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Mechanisms of arginine vasopressin-induced insulin secretion in RINm5F cells

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Mechanisms of arginine vasopressin-induced insulin secretion in RINm5F cells

by

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A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of the

Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Veterinary Physiology and Pharmacology
Major: Physiology

Approved:

In Charge of Major Work

For the Major Department

For the Graduate College

Iowa State University
Ames, Iowa

1996

UMI Number: 9620968

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Ann Arbor, MI 48103

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LIST OF ABBREVIATIONS

AC	adenylyl cyclase
ACA	N-(p-amylcinnamoyl)anthranilic acid
AVP	arginine vasopressin
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CD	cluster differentiation
CIF	Ca^{2+} influx factor
Da	dalton
DAG	diacylglycerol
EGTA	[ethylenebis (oxyethylenenitrilo)] tetraacetic acid
ER	endoplasmic reticulum
Fura-2AM	fura-2 acetoxymethyl ester
GLP	glucagon-like polypeptide
G protein	guanine nucleotide-binding protein
HEPES	4-(2-hydroxyethyl0-1-piperazineethanesulfonic acid
HIT	hamster insulin-secreting tumor
I_{CRAC}	Ca^{2+} release-activated Ca^{2+} current
I_{DC}	depletion of internal Ca^{2+} stores-activated Ca^{2+} current
$I_{Ca.IP3}$	IP_3-activated Ca^{2+} current
$I_{Ca.IP4}$	IP_4-activated Ca^{2+} current

$I_{Ca.ATP}$	ATP-activated Ca^{2+} current
$I_{Ca.Ca}$	Ca^{2+}-activated Ca^{2+} current
IP_3	inositol 1,4,5-trisphosphate
KRB	Krebs-Ringer bicarbonate buffer
OAG	1-oleoyl-2-acetyl-sn-glycerol
PA	phosphatidic acid
PC	phosphatidylcholine
PI3 kinase	phosphatidylinositol 3 kinase
PIP_2	phosphatidylinositide 4,5-bisphosphate
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
PPH	PA phosphohydrolase
PPI	phosphatidylinositide phosphate
PS	phosphatidylserine
RIA	radioimmunoassay
RIN	rat insulinoma
ROC	receptor-operated Ca^{2+} channel
TCR	T cell receptor
VDCC	voltage-dependent Ca^{2+} channel

WMN   wortmannin

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

CHAPTER I GENERAL INTRODUCTION

Dissertation Organization

This dissertation contains three research papers preceded by a general introduction, and followed by a general discussion, a summary and a list of references cited in the general introduction and discussion. The general introduction includes research objectives and background information including literature review. Chapter II represents a paper that has been published in the *Journal of Pharmacology and Experimental Therapeutics*, and Chapters III and IV correspond to manuscripts to be submitted for publication in the *Life Science* and *Journal of Pharmacology and Experimental Therapeutics*, respectively.

This dissertation contains most of the experimental results obtained by the author during the course of his graduate study under the supervision of his major professor, Dr. Walter H. Hsu.

Research Objective

Insulin is an important hormone in the regulation of carbohydrate metabolism. The pancreatic beta-cells, which secrete insulin, help the body convert ingested food into energy to be used by the organism. Insulin increases the storage

of glucose, fatty acid and amino acids by way of anabolic pathways. The physiological role of insulin is well known. However, the regulation of insulin secretion is not fully understood. Insulin deficiency in humans and animals, which is called diabetes, is a common and serious pathologic condition. Insulin deficiency commonly causes keto-acidosis and coma, but other complications associated with prolong hyperglycemia may also develop in long-standing diabetes. One of the factors which causes a deficiency of insulin secretion is a genetic modification during fetal development resulting in a loss of beta-cell generative capacity (Swenne, 1992). However, the intracellular regulation of insulin secretion and in the pancreatic beta-cells is still not well characterized (Ashcraft, 1994). Therefore, it is clinically and scientifically important to investigate the intracellular mechanisms of insulin secretion.

A number of studies have shown that changes in the intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, play a central role in the regulation of insulin secretion in pancreatic beta-cells (Ashcraft, 1994). Insulin secretion is triggered by a rise of $[Ca^{2+}]_i$ that results from the opening of Ca^{2+} channels on the beta-cell plasma membrane and/or release of Ca^{2+} from intracellular Ca^{2+} stores. However, activation of the effectors in the downstream of signal transduction such as phospholipases and protein kinases may regulate the changes in $[Ca^{2+}]_i$ and the secretion process.

Therefore, the purpose of this research is to characterize the events

following signal transduction in the pancreatic beta-cells by using arginine

vasopressin as a stimulator. To study vasopressin receptor-mediated signal

transduction, we applied specific inhibitors of downstream effectors to pinpoint the

role of each effector in insulin secretion. In addition, the interaction of effectors

and second messegers were investigated.

Background and Literature Review

This section provides background information for the studies presented in

this dissertation : (1) AVP-induced multiple signal transduction pathways in

pancreatic beta-cells; (2) Involvement of phospholipases in AVP-induced actions;

(3) Involvement of protein kinase C in AVP-elicited action in pancreatic beta-cells.

The organization of pancreatic islets

The islets of Langerhans are ovoid, approximately 76 x 175 μm collections

of cells scattered throughout the pancreas. The distribution of islets is more

plentiful in the tail than in the body or head of the pancreas. They make up 1-2%

of the weight of the pancreas. In humans, there are 1-2 million islets in each

pancreas. Each islet has a copious blood supply; and the blood from the islets, like

that from the gastrointestinal tract drains into the hepatic portal vein. The

endocrine pancreatic cells are distributed within the islet along fenestrated

capillaries.

The cells of the islet have been divided into several types based on their different staining properties and morphology. There are at least 4 distinct cell types: A, B, D, and F cells. The A cells secrete glucagon, the B cells (also called beta-cells) secrete insulin, the D cells secrete somatostatin, and the F cells secrete pancreatic polypeptide. The B cells, located in the center of the islets are the most common and make up 60-70% of the cells in the islet. The A cells make up 20% of the total and the D and F cells are less common. The A, D, and F cells surround the centrally located B cells. The organization of islet cells depends on cell adhesion molecules, which exist on the surface of endocrine cells. They share specific connections with each other and create a complex and heterogeneous micro-organ.

Structure and biosynthesis of insulin

Insulin is a polypeptide containing two chains of amino acids linked by disulfide bridges. There are minor differences (2 to 4 % of residues) in the amino acid composition of molecule from species to species. The differences are generally not sufficient to affect the biologic activity of a particular insulin in heterologous species but are sufficient to make the heterogenous insulin antigenic.

Insulin is synthesized in the endoplasmic reticulum (ER) of beta-cells. The initial polypeptide, preproinsulin, encoded by insulin mRNA translocates across the

membrane of rough ER. During translocation, the signal sequence is cleaved by a

signal peptidase which transforms preproinsulin to proinsulin, the precursor of

insulin. The proinsulin molecule begins to fold and undergoes a rapid formation of

disulfide bonds to gain its native structure. Proinsulin is then transported to the cis

region of the Golgi apparatus for further processing and packing (Fig. 1).

During the intracellular transport of proinsulin from ER to secretory

granules, the 31-residue connecting peptide fragment (C-peptide) is cleaved to

produce insulin. Both C-peptide and insulin are stored in the secretory granules.

The secretory granules move to the plasma membrane by a process involving

microtubules, their membranes fuse with the plasma membrane of the cell,

expelling insulin to the exterior by exocytosis.

Regulation of insulin secretion

Insulin secretion is regulated by a complex of many signals, the most important

of which is the concentration of glucose bathing the beta-cell. In addition, a

number of neurotransmitters and islet peptides can also exert paracrine effects on

insulin secretion (Gerich et al., 1976; Prentki and Matschinsky, 1987).

Glucose-induced insulin secretion is mediated by an increase in the intracellular

ATP concentration. ATP causes closure of ATP-sensitive potassium channels

located in the plasma membrane which depolarizes the membrane. Membrane

depolarization induces the opening of voltage-dependent calcium channels and

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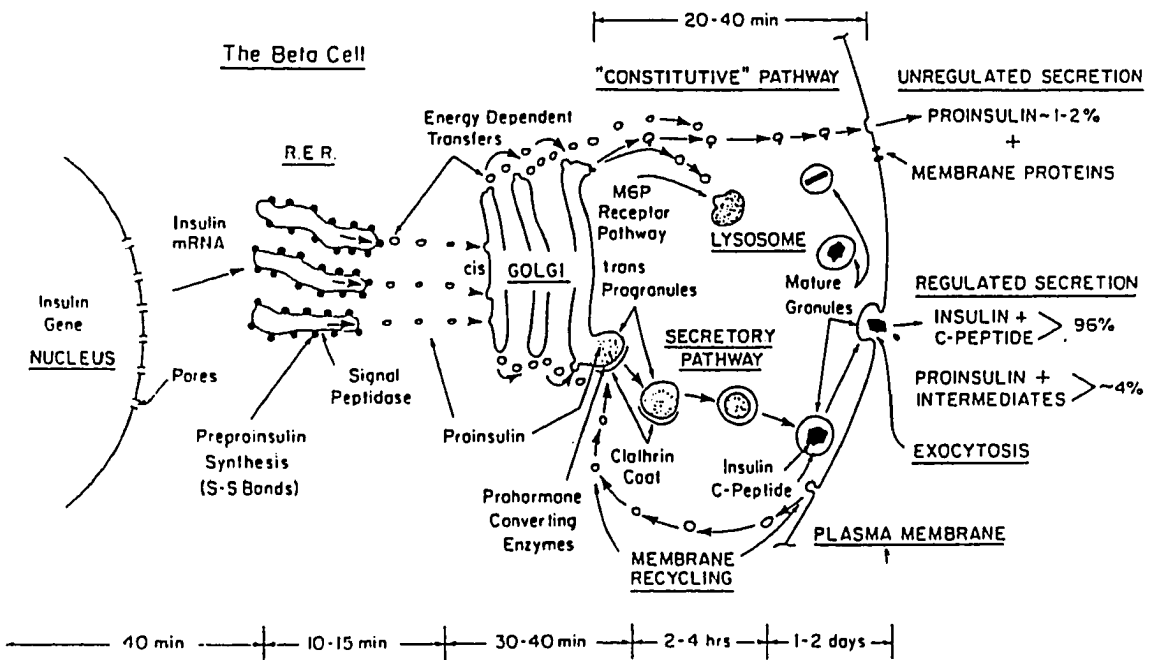
Fig. 1. The biosynthesis of insulin (Hazelwood, 1989).

beta-cell electric activity. The Ca^{2+} influx increases $[Ca^{2+}]_i$ and elicits exocytosis of insulin-containin secretory granules (Fig. 2).

Extracellular regulatory molecules of insulin secretion, such as hormones, neurotransmitters, and growth factors, interact with cells by binding to specific cell surface receptors. As a result of this interaction, the receptor may be activated thereby leading to the generation of second messenger molecules.

Several receptor families associated with different mechanisms have been characterized and classified. Most of the receptors are coupled to regulatory guanine nucleotide-binding proteins (G proteins). G proteins transmit the signal to effectors (such as enzymes or ion channels) which transduce the binding of a ligand into an intracellular signal, such as changes in the concentration of diffusible second messengers or the gating of ion currents (Offeermanns and Schultz, 1994). β-Adrenergic receptors and glucagon-like polypeptide (GLP) receptors activate a G protein, G_s, which activates the downstream effector adenylyl cyclase (AC) and thus generates cAMP. This second messenger may activate protein kinase A (PKA) or regulate Ca^{2+} channels directly (Ashcraft, 1994). Somatostatin receptors and α_2-adrenergic receptors activate a G protein, G_i, which inhibits the downstream effector AC and inhibits the generation of cAMP. Receptors for vasporessin, bombesin, cholecystokinin (CCK) and bradykinin activate a G protein, G_q, which activates the downstream effector phospholipase C (PLC) and thus generates IP_3 and diacylglycerol (DAG). These ligand receptor interactions selectively
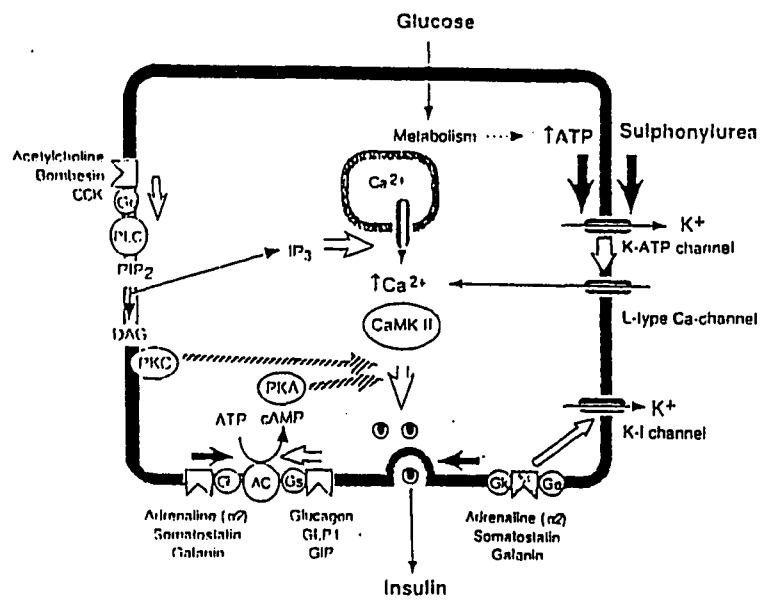
Fig. 2. The regulation of insulin secretion in pancreatic beta-cell (Ashcraft, 1994)

filter the incoming signals from extracellular stimuli. Extracellular stimuli converted by the plasma membrane network are in a complex of integration processing and allow the cells to generate an adequate response.

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Regulation of insulin secretion by vasopressin

Chemistry and synthesis of AVP

Arginine vasopressin (AVP) is a nonapeptide, with a molecular weight of 1084 Da, and is a strongly basic molecule (Schally, 1972). Synthesis of the AVP precursor occurs principally in the supraoptic and paraventricular nuclei of the hypothalamus. As the precursor complex migrates along the neuronal axons at a rate of about 2 mm/h, it undergoes specific cleavage, and the products that include AVP are stored as neurosecretory granules in the posterior pituitary gland (Russel et al., 1990). Release of AVP from the neural lobe is associated with an increase in the rate of phasic firing of electrical impulses (Dutton and Dyball, 1979).

Expression of the AVP gene has been observed in extrahypothalamic tissues, such as the adrenal gland, gonads, cerebellum and probably the pituicytes of the posterior pituitary gland (Richter et al., 1991).

Function of AVP

AVP, the antidiuretic hormone, plays a major role in the regulation of body fluid volume and osmolality. It also contributes to the maintenance of blood

pressure and other physiological functions. For example, AVP increases

glycogenolysis, corticotropin release, water reabsorption from the collecting duct,

platelet aggregation, release of coagulation factors, firing rate of certain neurons

(Thibonnier, 1992), proliferation of the pituitary gland and vascular smooth muscle

cells (McNicol et al., 1990; Sperti and Colucci, 1991) and increases secretion of

the glucagon and insulin (Dunning et al., 1984). All of these actions are mediated

through activation of specific receptors present on the surface of the target cells.

AVP receptors

There are two types of vasopressin receptors : V_1 and V_2 (Michell, et al.,

1979). Activation of vascular and hepatic AVP receptors, classified as V_1 receptors,

leads to an increase in IP_3 formation and a rise of $[Ca^{2+}]_i$ (Thibonnier, 1992).

Based on the fact that the AVP receptors in the anterior pituitary cells have a

slightly different pharmacological profile in terms of their binding characteristics of

AVP analogues, the term V_{1b} was proposed to distinguish them from the classical

vascular V_{1a} receptors (Schwartz et al., 1991). In contrast, renal AVP receptors,

classified as V_2 receptors, are involved in free water reabsorption via activation of

adenylyl cyclase (Thibonnier, 1992).

The presence of immunoreactive AVP, in nanomolar concentrations in the

mammalian pancreas (2-11 ng/g wet wt) (Amico et al., 1988), suggests a pancreatic

site of production since peripheral plasma levels are considerably lower (1-2 pg/ml)

in normal man (Scrier et al., 1979). In addition, administration of AVP has been
shown to increase plasma insulin concentrations in man (Karp et al., 1968). A
number of animal studies concerning the effects of AVP on insulin secretion
indicate that AVP is capable of influencing islet cell function (Altszuler and
Hampshire, 1981, Stock and Uvnas-Moberg, 1987, Dunning et al., 1984). AVP
stimulates insulin secretion from isolated mouse pancreatic islets (Gao et al., 1990),
cloned insulin-secreting cell lines RINm5F cells (Monaco et al., 1988) and hamster
insulin-secreting tumor (HIT) cells (Richardson et al., 1990). We have recently
found that AVP-induced insulin secretion is mediated by V_{1b} receptors in rat islets
and RINm5F cells (Lee et al., 1995).

Signal transduction pathway of V_1 receptors

AVP has been widely studied in several different cell models, such as smooth
muscle, endocrine cells, and endothelium (Spatz et al., 1994). The signal
transduction pathway of AVP receptors in insulin secretion has been investigated
by using cell lines (RINm5F and HIT) and pancreatic islets. AVP binds V_1
receptors which are coupled to the β_1-subtype of phospholipase C (PLC) by certain
GTP-binding (G) proteins of the G_q family (Lee and Severson, 1994). G proteins
are heterotrimers consisting of α, β and γ subunits. The α subunit binds guanine
nucleotide and appears to play the major role in activating the effector enzyme or
ion channel. A role for the β and γ subunits in activating or modifying the

activation of some effectors has been found in a limited number of cases. When

stimulated by AVP, this receptor couples to PLC and accelerates the rate of

phosphatidylinositide 4,5-bisphosphate (PIP_2) hydrolysis. This leads to the

formation of inositol 1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG)

molecules that act as second messengers by releasing Ca^{2+} from intracellular stores

and by activating protein kinase C (PKC), respectively (Nishizuka, 1992 and

Thibonnier, 1992). In addition, AVP activates other effectors, such as PLA_2 and

PLD (Fig.3). The AVP-induced Ca^{2+} influx through opening of Ca^{2+} channels is

mediated by PLA_2 in the smooth muscle cell line A_7r_5 (Thibonnier et al., 1992). In

the Swiss 3T3 fibroblasts (Briscoe and Wakelam, 1995) and L6 myoblasts

(Thompson et al., 1994), AVP stimulates PLD activity. The AVP-induced PLC-

mediated signal pathway is well characterized in several different insulin secreting

cell models (Gao et al., 1994, Li et al., 1990, and Richardson et al., 1990).

However, in the pancreatic beta-cells, the other signal transduction pathways

induced by AVP are not well characterized. This prompted us to study the role of

PLA_2 and PLD in AVP-induced $[Ca^{2+}]_i$ increase in the beta-cells.

Involvement of phospholipases in AVP's action

Phospholipase C

PLC is a family of enzymes that hydrolyze phospholipid at the 3-position

phosphodiester bond of the glycerol backbone. When PIP_2 is the substrate, PLC

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A

Agonist

PIP$_2$ PC

Phospholipase C   Phospholipase D   Phospholipase A$_2$

IP$_3$ ⊖   DG   ⊕   FFAs   LysoPC

Potentiation

Ca^{2+} PKC

Cellular Responses

B

Ca^{2+} Transient

Sustained PKC activation

DG from PC

DG from PIP$_2$

FFAs & LysoPC
from PC

IP$_3$
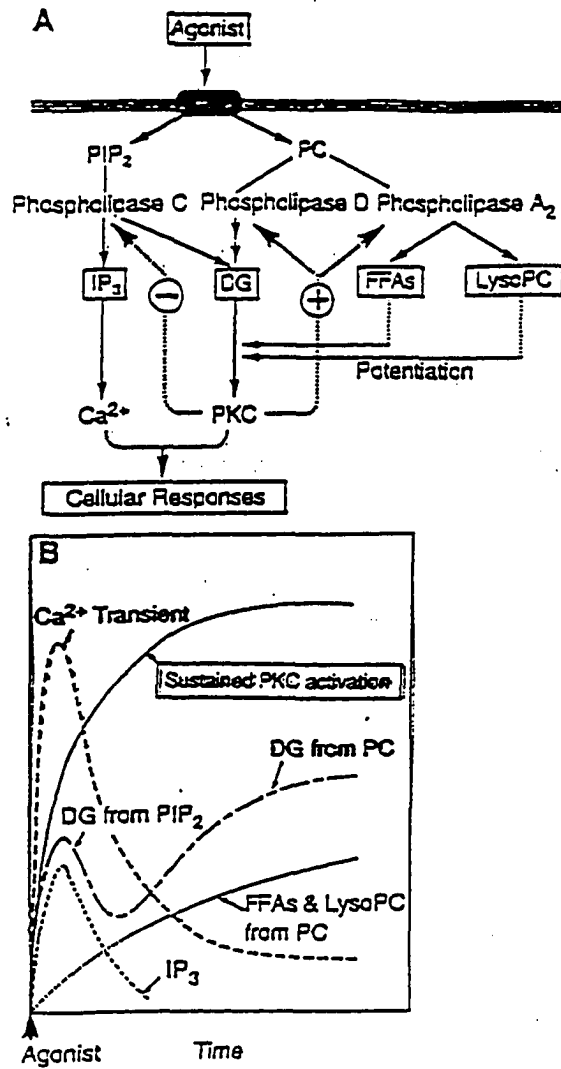
Agonist   Time

Fig. 3. Schematic representation of the agonist-induced membrane phospholipid

degradation (A). Time course of generation of various signalling molecules (B).

(Nishizuka, 1992)

action leads to the formation of IP_3 and DAG.

PPI-specific PLC (PPI-PLC) is a subfamily of PLC that acts specifically on inositol-containing lipids and does not hydrolyze other phospholipids, such as phosphatidylcholine (PC) (Lee and Severson, 1994). PPI-PLC can be divided into three types (β, γ and δ), which are distinct proteins that exhibit only a small amount of sequence identity. In cells, β and γ isoenzymes appear to be the ones that are involved in the receptor-activated PIP_2 hydrolysis, but the mechanisms of their activation are different. PLC-β is activated by G_q-mediated signal transduction, and PLC-γ is activated by tyrosine kinase receptor activation (Asaoka et al., 1992). A difference in the primary amino acid sequences between β and γ isoenzymes, which are related to their mechanisms of activation, is that PPI-PLC-γ contains src homology (SH2 and SH3) domains, whereas PPI-PLC-β does not. SH2 and SH3 domains mediate the binding to other proteins that contain phosphorylated tyrosine residues, for example, growth factor receptors (Rhee and Choi, 1992).

In insulin-secreting cells, AVP induces insulin secretion by promoting the production of IP_3 which increases $[Ca^{2+}]_i$ (Li et al., 1992). In addition, AVP may stimulate insulin secretion by closing ATP-sensitive K^+ channels, thereby promoting membrane depolarization (Martin et al., 1989). This in turn increases Ca^{2+} influx by opening voltage-dependent Ca^{2+} channels (VDCC) (Thorn et al., 1991).

Phospholipase A$_2$

PLA$_2$ enzymes catalyze the hydrolysis of the ester linkage in the membrane glycerophospholipids at the sn-2 position of the glycerol moiety. These enzymes can generally be divided into two major groups, the extracellular or low molecular mass (14-18 kDa) forms, secretory PLA$_2$ (sPLA$_2$), and the intracellular or high molecular mass (31-110 kDa) forms, termed cytosolic PLA$_2$ (cPLA$_2$) (Glaser et al., 1993). Table I shows the major differences between two groups of these enzymes. The sPLA$_2$ enzymes are the most highly characterized group of PLA$_2$ enzymes due to their universal nature and high concentration in venoms of snakes and pancreatic juices (Verheij et al., 1981). The characteristics of cPLA$_2$ are considered important for intracellular regulated enzymes responsible for the release of arachidonic acid for eicosanoid biosynthesis (Glaser et al., 1993). The cPLA$_2$ enzymes display a preference for arachidonic acid-contained phospholipid and have an almost absolute requirement for unsaturated fatty acids in the sn-2 position of glycerophospholipids.

In the pancreatic beta-cells, several studies have sought to identify positions in the stimulus-secretion signal pathway of insulin release affected by the action of PLA$_2$ (Fujimoto and Metz, 1987; Zawalich and Zawaich, 1985; Yamamoto et al., 1983). It has been demonstrated that three distinct points in the signal pathway of insulin secretion appear to be affected by PLA$_2$; one is Ca^{2+} influx, the second is Ca^{2+} release, and the third which is independent of a rise in $[Ca^{2+}]_i$ (Eddlestone, 1995). There are three types of PLA$_2$ enzymes expressed in pancreatic beta-cells;

Table I. Comparison of sPLA₂ and cPLA₂ (Glaser, 1995)

	sPLA₂	*cPLA₂*
Form	Secretory	Intracellular
Molecular weight	~14 kDa	~100 kDa (SDS-PAGE)
		~82 kDa (cDNA)
Ca^{+2} requirement	Absolute	Only for translocation
Regulation	Transcriptional (IL-1, IL-6-induced)	Transcriptional (IL-1 induced)
		Phosphorylation (MAPK)
	Ca^{+2} levels (enzyme activity)	
Catalytic residue	His	Ser[a]
Substrate specificity		
Head group	None	None
sn-2 fatty acid	None	Arachidonyl selective
Other enzymatic activities	None	Lysophospholipase
		Transacylase (?)[b]

[a] The role of an active site Ser was proposed by Trimble *et al.* (1993) based on NMR studies with cPLA₂ and a trifluoromethyl ketone inhibitor.

[b] The transacylase activity of cPLA₂ was described by Reynolds *et al.* (1993) and used specific assay conditions to observe this activity. It is not known whether this activity exists under normal physiological conditions.

two of them are membrane-associated, and one is cytoplasmic. In insulin-secreting cells, PLA_2 activity is increased in the presence of glucose (20 mM) (Ramanadham et al., 1993). Therefore, it has been proposed that PLA_2 may play a role in nutrient-induced insulin secretion (Konrad et al., 1992; Ramanadham et al., 1993). Also the endogenous polypeptide, gastric inhibitory polypeptide, inhibits the activity of PLA_2 (Lardinois et al., 1990). This suggests that PLA_2 is involved in the regulation of insulin secretion in the pancreatic beta-cell.

The PLA_2 enzymes have been shown to be involved in AVP-induced signal transduction (Spatz et al., 1994; Loxley, et al., 1993). However, the role of PLA_2 in AVP-induced insulin secretion from beta cells remains poorly defined.

Phospholipase D

PLD was described for the first time in 1947 in plants (Hanahan and Chalikoff, 1947) and was later found in mammals (Saito and Kanfer, 1973). PLD hydrolyses phosphatidylcholine (PC) and cleaves the phosphodiester bond distal to the glycerol backbone resulting in the formation of phosphatidic acid (PA) and choline. Its activation by various agonists has been described in many tissues and cell models (Boarder, 1994). The regulation of PLD activity seems to occur through several different mechanisms depending on the cell type and agonist. Various factors are involved in its activation such as G proteins, hydrolysis of PIP_2, activation of PKC, stimulation of calmodulin/myosin light chain kinase and

phosphorylation on tyrosine residues (Boarder, 1994).

There is clear evidence that a receptor-dependent PLD is present in a number of tissues. Agonists, such as bradykinin (Horwitz and Ricanati, 1992), bombesin (Briscoe et al., 1994) and prostaglandin $F_{2\alpha}$ (Sugiyama et al., 1994), stimulate the hydrolysis of PC via PLC and PLD. This leads to an increase in the production of DAG and phosphocholine in one pathway and PA and choline in the other (Rydzewska et al., 1993). PA is hydrolyzed by phosphatidic acid phosphohydrolase (PPH) to produce DAG. This signal transduction pathway provides an alternative source of DAG. The initial product of PLD activation is PA which has been suggested to be a second messenger (Thompson et al., 1991). PLD activation in the stimulation of insulin release was found in pancreatic islet cells (Dunlop and Metz, 1989; Metz and Dunlop, 1990). However, the role of PLD in agonist-induced insulin secretion is not well understood.

AVP-induced $[Ca^{2+}]_i$ increase

In most of cells, several polypeptide receptors such as AVP, bradykinin, bombesin, and oxytocin mediate a biphasic increase in $[Ca^{2+}]_i$ with a transient peak followed by a sustained phase. The initial phase is due to the Ca^{2+} release from intracellular stores, the sustained phase is mediated by Ca^{2+} influx which involves both the voltage-dependent Ca^{2+} channels and voltage-independent Ca^{2+} channels (Chen et al., 1994). Activation of VDCCs and voltage-independent Ca^{2+} channels

by PLC-coupled receptors has been reported (ZhuGe et al., 1995); however, the mechanisms underlying the receptor-activated sustained Ca^{2+} influx are not yet clear (Clapham, 1995).

In recent years, an attractive theory has been developed to account for the PLC-mediated Ca^{2+} influx. This is called capacitative Ca^{2+} entry mechanism (Putney, 1990). This theory indicates that the depletion of intracellular Ca^{2+} store evokes Ca^{2+} influx through opening of Ca^{2+} channels. Among the putative Ca^{2+} channels is a well-established ion channel that elicits Ca^{2+} release-activated Ca^{2+} current (I_{CRAC}). This channel has been characterized in several cell types such as mast cells (Hoth and Penner, 1992), pancreatic acinar cells (Bahnson et al., 1993), human T cells (Lewis and Cahalan, 1989), and A431 cells (Luckhoff and Clapham, 1994). I_{CRAC} has an extremely low conductance of most ion channels. The net current passing the channels in the whole cell is ~ 5 pA. I_{CRAC} is highly Ca^{2+}-selective and is inactivated by high intracellular Ca^{2+} levels (Fasolato et al., 1994). There are other types of Ca^{2+} currents triggered by Ca^{2+} store depletion such as depletion of internal Ca^{2+} stores-activated Ca^{2+} current (I_{DC}), IP_3-activated Ca^{2+} current ($I_{Ca.IP3}$), IP_4-activated Ca^{2+} current ($I_{Ca.IP4}$), ATP-activated Ca^{2+} current ($I_{Ca.ATP}$), Ca^{2+}-activated Ca^{2+} current ($I_{Ca.Ca}$), and voltage-dependent Ca^{2+} currents (Clapham, 1995). Except for the voltage-dependent Ca^{2+} currents, none of these putative ion channels have been purified or cloned.

The second major question in the capacitative theory is: how does the depleted

intracellular Ca^{2+} stores open the plasma membrane Ca^{2+} channels? One of the

popular hypotheses is that depletion of intracellular Ca^{2+} stores releases a novel

small messenger, Ca^{2+} influx factor (CIF) that activates Ca^{2+} channels

(Randriamampita and Tsien, 1993; Clapham, 1995). CIF is the most interesting

second messenger candidate to initiate I_{CRAC}. It was initially isolated from Jurkate

T cells after the depletion of Ca^{2+} stores by phytohemagglutinin treatment

(Randriamampita and Tsien, 1993). CIF caused Ca^{2+} influx when applied to

macrophages, astrocytoma cells, and fibroblasts. It has been partially characterized

as a < 500 Da phosphorylated pH-stable anion substance. CIF is probably released

or generated from the ER or adjacent regions after IP_3-induced Ca^{2+} release from

stores (Clapham, 1994).

Currently, key issues to be settled in this field include: 1) characterization of

Ca^{2+} entry pathways that are mediated by store depletion, and 2) characterization of

direct messengers that mediate the store-depletion signal.

The role of PKC on AVP-induced insulin secretion and changes in intracellular

$\underline{Ca^{2+}}$

One of the downstream effectors of both PLC and PLD pathways is the

generation of DAG which activates PKC. DAG, in combination with

phosphatidylserine (PS) activates PKC. PKC, in turn, phosphorylates a number of

regulatory proteins producing additional effects such as stimulation of secretion and

transcription (Nishizuka, 1988).

PKC is a serine and threonine kinase; it phosphorylates serine and threonine residues in protein substrates but does not phosphorylate tyrosine residues. PKC activation is thought to require translocation from cytosol to plasma membranes. Although it was initially thought that PKC was activated only when it becomes associated with the plasma membrane, there is evidence that PKC can also be associated with and be activated within the membrane of the nucleus (Nishizuka, 1992).

PKC belongs to a family of at least ten isoenzymes. Additional PKC isoenzymes are currently being identified by reverse transcription of total RNA (Chang et al., 1993). PKC isoenzymes have been grouped into three classes (Dekker and Parker, 1994). The conventional PKCs (cPKC) PKC- α, -βI, -βII, and -τ are Ca^{2+}- and phospholipase-dependent enzymes, the novel PKCs (nPKC) PKC -δ, -ϵ, -η and -θ are Ca^{2+}-independent but phospholipid-dependent. The atypical PKCs (aPKC) PKC-ξ and -λ are not activated by DAG or phorbol esters (Table II). The Ca^{2+}-dependent PKC may require both the generation of DAG and elevation of $[Ca^{2+}]_i$ of receptor mediated activation of PLC *in vivo*, whereas the Ca^{2+}-independent isoenzymes may be activated by phospholipid alone. It has been suggested that activation of different PKC isoenzymes *in vivo* may lead to distinct cellular responses (Dekker and Parker, 1994). This could occur if different isoenzymes phosphorylate distinct proteins. It is also possible that activation of

Table II Subspecies of protein kinase C (Dimitrijevic et al., 1995)

	Isoform	Lipid Activators
Ca^{2+}-dependent PKC		
Classical PKC	α^{*}	PS, DAG (PhE), FA, LPC
	β_I/β_{II}^{*}	PS, DAG (PhE), FA, LPC
	γ	PS, DAG (PhE), FA, LPC
Ca^{2+}-independent PKC		
New PKC	δ	PS, DAG (PhE), FA
	ϵ^{*}	PS, DAG (PhE), FA
	η	ND
	θ	ND
Atypical PKC	ζ^{*}	PS, FA
	ι	ND

PS, phosphatidylserine; PKC, protein kinase C; DAG, diacylglyc-
erol; PhE, phorbol ester; FA, fatty acid; LPC, lysophosphatidylcholine.
ND, not determined. *Present in vascular smooth muscle.

different isoenzymes may occur because they are present in different cellular

compartments. Differential regulation of PKC isoenzyme has been shown to occur

during stimulation of a single cell type by a single agonist (Dimitrijevic et al.,

1995). This suggested that PKC may play multiple roles in the regulation of

cellular processes.

All PKC isoenzymes consist of two functional domains, a carboxyl-terminal

catalytic domain which is involved in substrate phosphorylation and an amino-

terminal regulatory domain which is involved in the binding of regulatory cofactors

and activators (Nishizuka, 1988). These functional domains contain four conserved

regions (C1-C4) and five variable regions (V1-V5). A phorbol ester binds to the

C1 region of the PKC enzyme and acts by mimicking the action of DAG

(Nakamura et al., 1989). The binding of intracellular Ca^{2+} to the C2 region of

cPKC causes translocation of these enzymes to the plasma membrane where they

are activated following binding to DAG and PS.

PKC is involved as a modulator of multiple steps regulating the pancreatic

beta-cell stimulated-secretion coupling. The stimulation of PKC by DAG may

regulate the functional response in the cell and may play a negative feedback

control role in signal transduction (Nishizuka, 1992). In the normal mouse

pancreatic beta-cells, PKC plays a negative feedback control role in the responses

to acetylcholine and vasopressin to inactivate PLC (Gao et al., 1994). On the other

hand, activation of PKC with phorbol esters increases insulin release in normal

pancreatic cells and HIT cells (Arkhammer et al., 1994). However, the role of

PKC in beta-cells is controversial because the tools used in these studies are not

specific and, thus the results are difficult to interpret. For example, phorbol esters

that are used as to activate of PKC may exert additional effects that are not related

to PKC activation (Wilkinson and Hallam, 1994). Therefore, phorbol esters

stimulate insulin secretion in some studies (Manaco et al., 1988). In addition, the

PKC inhibitor (staurosporine) applied to confirm the specificity of PKC's effects,

also has additional effects which are not related to PKC activation (Wilkinson, and

Hallam, 1994). These include the inhibition of protein kinase A, calmodulin and

tyrosine kinase (Davis et al., 1989). Currently there are several specific PKC

inhibitors (e.g., Ro 31-8220) available to study the mechanism of PKC in

regulation of insulin secretion. Ro 31-8220 is a derivative of staurosporine which

is superior to staurosporine (Davis et al., 1989) and is a potent and selective

inhibitor of protein kinase C. As mentioned earlier, PKC may be a negative

regulator of cell responses. Ca^{2+} is an important intracellular signal which

regulates the cell responses and it has been reported that PKC activation regulates

Ca^{2+} channels (Mullen, 1995; Sena et al., 1995). Therefore, it is important to

characterize the role of PKC in the regulation of intracellular Ca^{2+} by using specific

PKC inhibitors. In a preliminary study, we also found that inhibition of PKC by

Ro 31-8220 enhanced AVP-induced Ca^{2+} influx. These results suggested that PKC

may inhibit the AVP-induced intracellular Ca^{2+} change in beta-cells. Ca^{2+} entry

through VDCC is a major pathway for raising $[Ca^{2+}]_i$ in most living cells including

beta-cells (Ashcroft et al., 1990 and Smith et al., 1993). It would be important to

characterize the types of Ca^{2+} channels that are involved in the inhibition of insulin

secretion by PKC.

CHAPTER II ARGININE VASOPRESSIN-STIMULATED INSULIN SECRETION AND ELEVATION OF INTRACELLULAR Ca^{2+} CONCENTRATION IN RINm5F CELLS: INFLUENCES OF A PHOSPHOLIPASE C INHIBITOR U-73122 AND PHOSPHOLIPASE A_2 INHIBITOR N-(P-AMYLCINNAMOYL)ANTHRANILIC ACID

A paper published in the Journal of Pharmacology and Experimental Therapeutics[1]

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ABSTRACT

The present study was undertaken to investigate the mechanism by which arginine vasopressin (AVP) increases insulin secretion in rat insulinoma (RINm5F) cells by using a specific phospholipase C (PLC) inhibitor, U-73122, and a phospholipase A_2 (PLA$_2$) inhibitor, N-(p-amylcinnamoyl)anthranilic acid (ACA). AVP (0.1-100 nM) increased insulin secretion and cytosolic free Ca^{2+} concentration

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($[Ca^{2+}]_i$) dose-dependently. Moreover, U-73122 (2-8 μM) inhibited the AVP-induced increases in the intracellular concentration of inositol 1,3,4-trisphosphate (IP$_3$) and $[Ca^{2+}]_i$ dose-dependently. At 8 μM U-73122 abolished the AVP's effect on IP$_3$ and $[Ca^{2+}]_i$, but it only reduced the AVP-induced increase in insulin secretion by 35%. In contrast, 8 μM U-73122 did not reduce the ionomycin (a Ca^{2+} ionophore, 100 nM)-induced increase in $[Ca^{2+}]_i$. The discrepancy between the results of $[Ca^{2+}]_i$ and insulin secretion may be due to the multiple signal transduction pathways associated with the activation of AVP receptors, specifically the Ca^{2+}-independent pathway. The phospholipase A$_2$ inhibitor ACA (100 μM) did not antagonize the AVP (10 nM)-induced increase in insulin release. These results suggested: 1) U-73122 blocks PLC activities but fails to block other signal transduction pathways that trigger insulin secretion in these cells, and 2) AVP increases insulin release from RINm5F cells through both the PLC mediated Ca^{2+}-dependent and Ca^{2+}-independent pathways.

INTRODUCTION

Arginine vasopressin (AVP) is a neurohypophyseal nonapeptide hormone with antidiuretic effects on the kidney and pressor action on the vascular smooth muscle. AVP binds to V_1-receptors and activates PLC, which in turn hydrolyzes phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and IP_3 (Nishizuka, 1992; Thibonnier, 1992). DAG activates protein kinase C, whereas IP_3 increases the release of calcium from the endoplasmic reticulum to elevate $[Ca^{2+}]_i$.

The aminosteroid U-73122 (1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione) inhibits a variety of PLC mediated events, in human neutrophils and platelets (Bleasdale et al., 1990; Smith et al., 1990; Vickers, 1993), human neuroblastoma cells (Thompson et al., 1991), human erythroleukemia cells (Wu et al., 1992), GH3 rat pituitary cells (Smallridge et al., 1992; Hawes et al., 1992), rat hepatocytes (Galan et al., 1992) and rat pancreatic acinar cells (Yule and Williams, 1992).

In insulin-secreting cells, AVP induces insulin secretion by promoting the production of IP_3 which increases $[Ca^{2+}]_i$ (Li et al., 1992). In addition, AVP may stimulate insulin secretion by closing ATP-sensitive K^+ channels and thus promoting membrane depolarization (Martin et al., 1989), which in turn may increase Ca^{2+} influx directly by opening voltage-dependent Ca^{2+} channels (Thorn et al., 1991).

The AVP-induced increase in Ca^{2+} influx is mediated by PLA_2 in the

smooth muscle cell line A_7r_5 (Thibonnier et al., 1991). Glucose activates PLA_2

thus increases the formation of arachidonic acid, which mobilizes Ca^{2+} from the

endoplasmic reticulum and regulates voltage-dependent Ca^{2+} channel in the

pancreatic-beta cell (Konrad et al., 1992). ACA, a PLA_2 inhibitor, inhibits

glucose-induced insulin secretion in rat pancreatic islets (Konrad et al., 1992).

The present study was undertaken to determine the mechanisms by which

AVP induces insulin secretion in pancreatic-beta cells by measuring $[Ca^{2+}]_i$, IP_3

production, and insulin secretion. Specifically, we studied the effects of this

inhibitor on PLC-dependent processes in RINm5F cells and the involvement of

PLA_2 in the AVP action by using the PLA_2 inhibitor ACA.

METHODS

Cell culture. RINm5F cells were maintained in RPMI 1640 containing 10% fetal

bovine serum and aerated with 5% CO_2-95% air at 37°C as previously described

(Thomas et al., 1989). All experiments were performed with cells in passages 45-

54.

Insulin secretion. RINm5F cells were plated into 24-well Costar plates at 2 x 10^5

cells/well and grown for 5 days. Growth medium was removed and the monolayer

cells were washed with modified Krebs-Ringer bicarbonate buffer (KRB)

containing (in mM): 136 NaCl, 4.8 KCl, 1.2 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5

NaHCO$_3$, 10 HEPES, 4 glucose, and 0.1% bovine serum albumin, and pH 7.4.

The cells were then preincubated for a 30-min basal period at 37°C, followed by a

15-min incubation in KRB with the test agent. When needed, U-73122 or ACA

was given 5 min before the administration of AVP. The supernatants were

collected for radioimmunoassay (RIA) of insulin (Hsu et al., 1990). To measure

insulin secretion in suspended cells, 1.5 x 10^6 cells were suspended in a test tube

(12 x 75 mm^2) with 1.5 ml of KRB, then incubated for 5 min with the test

reagents under gentle shaking with an orbit shaker (50 rpm) at 24°C. When

needed, U-73122 was given 100 s before the administration of AVP. The reaction

was terminated by centrifugating the cells and collecting the supernatants for RIA

of insulin.

Measurement of [Ca^{2+}]$_i$. To measure [Ca^{2+}]$_i$ in all suspensions, 30 x 10^6 cells were

loaded with 2 μM of fura-2 acetoxymethylester (fura-2AM) in KRB for 30 min at

37°C. The loaded cells were washed and kept at 24°C until use. The cells were

resuspended in a concentration of 10^6/ml and 1.5 ml of aliquot was used for [Ca^{2+}]$_i$

determination at 24°C. The 340/380 nm fluorescence ratios were monitored in a

SLM-8000 fluorescence spectrophotometer (SLM, Urbana, IL). The [Ca^{2+}]$_i$ was

calibrated after the cell lysis as described previously (Hsu et al., 1990). When

needed, U-73122 was given 100 s, and [ethylenebis (oxyethylenenitrilo) tetraacetic

acid (EGTA) or nimodipine was given 30 s before the administration of AVP.

Measurement of IP$_3$. IP$_3$ was quantified by use of a competitive radioreceptor-binding assay kit (Du Pont Co., Boston, MA). 1.5 x 10^6 cells in 1 ml of KRB were placed in polypropylene tubes and incubated in a shaking water bath at 37°C. Incubation with the test agent was terminated in 10 s by adding 20% (w/v) of ice-cold trichloroacetic acid. When needed, U-73122 was given 100 s before the administration of AVP.

Statistical Analyses. Results were analyzed by the Student's t test for paired and unpaired values. The significance level was set at $P < 0.05$.

Materials. The following were used: nimodipine (Research Biochemicals International, Natick, MA), ionomycin (Sigma Chemical Co., St. louis, MO), fura-2AM (Molecular Probes, Eugene, OR), U-73122 and ACA (Biomol Research Laboratory, Plymouth meeting, PA).

RESULTS

Effects of U-73122 and ACA on the AVP-induced increase in insulin secretion

AVP (0.1-100 nM) caused a dose-dependent increase in insulin secretion (Fig. 1). AVP (10 nM) induced an increase of about 2.5 times over basal insulin secretion. However, 1 μM of AVP caused significantly less insulin secretion than 100 nM of AVP. U-73122 (1-8 μM) alone did not change basal insulin secretion,

but it inhibited the AVP (10 nM)-induced insulin secretion dose-dependently. At

the highest concentration studied (8 μM), U-73122 inhibited approximately 30-35%

of AVP-induced insulin secretion in both the monolayer (Fig. 2a) and suspended

cell preparations (Fig. 2b). A PLA_2 inhibitor ACA (100 μM) did not affect AVP

(10 nM)-induced insulin secretion (ACA + AVP=138.4 ± 8.52 pg/min/well, n=4;

AVP=144 ± 7.4 pg/min/well, n=4, p>.05).

Effects of AVP on $[Ca^{2+}]_i$

When AVP (1 nM - 1 μM) was applied to suspended RINm5F cells loaded

with fura-2, it caused a dose-dependent increase in $[Ca^{2+}]_i$ (Fig. 3). The rise in

$[Ca^{2+}]_i$ after AVP administration usually reached a maximum within 30-40 s (The

first phase) and decreased to a sustained level for the following ≥ 4 min (The

second phase). The second phase of increase in $[Ca^{2+}]_i$ was seen in all doses

studied, but was diminished at 1 μM AVP (Fig. 4). In the presence of 2 mM

EGTA, AVP only evoked a single $[Ca^{2+}]_i$ transient which was smaller than AVP in

the absence of EGTA (Fig. 5). Nimodipine (1 μM), an L-type Ca^{2+} channel

blocker, reduced but did not abolish the AVP-elicited rise in $[Ca^{2+}]_i$ particularly in

the second phase (Fig. 6).

Effects of U-73122 on the AVP-induced increase in $[Ca^{2+}]_i$

U-73122 alone did not change $[Ca^{2+}]_i$ until 8 μM was given, which

increased $[Ca^{2+}]_i$ by 10%, and subsided within 100 s of administration.

Pretreatment with U73122 (1-8 μM) for 100 s inhibited the AVP (10 nM)-induced

increase in $[Ca^{2+}]_i$ dose-dependently. U-73122 at 8 μM abolished the AVP-elicited

increase in $[Ca^{2+}]_i$ including the peak and second phase (Fig. 7). Ionomycin (100

nM) elicited a biphasic increase in $[Ca^{2+}]_i$ with the pattern similar to that induced

by AVP (10 nM). U-73122 (8 μM) failed to affect the ionomycin-induced increase

in $[Ca^{2+}]_i$ (Fig. 8).

Inhibition by U-73122 of the AVP-induced production of IP_3

AVP (100 nM) increased IP_3 production by >200% of the basal level. U-

73122 (2, 4, and 8 μM) inhibited the AVP-stimulated production of IP_3 in RINm5F

cells in a dose-dependent manner (Fig. 9). U-73122 (8 μM) almost abolished the

AVP-induced increase in IP_3 production.

DISCUSSION

Ca^{2+} plays a central role in the stimulation of insulin secretion (Wollheim

and Pozzan, 1984). For example, glucose raises $[Ca^{2+}]_i$ by opening voltage-

dependent Ca^{2+} channels through depolarization induced by closing ATP-sensitive

K^+ channels (Findlay and Dunne, 1985; Lu et al., 1993). Also, some of the

neurotransmitters and hormones induce insulin secretion by activating adenylyl

cyclase or phospholipases to mobilize Ca^{2+} into the cytosol (Li et al., 1992). The

second phase of increase in $[Ca^{2+}]_i$ maybe due to a rise in Ca^{2+} influx because the

Ca^{2+} chelator EGTA blocked this increase in our studies. AVP opens Ca^{2+}

channels in excitable cells, particularly in the smooth muscle (Van Renterghem,

1988). The Ca^{2+} channels that are opened by AVP administration are not all L-

type channels, since nimodipine, an L-type channel blocker, only partially reduced

the Ca^{2+} influx elicited by AVP. Our results confirmed and extended those of

others (Li et al., 1992) and showed that AVP elicited an increase in $[Ca^{2+}]_i$ by

promoting the release of intracellular Ca^{2+} store and Ca^{2+} influx through channels

including L-type Ca^{2+} channels (Thorn et al., 1991).

U-73122 is a specific PLC blocker which has been used as a

pharmacological tool in many different cell preparations to block the formation of

IP_3 and DAG (Bleasdale, 1992). In the present study, we found that U-73122

inhibited the AVP-induced increase in $[Ca^{2+}]_i$ and IP_3 dose-dependently. The

highest dose of U-73122 studied (8 μM) abolished the AVP-induced increase in

$[Ca^{2+}]_i$ and IP_3. The effect of U-73122 was a specific one because it did not affect

the ionomycin-induced increase in $[Ca^{2+}]_i$. Ionomycin raises $[Ca^{2+}]_i$ in RINm5F

cells predominantly by increasing Ca^{2+} release from the endoplasmic reticulum

(Swope and Schonbrunn, 1988).

AVP increases the release of Ca^{2+} from the endoplasmic reticulum and Ca^{2+}

influx, and the high $[Ca^{2+}]_i$ promotes insulin release (Li et al., 1992). In the

present study, U-73122 inhibited the AVP-induced increase in $[Ca^{2+}]_i$ much more

than it affected insulin secretion. For example, U-73122 at 8 μM abolished the

increase in $[Ca^{2+}]_i$ induced by AVP, but only reduced the AVP-elicited increase in

insulin secretion by $\leq$ 35% in monolayer cells. We also used suspended cells, in the same condition as the $[Ca^{2+}]_i$ measurement, to investigate the effect of U73122 on AVP-elicited increase in insulin secretion. The results were similar to those by the use of monolayer cells. AVP causes multiple signal transduction mediated by V_1 receptor in the smooth muscle cell line A_7r_5 (Thibonnier et al., 1991). In the present study, the discrepant results between $[Ca^{2+}]_i$ and insulin secretion may be due to multiple signal transduction pathways involved in the AVP-induced increase in insulin secretion, particularly the Ca^{2+}-independent pathways. It is generally accepted that AVP activates V_1-receptors that are coupled to a G-protein-G_q. G_q, in turn, activates PLC leading to an increase in IP_3 and DAG (Thibonnier, 1992). However, cyclic AMP is not involved in the AVP receptor mechanism (Li et al., 1992).

Another possible mechanism by which AVP increases insulin release is through activation of PLA_2, which has been accounted for the AVP-induced increase in Ca^{2+} influx in the smooth muscle cell line A_7r_5 (Thibonnier et al., 1991). We used ACA, a PLA_2 blocker, in our experiments to determine if PLA_2 was involved in AVP's actions in RINm5F cells. ACA (100 μM) inhibits insulin secretion induced by glucose administration (Konrad et al., 1992). Our present results showed that ACA did not antagonize the AVP-induced increase in insulin release nor did it reduce the AVP-stimulated increase in $[Ca^{2+}]_i$ (data not shown), suggesting that PLA_2 was not involved in AVP's actions in RINm5F cells. Further work is needed to determine what pathways, besides those mediated by PLC,

mediate AVP's actions in pancreatic beta-cells. U-73122 blocked the peak as well as second phase of the increase in $[Ca^{2+}]_i$ in addition to its partial inhibition of insulin release. These findings suggested that activation of PLC but not PLA_2 is involved in the AVP-induced increase in insulin secretion in RINm5F cells.

Ca^{2+} is a major signal in the insulin secretion events (Martin et al., 1989; Li et al., 1992; Lu et al., 1993; Wollheim and Pozzan, 1984). In the present study, AVP still increased insulin secretion by 65% despite the fact that its increase in $[Ca^{2+}]_i$ was abolished by U-73122. Therefore, the Ca^{2+}-independent pathway(s) may have contributed to approximately 65% effect of AVP on insulin secretion in RINm5F cells. The AVP-activated Ca^{2+} independent pathway may not involve a rise in DAG, because U-73122 inhibits the PLC-elicited increase in DAG (Bleasdale et al., 1990; Bleasdale, 1992). Our observations raise concerns with regards to Ca^{2+} as the predominant second messenger in insulin secretion as mentioned by others (Findlay and Dunne, 1985; Wollhein and Pozzan, 1984). Further work is needed to determine the other signals, particularly the Ca^{2+}-independent ones that mediate the activation of V_1 receptors.

In summary, U-73122 antagonized the AVP-activated IP_3 and Ca^{2+} dose-dependently, but only partially inhibited the AVP-induced insulin secretion. We conclude that AVP activates multiple signal transduction pathways to trigger insulin secretion in RINm5F cells, one mechanism is via a Ca^{2+}-dependent pathway, and the others maybe via Ca^{2+}-independent pathway(s).

ACKNOWLEDGEMENTS

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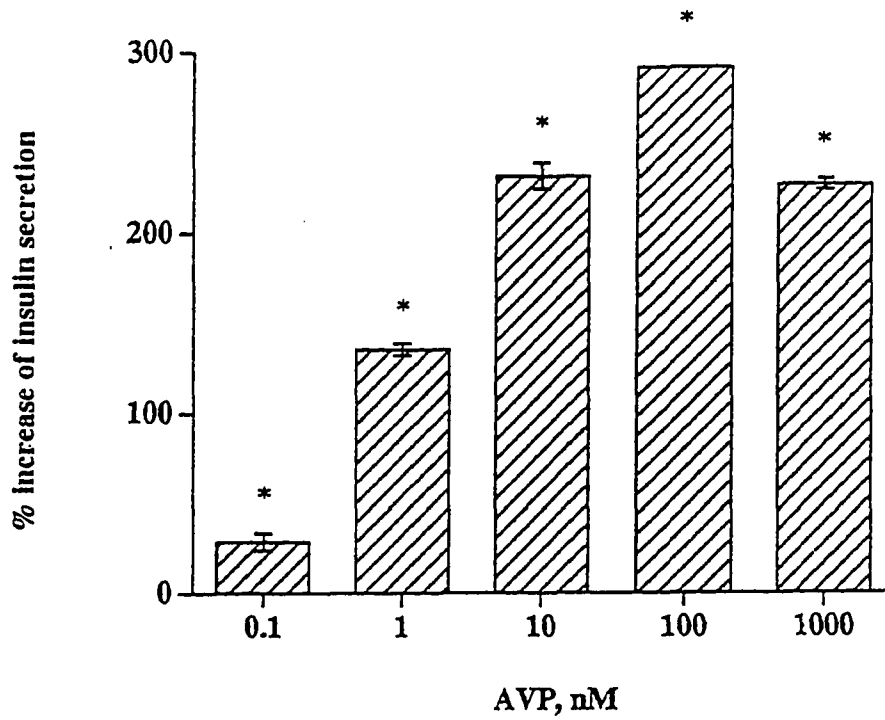
Fig. 1. Effects of AVP on insulin secretion in RINm5F cells. Static incubation for 15 min was performed to measure insulin secretion. Mean data ± S.E. (n=6) are shown. *p<.05, compared to the control group.
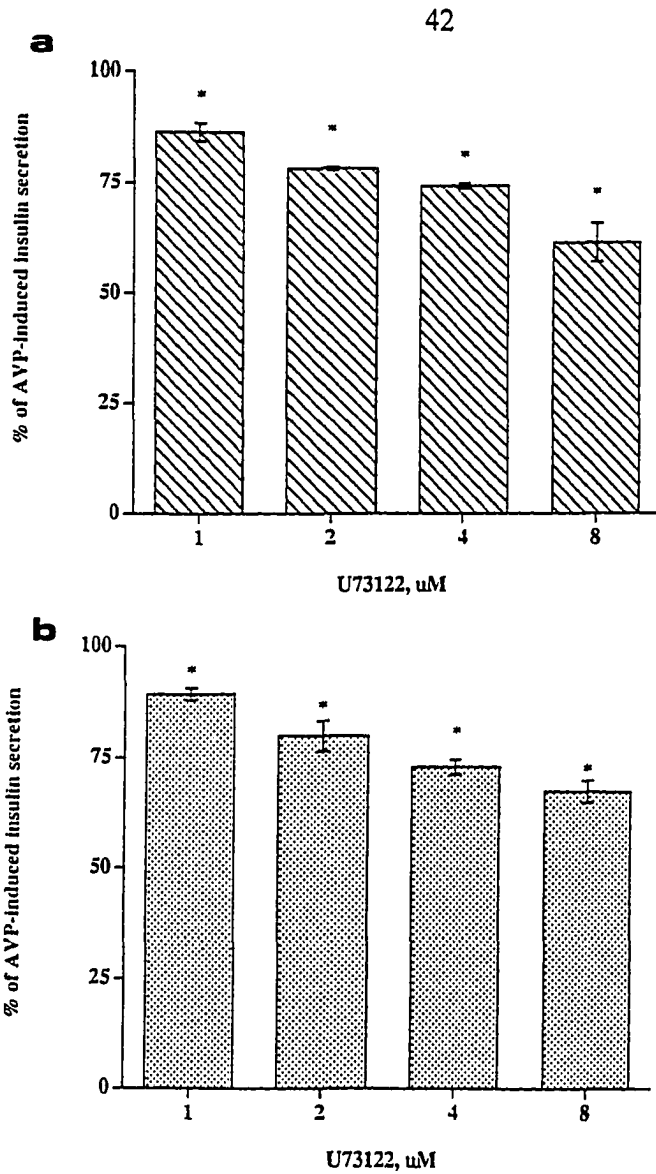
a

b

Fig. 2. Effect of U-73122 on AVP-induced insulin secretion in RINm5F cells. (a) Static incubation for 15 min was performed to measure insulin secretion. U-73122 was given 5 min before AVP (10 nM). (b) Incubation of suspended cells for 5 min was performed. U-73122 was given 100 s before AVP (10 nM). Mean data ± S.E. (n=4) are shown. *p<.05, compared to the AVP (10 nM)-alone group.

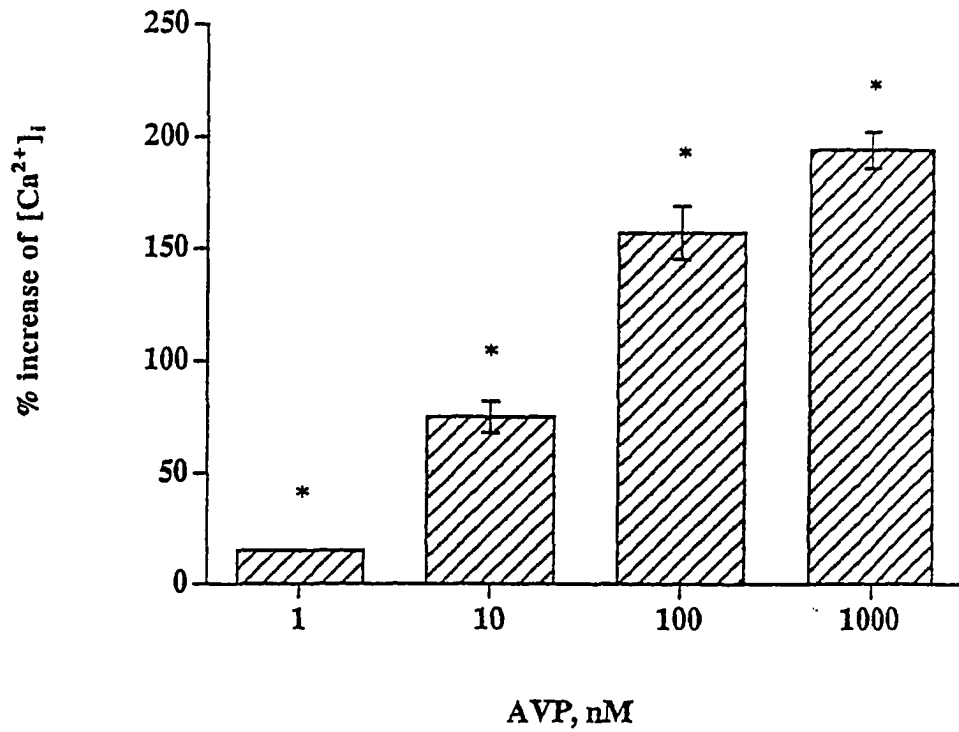Fig. 3. Effect of AVP on the peak $[Ca^{2+}]_i$ increase in RINm5F cells. Mean data $\pm$

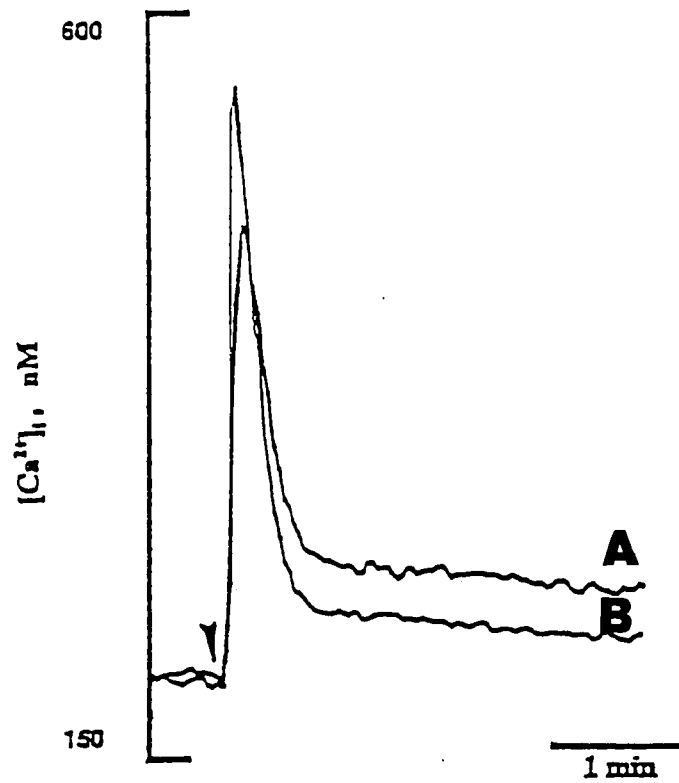S.E. (n=5) are shown. *p<.05, compared to the control group.

Fig. 4. Effect of AVP on $[Ca^{2+}]_i$ increase in RINm5F cells. Curve A shows AVP

(100 nM) and curve B shows AVP (10 µM). Arrow indicates the AVP

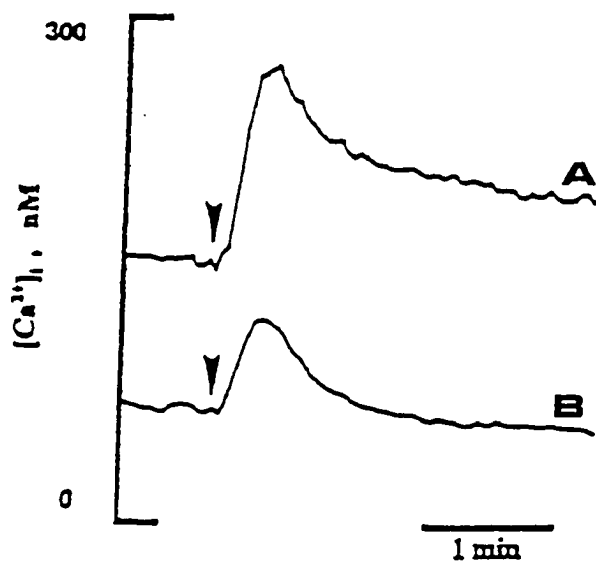adminstration. These are representative tracings, n=5.

Fig. 5. Effect of EGTA on AVP-induced $[Ca^{2+}]_i$ increase in RINm5F cells. Curve

A shows AVP (10 nM) alone as control. EGTA (2 mM) was given 30 s before

AVP (10 nM) in curve B. Arrow indicates the AVP adminstration. These are

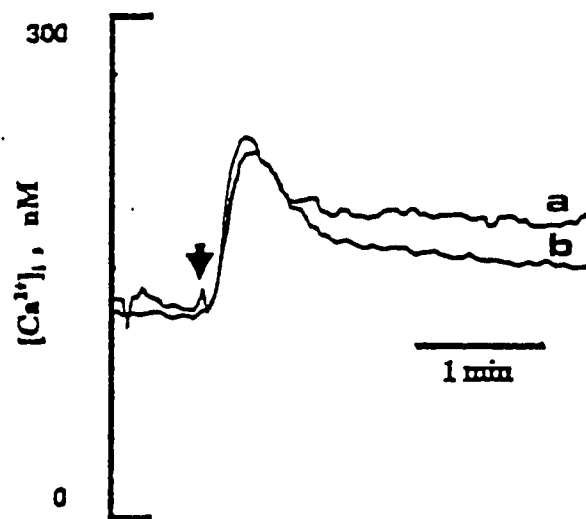representative tracings, n=5.

Fig. 6. Effect of nimodipine on AVP-induced $[Ca^{2+}]_i$ increase in RINm5F cells.

Curve a showed AVP (10 nM) alone as control. Nimodipine 10^{-6} M was given 30

s before AVP (10 nM) in curve b. Arrow indicates the AVP adminstration. These

are representative tracings, n=5.
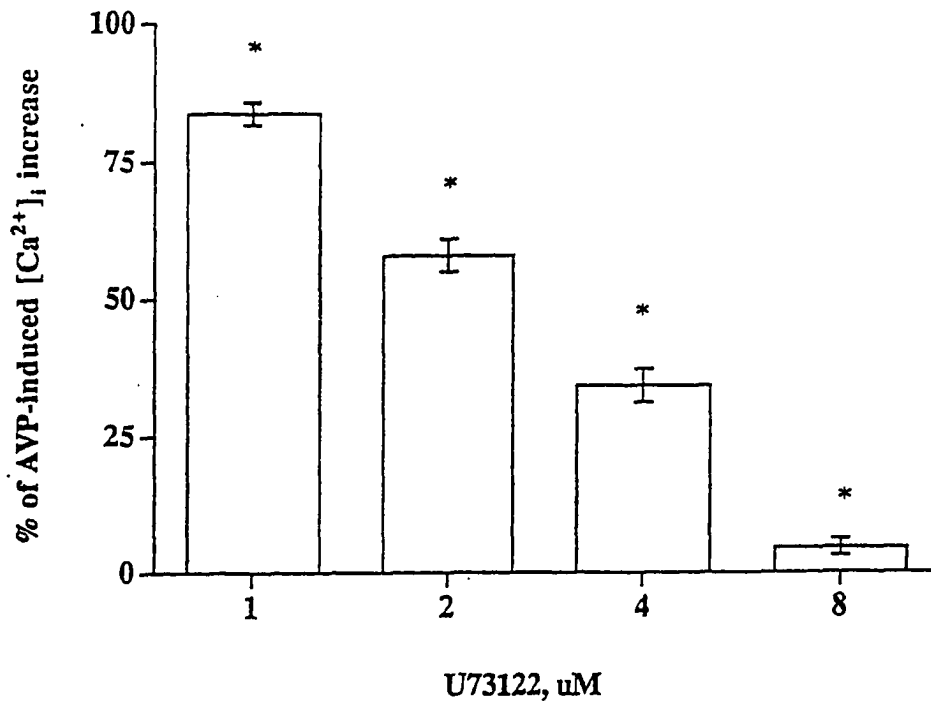
Fig. 7. Effect of U-73122 on AVP-induced peak $[Ca^{2+}]_i$ increase in RINm5F cells.

U-73122 was given 100 s before AVP (10 nM). Mean data ± S.E. (n=6) are

shown. *p<.05, compared to the AVP (10 nM)-alone group.
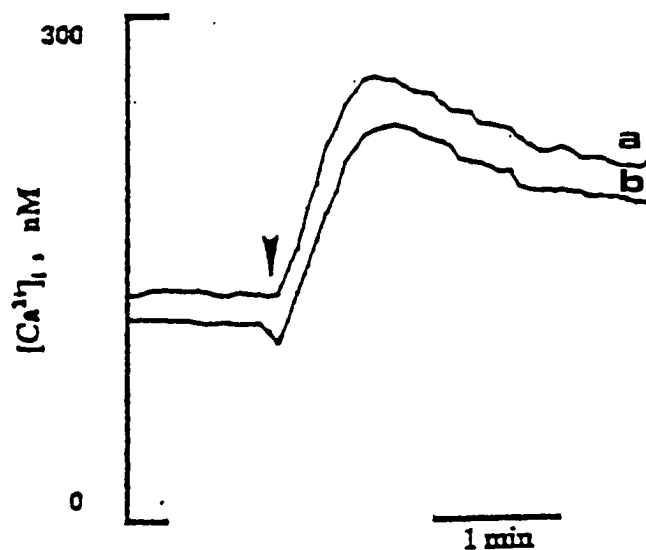
Fig. 8. Effect of U-73122 on ionomycin-induced $[Ca^{2+}]_i$ increase in RINm5F cells.

Curve a shows ionomycin (100 nM) alone as control. U-73122 was given 100 s

before ionomycin (100 nM) in curve b. Arrow indicates the ionomycin

adminstration. These are representative tracings, n=5.

Fig. 9. Inhibition by U-73122 of AVP-induced production of IP$_3$ from RINm5F

cells. U-73122 was given 100 s before AVP (100 nM). The reaction was

terminated after 10 s of AVP application. Mean data ± S.E. (n=4) are shown.

*p<.05, compared to the AVP (100 nM)-alone group.

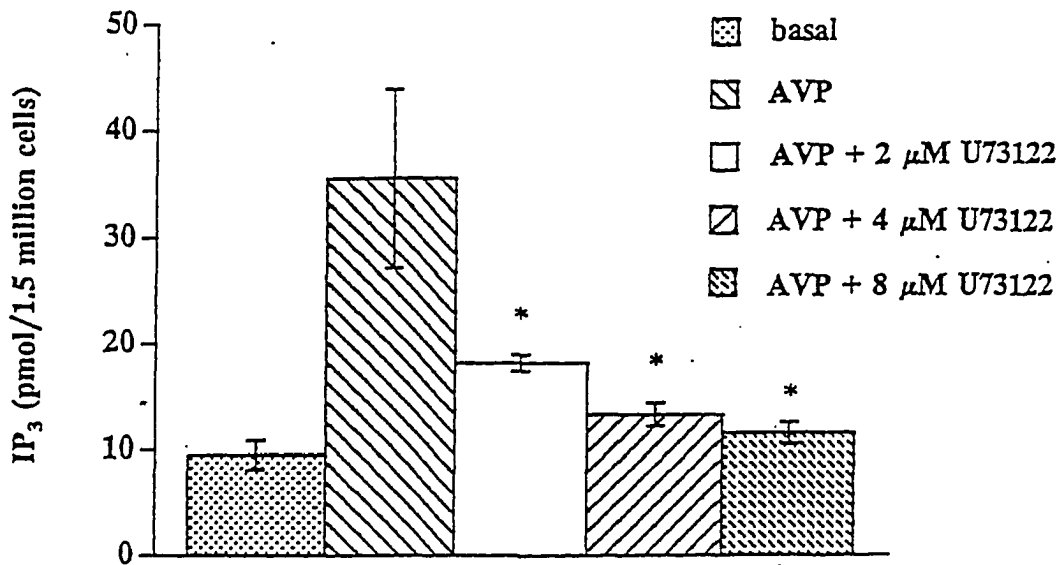# CHAPTER III THE CONTRIBUTION OF PHOSPHOLIPASE D TO AVP-INDUCED INSULIN SECRETION FROM RINm5F CELLS, A PANCREATIC BETA CELL LINE

Ter-Hsin Chen, Bumsup Lee and Walter H. Hsu

ABSTRACT

We studied the effect of wortmannin, an antifungal antibiotic that is a phospholipase D (PLD) inhibitor, on arginine vasopressin (AVP)-induced increases in insulin secretion and intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in RINm5F cells. Wortmannin (0.1 - 1 μM) inhibited AVP-induced increase of insulin secretion dose-dependently. Combination of wortmannin and U-73122, a phospholipase C (PLC) inhibitor, exerted an additive inhibition on AVP-induced increase of insulin secretion. However, wortmannin had no effect on AVP-induced increase of $[Ca^{2+}]_i$. These results suggested: (1) PLD is involved in AVP-induced increase of insulin secretion, (2) PLD does not influence AVP-elicited elevation of $[Ca^{2+}]_i$ and (3) both PLC and PLD participate in AVP-activated signal transduction independently.

INTRODUCTION

Agonists, such as AVP, bradykinin (1), bombesin (2), and prostaglandin $F_{2\alpha}$ (3) respectively activate the GTP-binding protein-coupled receptors and thus stimulate the hydrolysis of phosphatidylcholine (PC) via PLC and PLD. This process leads to an increase in the production of diacylglycerol (DAG) and phosphocholine in one pathway and phosphatidic acid (PA) and choline in the other (4). In a previous study, we found that AVP increased insulin secretion from an insulin-secreting cell line RINm5F (5). However, this effect of AVP was only reduced, but not abolished, when PLC was completely inhibited. Our results suggested that part of AVP-stimulated insulin secretion is mediated by another system (5). Also, PLD activation in the stimulation of insulin release was found in the pancreatic islet cells (6,7). Therefore, we hypothesize that PLD mediates part of the AVP-stimulated insulin secretion. In this present study, we employed a PLD inhibitor, wortmannin, to prove or disapprove our hypothesis. Wortmannin is an antifungal antibiotic isolated from *Penicillium Wortmannin Klocker*, and is widely used in the studies involving the PLD mechanism (8, 9, 10). The present study was performed in RINm5F cells, a clonal beta-cell line, that has been used as a model to study AVP-induced insulin secretion (5, 11, 12, 13).

METHODS

Cell culture. RINm5F cells were maintained in RPMI 1640 contained 10% fetal bovine serum and aerated with 5% CO_2-95% air at 37°C as previous described (5). The cells were subcultured every 7 days, and all experiments were performed with cells in passages 43-53.

Insulin release. RINm5F cells were plated into 24-well costar plates at 2 x 10^5 cells/well and grown for 5 days. Growth medium was removed and the monolayer cells were washed with modified Krebs-Ringer bicarbonate buffer (KRB) containing (in mM): 136 NaCl, 4.8 KCl, 1.2 $CaCl_2$, KH_2PO_4, 1.2 $MgSO_4$, 5 $NaHCO_3$, 10 Hepes, 4 glucose and 0.1% bovine serum albumin, pH 7.4. The cells were then preincubated for a 30-min basal period at 37°C, followed by a 10-min incubation in KRB with the test agent. When needed, U-73122 was given 5 min before the administration of AVP or KCl. The supernatants were collected for the radioimmunoassay (RIA) of insulin as previously described (14).

Measurement of $[Ca^{2+}]_i$. To measure $[Ca^{2+}]_i$ in all suspension, 30x10^6 cells were loaded with 2 μM of fura-2 acetoxymethylester (AM) in KRB for 30 min at 37°C. The loaded cells were washed and kept at 24°C until used. Cells were resuspended at a concentration of 10^6/ml and a 1.5-ml aliquot was used for $[Ca^{2+}]_i$ determination at 24°C. The 340/380 nm fluorescence ratios were monitored using a SLM-8000

Fluorescence spectrophotometer (SLM, Urbana, IL). The $[Ca^{2+}]_i$ was calibrated

after the cell lysis as described previously (14). When needed, U-73122 was given

100 s before the administration of AVP or KCl.

Statistical analysis. Results are presented as mean $\pm$ SE. Differences between

means were determined by using the Student's t test. The significance level was

set at $P<0.05$.

Materials. The following were used: fura-2AM (Molecular Probes, Eugene, OR);

AVP and wortmannin (Sigma Chemical Company, St. Louis, MO); and U-73122

(Biomol Research Laboratory, Plymouth Meeting, PA).

RESULTS

Effect of wortmannin in AVP-induced increase of insulin secretion and $[Ca^{2+}]_i$ in

RINm5F cells.

Pretreatment with wortmannin (0.1-1 μM) for 5 min inhibited AVP (0.1

μM)-induced insulin secretion dose-dependently (Fig. 1). Wortmannin alone did

not change basal insulin release (data not shown). At the highest dose studied,

wortmannin (1 μM) inhibited 0.1 μM AVP-induced insulin release by

approximately 30%. However, pretreatment with wortmannin (1μM) for 5 min

did not affect KCl (10 mM)-induced insulin secretion (data not shown). Although

$[Ca^{2+}]_i$ is the major signal to trigger insulin secretion (15), we found that wortmannin (1 μM) did not change AVP-elicited increase in $[Ca^{2+}]_i$ (data not shown).

Effect of the wortmannin - U-73122 combination in AVP-induced increase of insulin secretion in RINm5F cells.

In the previous study we found that the PLC inhibitor U-73122 partially inhibited AVP-induced insulin secretion and suggested that another component may be involved in AVP-induce insulin secretion (5). In the present study, pretreatment of RINm5F cells with wortmannin and U-73122 (8 μM) caused additive inhibition on AVP-induced insulin release (Fig. 2). As mentioned before, wortmannin (1 μM) inhibited AVP-induced insulin release by 30%, and U-73122 (8 μM) inhibited AVP-induced insulin release by approximately 50%. When wortmannin (1 μM) and U-73122 (8 μM) were combined, they inhibited AVP-induced insulin release by approximately 80%.

DISCUSSION

Phospholipid hydrolysis may play an important intermediary role in the stimulus-secretion coupling of insulin release (16). Physiologic agonists activate phospholipases and produce different lipid messengers which amplify insulin secretion (17). In our previous study, we found that AVP-induced insulin secretion

may be mediated by multiple signal transduction pathways (5). The downstream

effectors, such as PLC and PLA_2, have beeen investigated (5). Another possible

candidate in the AVP-induced signal transduction pathway is PLD. The result of

the present study suggested that PLD is involved in AVP-induced insulin secretion.

Our results further suggested that PLC and PLD are independent components in the

AVP's action, because the PLD inhibitor, wortmannin, and PLC inhibitor, U-

73122, caused an additive inhibition on AVP-induced insulin secretion. This is

consistent with the finding in liver cells (18, 19). However, the interaction

between PLC and PLD is different in different systems. In some systems,

activation of PLD is dependent on activation of PLC (21, 22, 23, 26). In other

systems, activation of PLD is independent of activation of PLC (18, 19). AVP

activates PLC and PLD in several models, such as hepatocytes (20), myoblasts

(21), Swiss 3T3 fibroblasts (22), smooth muscle cells (23), Leydig cells (24),

mesangial cells (25). Our finding suggested that AVP increases insulin secretion in

RINm5F cells by activating PLC and PLD independently.

Ca^{2+} plays a central role in cell signaling, both as a second messenger and

as a regulator of signaling effector enzymes (25). In RINm5F cells, AVP elicited

a peak followed by a sustained rise in $[Ca^{2+}]_i$ (5). The peak increase of $[Ca^{2+}]_i$ is

mediated by the release of Ca^{2+} from intracellular Ca^{2+} stores and the sustained rise

in $[Ca^{2+}]_i$ is maintained by influx of extracellular Ca^{2+} (5). The sustained rise in

$[Ca^{2+}]_i$ is essential for the activation of PLD (26). However, the effect of PLD on

$[Ca^{2+}]_i$ is not well understood. In the present study, we found that the PLD

inhibitor wortmannin inhibited AVP-induce increase in insulin secretion but did not

influence AVP-elicited increase in $[Ca^{2+}]_i$. This result suggested that PLD may be

only involved in the machinery of insulin secretion but not in the elevation of

$[Ca^{2+}]_i$. PLD catalyzes the formation of PA and DAG (4). Additional studies are

needed to further characterize the downstream signal transduction of PLD in the

regulation of insulin secretion or the role of PA and DAG on insulin secretion.

Wortmannin may exert a specific inhibitory effect on the activation of PLD

in beta-cells. Although wortmannin also is a potent inhibitor of

phosphatidylinositol 3 (PI3)-kinase inhibitor (27), PI3-kinase plays a negative feed-

back role on insulin secretion in the pancreatic beta-cell (28). If wortmannin had

inhibited both PLD and PI3-kinase in the present study, its real contribution to

AVP's action would have been underestimated. Therefore, our finding suggested

that PLD activation contributes to AVP-induced insulin secretion.

We concluded that in RINm5F cells: 1) PLD was involved in AVP-induced

increase of insulin secretion, 2) PLD did not influence AVP-elicited elevation of

$[Ca^{2+}]_i$, and 3) both PLC and PLD participated in the AVP-activated signal

transduction independently.

ACKNOWLEDGMENTS

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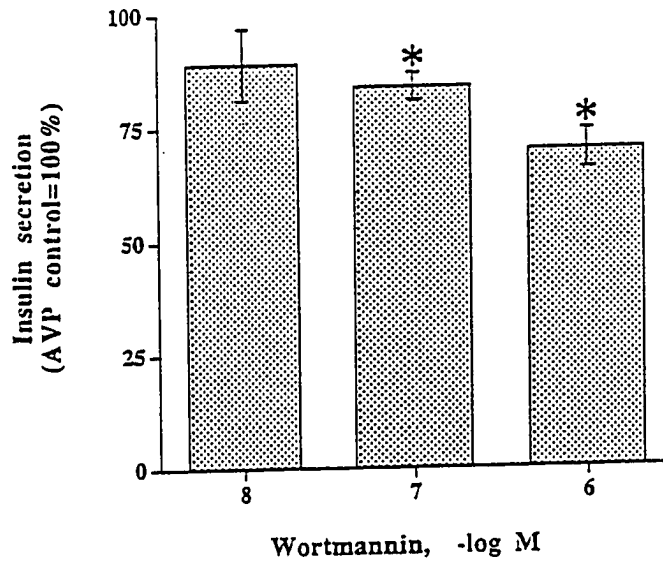
Fig. 1. Effect of wortmannin in AVP-induced increase of insulin release in

RINm5F cells. Static incubation for 10 min was performed to measure insulin

release. Wortmannin was given 5 min before the administration of AVP (0.1 μM).

Mean data $\pm$ SEM are shown (n=4). *p<0.05, compared with the control group.
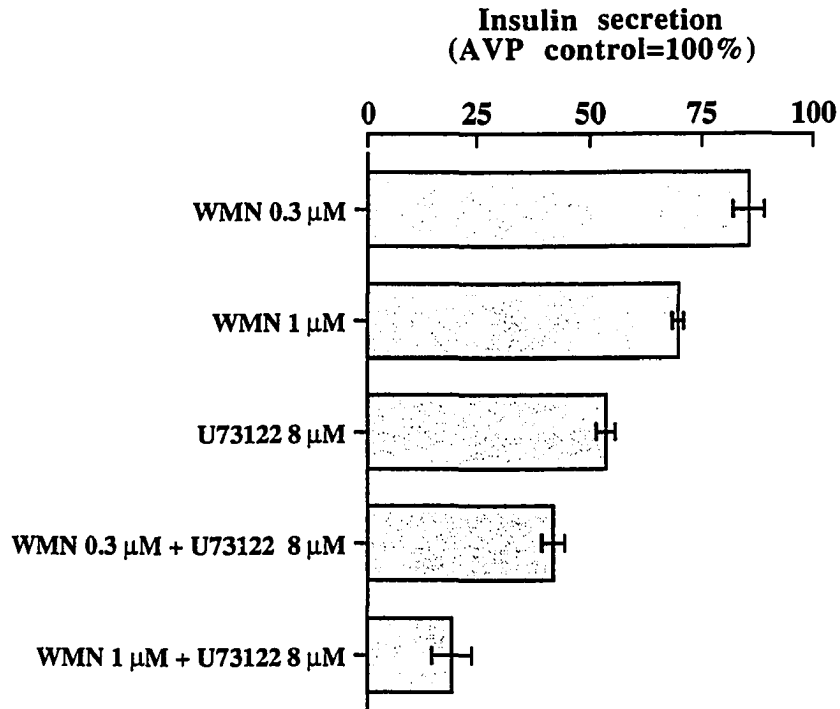
Insulin secretion
(AVP control=100%)

Fig. 2. Effect of wortmannin (WMN) - U-73122 combination on AVP-induced

increase of insulin secretion and in RINm5F cells. The experimental condition was

the same as in Fig. 1. U-73122 was given 5 min before the administration of AVP

(0.1 μM). Mean data ± SEM are shown (n=4). Each group is different from

others.

CHAPTER IV   THE INHIBITORY EFFECT OF PROTEIN KINASE C IN ARGININE VASOPRESSIN-INDUCED INSULIN SECRETION AND Ca^{2+} MOBILIZATION IN A PANCREATIC BETA-CELL LINE RINm5F

Ter-Hsin Chen, Bumsup Lee and Walter H. Hsu

ABSTRACT

The role of protein kinase C (PKC) in arginine vasopressin (AVP)-induced

insulin secretion was investigated in RINm5F cells. Ro 31-8220, a specific PKC

inhibitor, dose-dependently potentiated AVP-induced insulin secretion and elevation

of intracellular Ca^{2+} concentration ([Ca^{2+}]$_i$) when givn 30 min before AVP

administration. However, Ro 31-8220 did not enhance KCl-induced insulin

secretion or elevation of [Ca^{2+}]$_i$. In Ca^{2+}- or Na$^+$-free medium, Ro 31-8220 did not

change AVP-induced elevation of [Ca^{2+}]$_i$. Pretreatment with an analog of

diacylglycerol, 1-Oleoyl-2-acetyl-sn-glycerol (OAG) for 5 min, inhibited AVP-

induced insulin secretion dose-dependently, and slightly but significantly inhibited

AVP-induced elevation of [Ca^{2+}]$_i$. OAG alone did not change basal insulin

secretion or [Ca^{2+}]$_i$, nor did it change KCl-induced insulin secretion or elevation of

[Ca^{2+}]$_i$.

We conclude that: 1) PKC may serve as a negative regulator in RINm5F

cells to diminish AVP-induced insulin secretion, 2) PKC may decrease AVP-

induced insulin secretion at least partly by inhibiting Ca^{2+} channels, and 3) PKC

may not influence AVP-induced intracellular Ca^{2+} release.

INTRODUCTION

PKC is activated by diacylglycerol (DAG) which is generated via the

receptor-activated phospholipases by a number of hormones and neurotransmitters

such as vasopressin, oxytocin, acetylcholine and bombesin (Ashcroft, 1994). PKC

has been purified in beta-cells and exists as a monomer of 80 kD (Lord and

Ashcroft, 1984). There are 10 sub-types of Ca^{2+}-, phospholipid-dependent PKC

that have been identified, and at least two isoforms (α, β) are known to be present

in the pancreatic beta-cells (Ashcroft, 1994; Wilkinson and Hallam, 1994). Also a

number of endogenous substrates for PKC have been identified in rodent pancreatic

beta-cells (Hughes and Ashcroft, 1988).

In the mouse islet and two clonal pancreatic beta-cell lines (HIT and

RINm5F) activation of PKC may stimulate or inhibit insulin secretion depending

on the experimental conditions (Gao et al., 1994). Some reports showed that PKC

increased insulin secretion (Monaco et al., 1988; Yada et al., 1989), whereas others

concluded that PKC played a negative feedback role in insulin secretion (Li et al.,

1991). One cannot draw a clear-cut conclusion from these contradictory findings

with regard to the role of PKC in pancreatic beta-cells. The reasons for the

discrepancies among these reports are not known; however one possibility is that

the tools used in the investigation of PKC may have other effects (Wilkinson and

Hallam, 1994). For example, PKC inhibitors may have additional effects

independent of the PKC inhibition. Also, prolonged exposure to PKC activators

may cause downregulation of PKC. Furthermore, some PKC isoforms, such as

atypical PKC, are not downregulated by the pretreatment with phorbol-12-

myristate-13-acetate (PMA) and the absence of downregulation by phorbol esters

does not exclude the participation of PKC (Selbie et al., 1993). Phorbol esters, the

activators of PKC, usually produce a strong and prolonged activation of PKC when

compared with the transient activation of PKC generated by DAG analogs

(Wilkinson and Hallam, 1994). These existing pharmcological tools for studying

PKC may make the interpretation of findings more difficult. Recently, a series of

derivatives of staurosprine such as Ro 31-8220 have been developed as potent and

specific PKC inhibitors (Davis et al., 1989).

In the present study, we used a highly specific PKC inhibitor Ro 31-8220

and a DAG analog OAG to investigate the role of PKC on AVP-induced insulin

secretion and Ca^{2+} mobilization in RINm5F cells.

METHODS

Cell Culture. RINm5F cells were maintained in RPMI 1640 containing 10% fetal

bovine serum and aerated with 5% CO_2-95% air at 37°C as previous described

(Chen et al., 1994). All experiments were performed using passages 43-53.

Insulin Secretion. RINm5F cells were plated into 24-well Costar plates at 2 x 10^5

cells/well and grown for 5 days. Growth medium was removed and the monolayer

cells were washed with modified Krebs-Ringer bicarbonate buffer (KRB)

containing (in mM): 136 NaCl, 4.8 KCl, 1.2 $CaCl_2$, KH_2PO_4, 1.2 $MgSO_4$, 5

$NaHCO_3$, 10 Hepes, 4 glucose and 0.1% bovine serum albumin, pH 7.4. The cells

were then preincubated for a 30-min basal period at 37°C, followed by a 15-min

incubation in KRB with the test agent. The Ca^{2+}-free KRB was supplemented with

10 µM [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA). When needed,

Ro 31-8220 was given 30 min before the administration of AVP or KCl. The

supernantants were collected for the radioimmunoassay (RIA) of insulin (Hsu et

al., 1991).

Measurement of $[Ca^{2+}]_i$. To measure $[Ca^{2+}]_i$ in the cell suspension, 30 x 10^6 cells

were loaded with 2 µM of fura-2 actoxymethylester (AM) in KRB for 30 min at

37°C. The loaded cells were washed and kept at 24°C until use. Cells were

resuspended at a concentration of 10^6/ml and 1.5-ml aliquots were used for $[Ca^{2+}]_i$

determination at 24°C. The 340/380 nm fluorescence ratios were monitored using a SLM-8000 Fluorescence spectrophotometer (SLM, Urbana, IL). The $[Ca^{2+}]_i$ was calibrated after cell lysis as previously described (Hsu et al., 1991). When need, Ro 31-8220 was given 30 min before the administration of AVP or KCl.

Statistical Analysis. Results were analyzed by using the Student's t test for paired and unpaired values. The significance level was set at P<.05.

Materials. The following were used: Nimodipine (Research Biochemicals International, Natick, MA); Fura-2AM (Molecular probes, Eugene, OR); OAG (Sigma Chemical Co., St. louis, MO); SKF 96365 (Biomol Co., Plymouth meeting, PA); Ro 31-8220 (Roche Products, Hertfordshire, U.K.)

RESULTS

Enhancement by Ro 31-8220 of AVP-induced increase of $[Ca^{2+}]_i$ in RINm5F cells.

AVP induced a sharp and transient increase in $[Ca^{2+}]_i$ which was followed by a lower sustained phase in $[Ca^{2+}]_i$ (Fig. 1). Ro 31-8220 (1-10 μM) alone did not change $[Ca^{2+}]_i$ (data not shown). After the pretreatment with the PKC inhibitor Ro 31-8220 (1-10 μM), the sustained phase of AVP (0.1 μM)-induced increase of $[Ca^{2+}]_i$ was enhanced dose-dependently (Fig. 1). Ro 31-8220 (10 μM) did not change the AVP (0.1 μM)-induced transient increase of $[Ca^{2+}]_i$ (data not shown).

In a Ca^{2+}-free medium, AVP (0.1 μM) only induced a transient increase of $[Ca^{2+}]_i$ which is due to the Ca^{2+} release of Ca^{2+} from intracellular stores. The pretreatment with Ro 31-8220 (10 μM) did not enhance KCl (10 mM)-induced increase in $[Ca^{2+}]_i$ (Fig. 2). KCl (10 mM) increased $[Ca^{2+}]_i$ by depolarizing the membrane potential and opening voltage-dependent Ca^{2+} channels.

The sustained phase of AVP-induced increase of $[Ca^{2+}]_i$ is mediated by Ca^{2+} influx through Ca^{2+} channels (Chen et al., 1994). In order to determine if the enhancement by Ro 31-8220 of AVP-induced increase in $[Ca^{2+}]_i$ was due to opening of Ca^{2+} channels, SKF 96365, a receptor-operated Ca^{2+} channel (ROC) blocker, and nimodipine, a voltage-dependent Ca^{2+} channel blocker, were applied. Both of them significantly reduced Ro 31-8220 (10 μM)-elicited enhancement (Fig. 3).

Enhancement by Ro 31-8220 of AVP-induced increase of insulin secretion in RINm5F cells.

Pretreatment with Ro 31-8220 (1-10 μM) for 30 min enhanced AVP (0.1 μM)-induced insulin secretion dose-dependently (Fig. 4a). The results of the $[Ca^{2+}]_i$ experiment suggested that Ro 31-8220 enhanced the AVP-induced sustained phase of the $[Ca^{2+}]_i$ increase. In order to test the mechanism of the enhancement in insulin secretion, we used Ca^{2+}-free KRB in the insulin secretion experiment. Ro 31-8220 (10 μM) still enhanced AVP (0.1 μM)-induced insulin secretion in the Ca^{2+} free condition (Fig. 4b). But the amplitude of enhancement was reduced.

The basal insulin secretion (34 ± 5 ng/min/well) in the absence of extracellular Ca^{2+} was approximately 1/5 of the basal insulin secretion (150 ± 12 ng/min/well) in the presence of extracellular Ca^{2+}.

Nimodipine (1 µM), an L-type Ca^{2+} channel blocker, reduced but did not abolish the stimulatory effect of Ro 31-8220 on AVP-induced insulin secretion (Fig. 5). The pretreatment with Ro 31-8220 (10 µM) did not enhance KCl (10 mM)-induced insulin secretion (data not shown).

Effect of OAG on AVP-induced increase of $[Ca^{2+}]_i$ in RINm5F cells.

Stimulation of the cells with the membrane-permeable DAG analog OAG (30 µM) did not change the basal $[Ca^{2+}]_i$ (data not shown). Pretreatment with OAG (30 µM) produced a small but significant decrease in AVP-induced increase of $[Ca^{2+}]_i$ [AVP : AVP + OAG = 131.5 ± 4.3 nM : 149.2 ± 5.1 nM, $p<0.05$, (n=4)] (Fig. 6). In the Ca^{2+}-free medium, OAG (30 µM) did not change the AVP (0.1 µM)-induced transient increase of $[Ca^{2+}]_i$ (data not shown). Pretreatment with OAG (30 µM) did not influence KCl (10 mM)-induced increase in $[Ca^{2+}]_i$ (data not shown).

Inhibition by OAG of AVP-induced increase of insulin secretion in RINm5F cells.

OAG (30 µM) did not change basal insulin secretion (data not shown). Pretreatment with OAG (1-30 µM) for 5 min inhibited AVP (0.1 µM)-induced insulin secretion dose-dependently (Fig. 7), although OAG (30 µM) only slightly

reduced the AVP-induced increase of $[Ca^{2+}]_i$ (Fig. 6). Pretreatment with OAG (30

μM) did not change KCl (10 mM)-induced insulin secretion (data not shown).

DISCUSSION

The role of PKC in beta-cells has been studied with clonal beta-cell lines

and pancreatic beta-cells for a decade (Gao et al., 1994). However, most of the

findings are difficult to interpret. Even in an insulin secreting cell line (RINm5F

cells) and normal pancreatic beta-cells, PKC performs differently in response to

different PLC-mediated stimulators. The limitation in the study of PKC is the

lack of specific investigating tools. The members of the family of PKC have

different characteristics, such as Ca^{2+} sensitivity/insensitivity, and phorbol ester

sensitivity/insensitivity (Wilkinson and Hallam, 1994). Phorbol esters are

frequently used as a tool to study PKC. Phorbol esters stimulate PKC acutely but

overnight incubation of them inhibits PKC (Ashcroft, 1994). Phorbol esters may

cause some effects such as inducing Ca^{2+} influx which are not related to PKC

activation (Wilkinson and Hallam, 1994). A series of potent and highly selective

inhibitors have been developed based on the structure of staurosporine which is a

non-specific PKC inhibitor (Davis et al., 1989). Recently Ro 31-8220, a specific

PKC inhibitor, was used in the study of the cluster of differentiation (CD) 4- and

CD 8- induced intracellular signalling (Ravichandran and Burakoff, 1994) and

glucose-stimulated insulin secretion in the rat islets (Persaud and Jones, 1995). In

the present study, we applied Ro 31-8220 to investigate the role of PKC in AVP-induced insulin secretion in RINm5F cells. We found that Ro 31-8220 enhanced AVP-induced insulin secretion and elevation of $[Ca^{2+}]_i$. This result suggested that PKC negatively regulates AVP-induced insulin secretion and elevation of $[Ca^{2+}]_i$. This finding is consistent with that in RINm5F cells and normal mouse pancreatic beta-cells in that PKC plays a negative feedback role on the signal transduction mechanisms involving PLC (Gao et al., 1994, Li et al., 1990).

Ca^{2+}-free medium was used to further investigate the relationship of insulin secretion and $[Ca^{2+}]_i$ in Ro 31-8220-elicited enhancement. In the absence of extracellular Ca^{2+}, Ro 31-8220 failed to change AVP-induced increase in $[Ca^{2+}]_i$. This finding suggested that PKC decreases AVP-induced insulin secretion by inhibiting Ca^{2+} influx. The Ca^{2+} influx may be mediated by Ca^{2+} channels or the Na$^+$/Ca^{2+} exchanger. However, in the Na$^+$-free medium, Ro 31-8220 failed to change AVP-induced Ca^{2+} influx (data not shown). These results suggested that PKC inhibits Ca^{2+} influx through Ca^{2+} channels. In mouse pancreatic beta-cells, PKC plays a negative role in the regulation of VDCCs (Arkhammar et al., 1994). PKC inhibits the AVP-induced increase of $[Ca^{2+}]_i$ in HIT cells (Hughes et al., 1992). PKC activation is associated with a lowering of intracellular Ca^{2+} in HIT cells (Hughes et al., 1989). Also, activation of PKC inhibits T cell receptor (TCR)-mediated Ca^{2+} influx in HPB-ALL T cells (Shivnan and Alexander, 1995). In this study, we found the L-type Ca^{2+} channel blocker, nimodipine, reduced but did not abolish Ro 31-8220-enhanced AVP-induced Ca^{2+} influx. However, SKF

96365, a blocker of ROC, caused greater inhibition than nimodipine on Ro 31-8220's effect. This suggested that PKC inhibits not only VDCCs but also ROC.

The use of phorbol esters and DAG analogs to mimic the responses stimulated by physiological ligands has provided the evidence to support the potential role of PKC in ligand-induced cellular responses (Wilkinson and Hallam, 1994). In this study we used a DAG analog, OAG, to mimic the effect of DAG. We found that OAG (30 μM) had no effects on basal insulin secretion, but it inhibited AVP-induced insulin secretion. This finding suggested that PKC negatively regulates insulin secretion. Acute treatment with phorbol 12-myristate 13-acetate (PMA) ether enhanced (Monaco et al., 1988) or abolished (Li et al., 1991) AVP-induced insulin in RINm5F cells, but both studies showed that PMA inhibited AVP-stimulated production of inositol phosphate (Monaco et al., 1988, Li et al., 1991). Because phorbol esters stimulate insulin secretion by membrane depolarization and increase $[Ca^{2+}]_i$ in RINm5F cells (Yada et al., 1989) through mechanisms independent of PKC activation, this makes the interpretation of the PMA results difficult. As mentioned earlier, PKC may inhibit Ca^{2+} channel activity (Arkhammar et al., 1994); however, OAG only slightly reduced AVP-induced increase of $[Ca^{2+}]_i$ while it caused a greater inhibition on insulin secretion. Perhaps the effect of AVP-activated PKC on Ca^{2+} channels reached near maximum in the present study (as seen in the results of the Ro 31-8220 experiment). Under this circumstance, OAG may have caused a small activation of PKC and a small inhibition of AVP-induced increase of $[Ca^{2+}]_i$. More activation of PKC did slightly

enhance the inhibition on Ca^{2+} channels.

An alternative explanation would be that PKC inhibits partially insulin secretion through a Ca^{2+}-independent pathway in pancreatic beta cells.

In conclusion, our findings suggested that: 1) PKC is a negative regulator in AVP's action, 2) PKC inhibits the Ca^{2+} channels, thereby decreasing insulin secretion, and 3) PKC has no effect on AVP-induced intracellular Ca^{2+} release.

ACKNOWLEDGEMENTS

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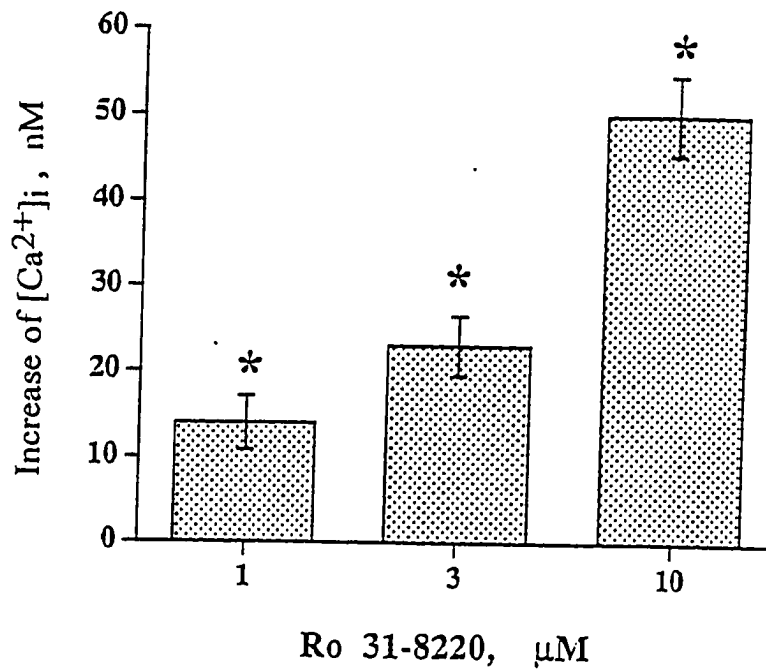
Fig. 1. Effect of Ro 31-8220 on AVP-induced sustained phase of increase in $[Ca^{2+}]_i$ in RINm5F cells. Ro 31-8220 was given 30 min before AVP (0.1 μM). $[Ca^{2+}]_i$ was measured 150 s after the administration of AVP. Mean ± S.E. (n = 4) are shown. *P<.05, compared to the AVP (0.1 μM) alone group.
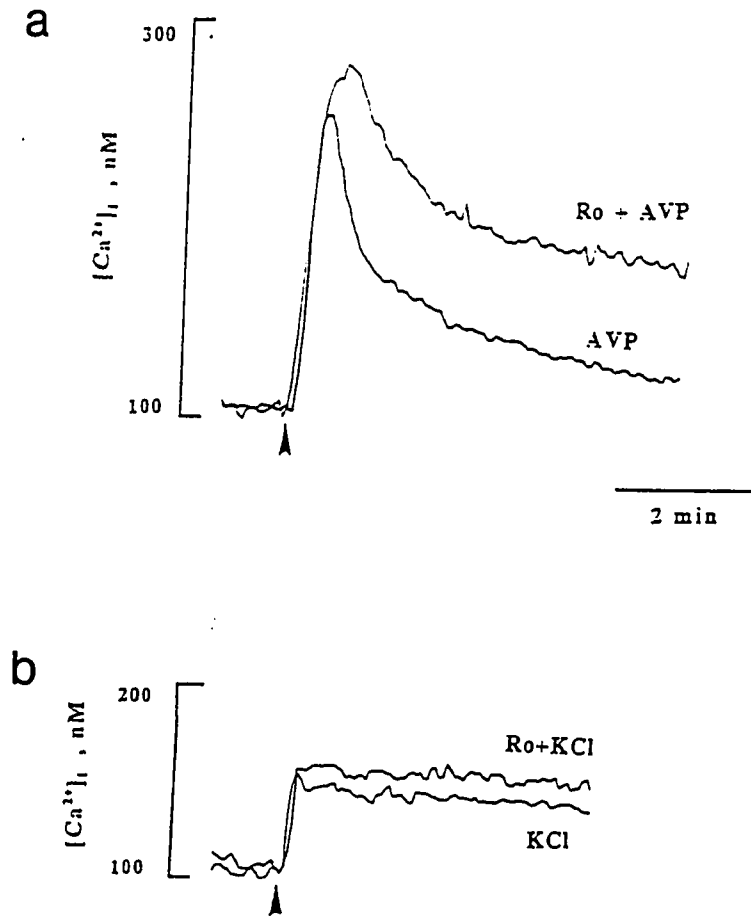
Fig. 2. Effect of Ro 31-8220 on AVP (a)- and KCl (b)-induced increase in $[Ca^{2+}]_i$. Ro 31-8220 (10 μM) was given 30 min before the administration of KCl (10 mM) or AVP (0.1 μM). These are representative tracings of four experiments.
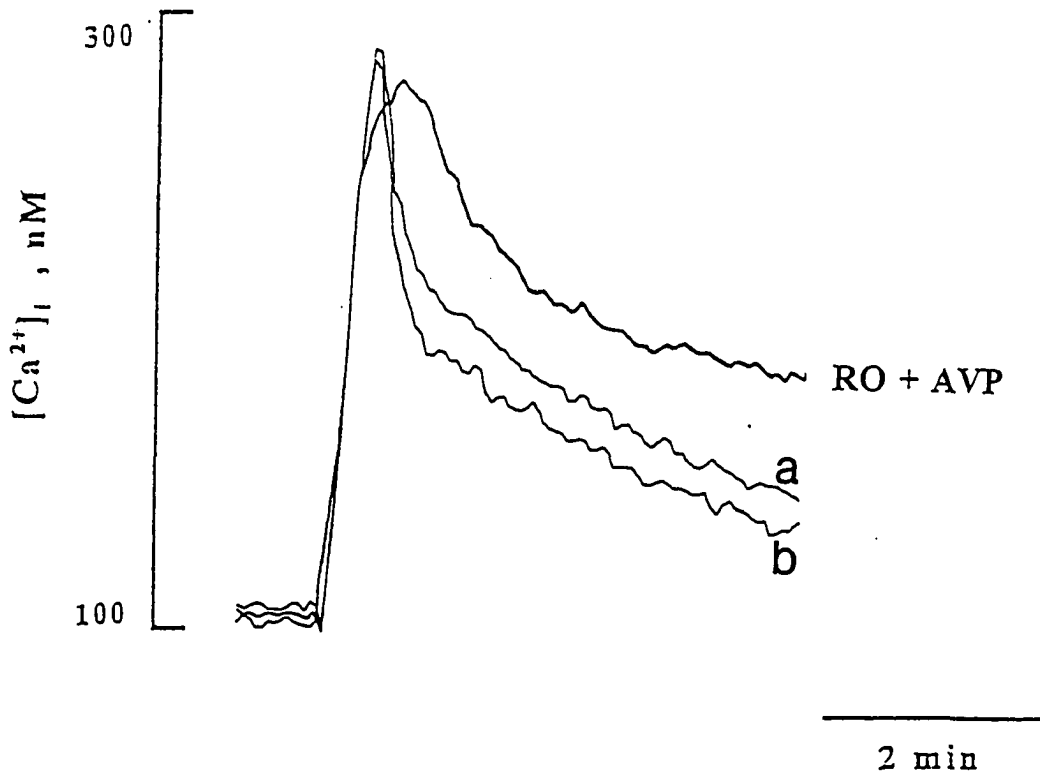
Fig. 3. Effect of SKF 96365 (a) and nimodipine (b) on Ro 31-8220 enhancement of AVP-induced increase of $[Ca^{2+}]_i$. Ro 31-8220 was given 30 min before the administration of AVP. SKF 96365 (30 μM) or nimodipine (1 μM) was given 100 s before the administration of AVP. These are representative tracings of four experiments.
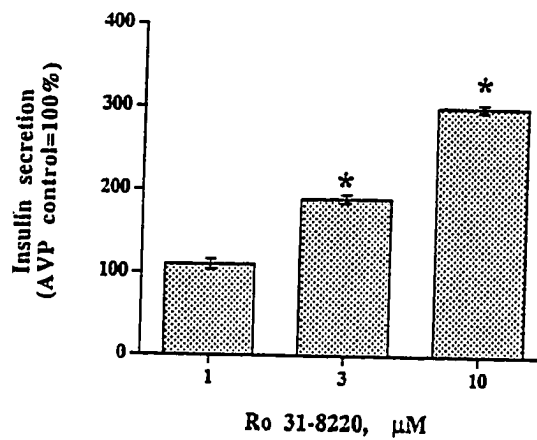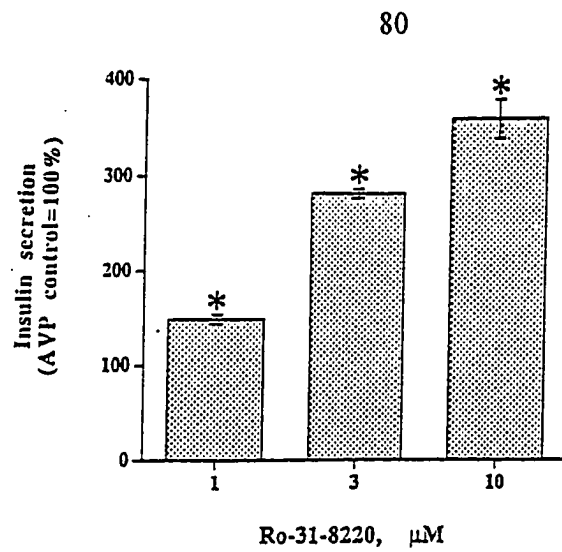
Fig. 4. Effect of Ro 31-8220 on AVP-induced insulin secretion. In the presence

(a) and absence (b) of extracellular Ca^{2+} static incubation for 10 min were

performed to measure insulin secretion. Ro 31-8220 was given 30 min before

AVP (0.1 μM). Mean data ± S.E. (n = 4) are shown. *P<.05, compared to the

AVP (0.1 μM) alone group.
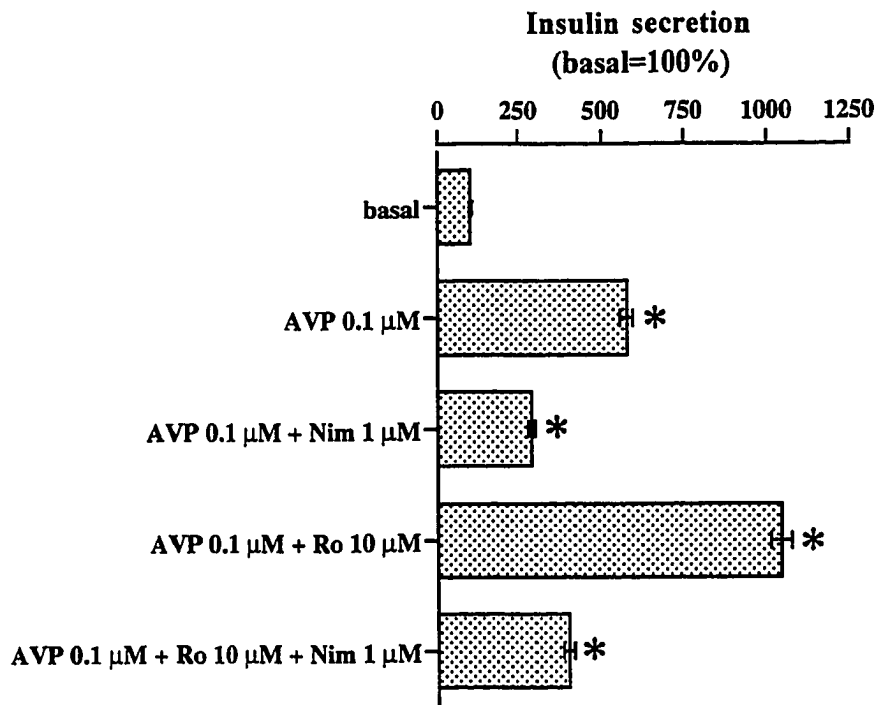
**Insulin secretion
(basal=100%)**

Fig. 5. Effect of nimodipine on the enhancement of Ro 31-8220 on AVP-induced
increase of $[Ca^{2+}]_i$. Ro 31-8220 was given 30 min before the administration of
AVP. Nimodipine (Nim) (1 μM) was given 5 min before the administration of
AVP (0.1 μM). Mean data $\pm$ S.E. (n = 4) are shown. *$P<.05$, compared to the
control (basal) group.
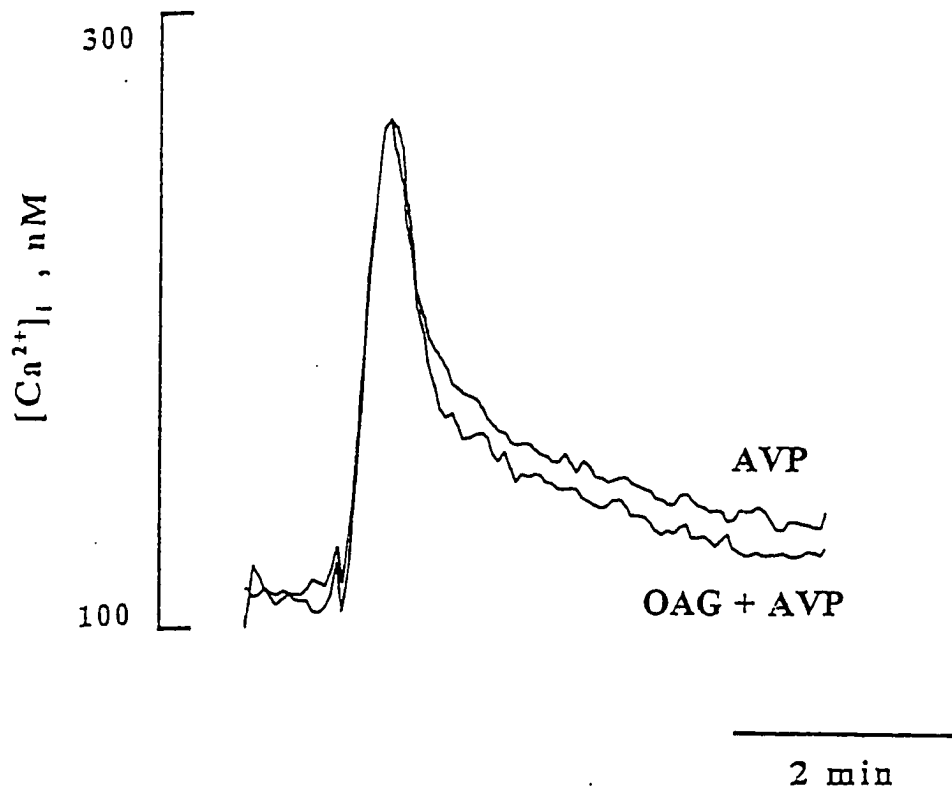
Fig. 6. Effect of OAG on AVP-induced $[Ca^{2+}]_i$ increase. OAG (30 μM) was given

30 s before the administration of AVP (0.1 μM). These are representative tracings

of four experiments.

Fig. 7. Effect of OAG on AVP-induced insulin secretion. Static incubation for 10 min was performed to measure insulin secretion. OAG was given 5 min before AVP (0.1 µM). Mean data ± S.E. (n = 4) are shown. *P<.05, compared to the AVP (0.1 µM) alone group.

CHAPTER V   GENERAL DISCUSSION
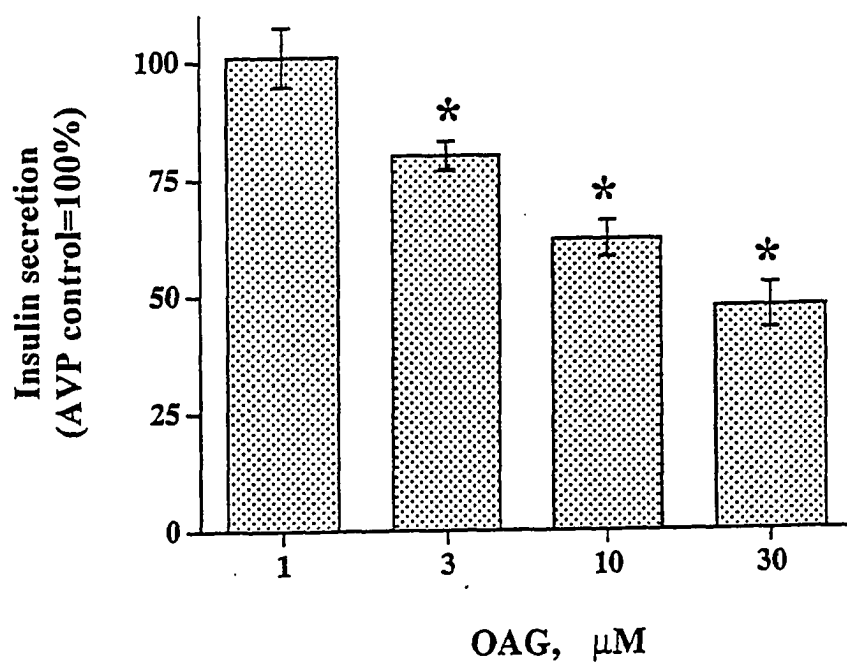
The results presented in this dissertation have already been discussed in the

Discussion section of each chapter. In this chapter, the delineation of these

chapters will be discussed in general.

AVP-induced multiple signal transduction pathways in pancreatic beta-cells

The hormone or neurotransmitter that carries information into the cells is

received by the cells when it binds to a specific receptor. This receptor then

mediates the activation of a secondary process to increase the concentration of the

second messenger in the cytosol of the cell. The cascade of signal transduction is

amplified in each step toward downstream effectors (Offermanns and Schultz,

1994). In the pancreatic beta-cell, AVP affects multiple signal transduction

pathways (Gao and Henquin, 1993). When AVP binds to the vasopressin receptor,

it activates the G protein which in turn activates PLC and PLD. In some other cell

models AVP also activates PLA_2 (Briley et al., 1994), but this phenomenon was

not seen in RINm5F cells. Activation of PLC hydrolyzes the PIP_2 to generate IP_3

and DAG. IP_3 releases Ca^{2+} from intracellular cellular stores, which in turn

induces Ca^{2+} influx. This is so called the "capacitative" mechanism (Putney, 1990).

However, the mechanism to cause Ca^{2+} influx after the rapid intracellular Ca^{2+}

release is still not well-understood and is under investigation (Fasolate et al., 1994).

According to the findings of this study, the capacitative mechanism is involved in

AVP-induced Ca^{2+} influx, and part of the Ca^{2+} influx is mediated through VDCCs. Similar observations have been found in several other cell models such as chromaffin cells and pituitary cells (Fasolate et al., 1994, Zheng et al., 1995).

Even though AVP activates multiple signal transduction pathways in the pancreatic beta-cell, the increase of $[Ca^{2+}]_i$ is still the major signal for triggering insulin release. When extracellular Ca^{2+} is absent, the AVP-induced insulin release is dramatically inhibited. This is consistent with other reports in beta cells (Ashcroft et al., 1994). Other endocrine cells such as chromaffin cells have similar dependency on extracellular Ca^{2+} (Augustine and Neher, 1994). In several systems, a Ca^{2+}-independent exocytosis exists (Arispe et al., 1992, Steuenkel and Nordmann, 1993). In the pancreatic beta-cell, it is generally accepted that the Ca^{2+}-dependent component is the major factor in the regulation of insulin release (Ammala et al., 1993). We found that the presence of extracellular Ca^{2+} is essential for AVP-induced insulin release, but part of the insulin release induced by AVP was independent of the increase of $[Ca^{2+}]_i$. This suggested that some other intracellular effectors operate to trigger insulin release when the $[Ca^{2+}]_i$ is at basal level. It is possible that a Ca^{2+}-independent component is involved in AVP-induced insulin secretion. However, this issue is still debatable, because it is possible that the concentration of intracellular Ca^{2+} under the plasma membrane was heterogenous and underwent changes which were not detected by the technique applied in this study. Further investigation is needed to study AVP-induced increase in $[Ca^{2+}]_i$ by using specific tools which can determine the local changes of the $[Ca^{2+}]_i$ (Etter et

al., 1994), or using confocal microscopy which can determine the 3-D dimension

of the cell structure. These advanced tools will provide more precise measurement

in $[Ca^{2+}]_i$ and reveal the accurate changes of $[Ca^{2+}]_i$ in a specific region of the cell.

The contribution of phospholipases to AVP-induced insulin secretion

In pancreatic beta-cells, different receptors which are coupled to the G_q

protein, also activate multiple signal transduction pathways (Regazz et al., 1990).

The results of this study suggest that PLC contributes to approximately 50% of the

AVP-induced insulin secretion and is the major cytosolic effector in AVP's action.

However, PLA_2 was not involved in the AVP-induced insulin secretion from

RINm5F cells. The activation of PLD contributes to approximately 30% of AVP-

induced insulin secretion. Similar observation were found in other endocrine

systems, such as chromaffin cells (Holbrook et al., 1992). PLD has become more

significant in signal transduction, because studies revealed that the products from

the catalysis by PLD, PA and DAG, have significant effects in the cellular

response (Exton, 1994, and Tronchere. et al., 1994). PLD activation generates PA

that is converted to DAG by the action of PA phosphohydrolase. PA has been

demonstrated to increase insulin secretion in pancreatic islets and RINm5F cells

(Rustenbeck et al., 1994). However, the mechanism by which PA increases insulin

secretion is still not clear. Atypical PKC is regulated by PA but not DAG

(Dimitrijevic et al., 1995). It is likely that the physiological effect of PA, and thus

PLD activation, is mediated through the activation of PKC or some unknown

mechanisms (Wakelam et al., 1993)

Although attention has been focused on the actions of the products of

phospholipases, it should be recognized that activation of phospholipases also

causes changes in the phospholipid content of their target membranes (Exton,

1994). If the action of phospholipases was localized to a particular area of the

membrane, the physical and chemical property of the membrane could be altered.

Such changes in certain secretory vesicles or constitutive membranes may be

involved in the regulation of secretion and neurotransmission. Also, it could

regulate the maintenance of the subcellular membranes. Lipid changes in the

plasma membrane resulting from the action of phospholipases may be responsible

for some effects of agonists on the membrane-dependent phenomena (Exton, 1994).

The role of PKC in the regulation of AVP-induced signal transduction

Evidence has emerged over the past decade to suggest that PKC is a

widespread family of kinases responsible for many diverse and critical cellular

functions (Wilknson and Hallam, 1994). Exocytosis is regulated by a dynamic

balance between protein phosphorylation and dephosphorylation. In the pancreatic

beta-cells, an increase in protein phosphorylation by an exogenously applied

activators of PKC enhance insulin secretion (Ammala et al., 1994). However,

receptor-mediated activation of PKC is controversial. A number of highly-specific

tools have been developed in recent years (Davis et al., 1989) and as a result, the

role of PKC in the cellular response is being re-evaluated. In this study, Ro 31-

8220, a specific PKC inhibitor, enhanced AVP-induced insulin secretion and the sustained phase of the increase $[Ca^{2+}]_i$. This suggested that PKC is a negative regulator in the action of AVP. Similar results were observed in bradykinin's action in pancreatic beta cells (C. Yang's unpublished data). However, Ro 31-8220 inhibits glucose-stimulated insulin secretion in rat islets (Persaud and Jones, 1995). It was speculated that phorbol ester-insensitive isoforms of PKC (atypical PKCs) are involved in glucose-stimulated insulin secretion (Persaud and Jones, 1995). It has been suggested that PKC plays a dual role in cellular responses (Nishizuka, 1992). Therefore, PKC may play multiple roles and thus interacts at different sites in the complex series of events constituting the beta-cell signal-transduction pathways (Arkhammar et al., 1994). We speculate that glucose and AVP, respectively, activate different signal transduction pathways and different subtypes of PKC. However, further investigation by characterizing the subtypes of PKC in AVP- and glucose-induced insulin secretion will provide answers to this question.

The target of PKC is a 145-kDa cytoplasmic protein that is essential for the Ca^{2+}-dependent secretion in the stimulation by PKC and has been identified in a number of neuroendocrine cells, including RINm5F cells (Nishizaki et al., 1992, Walent et al., 1992). Also, the VDCC is one of PKC-regulated targets (Arkhammar et al., 1994). PKC inhibits the AVP-induced $[Ca^{2+}]_i$ in HIT cells (Hughes et al., 1992), and PKC activation is associated with a lowering of intracellular Ca^{2+} in HIT cells (Hughes et al., 1989) and mouse pancreatic beta-

cells (Arkhammar et al., 1994). In the present study, we propose that PKC

activation is involved in the inhibition of Ca^{2+} influx which contributes to AVP-

induced increase of $[Ca^{2+}]_i$ in the pancreatic beta-cell. AVP-induced Ca^{2+} influx is

mediated through VDCCs and ROCs (Chen et al., 1994). Our results suggested

that PKC may inhibit both types of Ca^{2+} channels. In HPB-ALL T cells, PKC

activation inhibits agonist-stimulated Ca^{2+} influx but not intracellular Ca^{2+} release

(Shivnan and Alexander, 1995). The results indicate that PKC inhibits Ca^{2+} influx.

However, there is no specific ROC blocker; for example, SKF 96365 at the

concentration used in the study also inhibits VDCCs. Without a specific ROC

blocker, we can not clearly characterize this PKC-regulated effect. In our control

experiments, inhibition of PKC did not enhance KCl-induced increase of $[Ca^{2+}]_i$

which is mediated by Ca^{2+} influx through VDCCs. This suggested that there are

some interactions between the PLC-mediated signal transduction and PKC. If the

Ca^{2+} influx is not induced by PLC mediated signal transduction, the negative

regulation by PKC would not be real.

CHAPTER VI GENERAL SUMMARY

The present study was undertaken to investigate the mechanism by which AVP increases insulin secretion in RINm5F cells, a clonal beta-cell line. A specific PLC inhibitor, U-73122, and a PLA_2 inhibitor, ACA were used in the present study. AVP (0.1-100 nM) increased insulin secretion and $[Ca^{2+}]_i$ dose-dependently. Moreover, U-73122 (2-8 µM) inhibited the AVP-induced increases in the intracellular concentration of IP_3 and $[Ca^{2+}]_i$ dose-dependently. At 8 µM, U-73122 abolished AVP's effect on IP_3 and $[Ca^{2+}]_i$, but it only reduced the AVP-induced increase in insulin secretion by 35%. In contrast, 8 µM U-73122 did not reduce the ionomycin (a Ca^{2+} ionophore, 100 nM)-induced increase in $[Ca^{2+}]_i$. The discrepancy between the results of $[Ca^{2+}]_i$ and insulin secretion may be due to the multiple signal transduction pathways associated with the activation of AVP receptors, specifically the Ca^{2+}-independent pathway. The phospholipase A_2 inhibitor, ACA (100 µM) did not antagonize the AVP (10 nM)-induced increase in insulin release. These results suggested: 1) U-73122 blocks PLC activities but fails to block other signal transduction pathways that trigger insulin secretion in these cells, and 2) AVP increases insulin release from RINm5F cells through both the PLC mediated Ca^{2+}-dependent and Ca^{2+}-independent pathways.

Based on the above results, we speculated that PLD was involved in a Ca^{2+}-independent pathway of the AVP-induced insulin secretion.

To characterize the role of PLD in AVP's action, we investigated the effect

of the antifungal antibiotic wortmannin, a PLD inhibitor, on AVP-induced

increases in insulin secretion and $[Ca^{2+}]_i$ in RINm5F cells. Wortmannin (0.1 - 1

µM) inhibited AVP-induced increase of insulin secretion dose dependently. The

combination of wortmannin and U-73122, had an additive inhibition on the AVP-

induced insulin secretion. However, wortmannin failed to change the AVP-induced

increase of $[Ca^{2+}]_i$. These results suggested: (1) activation of PLD stimulated at

least partially the AVP-induced increase of insulin secretion, (2) PLD did not

influence the AVP-elicited elevation of $[Ca^{2+}]_i$ and (3) PLC and PLD stimulate

AVP-activated signal transduction in an independent manner.

The role of PKC in AVP-induced insulin secretion was investigated in

RINm5F cells. Ro 31-8220, a specific PKC inhibitor, dose dependently potentiated

the AVP-induced increase of insulin secretion and elevation of $[Ca^{2+}]_i$. In Ca^{2+} free

medium Ro 31-8220 had no effect on AVP-induced release of intracellular Ca^{2+}.

Thus, the potentiation of AVP-induced elevation of $[Ca^{2+}]_i$ by Ro 31-8220 was

mediated by an increase in Ca^{2+} influx through Ca^{2+} channels. However, Ro 31-

8220 did not enhance KCl (10 mM)-induced insulin secretion and elevation of

$[Ca^{2+}]_i$. Acute treatment with a DAG analog, OAG, inhibited AVP-induced insulin

dose-dependently, but reduced slightly the AVP-induced elevation of $[Ca^{2+}]_i$.

Activation of PKC by OAG had no effect on insulin secretion and $[Ca^{2+}]_i$. Also

OAG did not change KCl (10 mM)-induced insulin secretion or elevation of

$[Ca^{2+}]_i$. These results suggested that: 1) PKC is a negative regulator of AVP's

action in beta-cells, 2) PKC inhibits Ca^{2+} channels to regulate insulin secretion, and

3) PKC has no effect on AVP-induced intracellular Ca^{2+} release from pancreatic beta-cells.

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ACKNOWLEDGMENTS