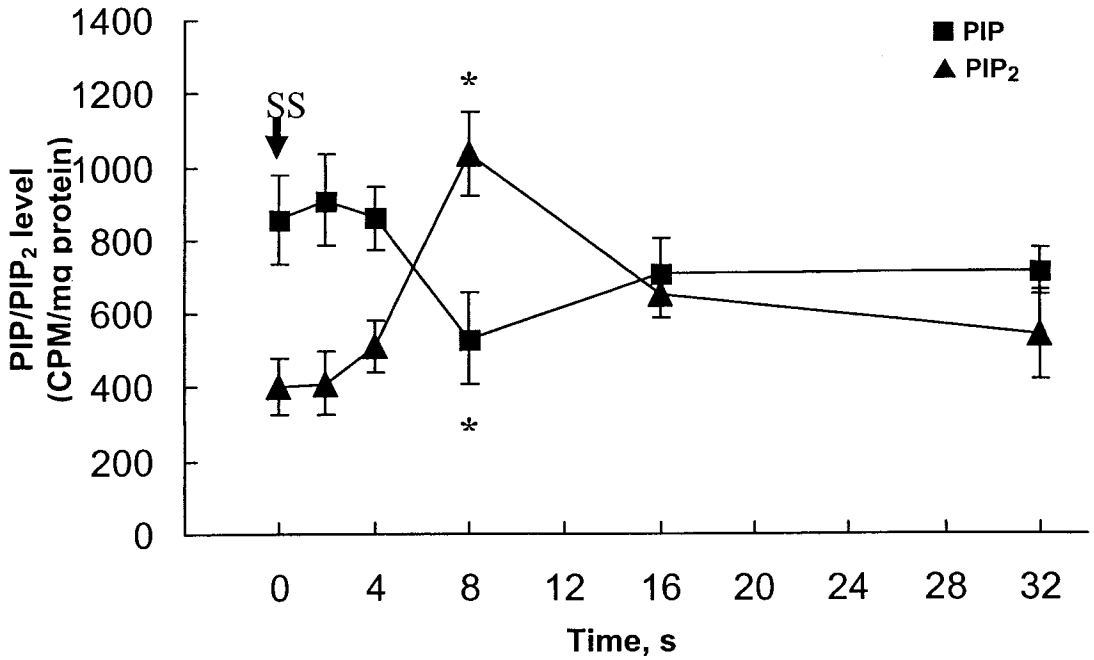
1400
1200
1000
800
600
400
200
0
PIP/PIP2 level
(CPM/mg protein)
SS
*
*
PIP
PIP2
0
4
8
12
16
20
24
28
32
Time, s

Fig. 4 A.

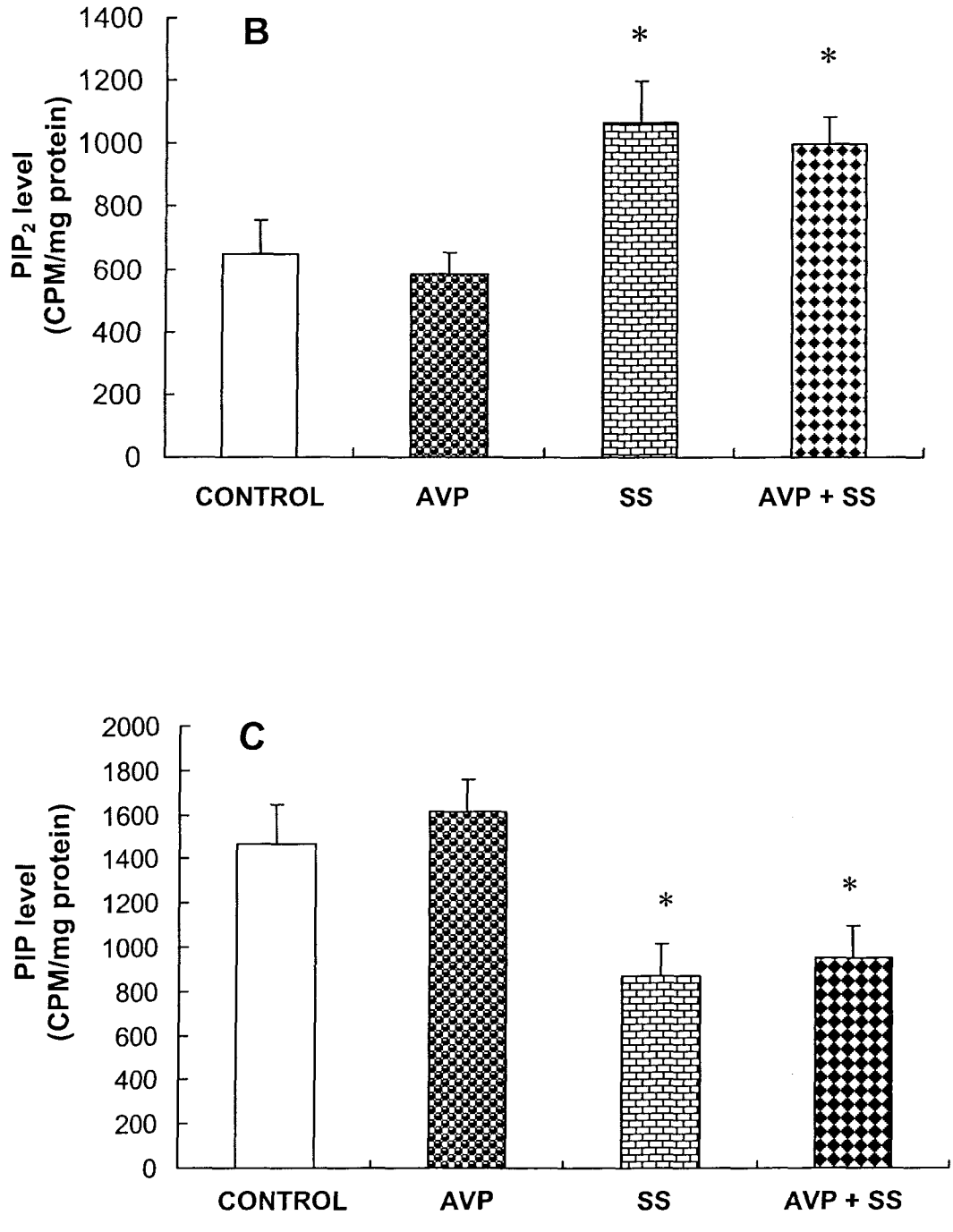
B
PIP2 level
(CPM/mg protein)
1400
1200
1000
800
600
400
200
0
CONTROL
AVP
SS
AVP + SS
*
*
C
PIP level
(CPM/mg protein)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
CONTROL
AVP
SS
AVP + SS
*
*

Fig. 4. B. C.

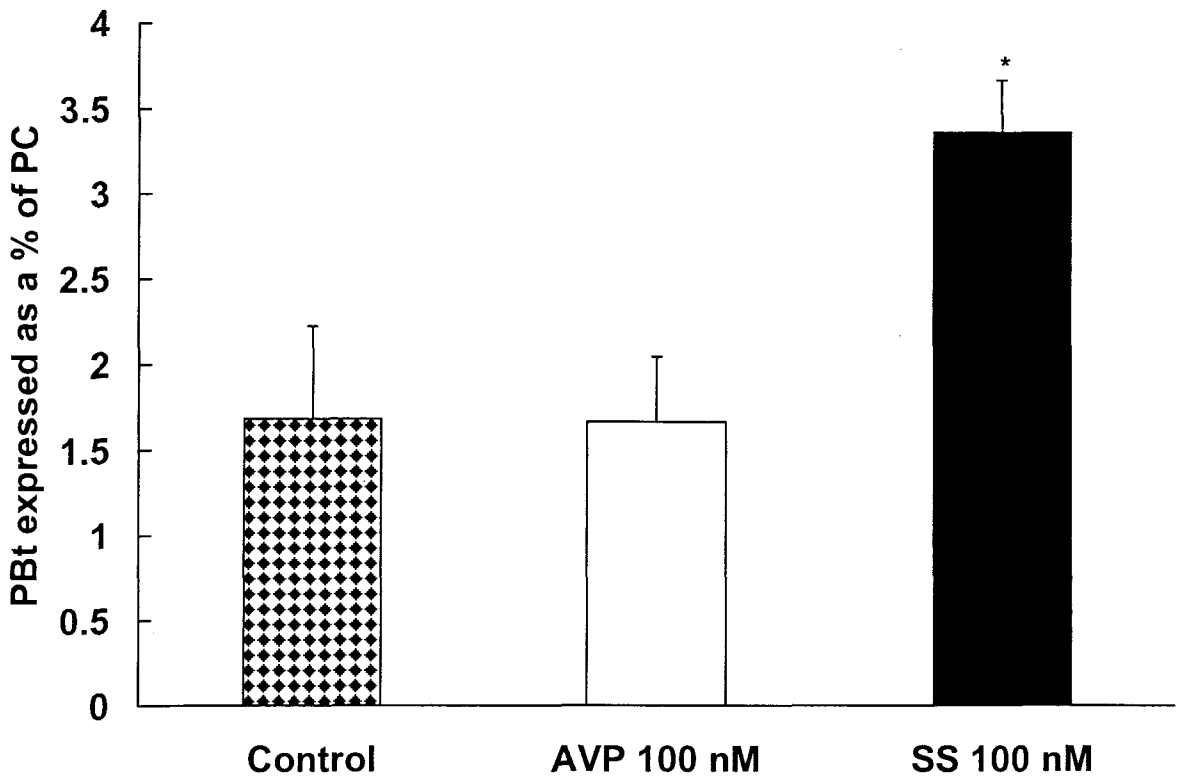
PBt expressed as a % of PC
4
3.5
3
2.5
2
1.5
1
0.5
0
*
Control
AVP 100 nM
SS 100 nM

Fig. 5. A.

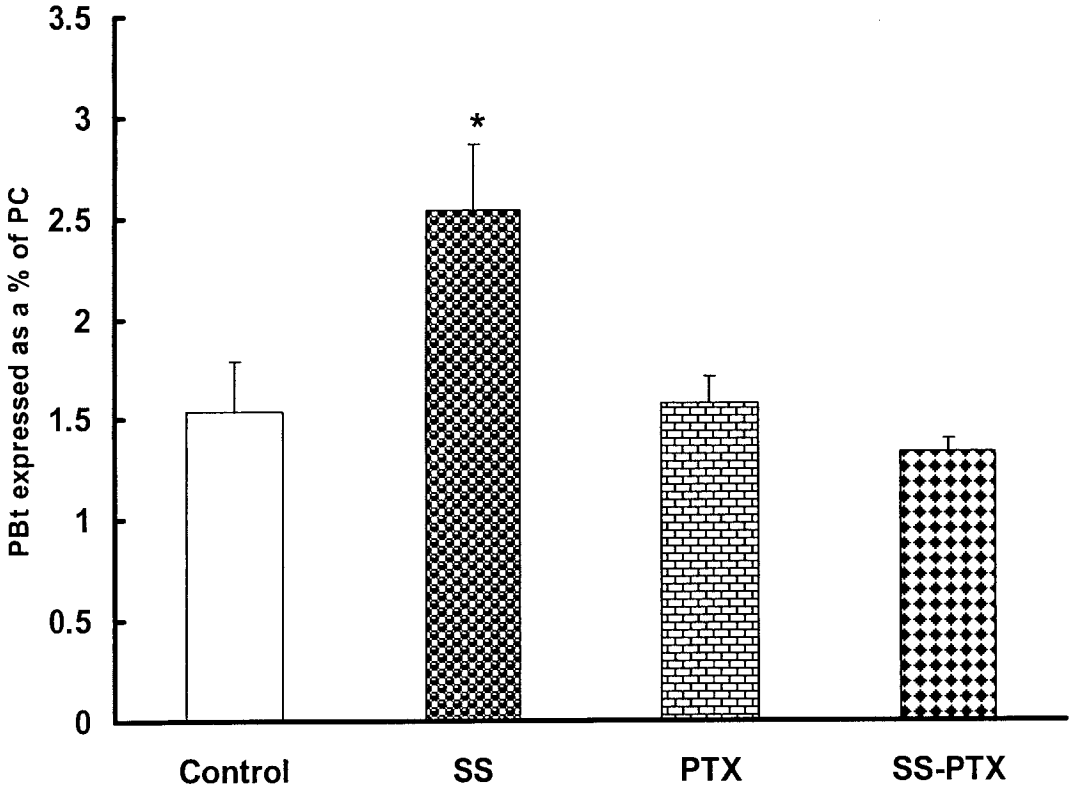

Fig. 5. B.

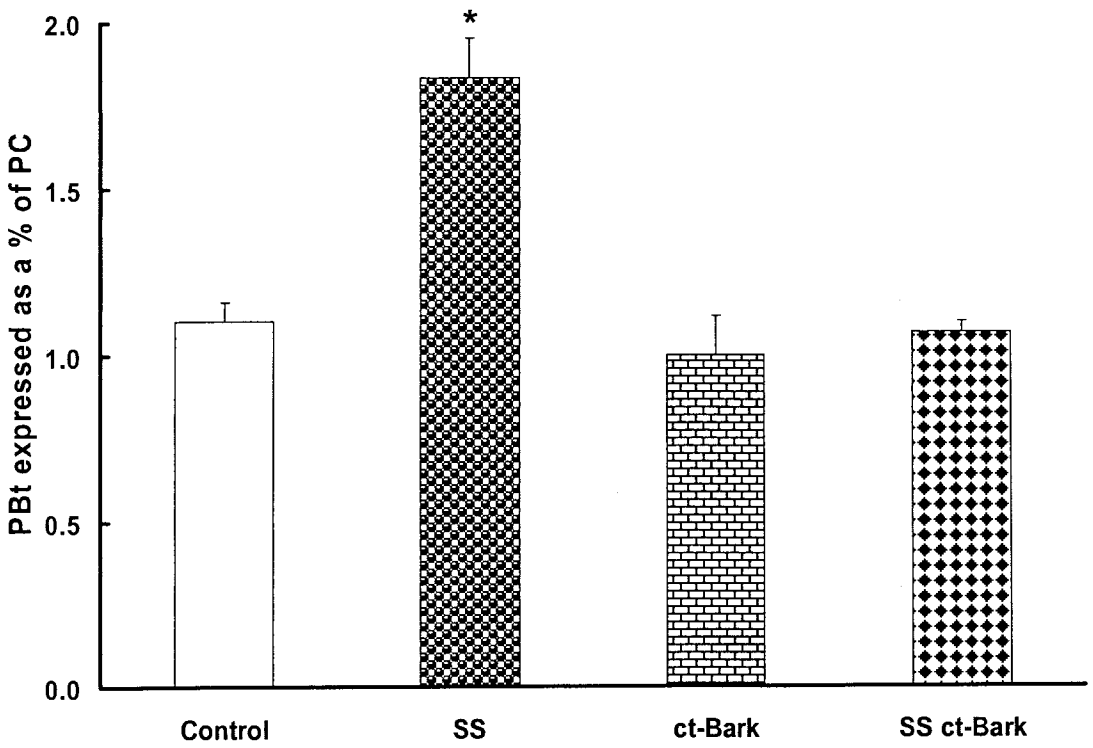
2.0
1.5
1.0
0.5
0.0
PBt expressed as a % of PC
*
Control
SS
ct-Bark
SS ct-Bark

Fig. 5. C.

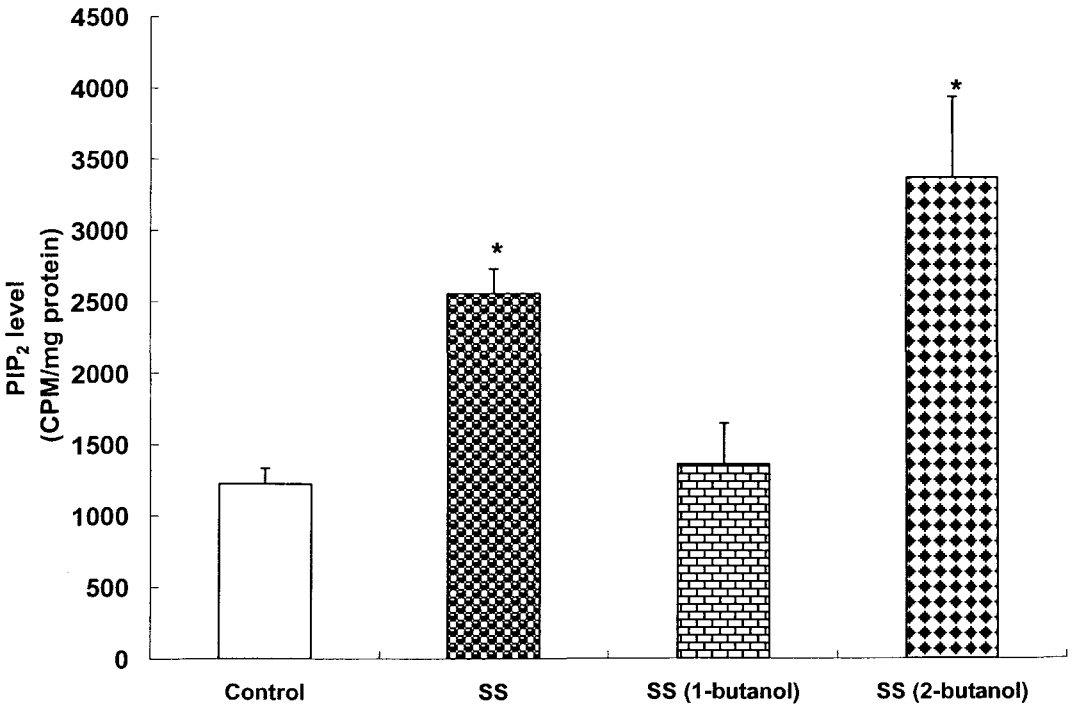
4500
4000
3500
3000
2500
2000
1500
1000
500
0
PIP2 level
(CPM/mg protein)
*
*
Control
SS
SS (1-butanol)
SS (2-butanol)

Fig. 6.

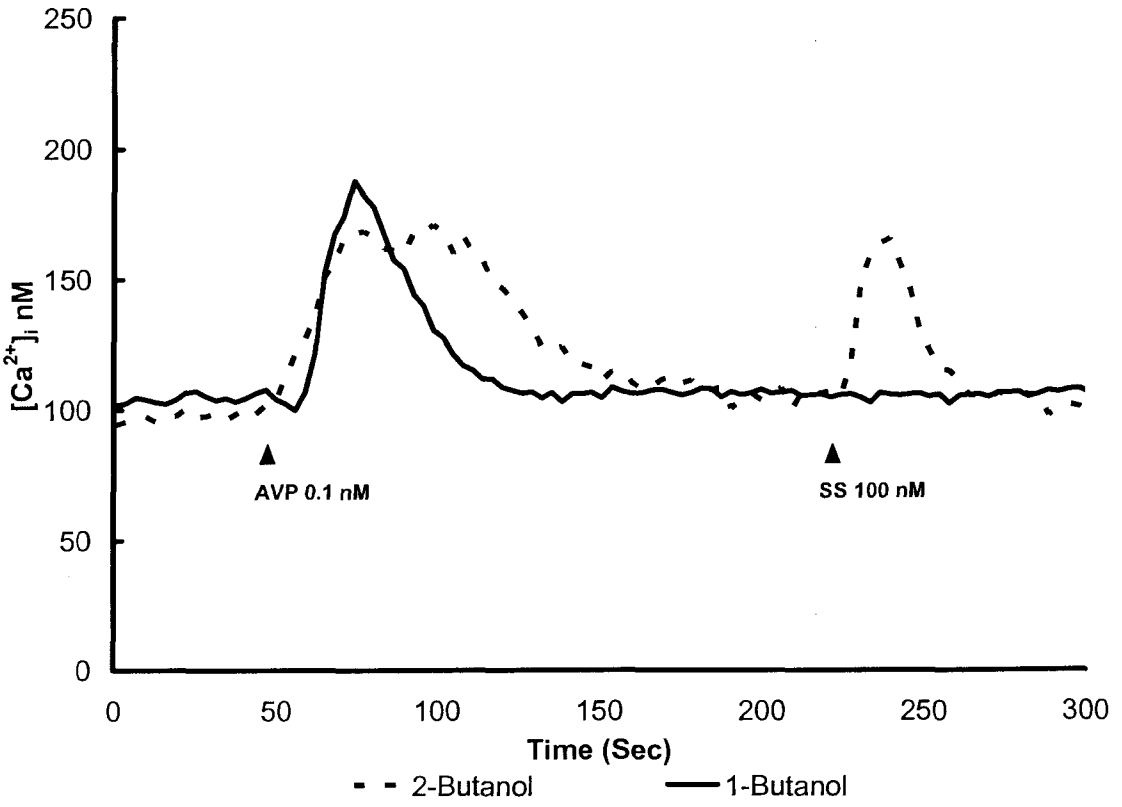
250
200
150
100
50
0
[Ca2+]i nM
AVP 0.1 nM
SS 100 nM
0
50
100
150
200
250
300
Time (Sec)
2-Butanol
1-Butanol

Fig. 7.

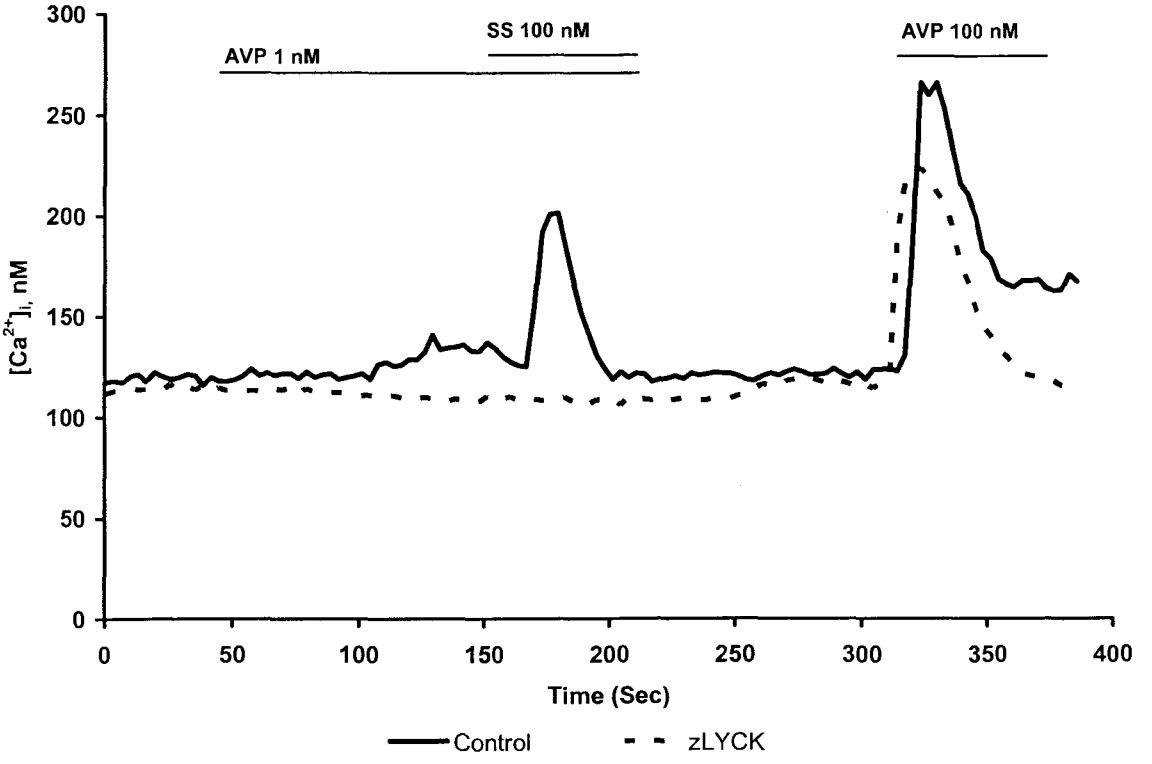
AVP 1 nM
SS 100 nM
AVP 100 nM
[Ca2+]i, nM
300
250
200
150
100
50
0
0
50
100
150
200
250
300
350
400
Time (Sec)
Control
zLYCK

Fig. 8.

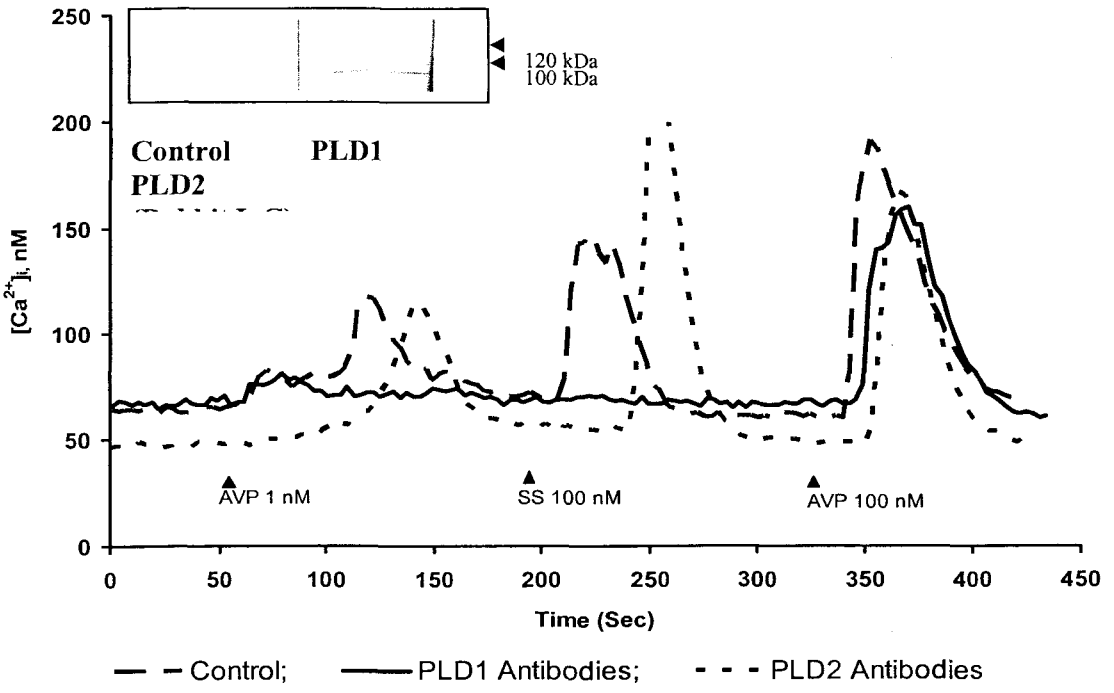
120 kDa
100 kDa
Control
PLD1
PLD2
[Ca2+]i, nM
250
200
150
100
50
0
AVP 1 nM
SS 100 nM
AVP 100 nM
0
50
100
150
200
250
300
350
400
450
Time (Sec)
— – Control;
——PLD1 Antibodies;
- - - PLD2 Antibodies

Fig. 9.

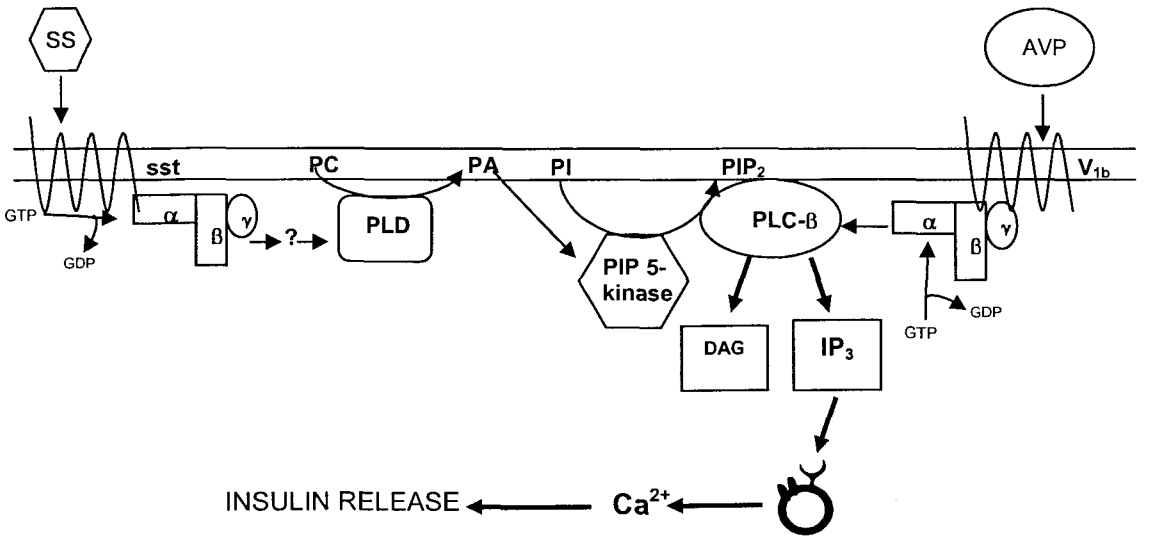
SS
AVP
sst
PC
PA
PI
PIP2
V1b
GTP
GDP
α
β
γ
?
PLD
PIP 5-
kinase
PLC-β
DAG
IP3
INSULIN RELEASE
Ca2+

Fig. 10.

CHAPTER 3: SOMATOSTATIN RECEPTORS SIGNALS THROUGH EFA6-ARF6 TO ACTIVATE PLD IN CLONAL β-CELLS, HIT-T15

Justin A. Grodnitzky[1], Michael J. Kimber[1], Tim A. Day[1], Julie Donaldson[2], Walter H. Hsu[1]

A paper to be submitted to the Journal of Biological Chemistry

[1]*Department of Biomedical Sciences, Iowa State University, Ames, Iowa 50011.*

[2]*Laboratory of Cell Biology, NHLBI, National Institutes of Health, Bethesda, Maryland 20892.*

Somatostatin (SS) is a peptide hormone that is known to inhibit insulin secretion in β-cells. This inhibitory effect occurs when SS activates its $G_{i/o}$-protein coupled receptors (GPCR). Our previous work indicated that βγ-dimer coupled to SS receptors can activate phospholipase D1 (PLD1)(12). The present study was to elucidate the mechanisms underlying SS-induced increase in PLD activity. We demonstrated the presence of ADP-ribosylation factor (Arf)1 and Arf6 in HIT-T15 cells. We also determined that this activation of PLD1 was mediated through Arf6. Over-expression of dominant-negative Arf6 mutant construct Arf6T27N completely abolished the ability of SS to activate PLD and over-expression of wild type-Arf6 further enhanced this PLD activation. Furthermore to determine if the effect of Arf6 on SS-PLD activity was non-specific, over-expression of

dominant-negative Arf1 mutant construct Arf1T31N was used in HIT-T15 β-cells. Finally we cloned and determined the involvement of the Arf6 guanine nucleotide exchange factor (GEF) EFA6A, a GEF previously thought to be found predominantly in the brain, in the activation of PLD1 in HIT-T15 β-cells. Over-expression of dominant negative EFA6A construct completely abolished the ability of SS to activate PLD, while over-expression of a dominant-negative mutant of ARNO, another GEF of Arf6, had no effect on SS-induced increase in PLD. Taken together, these results suggest that SS signals through EFA6 to activate Arf6-PLD cascade.

INTRODUCTION

SS is a peptide hormone that is well known for its ability to inhibit the secretion of growth hormone, glucagon, and insulin (1,2). This peptide hormone is secreted from many tissues in the body including the pancreatic δ-cells (1,2). SS secreted from δ-cells can exert an inhibitory paracrine effect on insulin producing β-cells and is released in two sizes; a 14-amino acid peptide and its prohormone N-terminal extended form, a 28 amino acid peptide (1,2). There are six SS receptors, all of which are G-protein coupled receptors (GPCRs) (3,4), which couple through a $G\alpha_{i/o}$ subunit. SS transduces its physiological signal through many effectors ranging from adenylyl cyclase (5), inwardly rectifying K^+ channels (6), tyrosine phosphatase (7), phospholipase C (8), voltage-dependent Ca^{2+} channel (9), Na^+-H^+ antiporter (10), and MAP kinase (11). We recently reported that SS can activate PLD, causing a subsequent increase in phosphoinsitol 4,5-bisphophate (PIP_2) concentration[12]. This receptor-mediated activation of PLD was attributed to the release of the βγ-dimer coupled to

SS-receptors and occurred in a pertussis toxin-sensitive manner. We also demonstrated that PLD1 was the only detectable isoform present in HIT-T15 cells and the downstream stimulatory effects of SS on PIP_2 levels and intracellular calcium concentration ($[Ca^{2+}]_i$) could be attributed to the activation of PLD1.

PLD is known to hydrolyze phosphatidylcholine (PC) to phosphatidic acid (PA). The conversion of PC to PA regulates many cellular processes such as endocytosis, exocytosis, cell proliferation, and cell migration (13,14,15,16,17). There are two mammalian PLD isoforms, PLD1 and PLD2. These isoforms differ in their subcellular location and regulation. PLD1 is located in the cytosol, Golgi, nucleus, and plasma membrane, while PLD2 seems to be largely found on the plasma membrane (18). Each isoform may exist as one of two a splice variants, i.e., PLD1a, PLD1b, PLD2a, and PLD2b (19,20). There are many potential regulators for PLD, but only a few of them can stimulate both isoforms. The membrane phospholipids, PIP_2, and protein kinase C (PKC) are both known to stimulate both isoforms of PLD (21,22). The family of small G-proteins, ADP-ribosylation factor (Arf) and Rho are potent stimulators of PLD1, but are unable to stimulate the endogenous forms of PLD2 (6,23). PLD activity can also be stimulated by the activation of various GPCRs. A plethora of GPCR agonists have been shown to stimulate PLD activity. Angiotensin II (24), bradykinin (25), carbachol (26), lysophosphatidic acid (27), gonadotropin releasing hormone (28), vasopressin (29), endothelin (30), thyroid-stimulating hormone (31), and prostaglandin $F_{2\alpha}$ (32) are examples of the prevalent nature of GPCR-mediated stimulation of PLD.

GPCRs can activate PLD in many ways. They can stimulate PLD through phospholipase C (PLC)-dependent signaling pathway. PLC catalyzes the conversion of PIP_2 to inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes Ca^{2+} from

endoplasmic reticulum stores. This increase in $[Ca^{2+}]_i$ is known to activate conventional isoforms of PKC. DAG, the other product of PLC activity, can also stimulate PKC through its interaction with the C1-domain of the conventional and novel PKC isoforms. Both PKC isoforms are known to enhance the activity of PLD. Researchers have also demonstrated that GPCR activation of small G-proteins can stimulate PLD (6,11). The signaling mechanisms responsible for small G-proteins regulation of PLD are diverse, but two small G-proteins, Rho and Arf, have been firmly established to regulate PLD activity through GPCRs (26,32,42,46,47,49). Stimulation of Rho by activation of GPCRs is attributed to the associated βγ-dimer binding to the pleckstrin homology (PH)-domain of a guanine nucleotide exchange factor for Rho (33) and its direct interaction with $G\alpha_{12/13}$-subunit (34, 35). These interactions cause GDP to dissociate from Rho, which allows Rho to bind GTP, switching it to the active form. Rho-GTP binds to the C-terminus of PLD1. In HIT-T15 cells, C3 exoenzyme (10 μg/ml), a Rho inhibitor isolated from *Clostridium botulinum* (36), and Ro31-8220 (10 μM), a PKC inhibitor, did not inhibit the SS-induced activation of PLD and its subsequent increase PIP_2 that leads to a synergistic increase in $[Ca^{2+}]_i$ in the presence of AVP (1 nM) (Grodnitzky and Hsu, unpublished data).

In this paper we focused on the role of Arf isoforms in the regulation of SS-induced increase in PLD activity in an insulin secreting cell line, HIT-T15. Arf proteins are small G-proteins that play an important role in vesicle transport, endocytosis, insulin secretion, and actin rearrangement. There are 3 classes of Arf proteins (37). Class I: Arf1, 2, 3 play an important role in Golgi vesicle transport. Class II: the role of Arf4 and Arf5 in cell signaling is not fully elucidated. Class III: Arf6 is located in the plasma membrane and facilitates endocytosis pathways. Arf6 also regulates cortical actin cytoskeleton arrangement and has a

profound effect on cell migration (38), wound healing (39) and phagocytosis (40). Like all small G-proteins, Arf proteins switch from their inactive GDP-bound state to their active GTP-bound state. The intrinsic GTPase activity of Arf proteins is mainly affected by guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). GEFs enhance the dissection rate of GDP from Arfs, which is the rate limiting step in Arf activation. GAPs increase the intrinsic GTPase activity of Arf, switching them to their GDP inactive state.

There are several well-defined hypotheses for GPCR activation of Arf proteins. There is evidence suggesting that Arf1 and Arf6 directly interact with the third intracellular loop of GPCRs and become activated upon stimulation of the receptors (41, 42). Other studies indicate that Arf6 binds to β-arrestin and becomes activated by the stimulation of ARNO, a GEF for Arf1 and Arf6 (14). In this paper we report that in HIT-T15 cells, SS utilizes a novel signaling pathway to regulate PLD activity. We hypothesize that in HIT-T15 cells 1) SS receptor-mediates release of βγ-dimer signals through Arf6 to activate PLD and 2) Arf6 activation by SS receptors is mediate by EFA6A, a low-molecular weight guanine nuclear exchange factor of Arf6, thought to be primarily found in the brain (43).

EXPERIMENTAL PROCEDURES

Cell culture and transfection. HIT-T15 cells were maintained in RPMI 1640 with 10 % FBS and aerated with 5 % CO_2, 95 % air at 37°C. All experiments were performed using cells from passages 80-90. pXS plasmids expressing wild type Arf6 (wtARF6), and dominant negative constructs of Arf6 (ARF6T27N), and Arf1 (ARF1T31N) were generated as described previously (44). All plasmid were transfected into HIT-T15 cells using

Lipofectamine™ (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Protein expression of the FLAG-tagged EFA6 and ARNO constructs and HA-tagged Arf constructs was determined by immunocytochemistry using anti-FLAG and HA monoclonal antibodies with 80-90% of HIT T-15 cells expressing the FLAG and HA epitopes (data not shown).

Western blot anlysis. Rabbit polyclonal antibodies were raised against a COOH-terminal peptide of Arf6, residues 164-175. Antibodies raised against Arf1 were a generous gift supplied by Dr. Richard Kahn of Emory University School of Medicine. Ten μg of whole cell HIT-T15 protein was separated by reduced SDS-PAGE (10%). Protein was transferred to PVDF membranes in transfer buffer (35 mM Tris, 190 mM glycine, 20% methanol). The PVDF membranes were blocked with 5% non-fat dry milk in PBS for 1 h at room temperature. The primary Arf antibodies were diluted 1:50 in wash buffer (0.01% Tween 20 in PBS) and incubated with the PVDF membranes for 1 h at room temperature. The blots were washed 3 times for 10 min each with wash buffer. The secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, Pierce, Rockford, IL) was diluted 1:2,000 in wash buffer and incubated with the PVDF membranes for 1 h at room temperature. The blots were then washed and developed using diaminobenzidine.

Determination of PLD activity. PLD assay was performed using a previously described method [45]. Briefly, HIT-T15 cells were grown in 24-well plates overnight in RPMI 1640 medium with 10% FBS. The medium was then discarded and 500 μl of 3 μCi/ml of [^{3}H]myristic acid was added to each well for 60 min. Cells were then washed twice with

KRB before 400 µl of KRB was added to each well. Treatments were applied 15 s after the addition of 1-butanol (0.5%). The reactions were terminated 30 s after treatments had been administered. Phosphatidylcholine (PC) and phosphatidylbutanol (PBt) were identified by migration with unlabeled standards, visualized by iodine staining and radiographed into 1-cm blocks and were subsequently scraped and radioactivity quantified by liquid scintillation counting.

Cloning of EFA6. Total RNA was extracted from HIT-T15 cells using TRI-REAGENT (Sigma) as described by the manufacture. Poly-A^+ RNA was extracted using Dynabeads mRNA DIRECT Kit (DYNAL) as described by the manufacture. mRNA was then used to synthesize separate 5' and 3' RACE-ready cDNA using the SMART RACE cDNA Amplicfication Kit (BD Biosciences). Rat EFA6A gene specific sense and antisense primers were designed, based on the sequence information obtained using NCBI gene bank (GenBank Number NM 134370). These primers and cDNA templates were used in RACE PCR using SMART RACE cDNA Amplification Kit (BD Biosciences). The components of the reactions were as follows: 5.0 µl universal Primer Mix (10x), 1.0 µl gene specific primer (10 µM), 2.5 µl cDNA, 1.0 µL dNTP(10 µM), 5.0 µl 10x PCR buffer, 34.5 µl PCR grade water and 1.0 µl Advantage 2 polymerase Mix. The reactions were visualized on a 1.2% agarose gel (0.5 µg/ml ethidium bromide). Discrete amplicons were excised from the agarose gel and purified using the MinElute Gel Extraction Kit (QIAGEN). The amplicons were then ligated into pGEM-T Easy Vector (Promega) prior to transformation into *E. coli* competent cells (JM109). Positive clones were cultured overnight and plasmid prepped using Wizard Plus SV Minipreps DNA purification System (Promega). DNA insert was

sequenced by Iowa State University's DNA Squencing facility using standard protocols. Sequences were edited using Vector NTI software (Informax).

Data analyses. All values are presented as mean ± SE. Results were analyzed using ANOVA and individual mean comparisons were made using the Least Significant Difference test. The significance level was set at $p < 0.05$.

RESULTS

Western blot analysis

Both Arf1 and Arf6 have shown to stimulate PLD activity through GPCR signaling. To determine the particular Arf isoform that may play a role in this SS-receptor signaling pathway, Western blot analysis was performed to determine the Arf isoforms present in HIT-T15 cells. Arf1 and Arf6 with sizes of 20 kDa were both detected in HIT-T15 cells (Fig.1). Western blot analysis demonstrated the presence Arf1 and 6 specific antibodies indicating both Arf1 and Arf6 are present in HIT-T15 cells.

Over-expression of Arf6 constructs

Arf6 has been previously shown to stimulate PLD in other systems via a GPCR-mediated pathway (26,42,46,47). To determine the involvement of Arf6 in PLD stimulation in HIT-T15 cells, we over-expressed various Arf6 constructs and analyzed their effect on SS-induced increase in PLD activity. SS (100 nM) increased PLD activity ~2 fold (Fig. 2). Over-expression of Arf6 dominant-negative mutant Arf6T27N completely blocked SS induced increase in PLD (Fig. 2). Furthermore, over-expression of dominant-negative

mutant Arf6T27N did not affect baseline PLD activity (Fig. 2). These results suggest that Arf6 mediates SS-induced activation of PLD.

Over-expression of wild type (wt) Arf6 enhanced SS (100 nM)-induced increase in PLD activity, but did not change baseline PLD activity (Fig. 3). These results suggested that SS stimulation of PLD activity is mediated through an Arf6 dependent pathway.

Over-expression of Arf1 dominant-negative mutant

Arf1 is another well known regulator of PLD1 and has also been demonstrated to stimulate PLD activity via a GPCR-mediated pathway and was present in HIT-T15 cells (Fig 1). To determine the involvement of Arf1 in this signaling pathway, an Arf1 dominant-negative mutant Arf1T31N was over-expressed to determine its effects on SS-mediated increase in PLD activity. SS (100 nM) stimulation of PLD was not affected by the over-expression of Arf1T31N (Fig. 4). Over-expression of Arf1T31N had no effect on baseline PLD activity either (Fig. 4). The above data suggested that Arf1 does not play a significant role in SS-induced increase in PLD activity.

Over-expression of dominant-negative mutants of EFA6 and ARNO

ARNO is one of three low molecular weight GEFs that are known to increase Arf activity and cause a subsequent increase in PLD activity through a GPCR-signaling pathway (46,47,48). To determine the role of ARNO in the SS-induced increase in PLD, over-expression of ARNO dominant-negative mutants (ARNOE156K) was used to determine its role in this system. Over-expressing ARNOE156K had no effect on SS-induced increase on PLD activity, nor was PLD basal activity affected (Fig. 5). These results suggested that

ARNO does not play a significant role in SS-mediated activation of PLD. EFA6, another GEF of Arf6, has not been reported to stimulate PLD through GPCR stimulation. In order to determine a possible role for EFA6, the other main GEF for Arf6, in this signaling pathway, cells were transfected with the EFA6 dominant-negative mutant, EFA6E242K. Over-expressing EFA6E242K abolished the ability of SS to stimulate PLD activity (Fig. 6), but did not change basal PLD activity (Fig. 6). These results suggested that EFA6 may play role in SS-induced activation of PLD and they provide further evidence for the involvement of Arf6 in signaling pathway of SS receptors.

Cloning of EFA6 in HIT-T15 cells

Previous reports regarding the expression pattern of EFA6 indicated that this GEF is found predominantly in the brain (43). To implicate the involvement of EFA6 in our observed signaling pathway, it was imperative to confirm the presence of EFA6 in HIT-T15 cells. PCR primers designed from rat EFA6 sequence were used in conjunction with HIT-T15 cDNA to identify a 1256-base pair fragment with high homology to EFA6. The fragment encodes a 417-amino acid open reading frame that has 97% indentity to rat EFA6 at the amino acid level. Such homology strongly suggests that this fragment constitutes at least part of an endogenous hamster EFA6 molecule expressed in HIT-T15 cells.

DISCUSSION

The above findings strongly support the hypothesis that Arf6 mediates the SS-induced increase in PLD activity in clonal β-cells HIT-T15. The effects of over-expression of Arf6T27N and wt-Arf6 indicate that Arf6 is essential for this pathway. In addition, this

effect does not seem to be an artifact of the over-expression of an Arf protein, because over-expression of Arf1T31N did not have any effect on SS receptor signaling pathway. The ability of the EFA6 dominant-negative mutant, EFA6E246K to block SS-induced increase in PLD activity further implicated the involvement of Arf6. EFA6 is a specific GEF for Arf6 and alteration of this GEF would have a profound effect on Arf6. Over-expressing ARNOE156K, a dominant-negative mutant of a GEF for Arf1 and Arf6, did not alter SS-induced activation of PLD. These findings taken together with EFA6E246K ability to hinder SS activation PLD strongly suggested the involvement of EFA6 in this signaling pathway.

We previously demonstrated that SS was able to increase PLD activity in HIT-T15 cells, leading to a subsequent increase in PIP_2 levels (21). In this paper we report a novel mechanism for PLD stimulation. In our proposed model, the βγ-dimer that couples to SS receptors interacts with EFA6, which is known to increase the nucleotide exchange rate of Arf6. Arf6-GTP would activate PLD1 in HIT-T15 cells. Although our proposed model for GPCR-mediated activation of the Arf6-PLD signaling cascade is considerably similar to other previously reported models, distinct differences between the models are apparent. In one proposed model βγ-dimer directly interact with Arf1 and Arf4 (49,50). In our system, Arf1 did not play a major role in the SS-induced activation of PLD, indicating that the direct interaction of βγ-dimer with Arf1 does not occur in the HIT-T15 cell system for SS-Arf6 signaling.

There have been several reports of GPCR activation of Arf6 occurring due to the ability of Arf6 to directly interact with activated GPCRs. This signaling cascade has been demonstrated in 5-hydroxytryptamine-2a receptors (41), gonadotropin-releasing hormone receptors (42) and M_3 muscarinic receptors (14). These studies show the involvement of

Arf1, Arf3, and Arf6 in the GPCR-mediated activation of PLD. This signaling cascade is dependent on Arf proteins interacting with the NxxPY motif located in the third intracellular loop of these receptors. NPxxY motif is a common motif found in most GPCRs Class A family and is found in SS_2 receptor (SSTR2), the SS receptor present in HIT-T15 cells that mediate the effect of SS in these cells (51). These studies demonstrate that the activation of a GPCR can stimulate the conversion of Arf-GDP to its active Arf-GTP bound state, Arf-GTP can then bind to the NxxPY region of the receptor. The direct binding model does not conflict with the results obtained in this study. The focus of this study was to determine the proteins involved in SS-induced activation of PLD and we did not address the translocation or scaffolding properties of activated Arf6 in this paper. It is possible that the βγ-dimer interacts with EFA6 to switch Arf6 to the GTP bound state. Furthermore, the active Arf6 could then bind to NxxPY motif of an activated SS receptor, which may be essential for PLD activity. Additional studies are required to determine if a direct interaction transpires between SS receptors and Arf6-GTP.

In our proposed model, the mechanism of SS-induced activation of PLD in HIT-T15 cells signals through βγ-dimer (12). We hypothesize that the βγ-dimer coupling to SS receptors interacts with EFA6, which will cause a subsequent stimulation of PLD1 in HIT-T15 cells through Arf6 activation (Fig. 8). This study provides an exciting novel signal mechanism upon which SS can activate PLD. Another well-documented model for GPCR mediated activation of Arf6 involves the stimulation of β-adrenergic receptors and other GPCRs (52,53,54). Activation of these receptors leads to the dissociation of βγ-dimer and subsequent activation of G-protein receptor kinases (GRK). GRK then phosphorylates the receptors, which recruits β-arrestin to the receptor and leads to receptor desensitization

(52,53,54). Upon β-arrestin binding, ARNO scaffolds with the desensitized receptor complex, thereby activating Arf6. The two main differences between the models are the GEFs involved in Arf6 activation and the additional downstream signaling events that occur after the dissociation of βγ-dimer. In our system, EFA6 controls this SS-induced activation of PLD. In the other model ARNO is responsible for Arf6 activation. Surprisingly, in HIT-T15, EFA6 dominated this signaling cascade and ARNO did not significantly contribute to this pathway. These results indicate that in HIT-T15 cells EFA6 is essential for this pathway. However, it is possible that additional signaling events occurring between the βγ-dimer and EFA6. Such events are likely to include the involvement of GRKs and arrestins, as suggested by the alternative signal pathway.

Although this study provides an exciting novel signal mechanism upon which SS can activate PLD1 and cause a subsequent increase in PIP_2, the physiological significance for SS-induced activation of Arf6 and PLD1 is unknown. Arf6-induced increase in PIP_2 levels has been reported in another pancreatic β-cell line. In MIN6 cells, the ability of Arf6 to increase PIP_2 levels was essential to the slow phase of insulin secretion but had no effect on initial fast phase of insulin secretion (55). In addition, PLD1 activation has been shown to be essential for insulin secretion for both glucose and cholinergic receptor-mediated responses (56,57). The action of PLD1 on exocytosis appears to mediate its effect through a distal step of exocytosis, somewhere beyond vesicle recruitment and the readily releasable pool. Both Arf6 and PLD1 activity enhance exocytotic processes in insulin secreting β-cells, so we are perplexed to why an inhibitor hormone like SS would signal through Arf6-PLD1 pathway. Although we do not understand the physiological significance of SS signaling through EFA6 to activate Arf6 and PLD, it does provide a novel signal mechanism for SS.

This study also describes for the first time the ability of a GPCR to interact with EFA6A and the presence of EFA6A in insulin secreting cells. In HIT-T15 cells, a partial sequence of EFA6A was detected. We are consistently unable to characterize the N-terminal third of the putative EFA6A molecule as compared to the rat EFA6A. In addition, Western blot analysis of using antibodies that recognize the N-terminus of EFA6A was unable to detect endogenous EFA6A in HIT-T15 cells (Grodnitzky, unpublished data). The high sequence homology between rat EFA6A and our hamster HIT-T15 EFA6 fragment, indicates the presence of EFA6A in HIT-T15 cells. Other researchers have demonstrated a short form of EFA6A in the small intestine, ovary, and colon in human tissue (58). Taken together with our cloning efforts and Western blot analysis, there is tentative evidence for the existence of this truncated EFA6A isoform in HIT-T15, although further studies will be required to confirm this hypothesis (58).

LEGENDS FOR FIGURES

Fig. 1. Western blot analysis of Arf1 and Arf6 in HIT-T15 cells. 10 μg of the whole cell protein was separated using SDS-PAGE, transferred to PVDF membrane, and blotted with 1:50 Arf1 and 1:50 Arf6 antibodies.

Fig. 2. Effect of Arf6(T27N) expression on SS-induced increase on PLD activity. HIT-T15 cells were transfected 24 h prior to PLD assay. Cells were then pretreated with 0.5% 1-butanol 15 s prior to SS treatment. Experiments were terminated 30 s after SS had been

administered. Values are the mean ± SE (n=4 independent cell preparations). *$p < 0.05$ compared to controls. PBt = phosphatidylbutanol, PC=phosphatidylcholine.

Fig. 3. Effect of wt-Arf6 expression on SS-induced increase on PLD activity. HIT-T15 cells were transfected 24 h prior to PLD assay. Cells were then pretreated with 0.5% 1-butanol 15 s prior to SS treatment. Experiments were terminated 30 s after SS had been administered. Values are the mean ± SE (n=4 independent cell preparations). *$p < 0.05$ compared to controls. PBt = phosphatidylbutanol, PC=phosphatidylcholine.

Fig. 4. Effect of Arf1(T31N) expression on SS-induced increase on PLD activity. HIT-T15 cells were transfected 24 h prior to PLD assay. Cells were then pretreated with 0.5% 1-butanol 15 s prior to SS treatment. Experiments were terminated 30 s after SS had been administered. Values are the mean ± SE (n=4 independent cell preparations). *$p < 0.05$ compared to controls. PBt = phosphatidylbutanol, PC=phosphatidylcholine.

Fig. 5. Effect of ARNO(E27K) expression on SS-induced increase on PLD activity. HIT-T15 cells were transfected 24 h prior to PLD assay. Cells were then pretreated with 0.5% 1-butanol 15 s prior to SS treatment. Experiments were terminated 30 s after SS had been administered. Values are the mean ± SE (n=4 independent cell preparations). *$p < 0.05$ compared to controls. PBt = phosphatidylbutanol, PC=phosphatidylcholine.

Fig. 6. Effect of EFA6A(E242K) expression on SS-induced increase on PLD activity. HIT-T15 cells were transfected 24 h prior to PLD assay. Cells were then pretreated with 0.5% 1-butanol 15 s prior to SS treatment. Experiments were terminated 30 s after SS had been administered. Values are the mean ± SE (n=4 independent cell preparations). *$p < 0.05$ compared to controls. PBt = phosphatidylbutanol, PC=phosphatidylcholine.

Fig. 7. Comparison of amino acid sequence deduced from known rat EFA6A to HIT-T15 cell EFA6a. HIT-T15 cell EFA6 has 97% identity with rat EFA6 at the amino acid level.

Fig. 8. Proposed model of SS-induced increase in PLD activity. SS type 2 receptor (SSTR2) activation releases the βγ-dimer activates EFA6, a GEF for Arf6. This causes Arf6 to release GDP and to bind GTP. The active Arf6 will then bind to and activate PLD. PLD will convert phosphatidylcholine (PC) into phosphatidic acid (PA).

FIGURES

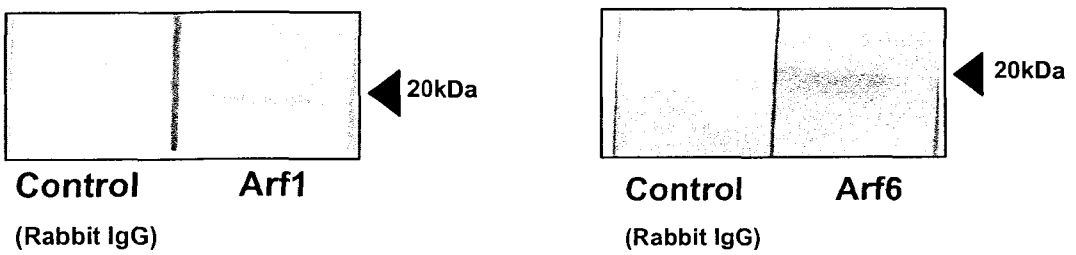

Fig. 1.

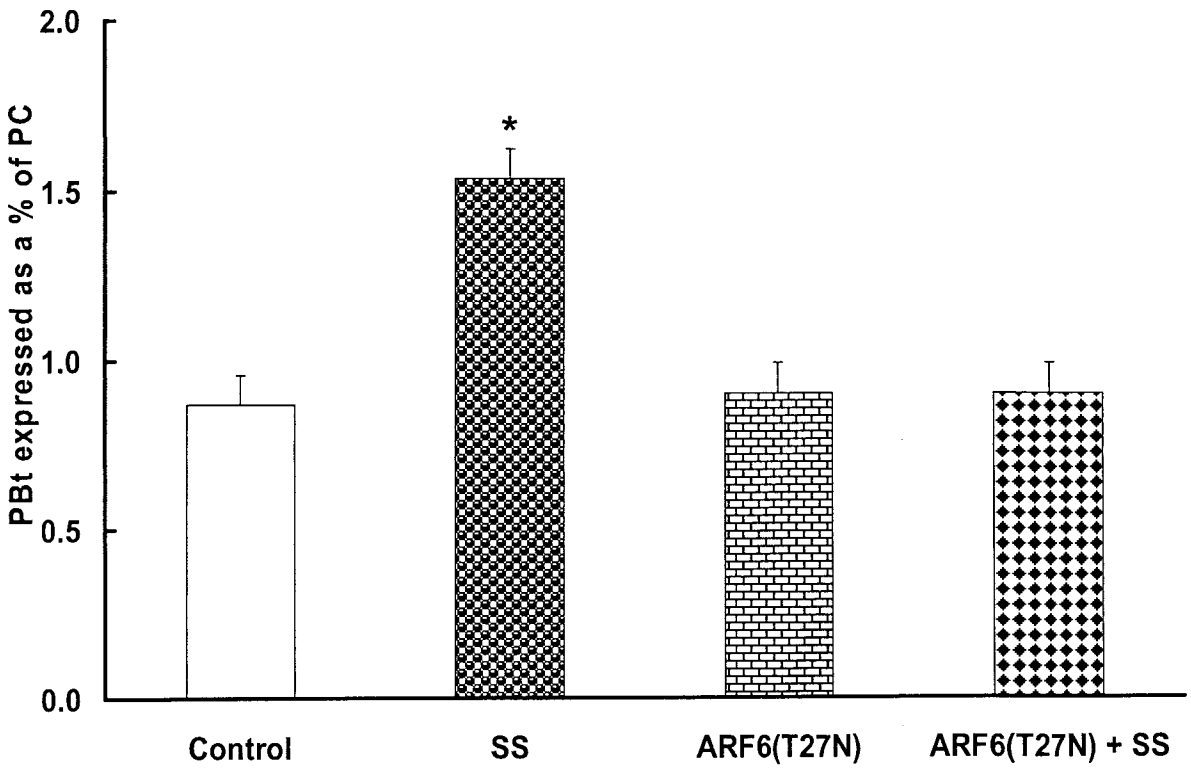
2.0
1.5
1.0
0.5
0.0
PBt expressed as a % of PC
*
Control
SS
ARF6(T27N)
ARF6(T27N) + SS

Fig. 2.

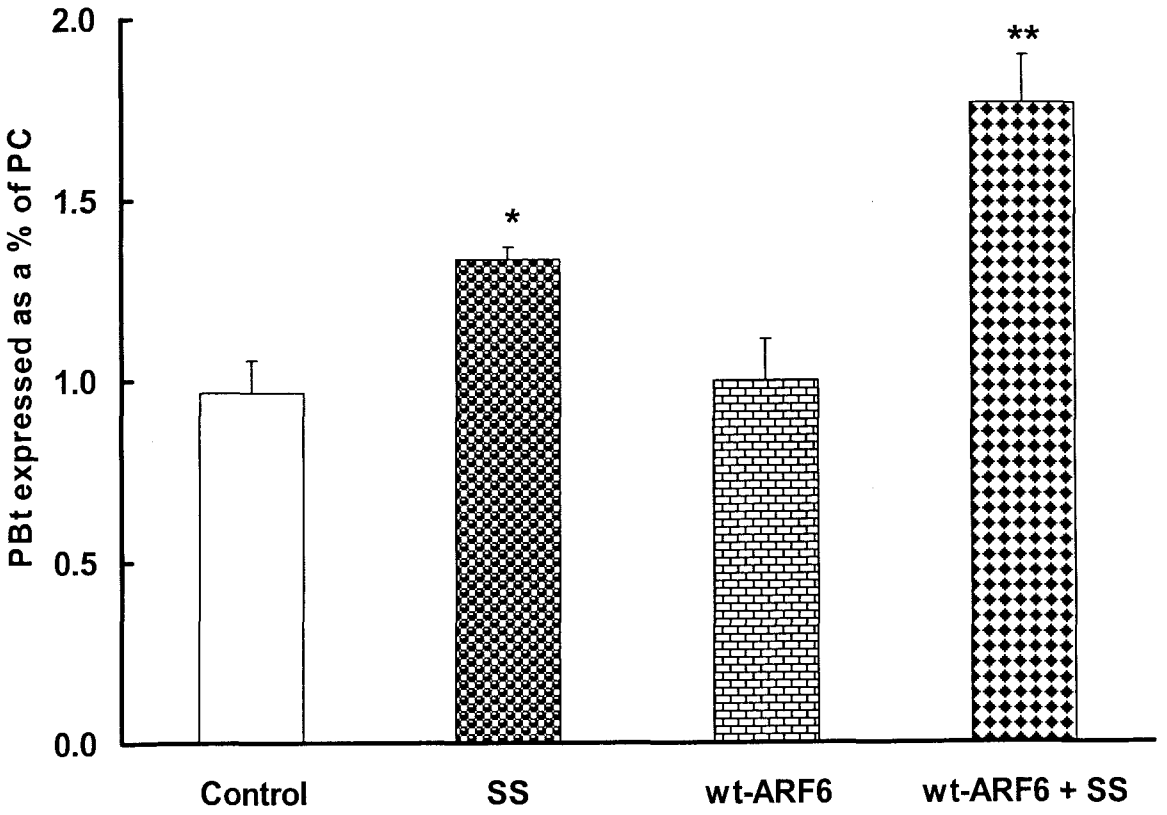
2.0
1.5
1.0
0.5
0.0
PBt expressed as a % of PC
*
**
Control
SS
wt-ARF6
wt-ARF6 + SS

Fig. 3.

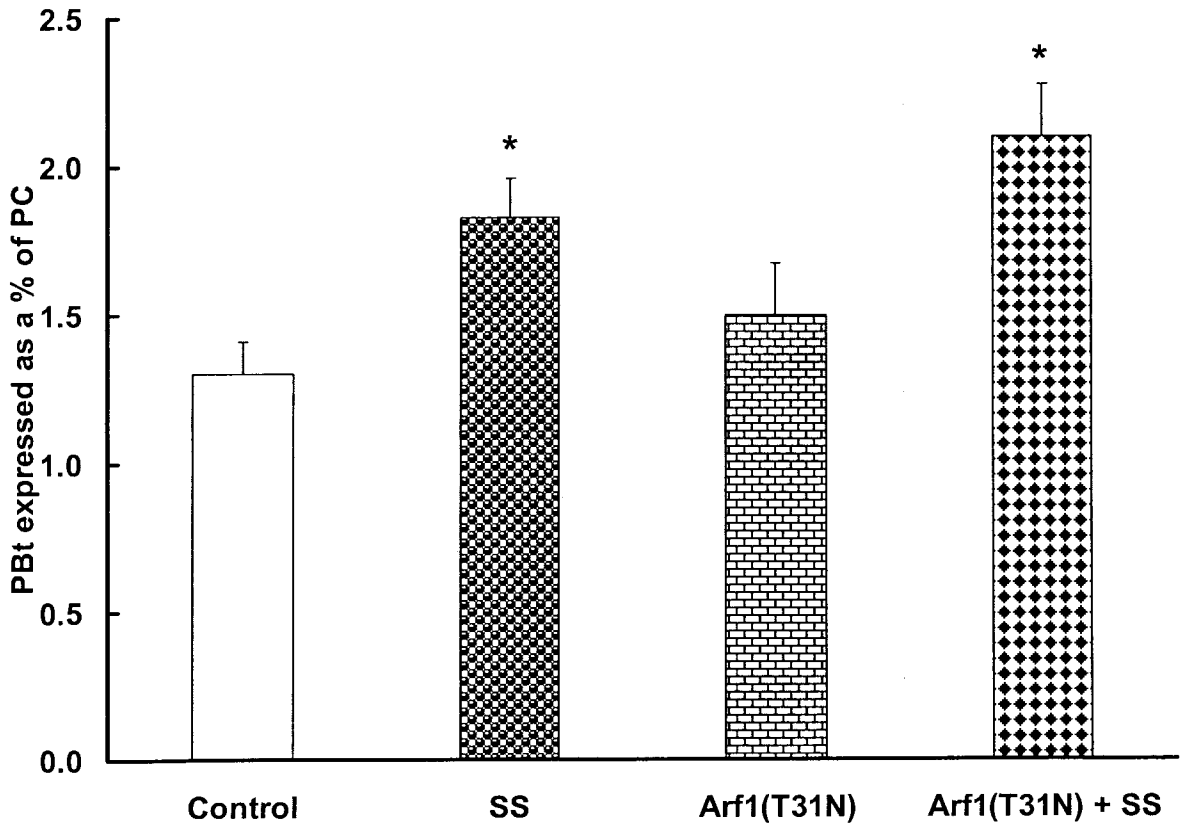
2.5
2.0
1.5
1.0
0.5
0.0
PBt expressed as a % of PC
*
*
Control
SS
Arf1(T31N)
Arf1(T31N) + SS

Fig. 4.

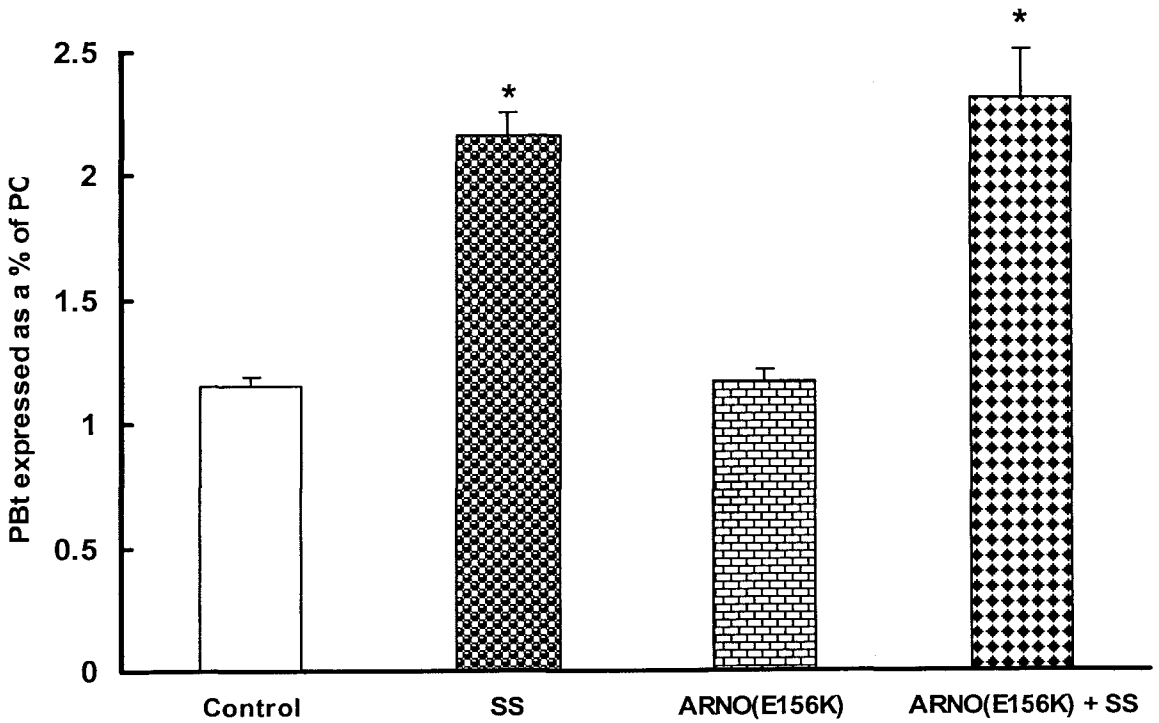
2.5
2
1.5
1
0.5
0
PBt expressed as a % of PC
*
*
Control
SS
ARNO(E156K)
ARNO(E156K) + SS

Fig. 5.

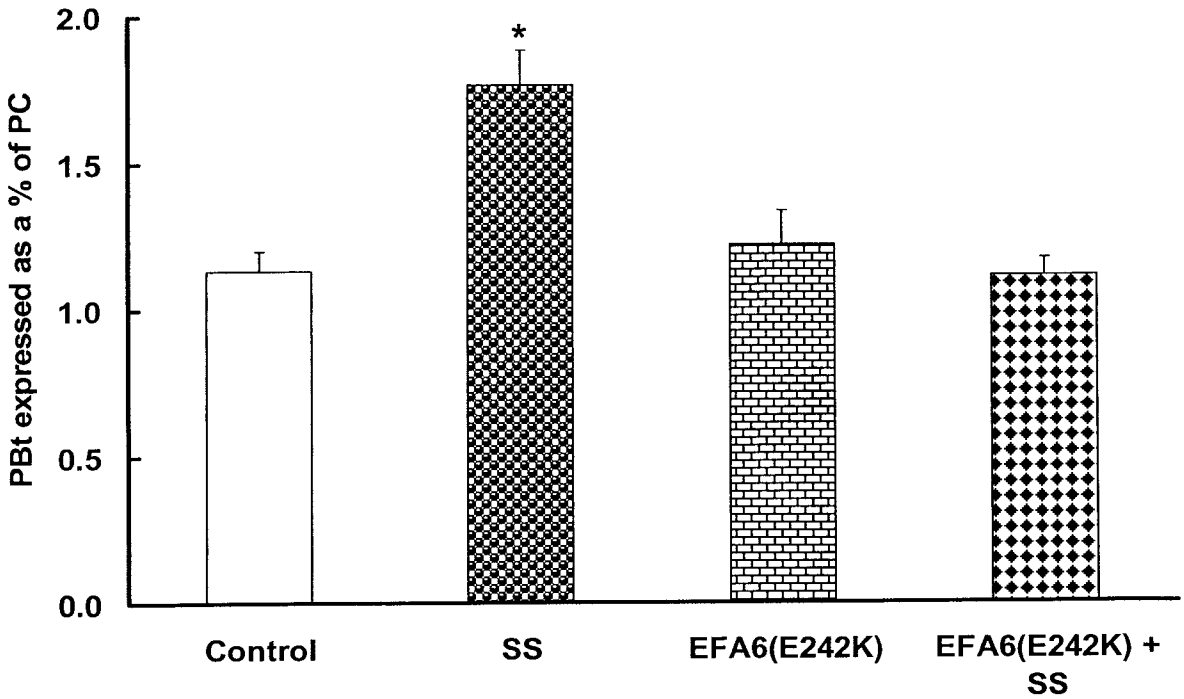
2.0
1.5
1.0
0.5
0.0
PBt expressed as a % of PC
*
Control
SS
EFA6(E242K)
EFA6(E242K) + SS

Fig. 6.


Rat (235) RVFLKELALMGETQERERVLAHFSQRYFQCNPEALSSEDGAHTLTCALMLLNTDLHGHNIGKRMT
HIT-T15 RVFLKELALMGETQERERVLAHFSQRYFQCNPEALPSEDGAHTLTCALMLLNTDLHGHNIGKRMT
Rat (300) CGDFIGNLEGLNDGGDFPRELLKALYSSIKNEKLQWAIDEEELRRSLSELADPNPKVIKRVSGGS
HIT-T15 CGDFIGNLEGLNDGGDFPRELLKALYSSIKNEKLQWAIDEEELRRSLSELADPNPKVIKRVSGGS
Rat (365) GSSSSPFLDLTPEPGAAVYKHGALVRKVHADPDCRKTPRGKRGWKSFHGILKGMILYLQKEEYQP
HIT-T15 GSSSSPFLDLTPEPGAAVYKHGALVRKVHADPDCRKTPRGKRGWKSFHGVLKGMILYLQKEEYQP
Rat (430) GKALSEAELKNAISIHHALATRASDYSKRPHVFYLRTADWRVFLFQAPSLEQMQSWITRINVVAA
HIT-T15 GKALSEAELKNAISIHHALATRASDYSKRPHVFYLRTADWRVFLFQAPSLEQLQSWITRINVVAA
Rat (495) MFSAPPFPAAVSSQKKFSRPLLPSAATRLSQEEQVRTHEAKLKAMASELREHRAAHLGKKARGKE
HIT-T15 MFSAPPFPAAVSSQKKFSRPLLPSAATRLSQEEQVRTHEAKMKAMASELREHRAAHLGKKARGKE
Rat (560) AEEQRQKETYLEFEKSRYGTYAALLRVKMKAASEELDAIEAALAQAGSTEEGCPPPHSSPSLQPN
HIT-T15 ADEQRQKEAYLEFEKSRYGTYAALLRVKMKAASEELDAVEAALAQAGSTEDGCPPPHSSPSLQPN
Rat (625) P--TSQPRAQRPGSEARAGAGSTRPKP
HIT-T15 PNPTSQTRAQRSGSEARAGAGSTRPKP

Fig. 7.

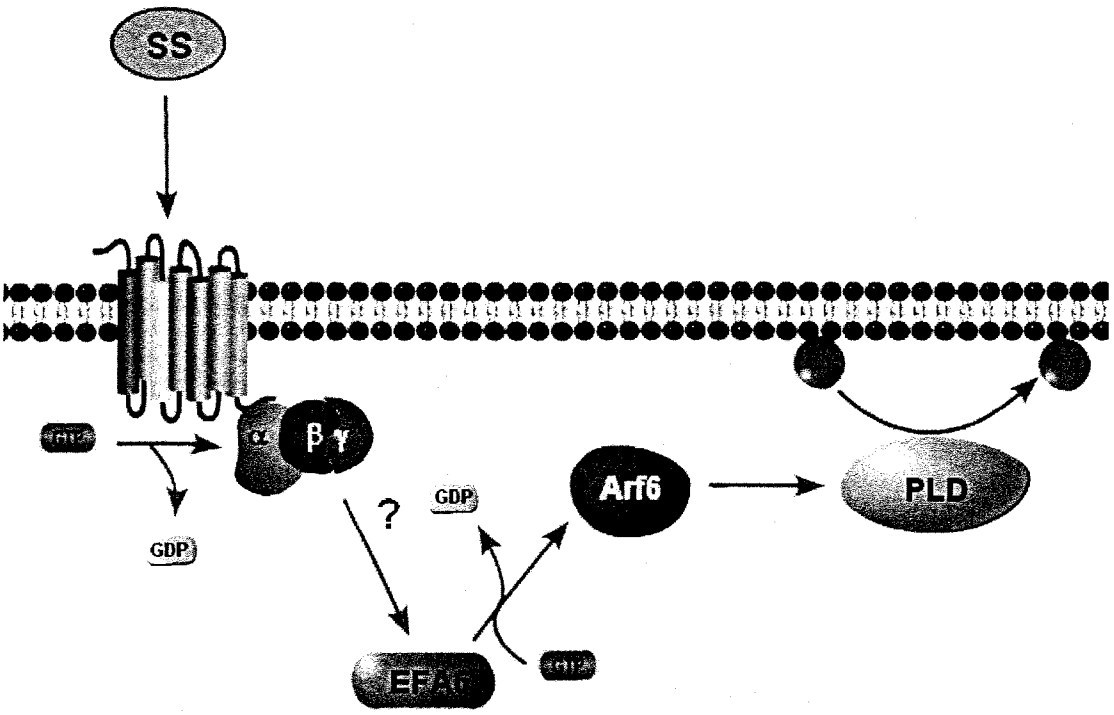

Fig. 8.

REFERENCES

[1] Gangong W. F. Endocrine functions of the pancreas & regulation of carbohydrate metabolism. In review of medical physiology, 20th ed. Appleton and Lange, Norwalk. 2001, pp 322-343.

[2] Patel YC. (1999) Somatostatin and its receptor family. *Front Neuroendocrinol.*, **20**: 157-98.

[3] Reisine T., Bell G. I., 1995. Molecular biology of somatostatin receptors. *Endocr. Rev.*, **16**: 427-42.

[4] Yamada Y., Post S. R., Wang K., Tager H. S,. Bell G. I., Seino S. (1995) Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract and kidney of human. *Proc Natl. Acad. Sci. U.S.A.*, **89**: 251-255.

[5] Hadcock J., Strnad J., Eppler C. (1994) Rat somatostatin receptor type 1 couples to G proteins and inhibition of cyclic AMP accumulation. *Mol. Pharmacol.*, **45**: 410-416.

[6] Karschin A. (1995) Molecular single-cell analysis identifies somatostatin tpe 1 (sst1) receptors to block inwardly rectifying K+ channels in rat brain oligodendrocytes. *Neuroreport*, **7**: 121-124.

[7] Buscail L., Delesque N., Esteve J. P., Saint-Laurent N., Prats H., Clerc P., Robberecht P., Bell G. I., Liebow C., Schally A., Vaysse N., Susini C. (1994) Stimulation of tyrosine phosphatase and inhibition of cell proliferation by somatostatin analogues: mediation by human somatostatin receptor subtypes SSTR1 and SSTR2. *Proc Natl. Acad. Sci. U.S.A.*, **91**: 2315-2319.

[8] Akbar M., Okajima F., Tomura H., Majid M.A., Yamada Y., Sino S., Kondo Y. (1994) Phospholipase C activation and Ca^{2+} mobilization by cloned human somatostatin receptor subtypes 1-5 in transfected COS-7 cells. *FEBS letters,* **348**: 192-196.

[9] Roosterman D., Glassmeier G., Baumeister H., Scherubl H., Meyerhof W.A. (1998) A somatostatin receptor 1 selective ligand inhibits Ca2+ currents in rat insulinoma 104639 cells. *FEBS Letters,* **425**: 137-140.

[10] Hou C., Gilbert R., Barber D. (1994) Subtype-specific signaling mechanisms of somatostatin receptors SSTR1 and SSTR2. *J. Biol. Chem.,* **269**: 10357-10362.

[11] Florio T., Yao H., Carey K., Dillon T., Stork P. (1999) Somatostatin activation of mitogen-activated protein protein kinase via somatostatin receptor 1 (SSTR1). *Mol. Endocrinology,* **13**: 24-37.

[12] Cheng H., Grodnitzky J. A., Yibchok-Anun S., Ding J., Hsu W. H. (2005) Somatostatin increases phospholipase D activity and PIP2 synthesis in clonal β-cells HIT-T15. *Mol. Pharmacol.*. **67**: 2162-2172.

[13] Exton J. H. (1997) New developments in phospholipase D. *J. Biol. Chem.*, **272**: 15579-15582.

[14] Stutchfield J., Cockcroft S. (1993) Correlation between secretion and phospholipase D activation in differentiated HL60 cells. *Biochem. J.*, **293**: 649-655.

[15] Xie M., Jacobs L. S., Dubyak G. R. (1991) Regulation of phospholipase D and primary granule secretion by P2-purinergic and chemotactic peptide-receptor agonists is induced during granulocytic differentiation of HL-60 cells. *J. Clin. Invest.*, **88**: 45-54.

[16] Foster D. A., Lizhong X. 2003. Phospholipase D in cell proliferation and cancer. *Molecular Cancer Research.*, **1**: 789-800.

[17] Cui Y., English D. K., Siddiqui R. A., Heranyiova M., Garcia J. G. (1997) Activation of endothelial cell phospholipase D by migrating neutrophils. *J. Investig. Med.*, **45:** 388-93.

[18] Liscovitch M., Czarny M., Fiucci G., Lavie Y.,Tang X. (1999) Localization and possible functions of phospholipase D isozymes. *Biochim. Biophys. Acta.*, **1439**: 245-263.

[19] Hammond S. M., Jenco J. M., Nakashima S., Cadwaller K., GU Q. M., Cook S., Nozawa Y., Prestwich G. D., Froman M. A., & Morris A. J. (1997) Characterization of two alternately spliced forms of phospholipase D1. *J. Biol. Chem.*, **272**: 3860-3868.

[20] Steed P. M., Clark K. L., Boyar W. C., Lasala D. J. (1998) Characterization of human PLD2 and analysis of PLD isoform splice variants. *FASEB J.*, **12**: 1309-1317.

[21] Hodgkin M. N., Masson M. R., Powner D. J., Saqib K. M., Ponting C. P., Wakelam M. J. O. (2000) Phospholipase D regulation and localization is dependent upon a phosphatidylinositol 4,5-bisphosphate-specific PH domain. *Curr. Biol.*, **10**: 43-46.

[22] Divecha N., Roefs M., Halstead J. R., D'Andrea S., Fernandez-Borga M., Oomen L., Saqib K. M., Wakelam M. L. O, D'Santos C. (2000) Interaction of the type 1a PIPkinase with phospholipase D: a role for the local generation of phosphatidylinositol 4,5-bisphosphate in the regulation of PLD2 activity. *EMBO J.*, **19**: 5440-5449.

[23] Lopez I., Arnold R. S., Lambeth J. D. (1998) Cloning and initial characterization of a human phospholipase D2 (hPLD2). *J. Biol. Chem.*, **273**: 12846-12852.

[24] Bollag W. B., Jung E. M., Calle R. A. (2002) Mechanism of angiotensin II-induced phospholipase D activation in bovine adrenal glomerulosa cells. *Mol. Cell. Endocrinol.*, **192**: 7-16.

[25] Lee S. D., Lee B. D., Kim Y., Suh P. G., Ryu S. H. (2000) Bradykinin activates phospholipase D2 via protein kinase C delta in PC12 cells. *Neurosci. Lett.*, **294**: 130-132.

[26] Mitchell R., Robertson D. N., Holland P. J., Collins D., Lutz E. M., Johnson M. S. (2003) ADP-ribosylation factor-dependent phospholipase D activation by the M_3 muscarinic receptor. *J. Biol. Chem.*, **278**: 33818-33830.

[27] Ha K. S., Yeo E. J., Exton J. H. (1994) Lysophosphatidic acid activation of phosphatidylcholine-hydrolysing phospholipase D and actin polymerization by a pertussis toxin-sensitive mechanism. *Biochem. J.*, **303**: 55-59.

[28] Liscovitch M., Amsterdam A. (1989) Gonadotropin-releasing hormone activates phospholipase D in ovarian granulose cells. Possible role in signal transduction. *J. Biol. Chem.*, **264**: 11762-11767.

[29] Miwa M., Kozawa O., Suzuki A., Watanabe Y., Shinoda J., Oiso Y. (1995) Vasopressin activates phospholipase D through pertussis toxin-insensitive GTP-binding

protein in aortic smooth muscle cells: function of Ca2+/calmodulin. *Biochem. Cell. Biol.*, **73**: 191-199.

[30] Fahimi-Vahid M., Gosau N., Michalek C., Han L., Jakobs K. H., Schmidt M., Roberts N., Avkiran M.,Wieland T. (2002) Distinct signaling pathways mediate cardiomyocyte phospholipase D stimulation by endothelin-1 and thrombin. *J. Mol. Cell. Cardiol.*, **34**: 441-453.

[31] Gupta S., Gomez-Munoz A., Matowe W. C., Brindley D. N., Ginsberg J. (1995) Thyroid-stimulating hormone activates phospholipase D in FRTL-5 thyroid cells via stimulation of protein kinase C. *Endocrinology.*, **136**: 3794-3799.

[32] Kato Y., Banno Y., Dohjima T., Kato N., Watanabe K., Tatematsu N., Nozawa Y. (1997) Involvement of Rho family proteins in prostaglandins F2 alpha-induced phospholipase D activation in the osteoblast-like cell line MC3T3-E1. *Prostaglandins,* **54**: 475-492.

[33] Niu J., Profirovic J., Pan H., Vaiskunaite R., Voyno-Yasenetskaya T. G. (2003) Protein betagamma subunits stimulate p114RhoGEF, a guanine nucleotide exchange factor for RhoA and Rac1: regulation of cell shape and reactive oxygen species production. *Circ. Res.*, **93**: 848-56.

[34] Kozasa T., Jiang X., Hart M. J., Sternweis P. M., Singer W. D., Gilman A. G., Bollag G., Sternweis P. C. (1998) p115 RhoGEF, a GTPase activating protein for $G\alpha_{12}$ and $G\alpha_{13}$. *Science,* ***280***: 2109–2111.

[35] Hart M. J., Jiang X., Kozasa T., Roscoe W., Singer W. D., Gilman A. G., Sternweis P. C., Bollag G. (1998) Direct stimulation of the guanine nucleotide exchange activity of p115 RhoGEF by $G\alpha_{13}$. *Science,* **280**: 2112–14.

[36] Jalink K., van Corven E. J., Hengeveld T., Mori N., Narumiya S., Moolenaar W. H. (1994) Inhibition of lysophosphatidate-and thrombin-induced neurite retraction and neuronal cell rounding by ADP ribosylation of the small GTP-binding protein Rho. *J. Cell Biol.* **126**: 801-810

[37] Nie Z., Hirsch D. S., Randazzo P. A. (2003) Arf and its many interactors. *Curr. Opin. Cell Biol.,* **15**: 396-404.

[38] Palacios F., Price L., Schweitzer J., Collard J. G., D'Souza-Schorey C. (2001) An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. *EMBO J.,* **20**: 4973-4986.

[39] Santy L. C., Casanova J. E. (2001) Activation of ARF6 by ARNO stimulates epithelial cell migration through downstream activation of both Rac1 and phospholipase D. *J Cell Biol.*, **154**: 599-610.

[40] Niedergang F., Colucci-Guyon E., Dubois T., Raposo G., Chavrier P. (2003) ADP ribosylation factor 6 is activated and controls membrane delivery during phagocytosis in macrophages. *J. Cell Biol.*, **161**: 1143-1150.

[41] Robertson D. N., Johnson M. S., Moggach L. O., Holland P. J., Lutz E. M., Mitchell R. (2003) Selective interaction of ARF1 with the carboxy-terminal tail domain of the 5-HT2A receptor. *Mol. Pharmacol.*, **64**:1239-1250.

[42] Mitchell R., McCulloch D., Lutz E., Johnson M., MacKenzie C., Fennell M., Fink G., Zhou W., Sealfon S. C. (1998) Rhodopsin-family receptors associated with small G-proteins to activate phospholipase D. Nature, **392**: 411-414.

[43] Derrien V., Coullaul C., Franco M., Martineau S., Montcouttier P., Houlgatte R., and Chavrier P. (2002) A conserved C-terminal domain of EFA6-family ARF6-guanine nucleotide exchange factors induces lengthening of microvilli-like membrane protusions. *J. Cell Sci.*, **115**: 2867-2879.

[44] Radhakrishna H., Donaldson J. G. (1997) ADP-ribosylation factor 6 regulates a novel plasma membrane recycling pathway. *J Cell Biol.*, **139**: 49-61.

[45] O'Launaigh N., Pardo R., Fensome A., Allen-Baume V., Jones D., Holt M. R., and Cockcroft S. (2002) Continual production of phosphatidic acid by phospholipase D is essential for antigen-stimulated membrane ruffling in cultured mast cells. *Mol. Biol. Cell,* **13:** 3730-3746.

[46] Claing A., Chen W., Miller W. E., Vitale N., Moss J., Premont R. T., Lefkowitz R. J. (2001) β-Arrestin-mediated ADP-ribosylation factor 6 activation and β2-adrenergic receptor endocytosis. *J. Biol. Chem.*, **276**: 42509-42513.

[47] Mukherjee S., Gurevich V. V., Jones J. C. R., Casanova J. E., Frank S. R., Maizels E. T., Bader M. F., Kahn R. A., Palczewski K., Aktories K., Hunzicker-Dunn M. (2000) The ADP ribosylation factor nucleotide exchange factor ARNO promotes β-arrestin release necessary for luteinizing hormone/choriogonadotropin receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **97:** 5901-5906.

[48] Andresen B. T., Jackson E. K., Romero G. G. (2001) Angiotensin II signaling to phospholipase D in renal microvascular smooth muscle cells in SHR. *Hypertension*, **37**: 635-639.

[49] Colombo M. I., Inglese J., D'Souza-Schorey C., Beron W., Stahl P. D. (1995) Heterotrimeric G proteins interact with the small GTPase ARF. Possibilities for the regulation of veicular traffic. *J. Biol. Chem.*, **270**: 24564-24571.

[50] Franco M., Paris S., Chabre M. (1995) The small G-protein $ARF1_{GDP}$ binds to the $G_t\beta\gamma$ subunit of transducin but not to $G_t\alpha_{GDP}$-$G_t\beta\gamma$. *FEBS Letters*, **362**: 286-290.

[51] Cheng H., Yibchok-anun S., Coy D. H., Hsu W. H. (2002) SSTR2 mediates the somatostatin-induced increase in intracellular (Ca2+) concentration and insulin secretion in the presence of arginine vasopressin in clonal beta-cell HIT-T15. *Life Sci.*, **71**: 927-936.

[52] Claing A., Chen W., Miller W. E., Vitale N., Moss J., Premont R. T., Lefkowitz R. J. (2001) β-Arrestin-mediated ADP-ribosylation factor 6 activation and β2-adrenergic receptor endocytosis. *J. Biol. Chem.*, **276**: 42509-42513.

[53] Mukherjee S., Gurevich V. V., Jones J. C. R., Casanova J. E., Frank S. R., Maizels E. T., Bader M. F., Kahn R. A., Palczewski K., Aktories K., Hunzicker-Dunn M. (2000) The ADP ribosylation factor nucleotide exchange factor ARNO promotes β-arrestin release necessary for luteinizing hormone/choriogonadotropin receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **97:** 5901-5906.

[54] Andresen B. T., Jackson E. K., Romero G. G. (2001) Angiotensin II signaling to phospholipase D in renal microvascular smooth muscle cells in SHR. *Hypertension*. **37**: 635-639.

[55] Lawrence J. T., Birnbaum M. J. (2003) ADP-ribosylation factor 6 regulates insulin secretion through plasma membrane phosphatidylinositol 4,5-bisphosphate. *Proc. Natl. Acad. Sci. U.S.A.,* **100**: 13320-13325.

[56] Hughes W. E., Elgundi Z., Huang P., Frohman M. A., Biden T. J. (2004) Phospholipase D1 regulates secretagogue-stimulated insulin release in pancreatic beta-cells. *J Biol Chem.,* **279**: 27534-27541.

[57] Metz S.A., Dunlop M. (1990) Stimulation of insulin release by phospholipase D. A potential role for endogenous phosphatidic acid in pancreatic islet function. *Biochem. J.,* **270**: 427-435.

[58] Derrien V., Couillault C., Franco M., Martineau S., Montcourrier P., Houlgatte R., Chavrier P. (2002) A conserved C-terminal domain of EFA6-family Arf6-guanine nucleotide exchange factors induces lengthening of microvilli-like membrane protrusions. *J. Cell Sci.*, **115**: 2867-2879.

CHAPTER 4: GENERAL DISCUSSION

In this dissertation we demonstrate a novel signaling pathway within pancreatic β-cells. This project started because of a previous report in HIT-T15 cells that somatostatin (SS) increased $[Ca^{2+}]_i$ and transiently stimulated insulin release in the presence of AVP[1]. We have shown that after SS treatment, phosphosinositol 4-phosphosate (PIP) levels decreased but phosphoinositol 4,5-bisphosphate (PIP_2) levels increased, suggesting that SS increases PIP_2 synthesis. Our findings are further supported by the administration of PIP_2 into the cell, in which PIP_2 alone failed to increase $[Ca^{2+}]_i$, but PIP_2 in the presence of a small concentration of AVP (1 nM) increased $[Ca^{2+}]_i$. Because PIP_2 and PIP failed to increase $[Ca^{2+}]_i$ in the absence of AVP (1 nM), we believe that these phospholipids alone are unable to elicit any $[Ca^{2+}]_i$ response in HIT-T15 cells. The failure of microinjected PIP (50 amol) to induce changes in $[Ca^{2+}]_i$ in the presence of AVP (1 nM) demonstrated that the microinjection of PIP_2 in the presence of AVP, which caused an increase in $[Ca^{2+}]_i$, was due to the use of PIP_2 as a substrate for low-grade PLC activation and not an artifact of the system. Thus, we have demonstrated that SS can increase PIP_2 synthesis, which in turn provides extra substrate for preactivated PLC-β by AVP to generate high levels of IP_3. Without a preactivated PLC-β, SS failed to increase IP_3 levels (Fig. 3) and $[Ca^{2+}]_i$[1].

In this dissertation we also demonstrated for the first time that SS can increase PLD activity. This effect of SS was PTX-sensitive, and was blocked by expression of ct-βARK, which is known to sequester βγ-dimer, suggesting that $G_{i/o}$-βγ dimer mediates this effect. We further demonstrated that SS-induced increase in PLD activity resulted in an increase in PIP_2 formation. PLD is the enzyme that catalyzes the formation of phosphatidic acid (PA), which

formation. PLD is the enzyme that catalyzes the formation of phosphatidic acid (PA), which in turn activates PIP 5-kinase to increase PIP_2 synthesis[2]. In this dissertation, we demonstrated that microinjection of PA in the presence of AVP increased $[Ca^{2+}]_i$, which has been used as an indicator for IP_3 levels increase. In addition, PLD inhibitors, 1-butanol and zLYCK, and antibody against PLD1 all blocked SS-induced $[Ca^{2+}]_i$ increase in the presence of AVP. 1-Butanol further abolished SS-induced PIP_2 increase. Therefore, the present findings strongly supported the hypothesis that SS increases PA synthesis, which in turn activates PIP-5 kinase, the enzyme catalyzing the formation of PIP_2. In this system SS was able to increase PIP_2 levels, providing extra substrate for PLC-β.

We have further demonstrated (Chapter 3) the involvement of mediators upstream from PLD activation. The activation of SS receptors that are coupled to $G_{i/o}$, leads to an increase in PIP_2 synthesis through Gβγ activation of PLD. In Chapter 3 we demonstrated that Arf6 mediates the SS-induced increase in PLD activity. The effects of over-expression of Arf6T27N (a dominant-negative Arf6 mutant) and wt-Arf6 (Arf6 wild type) indicate that Arf6 is essential for SS-mediated PLD activation. In addition, this effect does not seem to be an artifact of the over-expression of an Arf protein, because over-expression of Arf1T31N (a dominant-negative Arf1 mutant) did not have any effect on SS-mediated pathway. The ability of the EFA6 dominant-negative mutant, EFA6E246K, to block SS-induced increase in PLD activity further implicates the involvement of Arf6. EFA6 is a specific GEF for Arf6 and disruption of this GEF would have a profound effect on Arf6. Over-expressing ARNOE156K, a dominant-negative mutant of a GEF for Arf1 and Arf6, did not affect SS activation of PLD. This result taken together with the ability of EFA6E246K to hinder SS activation of PLD strongly suggests the involvement of EFA6 in this pathway.

This dissertation demonstrates a novel receptor-mediated mechanism for PIP_2 increase in insulin secreting β-cells. Binding of SS to its $G_{i/o}$ coupled receptor activates the small G-protein, Arf6, with modulation by the GEF, EFA6. Activated Arf6 stimulates PLD1 to hydrolyze phosphatidylcholine (PC) to PA, which in turn will stimulate PIP-5 kinase to PIP, forming PIP_2 (Fig. 1). PIP_2 is the substrate for generation of IP_3 and subsequently the liberation of Ca^{2+} from ER (Fig.1). Although this pathway represents a novel signal transduction mechanism for SS in insulin secreting β-cells, the physiological significance of this pathway is unknown. The crosstalk effect in the presences of a $G\alpha_q$-coupled receptor agonist is paradoxical to SS's role as an inhibitor. The action of SS to function as a secretagogue for insulin release led critics of our system to believe that the cross-talk effect was just an artifact of our cell culture system and not a realistic effect for the in vivo activity of SS. Other than our previous findings, there are no reports that SS can increase insulin release from cell culture models or isolated pancreatic islets. In normal physiology, cells are exposed to many hormones and the cross-talk effects of these hormones are almost always overlooked. Conclusions are then drawn about the physiological effects of a hormone in the absence of other signaling molecules. Although it is important to evaluate the effect of the hormone in isolation, it should not be forgotten that cells are surrounded by many signaling molecules that may affect its response to that hormone. In the HIT-T15 cell system, our results were dismissed by some of our critics because we showed a stimulator effects of SS in the presences of a $G\alpha_q$-coupled agonist, which goes against the normal paradigm of the inhibitory nature of SS. Interestingly, SS receptor 5 (sstr5) knockout mice have lower plasma insulin levels when compared to the controls although their β-cells contained more insulin and secreted more insulin from their isolated

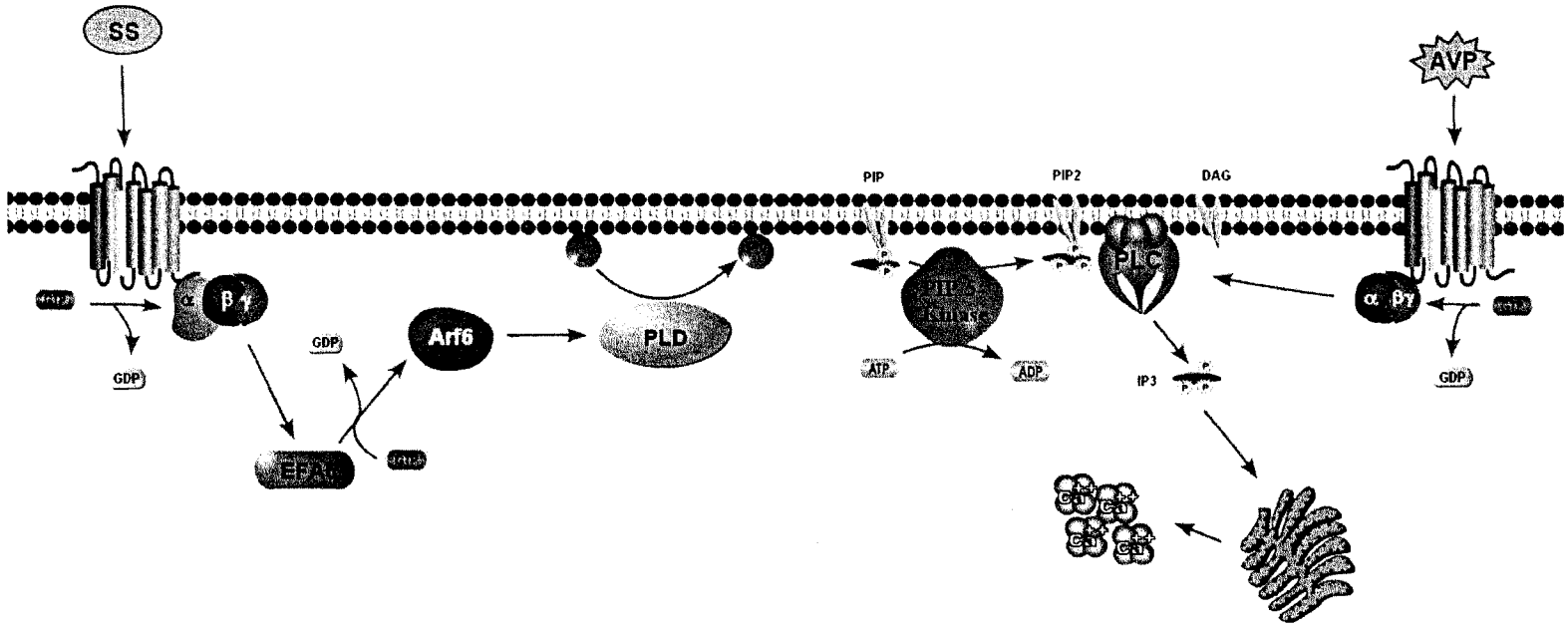

Figure 1. Proposed model of SS-induced increase in PIP_2 and cross-talk with AVP. SS receptors activation releases the bg-dimer and directly activates EFA6, a GEF for Arf6. This causes Arf6 to release GDP and to bind GTP. The active Arf6 will then bind to and activate PLD. PLD will convert phosphatidylcholine (PC) into phosphatidic acid (PA). PA, a known activator of PIP 5-kinase will activate this enzyme synthesizing more PIP_2, providing extra substrate for preactivated PLC-β by AVP. This increase in DAG and IP_3 levels and $[Ca^{2+}]_i$ leads to insulin release.

pancreatic islets[3]. These results are also paradoxical in nature. If mice missing sstr5 had low insulin release and their β-cells contain more insulin than the control mice, one would expect to find higher plasma insulin levels because there would be no inhibitory control in these SS-knockout mice. However, the opposite results were found. These data suggested that β-cells from isolated islets are inhibited by SS but input from other tissue or hormone enables SS to have a stimulatory effect on insulin secretion. Although we cannot determine if SS paradoxical effect plays a true role in normal physiology, the above study suggests that the HIT-T15 cell model is at least plausible. It also clearly exemplifies the complex nature of system physiology and the effects of multiple interactions on cells.

The paradoxical cross-talk, which occurs in HIT-T15 cells is an interesting signaling event that may not be the only consequence of SS-mediated increase in PIP_2 levels. PIP_2 can regulate a wide range of cellular processes, including exocytosis[4], clathrin-mediated endocytosis[5], actin rearrangement[6], vesicle docking[7], opening of G-protein-gated inwardly rectifying K^+ channels (GIRK) [8], K_{ATP} channels[9], membrane ruffling and trafficking[10]. Because of the diverse role of PIP_2 in cellular processes, it is uncertain what impact SS-induced increase in PLD activity and its subsequent increase in PIP_2 levels have on normal β-cell physiology. However, SS has been shown to regulate many of the same effectors as PIP_2. Out of the copious number of signaling events that PIP_2 can elicit, the ones that may play an important role in SS-mediated signaling in β-cells are GIRK activation and receptor desensitization and sequestration and activation of GIRK. The inhibitory effect of SS has been shown to act independently of its receptor coupled α-subunit. It is interesting to note that opening of GIRK is enhanced in the presence of both βγ-dimer and PIP_2. In our system

SS-induced increase in PIP_2 levels is dependent on βγ-dimer function. It is also possible that activation of GIRK to produce the classical inhibitory effect of SS is regulated by SS ability to increase Arf6, PLD and PIP_2 levels.

The SS-induced stimulation of the PLD pathway through Arf6 that causes a subsequent increase in PIP_2 signaling may elicit other effects such as receptor desensitization and sequestration. Arf6 activation has been demonstrated to be essential to receptor desensitization and sequestration of receptors[11]. PIP_2 has also been shown to be critical for endocytotic pathways[12]. It is likely the ability of SS to increase activation of Arf6, PLD and PIP_2 levels will impact on to receptor desensitization and sequestration. The crosstalk effect produced by SS maybe just a coincidental effect of SS's ability to increase PIP_2 for alternative functions.

We have demonstrated that the βγ-dimer coupled to SS receptors causes EFA6 to activate Arf6, which stimulates PLD1 activity, producing a subsequent increase in PIP_2. However, the nature of the interaction between βγ-dimer and EFA6 is unknown. βγ-dimer could interact directly with EFA6 or indirectly affect EFA6. EFA6 is a 71-kDa protein that acts as a GEF and catalyzes the exchange of Arf6-GDP to Arf6-GTP, its active state.

The Sec 7 domain of EFA6 and all other GEFs of Arf enhance nucleotide exchange rate in Arfs[13]. Sec7 and PH domain is tandem to each other in EFA6. Phosphatidylinositide binding site of EFA6 is a PH domain, which is the eleventh most common domain found in humans[14]. A PH domain within proteins is responsible for translocating proteins to membranes due to their high affinity to PIP_2 and PIP_3. The PH domain is also an effective binding site for βγ-dimer. GRKII is preferentially activated by specific βγ-dimer combinations upon their binding to GRKII PH domain[15, 16, 17]. PLC-β is also activated by βγ-

dimer binding to their PH-domain located on their C-terminus. GEFs in general are attractive effectors for βγ-dimer because of this PH domain. The PH domain of the GEF, ARNO, can act as an electrostatic switch to regulate nucleotide exchange of Arf1, indicating that the PH domain in ARNO is critical for activating Arf1,[18] which could extend to all Arf GEFs. For other small G-proteins, Rho, Ras, and Rac, GEFs are known to be stimulated by βγ-dimer by directly binding to their PH-domain[19, 20]. This line of logic may transverse to Arfs and their GEFs.

Another possible mechanism for βγ-dimer-EFA6 mediated activation of Arf6 is exemplified by the stimulation of β-adrenergic receptors and other GPCRs, which leads to βγ-dimer dissociation and activation of G-protein receptor kinases (GRK). GRK then phosphorylates the receptors, which recruits β-arrestin to the receptor and leads to receptor desensitization[21, 22, 23]. Upon β-arrestin binding, ARNO scaffolds with the desensitization-receptor complex activating Arf6[22]. The main differences between the models are the GEFs involved in Arf6 activation. These data suggest that the βγ-dimer that couples to SS receptors could interact with GRK before interacting with EFA6. It will be exciting to elucidate how βγ-dimer activates EFA6. Although we have not investigated the role of small G-protein cross-talk, it will be interesting if this crosstalk plays a role in this system. Small G-proteins coordinate their activity to synergize downstream effectors. Arf and Rho families of small G-proteins can cooperatively enhance the activity of PLD[24], cell motility[25], and Golgi function[26]. It is interesting to note that EFA6A and Rac, a member of the Rho family, can increase membrane ruffling and actin rearrangement in TRVb-1 cells[27]. Deletion of EFA6A C-terminus completed inhibited membrane ruffling, suggesting that Rac may bind to the

proline-rich and coiled-coil motif found on EFA6A C-terminus. In HIT-T15 cells, EFA6A enhances PLD activity. Another documented small G-protein that coordinates its activity with the Arf family on PLD activity is Ral. RalA and Arf6 can synergistically activate PLD1[28]. Further experiments will be needed to determine the role of other small G-proteins on PLD1 activity in our system.

We demonstrated a novel signaling mechanism and cellular responses for SS signaling in β-cells and a multitude of new signaling effectors that were not previously associated with SS activity. This novel mechanism has us very excited to determine the physiological significance of SS-induced increase in PLD activity and PIP_2 levels in β-cells and to determine the complete signaling cascade responsible for these cellular responses. In the future, I would like to pursue these research questions to help elucidate this novel signaling pathway.

REFERENCE

[1] Cheng H, Yibchok-anun S, Park S-C, and Hsu WH (2002) Somatostatin-induced paradoxical increase in intracellular Ca^{2+} concentration and insulin release in the presence of arginine vasopressin in clonal HIT-T15 β-cells. *Biochem. J.*, **364**:33-39.

[2] Hawkins PT, Jackson TR, and Stephens LR (1992) Platelet-derived growth factor stimulates synthesis of PtdIns(3,4,5)P3 by activating a PtdIns(4,5)P2 3-OH kinase. *Nature,* **358:**157-159.

[3] Strowski M. Z., Kohler M., Chen H. Y., Trumbauer M. E., Li Z., Szalkoski D., Gopal-Truter S., Fisher J. K., Schaeffer J. M., Blake A. D., Zhang B. B., Wilkinson H. A. (2003) Somatostatin receptor subtype 5 regulates insulin secretion and glucose homeostasis. *Molecular Endocrine*, **17**: 93-106.

[4] Cremona O and De Camilli P (2001) Phosphoinositides in membrane traffic at the synapse. *J. Cell Sci.*, **114:**1041-1052.

[5] Gillooly DJ and Stenmark H (2001) Cell biology. A lipid oils endocytosis machine. *Science,* **291:**993-994.

[6] Tolias KF, Hartwig JH, Ishihara H, Shibasaki Y, Cantley LC, and Carpenter CL (2000) Type Iα phosphatidylinositol-4-phosphate 5-kinase mediates Rac-dependent actin assembly. *Curr. Biol.*, **10:**153-156.

[7] Brown FD, Rozelle AL, Yin HL, Balla T, and Donaldson JG (2001) Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. *J. Cell Biol.*, **154:**1007-1017.

[8] Zhang H, He C, Yan X, Mirshahi T, and Logothetis DE (1999) Activation of inwardly rectifying K^+ channels by distinct PtdIns(4,5)P2 interactions. *Nature Cell. Biol.*, **1:**183-188.

[9] Baukrowitz T, Schulte U, Oliver D, Herlitze S, Krauter T, Tucker SJ, Ruppersberg JP, and Fakler, B (1998) PIP_2 and PIP as determinants for ATP inhibition of K_{ATP} channels. *Science,* **282:**1141-1144.

[10] Honda A, Nogami M, Yokozeki T, Yamazaki M, Nakamura H, Watanabe H, Kawamoto K, Nakayama K, Morris AJ, Frohman, MA, et al. (1999) Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. *Cell,* **99:**521-532.

[11] Houndolo T., Boulay PL, Claing A. 2005. G protein-coupled receptor endocytosis in ADP-ribosylation factor 6-depleted cells. *J. Biol. Chem.*, **280**: 5598-5604.

[12] De Camilli P., Emr S. D., McPherson P. S., Novick P. 1996 Phosphoinositides as regulators in membrane traffic. Science, 271: 1533-1538.

[13] Jackson CL, Casanova JE. 2000. Turning on ARF: the Sec7 family of guanine-nucleotide-exchange factors. *Trends Cell Biol.*, **10**: 60-67.

[14] Cullen PJ, Cozier CE, Banting G, Mellor H. 2001. Modular phosphoinositide-binding domains--their role in signalling and membrane trafficking. *Curr. Biol.,* **11**: R882-893.

[15] Pitcher JA, Inglese J, Higgins JB. 1992. Role of βγ subunits of G proteins in targeting the β-adrenergic receptor kinase to membrane-bound receptors. *Science,* **257**: 1264-1267.

[16] Muller S, Hekman M, Lohse MJ. 1997. Specific enhancement of β-adrenergic receptor kinase activity by defined G-protein β and γ subunits. *Proc. Natl. Sci. U.S.A.*, **90**: 10439-10443.

[17] Clapham DE, Neer EJ. 1997. G protein βγ subunits. *Annu. Rev. Pharmacol. Toxicol.,* **37**:167-203.

[18] Santy LC, Frank SR, Hatfield JC, Casanova JE. 1999. Regulation of ARNO nucleotide exchange by a PH domain electrostatic switch. *Curr. Biol.,* **9**: 1173-1176.

[19] Haga K, Haga T. 1992. Activation by G protein βγ subunits of agonist-or light-dependent phosphorylation of muscarinic acetylcholine receptors and rhodopsin. *J. Biol. Chem.,* **267**: 2222-2227.

[20] Niu J, Profirovie J, Haiyum Pan, Vaiskunaite R, Tatyana Voyno-Yasenetskaya. 2003. G protein βγ subunits stimulate p114RhoGEF, a guanine nucleotide exchange factor for RhoA and Rac1: regulation of cell shape and reactive oxygen species production. *Cir. Res.,* **31**: 848-856.

[21] Claing A., Chen W., Miller W.E., Vitale N., Moss J., Premont R.T., Lefkowitz R.J. 2001. β-Arrestin-mediated ADP-ribosylation factor 6 activation and β2-adrenergic receptor endocytosis. *J. Biol. Chem.*, **276**: 42509-42513.

[22] Mukherjee S, Gurevich VV, Jones JCR, Casanova JE, Frank SR, Maizels ET, Bader MF, Kahn RA, Palczewski K, Aktories K, Hunzicker-Dunn M. 2000. The ADP ribosylation factor nucleotide exchange factor ARNO promotes β-arrestin release necessary for luteinizing hormone/choriogonadotropin receptor. *Proc. Natl. Acad. Sci. U.S.A.*, **97:** 5901-5906.

[23] Andresen BT, Jackson EK, Romero GG. 2001.Angiotensin II signaling to phospholipase D in renal microvascular smooth muscle cells in SHR. *Hypertension.*, **37**: 635-639.

[24] Fensome A., Whatmore J., Morgan C., Jones D., Cockroft S. (1998) ADP-ribosylation factor and Rho proteins mediate fMLP-dependent activation of phospholipase D in human neutrophils. *J. Biol. Chem.*, **273**: 13157-13164.

[25] Zhang Q., Calafat J., Janssen H., Greenberg S. (1999) ARF6 is required for growth factor- and rac- mediated membrane ruffling in macrophages at a stage distal to rac membrane targeting. *Mol. Cell. Biol.*, **19**: 8158-8168.

[26] Wu W. J., Erickson J. W., Lin R., Cerione R. A. (2000) The g-subunit of the coatmer complex binds Cdc42 mediate transformation. *Nature,* **405**: 800-804.

[27]Franco M., Peters P. J., Boretto J., Donselaar E. V., Neri A., D'Souza-Schorey C., Chavrier P. (1999) EFA6, a sec7 domain-containing exchange factor for ARF6, coordinates membrane recycling and actin. *EMBO J.,* **18**: 1480-1491.

[28] Xu L., Frankel P., Jackson D., Rotunda T., Boshans R. L., D'Souza-Schorey C., Foster D. A. (2003) Elevated phospholipase D activity in H-Ras- but not K-Ras-transformed cells by the synergistic action of RalA and Arf6. *Mol. Cell. Biol.,* **23**: 645-654.