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Mechanisms of arginine vasopressin- and oxytocin-induced glucagon release

by

Sirintorn Yibchokanun

A dissertation submitted to the graduate faculty

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This is to certify that the Doctoral dissertation of

Sirintorn Yibchokanun

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

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For the Major Program

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For the Graduate College

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LIST OF ABBREVIATIONS

AA	*arachidonic acid*
AC	adenylyl cyclase
ACA	N-(p-amylcinnamoyl)anthranilic acid
ARF	ADP-ribosylation factor
AVP	arginine vasopressin
BAPTA	1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
$[Ca^{2+}]_i$	intracellular calcium concentration
CCK	cholecystokinin
CO	cyclooxygenase
CTX	chorela toxin
Da	dalton
DAG	diacylglycerol
EGTA	*[ethylenebis (oxyethylenenitrilo)]* tetraacetic acid
EP	epoxyeicosatrienoic acid
EPO	epoxygenase
ER	endoplasmic reticulum
FFA	free fatty acid
Fura-2AM	fura-2 acetoxymethyl ester
GABA	γ-aminobutyric acid
GLP	glucagon like peptide
G-protein	heterotrimeric guanine nucleotide-binding protein
HEPES	*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid
I_{crac}	Ca^{2+} release-activated Ca^{2+} current
In-R1-G9	hamster glucagonoma cell line
IP_3	inositol 1,4,5-trisphosphate
KRB	Krebs-Ringer bicarboante buffer
MLCK	*myosin light chain kinase*

OAG	1-oleoyl-2-acetyl-sn-glycerol
PA	*phosphatidic acid*
PC	phosphatidylcholine
PDE	*phosphodiesterase*
PE	phosphatidylethanolamine
PI3 *kinase*	*phosphatidylinositol 3 kinase*
PIP_2	phosphatidylinositide 4,5-bisphosphate
PKA	*protein kinase A*
PKC	protein kinase C
PLA_2	*phospholipase* A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol-12-myristate-13-acetate
PPH	phosphatidate phosphohydrolase
PPI	phosphatidylinositide phosphate
PS	phosphatidylserine
PTX	pertussis toxin
RACK	receptors for activated C-kinase
RIA	radioimmunoassay
RICK	receptors for inactive C-kinase
ROC	receptor-operated Ca^{2+} channel
VDCC	voltage-dependent Ca^{2+} channel
zLYCK	carbobenzyloxy-leucine-tyrosine-chloromethylketone

ABSTRACT

We studied the effects of arginine vasopressin (AVP) and oxytocin (OT) on glucagon release and characterized the receptors that mediate the effects of these two peptides by use of a number of antagonists in the perfused rat pancreas, clonal α-cells In-R1-G9, and fluorescence imaging of the receptors in rat islets. AVP and OT (3 pM-3 nM) increased glucagon release in a concentration-dependent manner from the rat pancreas. The antagonist with potent V_{1b} receptor-blocking activity abolished AVP-induced glucagon release, but did not alter OT-induced glucagon release. In contrast, the OT receptor antagonist abolished OT-induced glucagon release, but did not change the effect of AVP. Fluorescent microscopy of rat pancreatic sections also showed that fluorescence-labeled vasopressin and OT bound specifically to V_{1b} and OT receptors, respectively. Therefore, in the rat pancreas, AVP and OT increased glucagon release through the activation of V_{1b} and OT receptors, respectively. However, in clonal α-cell line In-R1-G9, V_{1b} receptors mediated both AVP- and OT-induced glucagon release, because the antagonists with V_{1b} blocking activity, but not the OT receptor antagonists, inhibited AVP- and OT-induced glucagon release in In-R1-G9 cells in a concentration-dependent manner. A clonal α-cell line In-R1-G9 was used to study the mechanisms underlying AVP-induced glucagon release. AVP (100 nM) increased $[Ca^{2+}]_i$ in a biphasic pattern; a peak followed by a sustained plateau. When $[Ca^{2+}]_i$ was stringently deprived by BAPTA, a Ca^{2+} chelator, AVP still significantly increased glucagon release. These results suggest that AVP caused glucagon release through both Ca^{2+}-dependent and -independent pathways. For the Ca^{2+}-dependent pathway, our results were consistent with the current concept that the G_q protein activates phospholipase C, which catalyzed the formation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induced Ca^{2+} release from the endoplasmic reticulum, thereby triggering Ca^{2+} influx via receptor-operated Ca^{2+} channel and increasing glucagon release. Our results further suggest that, DAG activates novel (nPKCs) and atypical protein kinase C (aPKCs). nPKCs may exert negative feedback on AVP-induced increase in IP_3 production, leading to an attenuation of $[Ca^{2+}]_i$, which, in turn, attenuated AVP-induced

glucagon release. On the other hand, aPKCs may contribute to the stimulatory effect of AVP on glucagon release.

CHAPTER I GENERAL INTRODUCTION

Dissertation Organization

This dissertation is written in an alternative thesis format. It contains a general introduction, five research papers, a general discussion, a list of references cited in the general introduction and discussion, and acknowledgments. The general introduction includes a research objective, background information, and literature review. Chapter II, "Characterization of receptors mediating AVP- and OT-induced glucagon release from the rat pancreas", and Chapter III, "Effects of arginine vasopressin and oxytocin on glucagon release from clonal α-cell line In-R1-G9: involvement of V_{1b} receptors", have been published in the *American Journal of Physiology* and the *Life Sciences*, respectively. Chapter IV, "Mechanisms of AVP-induced glucagon release in clonal α-cells In-R1-G9: involvement of Ca^{2+}-dependent and -independent pathways", and Chapter V, "Protein kinase C attenuates arginine vasopressin-induced increases in IP_3 and $[Ca^{2+}]_i$ in clonal α-cells In-R1-G9" have been submitted for publication in the *British Journal of Pharmacology* and the *European Journal of Pharmacology*, respectively. Chapter VI, "Novel protein kinase C isozymes inhibit and atypical protein kinase C isozymes stimulate arginine vasopressin-induced glucagon release in clonal α-cells In-R1-G9", will be submitted for publication in *Diabetes*.

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

Research Objective

Glucagon is an important hormone for the regulation of glucose homeostasis. It has been clearly established that glucagon increases plasma glucose concentrations via direct effect upon fuel metabolism, which indicates that glucagon is a provider of energy for cerebral function. Although intestinal and neural cells express the preproglucagon gene and process preproglucagon, only the normal intact pancreatic α-

cells can secrete true glucagon, the 29-amino acid hormone that evokes glycogenolysis and gluconeogenesis. Glucagon helps to increase the flow of glucose from hepatic glycogen stores to the brain during stress or shock caused by trauma, infection, and burns or myocardial damage. It also inhibits glucose utilization by peripheral tissues. This hormone, therefore, is crucial for survival. The physiological function of glucagon is well understood, but the physiological regulation of glucagon release is controversial.

Diabetes mellitus is a common and serious metabolic disease that causes ketoacidosis and coma. In addition to a decrease in insulin release in diabetic patients, leading to hyperglycemia, most of these patients have excessive glucagon release, which further aggravates hyperglycemia in diabetes (Unger and Orci, 1995). A variety of nutrients and neurotransmitters, including arginine vasopressin (AVP) and oxytocin (OT), increase glucagon release from the pancreas (Itoh et al., 1981; Dunning et al., 1984). In addition, AVP and OT at concentrations of ~2,000 to 10,000 pM are found in the pancreas (Amico et al., 1988). Because plasma concentrations of AVP and OT are in the range of 3-25 pM (Franchini et al, 1996; Kjaer et al., 1995), these findings suggest the local synthesis of both peptides in the pancreas. Therefore, the pancreatic AVP and OT rather than systemic ones may play a major role in the regulation of glucagon release. Since the role of AVP and OT in the physiological regulation of glucagon release is not well established, it is clinically and scientifically important to investigate their effects and intracellular mechanisms underlying the effects of these two hormones on glucagon release.

Ca^{2+} plays a central role in triggering glucagon release from rat pancreas (Hii and Howell, 1986; Pipeleer et al., 1985). An elevation of $[Ca^{2+}]_i$, derived from the opening of Ca^{2+} channels on plasma membrane of α-cells and/or release of Ca^{2+} from intracellular Ca^{2+} stores, increases glucagon release. However, the activation of specific effectors upstream and downstream from the Ca^{2+} signal remain to be determined; for instance phospholipases and protein kinases may regulate the changes of $[Ca^{2+}]_i$ leading to an increase or a decrease in glucagon release.

In this study, in situ pancreatic perfusion and pancreatic fluorescence imaging have been used to examine the physiological role of AVP- and OT-induced glucagon release as well as the characterization of receptors mediating their effects. However, it is difficult to isolate and purify α-cells from the pancreatic islets, which account for

only 10-25% of islet cells. Therefore, we used a clonal α-cell line, In-R1-G9 cells, which is derived from the In-111-R1 hamster insulinoma cell line (Takaki et al., 1986), as a model for the rest of this study. Glucagon release from In-R1-G9 cells has been observed and the characteristics of this cell line have been suggested to be similar to the α-cells of the endocrine pancreas (Rorsman, et al., 1991); for example forskolin, arginine, and theophylline stimulate, but somatostatin and insulin inhibit glucagon release from these cells (Fehmann et al., 1995). In addition, Ca^{2+} and phospholipid-dependent protein kinase C plays important roles in glucagon release in this cell line (Ono et al., 1986).

In summary, the objectives of this study are: 1) to characterize the receptors mediating AVP- and OT-induced glucagon release in the rat pancreas and In-R1-G9 cells, and 2) to elucidate the mechanisms underlying the effect of AVP-induced glucagon release in In-R1-G9 cells.

Background and Literature Review

This section provides background information related to the studies that are presented in the dissertation: 1) Classification of AVP and OT receptors; 2) AVP-activated several signal transduction pathways; 3) Involvement of phospholipases in the mechanism of AVP; 4) Involvement of protein kinase C in the mechanism of AVP.

The pancreatic islets

The pancreas is an exocrine and endocrine organ, which is located in the dorsal part of both the epigastric and the mesogastric abdominal segments, caudal to the liver. The rat pancreas, which has frequently been used as an animal model to study changes in diabetes mellitus and islets cell tumors, is divided into four regions: lower duodenal (derived from the ventral primordium) and upper duodenal, gastric and splenic regions (derived from the dorsal primordium) (Elayat et al., 1995). The exocrine pancreas secretes pancreatic juice, the most important part of digestive secretion, which contains three principal enzymes for digestion of proteins, fats and carbohydrates. The endocrine pancreas, which composed of the islets of Langerhans, dispersed among the much larger mass of exocrine pancreas. The islets contain four major types of

endocrine cells: α (or A) cells, β (or B) cells, δ (or D) cells and F (or PP) cells (Pelletier, 1977). The α cells, which secrete glucagon, are generally located in the periphery of the islets and represent ~20% of the islet cells. The β cells, which secrete insulin, are located in the center of the islets and represent ~75% of the islet cells. The δ cells, which secrete somatostatin, are located between α and β cells and represent ~3-5% of the islet cells. The F cells, which secrete pancreatic polypeptide (PP), are located near the α cells and represent $<2\%$ of the islet cells (Karam, 1995).

The arrangement of islet cells may have functional implications for within-islet communication and the pattern differs from species to species. In the rat islets, the β cells occupy the central region and are surrounded by α and δ cells. In the human islets, the arrangement is similar, in which it has centrifugal arrangement of α cells relative to β cells within the pseudo-lobules (Fig. 1). The pancreas receives blood supply from the pancreatic branches of the cranial and caudal pancreaticoduodenal arteries and from the pancreatic branches of the splenic artery (Evans, 1993). The arterioles anastomose and form a capillary network in the islets; thus the blood flows from the center to the periphery and carries insulin from the central β cells to the peripheral α and δ cells. This system is called "portal microcirculation" and it is possible that insulin may exert the within-islet control over glucagon secretion via microcirculation within the islets (Unger and Lelio, 1995). Gap junctions have been demonstrated among β cells and between α and β cells, which may create a close contact with each other among the islet cells. The islets of Langerhans are innervated by the autonomic nervous system (Hadley, 1992). The sympathetic fibers that innervate pancreas come from the celiac plexus and the parasympathetic fibers come from the vagal nerve, which reach the organ by following the arteries (Evans, 1993).

Properties and structure of glucagon

Glucagon is a polypeptide hormone consisting of a single chain of 29 amino acids, with a molecular weight of 3,485 (Fig. 2). The sequence of glucagon in most mammals is identical, except for guinea pig, which differs at five positions in its C-terminal region (Huang et al., 1986; Fig. 3). The sequence in chicken (Pollock and Kimmel, 1975) and duck (Sundby et al., 1972) also differ from human glucagon at one

and two positions, respectively (Fig. 3). Neither of these changes, except that of guinea pig glucagon, appears to have a major impact on bioactivity. The bioactivity of guinea pig glucagon is reduced by 10% compared to other species, which may represent an adaptation to a lowered biological potency of glucagon in this species.

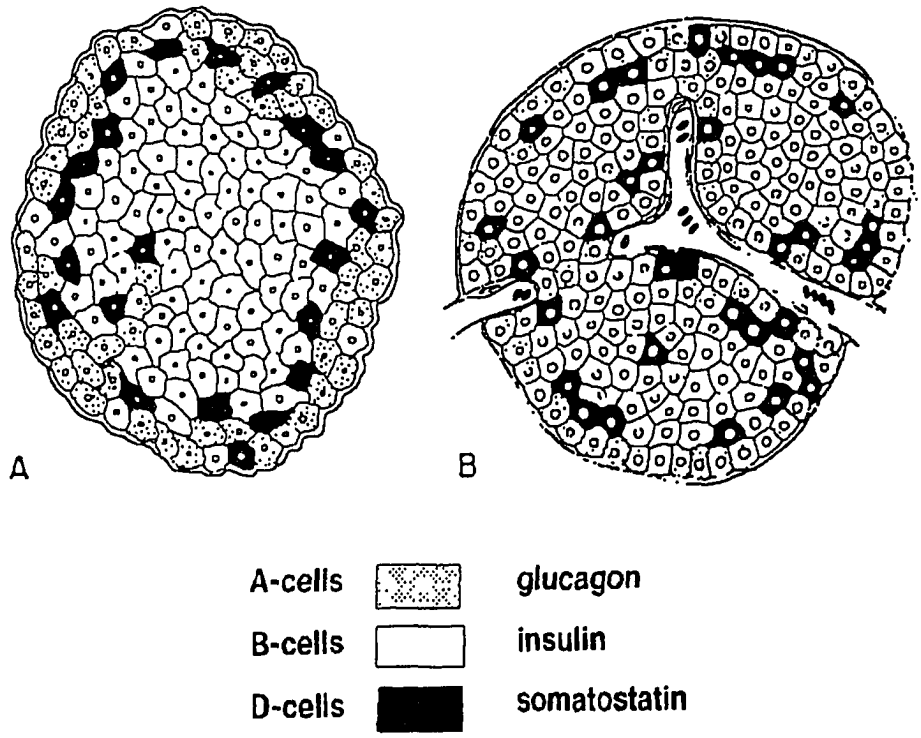


Fig. 1. Schematic representation of the typical rat islet (A) and human islet (B) showing the arrangement of α, β, and δ cells (modified from Hsu and Crump, 1989; Unger and Lelio, 1995).

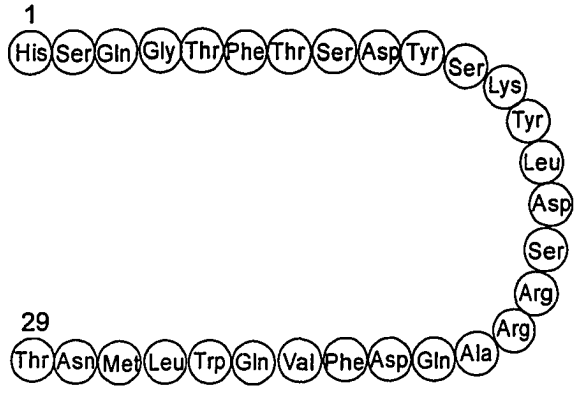


Fig. 2. Amino acid sequence of glucagon polypeptide (modified from Karam, 1995).

Human	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
Guinea pig	----------Q-LK--L-V
Chicken	----------N
Duck	----------T----------N

Fig. 3. Primary structures of glucagons in several vertebrate species (modified from Steiner et al., 1995).

Biosynthesis of glucagon

Glucagon is derived from a preprohormone with a molecular weight of 18 kDa. The glucagon preprohormone is found in the pancreatic α cells, intestinal glucagon cells (L cells), and in the brain. The 179 amino acid-preproglucagon contains glicentin near its N-terminal and the major proglucagon fragment (MGPF) near its C-terminal (Fig. 4). Glicentin is a polypeptide that consists of glicentin-related polypeptide (GRPP) and amino acid-extended glucagon. The MGPF contains two major glucagon like peptides (GLP), which are GLP-1 and GLP-2 (Fig. 4). Preproglucagon is the product of a single mRNA, which is processed differently in different tissues. Generally, it is processed

primarily to the 29-amino acid glucagon and the MPGF in the pancreatic α cells, whereas it is processed to glicentin, GLP-1, GLP-2 and some oxyntomodulin in the L cells. In addition, GRPP is left in both α and L cells (Ganong, 1995). Glicentin has some glucagon activity, but the definite functions of GLP-1, GLP-2 and GRPP have not been identified yet. However, when GLP-1 is further processed by removal of its 7 N-terminal residues and amidation, the product, GLP-1 amide, becomes a potent stimulator of insulin release. Oxyntomodulin inhibits gastric acid secretion.

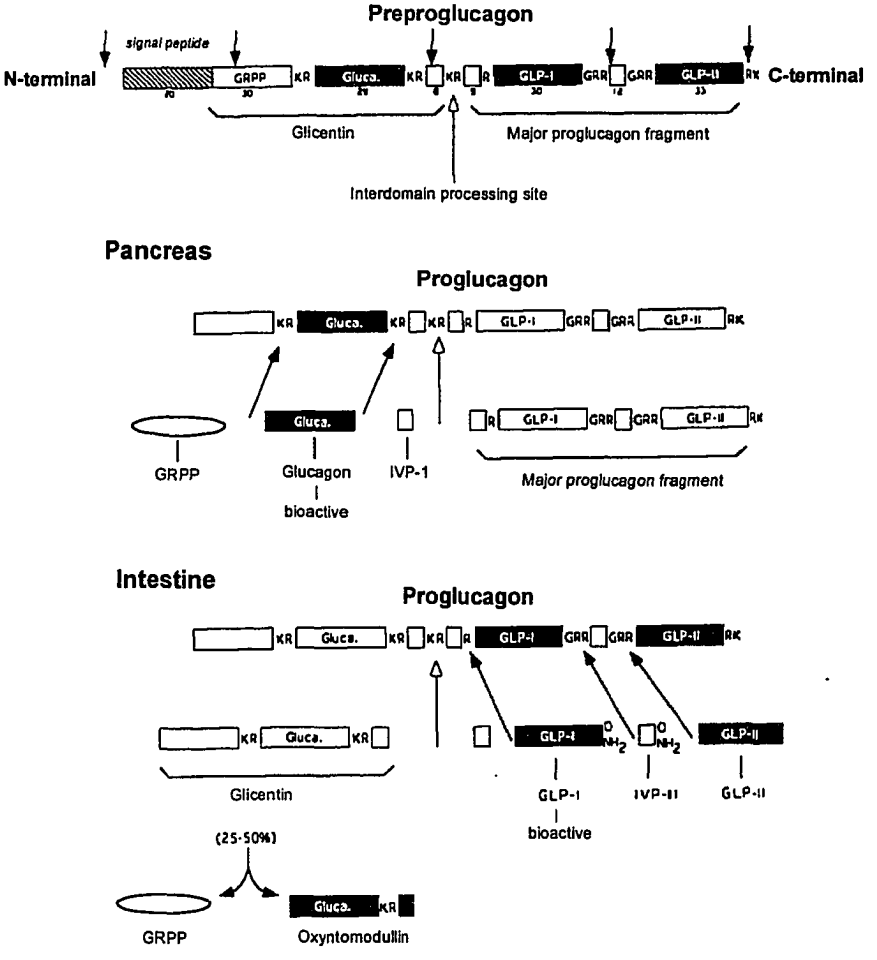


Fig. 4. Schematic of the human glucagon precursor and its tissue-specific processing of pancreatic α cells and intestinal glucagon cells (modified from Steiner et al., 1995).

Metabolism and action of glucagon

The half-life of glucagon in the circulation is ~5-10 min. It is degraded predominantly by the liver. The physiological actions of glucagon include glycogenolysis, gluconeogenesis, lipolysis and ketogenesis. There are two kinds of glucagon receptors, GR-1 and GR-2. The GR-2 receptor, a major hepatic glucagon receptor, is a glycoprotein of 63 kDa containing both the glucagon-binding function and the ability to interact with a heterotrimeric G-protein, G_s (Bonnevie and Tager, 1983). This receptor exists in two different functional forms, a high-affinity form that represents 1% of the glucagon-binding sites and a low-affinity form that comprises 99% of the molecules. It is likely that the high-affinity GR-2 receptor mediates the inhibitory effect of glucagon upon hepatic glycogen formation.

Glucagon binds to GR-2 receptors, couples to G_s, then activates adenylyl cyclase to increase cAMP formation (Rodbell, 1983). cAMP activates cAMP-dependent protein kinase (PKA), leading to the activation of phosphorylase b kinase. This enzyme, then phosphorylates phosphorylase b to the active from, phosphorylase a (a rate-limiting enzyme of glycogenolysis), resulting in an increase in liver glycogenolysis (Fig. 5). At the same time, phosphorylation of active glycogen synthase-a inactivates phosphorylase a to the b form, thereby attenuating glycogen formation (Stalmans, 1983).

Activated PKA also mediates hepatic gluconeogenesis, ketogenesis and lipolysis by increasing phosphorylation of fructose-2,6 bisphosphatase (FBPase-2) and degrading fructose-2,6-bisphosphate (F-2,6-P_2) to fructose-6-phosphate (F-6-P), thus reducing glycolysis and promoting gluconeogenesis. For ketogenic and antilipogenic actions of glucagon, the decrease of F-2,6-P_2 reduces glucose-derived fatty acid synthesis. Glucagon decreases Malonyl-CoA levels, the first product in the lipogenic pathway, by inhibiting both glycolysis and acetyl-CoA carboxylase through phosphorylation process (McGarry et al., 1978). Malonyl-CoA inhibits carnitine palmitoyl transferase-1 (CPT-1), the enzyme that transesterifies fatty acyl CoA to fatty acyl carnitine, and the carnitine enters the mitochondria, which is the site of fatty acid oxidation to ketones. To summarize, glucagon causes an increase in intrahepatic levels of fatty acyl CoA and carnitine, which coupled with activation of CPT-1, resulting in an increase in ketogenesis (Fig. 5).

The GR-1 receptor is the second glucagon receptor that has been found to mediate the effect of glucagon through the cAMP-independent pathway (Wakelam et al., 1986). Glucagon binds to GR-1 receptor, activates PLC, thus enhancing inositol phospholipid breakdown to generate inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 increases Ca^{2+} release from the endoplasmic reticulum (ER) and DAG activates protein kinase C (PKC); however, it remains unknown as to which proteins are phosphorylated by the activation of PKC. It now appears that glucagon acts via both cAMP-mediated and IP_3-mediated pathways to induce glycogenolysis, glyconeogenesis and ketogenesis.

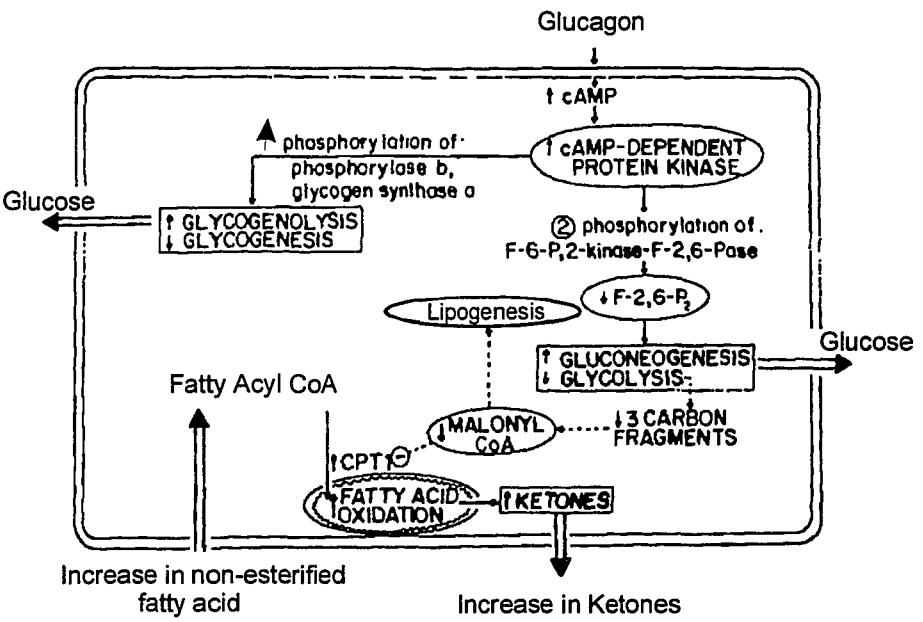


Fig. 5. A panoramic perspective of the major sites of gluacgon actions at the hepatocyte. CPT-Carnitine palmitoyl transferase-1 (modified from Steiner et al., 1995).

Regulation of glucagon release

Glucagon is one of the regulatory hormones for glucose homeostasis that causes hyperglycemia. The release of glucagon from the pancreatic α cells can be inhibited or stimulated by a variety of nutrients and hormones, which are summarized in Table 1.

Table 1. Factors affecting glucagon release (modified from Ganong, 1995)

Stimulators	Inhibitors
Amino acids (particularly the glucogenic amino acids: arginine, alanine, serine, glycine, cysteine and threonine)	Glucose
Cholecystokinin (CCK), gastrin, secretin	Somatostatin
Cortisol	Insulin
Stresses (Exercise and infections)	Free fatty acid (FFA)
α_2-Adrenergic stimulators	Ketones
β-Adrenergic stimulators	γ-aminobutyric acid (GABA)
Acetylcholine	Phenytoin
Theophyllline	

Inhibitors of glucagon release

It is well established that glucose is the major nutrient to inhibit glucagon release (Gerich, 1983); however, its effects on the α cells are mediated both directly and via glucose-stimulated insulin release. The relative importance of glucose versus insulin in suppressing glucagon release has been debated because glucagon secretion by isolated α-cells is suppressed in vitro in the total absence of insulin (Weir and Bonner-Weir, 1990). However, glucagon released from the α-cells that do not contact the β-cells, such as dog gastric α-cells (Blazquez et al., 1976), glucagonomas, or islets following β-cells destruction (Muller et al., 1971), appears not to be inhibited by an increase in glucose concentration, but it is inhibited by insulin. The mechanisms underlying insulin-inhibited glucagon release are unknown. Although no insulin-binding sites are identified in pancreatic α cells (Schravendijk et al., 1985), insulin receptors are found in glucagonoma In-R1-G9 cells (Fehmann et al., 1994 and Kisanuki et al., 1995). In addition, the arterial infusion of insulin suppresses and anti-insulin antibodies increase glucagon release from the isolated islets (Samols, 1988). Insulin also inhibits glucagon release and proglucagon gene expression via an inhibition of the proglucagon gene transcription (Fehmann et al., 1994).

Glucose uptake into the pancreatic α cells is not stimulated by insulin and the rate of glucose metabolism in these cells is ~ 15-20% of that in the β cells (Gorus et al., 1984). Generally, the inhibitory effect of glucose is shared by other glucose metabolites and related sugar and its capacity is determined by the ability of the cells to metabolize the sugar, suggesting that the effect of glucose is mediated by its metabolites. Thus, the inhibitors of cellular metabolism, such as 2,4-dinitrophenol (Gerich, 1983) or anoxia (Narimiya et al., 1982) can counteract the glucose-induced inhibition of glucagon release. Glucose inhibits glucagon release by stimulating the mobilization of Ca^{2+} from the cytosol, leading to a decrease in $[Ca^{2+}]_i$ (Johasson, et al., 1987; Johasson, et al., 1989). It also inhibits amino acid-induced glucagon release (Pipeleers, 1985).

The pancreatic β cells contains the inhibitory neurotransmitter γ-aminobutyric acid (GABA) and there is evidence that glucose concomitantly increases the release of insulin and GABA. GABA activates $GABA_A$ receptors in the α cells to inhibit glucagon release. The $GABA_A$ receptors are Cl^- channels, which cause Cl^- influx, resulting in hyperpolarization of the α cells. GABA was found to inhibit the effect of arginine-induced glucagon release and this effect was blocked by the $GABA_A$ receptor antagonist, bicuculline (Rorsman et al., 1989). In addition, GABA plays a role in glucose-induced inhibition of glucagon release. The inhibitory effect of glucose is attenuated when $GABA_A$-activated Cl^- receptors are blocked by bicuculline (Rorsman et al., 1989). The interrelationship between glucose, insulin and GABA on the regulation of glucagon release is shown in Fig. 6.

Somatostatin is known to inhibit both glucagon and insulin releases. It is secreted from the pancreatic δ cells as two peptides; one consists of 28 amino acids (S28) and the other consists of 14 amino acids (S14) (Efendic, 1980). The mechanism underlying the effect of somatostatin is unknown. However, two possible mechanisms have been proposed: 1) interference with second messenger system or the exocytotic machinery of the α cells; 2) alterations of membrane ion conductances.

FFA and ketone also inhibit glucagon release. However, the inhibition by FFA and ketone may not be obvious because plasma glucagon levels are high in diabetic ketoacidosis.

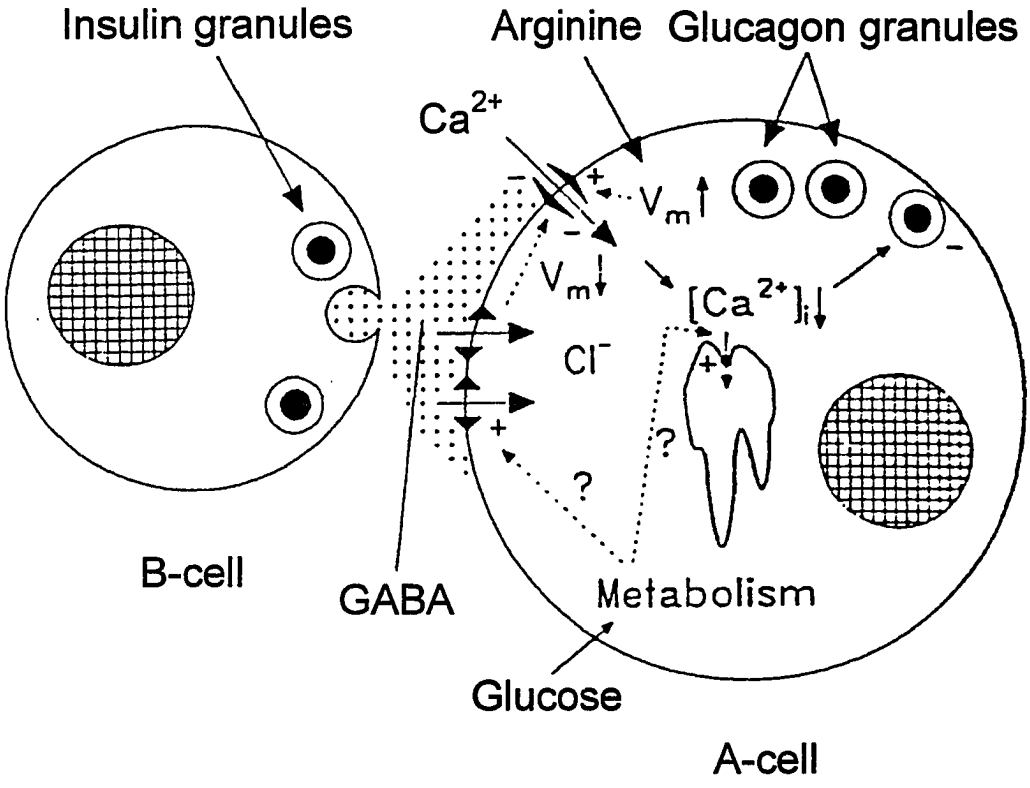


Fig. 6. A model for the regulation of glucagon release. An increase in glucose concentration leads to stimulation of insulin and GABA release from the β cells. GABA binds to $GABA_A$ receptors on the α cells and opens Cl^- channels, leading to membrane hyperpolarization and inactivation of Ca^{2+} channels. This results in a reduction of $[Ca^{2+}]_i$ and glucagon release (modified from Rorsman et al., 1991).

Stimulators of glucagon release

Glucagon release is increased by the activation of the sympathetic nerve supply to the pancreas. This sympathetic effect is mediated through β- and α_2-adrenergic receptors, and cyclic AMP (cAMP). Hormones that are secreted during stress, such as epinephrine, norepinephrine, growth hormone, β-endorphin, vasopressin and cortisol stimulate glucagon release. Catecholamines play a role in metabolic adjustments by directly increasing liver glycogenenolysis via α_1- and β_2-adrenergic

effects on the liver. They also indirectly enhance glucose production by stimulating glucagon release and simultaneously inhibiting insulin release. The net result is to enhance glucose flow from hepatic glycogen stores to non-insulin-requiring tissue, such as the brain, which is called as "stress hyperglycemia". Stress hyperglycemia serves as a vital survival function of glucagon by maximizing glucose delivery to the brain during a stress or shock.

Exercise increases glucagon release via impulses from the ventromedial nucleus of the hypothalamus through autonomic fibers that innervate the islets. In addition to the signal from hypothalamus, the pancreatic α cells may be locally regulated by adrenergic innervation because the α-adrenergic receptor antagonists block the response of glucagon release to glucopenia. The release of norepinephrine during hypoxia may be a local manifestation in autonomic nerve endings near islets. Acetylcholine stimulates both glucagon and insulin release.

The ingestion of protein such as beef, casein, and amino acids, or fat meal stimulates glucagon release, but has only little impact on insulin release. However, a carbohydrate-rich meal stimulates insulin release, but has slight impact on glucagon release. Hormones that are released during the protein meal, for example gastrin, cholecystokinin (CCK) and gastric inhibitory polypeptide (GIP) stimulate glucagon release. In addition, vasoactive intestinal polypeptide (VIP) and secretin stimulate glucagon release (Ahren, 1991). Accumulation of cAMP in the islet has been observed in parallel with an increase in glucagon release after treating isolated mouse pancreatic islets with secretin, suggesting that it may increase glucagon release through the activation of adenylyl cyclase (Kofold et al., 1991). The mechanisms underlying other GI hormones-induced glucagon release remain unknown.

The amino acids arginine, glutamine and alanine stimulate glucagon release (Pipeleers et al., 1985). Arginine is the most potent amino acid that induces glucagon release. Other amino acids have either no, or only slight, stimulatory action on their own, but they can potentiate the effect of another amino acid-induced glucagon release. Arginine is a cationic amino acid that can depolarize the membrane of the α cells by its electrogenic entry (Rorsman and Hellman, 1988).

The role of Ca^{2+} and the α cell electrical activity

It is now generally accepted that Ca^{2+} is a signal to initiate glucagon release. The observations that support this concept are as follows: 1) Physiological secretagogues-induced glucagon release is dependent on the presence of extracellular Ca^{2+} (Pipeleers et al., 1985); 2) Ca^{2+} ionophore A23187 stimulates glucagon release (Hii and Howell, 1986); 3) Glucose-induced inhibition of glucagon release involves reduction of $^{45}Ca^{2+}$ uptake in islets from streptozotocin-treated guinea pigs (Berggren et al., 1979); 4) In permeabilized rat pancreatic islet cells, glucagon release increases are parallel to the Ca^{2+} concentrations (Niki et al., 1986); 5) microfluorometric measurements using fura-2 demonstrate that stimulations of glucagon release are associated with increases in $[Ca^{2+}]_i$ (Johansson, et al., 1989).

Patch clamp experiments have demonstrated that the pancreatic α cells produce a spontaneous action potential, which is initiated from a membrane potential of about −65 mV and peak at +20 mV. From voltage-clamp measurements, α cells have been found to be equipped with voltage-dependent Na^+, Ca^{2+} and K^+ channels. Activation of inward Na^+ and Ca^{2+} currents is responsible for the depolarization of the plasma membrane, whereas activation of outward K^+ current is responsible for the repolarization (Rorsman and Hellman, 1988). In addition, L and T types of voltage-dependent Ca^{2+} channels have been found to be present in the α cells (Rorsman, 1988). The activation of T-type channels can be elicited at membrane potentials as negative as −60 mV, whereas the opening of L-type channels requires more positive voltages. This suggests the different roles of these two channels in the α cells. The L-type Ca^{2+} channel opens at the rapid rising phase of action potential and it is likely to participate in this process. The T-type Ca^{2+} currents occur at the membrane potentials close to the threshold for initiation of action potential and may play a role in the pacemaking of the α cells (Rorsman and Hellman, 1988).

Regulation of glucagon release by arginine vasopressin and oxytocin

Chemistry and biosynthesis of arginine vasopressin and oxytocin

Vasopressin (VP) and oxytocin (OT) are nonapeptides with closely related structure. There are a number of VP and OT-like peptides occurring naturally in different species. All of them contain cysteine residues in positions 1 and 6 and have a disulfide bridge between the two cysteine residues, which is essential for agonist activity. In addition, they have conserved amino acids in positions 5, 7 and 9 that are asparagine, proline and glycine, respectively (Table 2). In all mammals except swine, these peptides contain arginine in position 8 and thus the terms vasopressin, arginine vasopressin (AVP) and antidiuretic hormone (ADH) are used interchangeably. In swine, the arginine residue in position 8 is replaced with the lysine residue; thus it is called as lysine vasopressin (LVP; Table 2). OT differs from AVP in possessing an isoleucine and a leucine at the 3 and 8 positions, respectively (Table 2).

Table 2. Primary structure of the vertebrate vasopressin and oxyotcin (modified from Hadley, 1992).

Hormone	Positions of amino acid residues	Animal group
	1 2 3 4 5 6 7 8 9	
Ancestral molecule	Cys-Tyr-X-X-Asn-Cys-Pro-X-Gly-(NH_2)	
	1 2 3 4 5 6 7 8 9	
Oxytocin	Cys-Tyr-Ile-Gln-Asn-Cys-Pro-Leu-Gly-(NH_2)	Mammals
	1 2 3 4 5 6 7 8 9	
Arginine vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-Gly-(NH_2)	Mammals except domestic pigs and Macropodidae
	1 2 3 4 5 6 7 8 9	
Lysine vasopressin (Lysipressin)	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-Gly-(NH_2)	Placental mammals (Suidae) Marsupials (Macropodidae, Didelphidae)

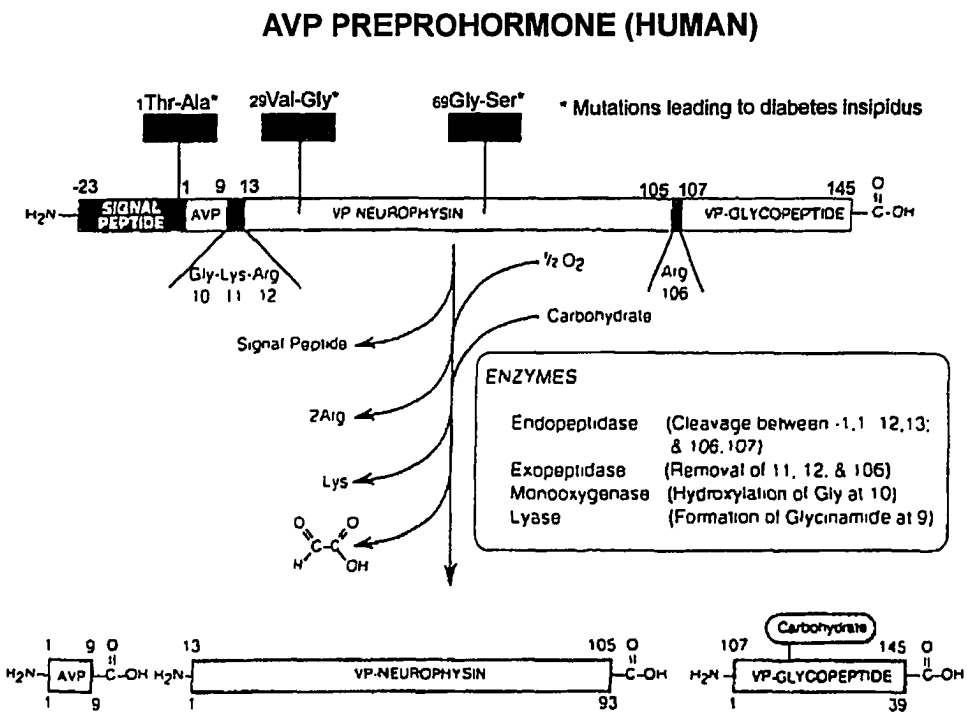
AVP and OT are synthesized in the perikarya of magnocellular neurons in the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) of the hypothalamus. Each nucleus synthesizes both hormones (Dierickx et al., 1978), with some differential distribution within the nuclei; for example, there are more AVP cells in the caudal part and more OT cells in the rostral part of both SON and PVN (Swaab et al., 1975). AVP, a product of a preprohormone with 168 amino acids, (Fig. 7A) is synthesized and incorporated into ribosome. During synthesis, a signal peptide (residues −23 to −1) is removed to form pro-VP, then it is translocated through the rough endoplasmic reticulum, consequently incorporated into large membrane-enclosed granules. The prohormone consists of three domains: VP (residues 1-9), VP-neurophysin or neurophysin II (residues 13-105) and VP-glycopeptide or copeptin (residues 107-145). In the secretory granules, the prohormone will be cleaved sequentially by endopeptidase, exopeptidase, monooxygenase and lyase to form VP.

OT is also formed by the processing of a precursor molecule (Fig. 7B) that contains OT-neurophysin, a specific binding protein for the hormone. The precursors are packed into the granules and processed into the secretory products within the granules. OT-neurophysin and VP-neurophysin contain a sequence of more than 90 amino acids that is identical (Land et al., 1983). AVP, OT and their own neurophysins are stored separately as the neurosecretory granules and move along the axon toward the terminals in the posterior pituitary (Russel et al., 1990).

The principal physiological stimuli of AVP release are an increase in plasma osmolality, hypovolemia/hypotension, pain, nausea, hypoxia, and some agents such as acetylcholine, histamine, dopamine, glutamine, cholecystokinin, and angiotensin II. About 2% elevation of plasma osmolality causes a two to three-fold increase in plasma VP levels. For OT, the sensory stimuli arising from the reproductive tract or mammary gland induce OT release from the posterior pituitary gland.

AVP and OT are present in the extrahypothalamic tissues, such as the adrenal gland, cerebellum (Richter et al., 1991), ovary (Guldenaar et al., 1984), thymus (Geenan et al., 1986), testis (Guldenaar and Pickering, 1985) and pancreas (Amico et al., 1988).

(A)



(B)

Signal peptide-OXYTOCIN-**Gly.Lys.Arg**-neurophysin-His

Fig. 7. (A) Processing of the 168-amino acid prepro-AVP to AVP, VP-neurophysin and VP-glycopeptide (modified from Jackson, 1996) and (B) the oxytocin precursor (modified from Pickering, 1995).

Actions of AVP and OT

The major physiological function of AVP is the regulation of body fluid volume, osmolality, and maintenance of blood pressure. However, it also affects other systems; for example, AVP increases glycogenolysis (Kirk et al., 1979), proliferation of the pituitary gland (McNichol et al., 1990), secretion of clotting factors (Fuchs and Fuchs, 1984; Gibbens and Chard, 1976), ACTH (Fuchs and Fuchs, 1984), catecholamines

(Grazzini et al., 1996), glucagon, and insulin (Dunning et al., 1984). AVP increases the permeability to water of the collecting tubules, allowing water to move along the gradient from lumen to renal medulla, resulting in urinary concentration (Bankir, 1991).

OT plays a major role in the regulation of milk ejection and uterine contraction; however, it also increases ACTH (Schlosser et al., 1994), glucagon and insulin release (Dunning et al., 1984). OT contracts or relaxes vascular smooth muscle depending on species, vascular bed, and region within the vascular bed (Rouille et al., 1991). In addition, both AVP and OT are involved in central processes of higher cognitive function such as memory and learning (Barberis and Tribollet, 1996).

AVP and OT receptors

The classification of AVP and OT receptors is based upon both the second messenger system coupled to the receptors and the affinity of various AVP and OT analogues to a certain receptor type. The effects of AVP are mediated by two principal types of receptors, V_1 and V_2 receptors (Guillon et al., 1980). The V_1 receptors have been further subclassified into V_{1a} and V_{1b} receptors because the binding properties of the V_{1b} to various vasopressin agonists and antagonists differ from those of V_{1a} receptors (Schwartz et al., 1991). The V_{1a} receptor, the most widespread subtype of AVP receptors, has been found in vascular smooth muscle, myometrium, the bladder, adipocytes, hepatocytes, platelets, renal medullary interstitial cells, vasa recta in the renal microcirculation, epithelial cells in the renal cortical collecting duct, spleen, testis, and many CNS structures (Jackson, 1996). The V_{1b} receptor is primarily located in the adenohypophysis. However, V_{1b} receptor mRNA has been detected in peripheral tissues (kidney, thymus, heart, lung, spleen, uterus and breast) and some areas of the brain in the rat (Lolait et al., 1995) as well as in the pancreas (Saito et al., 1995). In addition, this receptor subtype has been pharmacologically characterized in the rat adrenal medulla (Grazzini et al., 1996), rabbit tracheal epithelium (Tamaoki et al., 1998) and rat pancreas (Lee et al., 1995). These findings suggest that the V_{1b} receptors may have additional and unknown functions in the brain and at the periphery. The V_2 receptor is found principally in cells of the renal collecting duct system. OT receptor is predominantly located in the uterus and mammary gland. In addition, a high density of [^{3}H]oxytocin binding is present in the periphery of rat pancreatic islets suggesting the

presence of OT receptors in the pancreas (Stock et al., 1995). The amino acid sequences of four different types of AVP and OT receptors have been identified and they display a high homology of 102 conserved amino acids among the 370-420 amino acids. Both AVP and OT receptors are typical G protein-coupled receptors (GPCR), containing seven hydrophobic transmembranes α-helices joined by different intracellular N-terminal and extracellular C-terminal domains (Fig. 8). The ligand or agonist binding site is predicted to appear in a pocket formed by the seven transmembrane domains (Mouillac et al., 1995). The V_1 (Mitchell et al., 1979) and OT receptors are coupled to $G_{q/11}$ to activate phospholipase C (Marc et al., 1986), whereas the V_2 receptor is coupled to G_s to activate adenylyl cyclase, leading to generation of cAMP (Thibonnier, 1992). The third intracellular loop of the V_2 receptor is responsible for recognition and activation of G_s, and the second intracellular loop of V_1 receptor plays a key role in the selective activation of $G_{q/11}$ (Barberis et al., 1998).

High concentration of immunoreactive AVP and OT (0.9 ng/g wet weight to 3.7 ng/g wet weight tissues) are present in human and rat pancreata (Amico et al., 1988). Since this range of concentrations of AVP and OT in the pancreas is much higher than the one from the neurohypophysis that reaches the pancreas (3-25 pM; Franchini et al, 1996 and Kjaer et al., 1995)) via the circulation, the pancreas may be a site of local synthesis of both AVP and OT (Amico et al., 1988). Administration of OT increases plasma glucagon and insulin levels in normal dogs (Altszuler and Hampshire, 1981). A rise in the plasma glucagon level was found to be mediated by the increases in AVP and OT in rats subjected to hemorrhage (Dunning et al., 1985). OT and AVP (20 pg/ml) increase glucagon release from in situ perfusion of rat pancreas (Dunning et al., 1984). These findings suggest a paracrine function of AVP and OT in the induction of glucagon release.

Receptor antagonists

Shortly after the structure of AVP was synthesized, du Vigneaud et al. (1954), started to designate antagonists against AVP's pharmacological effects. Since that time, a number of highly selective V_{1a}, V_2 and OT receptor antagonists have been synthesized (Manning et al., 1993; László et al., 1991 and Williams et al., 1992), including both cyclic and linear peptides. [1-(β-mercapto-β,β-

cyclopentamethyleneproprionic acid),2-O-methyltyrosine]AVP, also known as $d(CH_2)_5[Tyr(Me)^2]AVP$ (pA_2 = 8.62; Fig. 9 A; Manning and Sawyer, 1989) and $desGly^9d(CH_2)_5[Tyr(Et)^2]AVP$ (WK-3-6) (pA_2 = 8.17; Jard et al., 1992) are more potent antagonists for V_{1a} receptors than for either V_{1b} or V_2 receptors. These antagonists have been widely used in physiological and pharmacological studies.

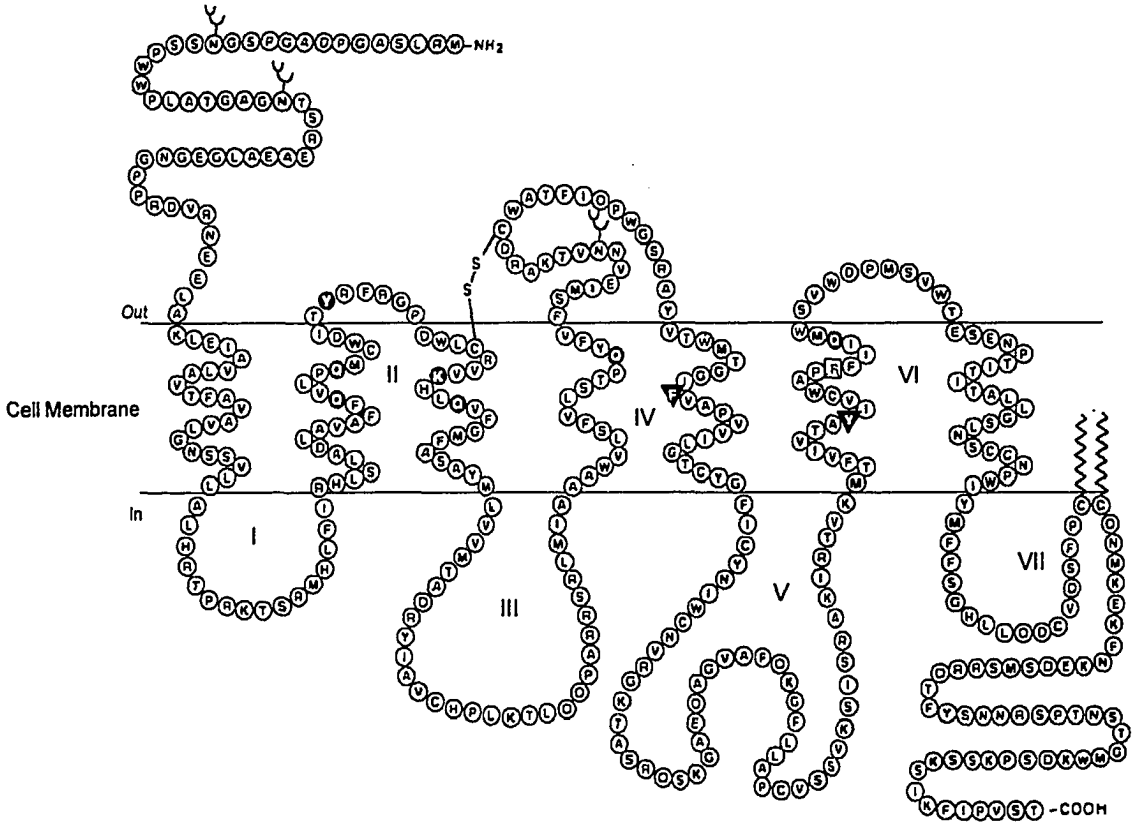


Fig. 8. Transmembrane topology of the human vasopressin V_{1a} receptor. Black-circled amino acids indicate the residues that are involved in the agonist binding. The amino acid presented in triangle (F) is possibly involved in the antagonist binding (modified from Barberis et al., 1998).

Although [1-deaminopenicillamine, 2-O-methyltyrosine]AVP, also known as dP[Tyr(Me)^2]AVP, is a potent V_{1b} receptor antagonist (pA_2 = 7.98; Fig. 9 B; Manning and Sawyer, 1989) with a low affinity for the V_2 receptors, it also blocks V_{1a} receptors (Schlosser et al. 1994).

4-OH-phenacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (CL-4-84) is a linear AVP antagonist that has a high affinity for V_{1b} receptor (K_i = 2.2 ± 0.1 nM; Thibonnier et al., 1997). However, it is also a potent antivasopressor (pA_2 = 8.74 and K_i = 0.45 ± 0.04 nM; Jard et al., 1986; Thibonnier et al., 1997). Unfortunately, there is no truly selective V_{1b} receptor antagonist currently available.

d(CH$_2$)$_5$[D-Phe^2, Ile^4]-AVP (AO-2-44) is a potent $V_2/V_{1a}/V_{1b}$ receptor antagonist (Jard et al., 1986). Its pA_2 values are 7.83 and 8.16 for anti-V_{1a} and anti-V_2, respectively. It is about 350 times more potent for binding V_{1b} receptors than WK-3-6 (Jard et al., 1986).

A number of structurally novel hexapeptides have been characterized as potent and selective antagonists for OT receptors. Cyclo-(L-Pro-D-2-naphthyl-Ala-L-Ile-D-pipecolic acid-L-pipecolic acid-D-His) (L-366,948; Fig. 9 C), as a representative of this class of compounds, exhibits a high binding affinity for OT receptors in rat uterus (K_i = 0.7 ± 0.21 nM) and mammary tissue (K_i = 0.8 ± 0.11 nM) with a much lower affinity against V_{1a} (K_i = 760 ± 100 nM) and V_2 (K_i = 320 ± 25 nM) receptors in the rat (Pettibone et al., 1991). It is a pure and highly potent OT antagonist that blocks both OT-stimulated uterine contraction (PA_2 = 8.53 ± 0.08) and phophatidylinositol turnover in uterine slices (Pettibone et al., 1991).

Signal transduction pathway of V_1 receptors

The signal transduction pathway of AVP has been investigated in various tissues, including smooth muscle, endothelium and endocrine cells (Spatz et al., 1994). AVP- and OT-induced ACTH release from the pituitary (Antoni et al., 1984 and Schosser et al., 1994) and insulin release from the rat pancreas and clonal β-cell lines RINm5F (Lee et al., 1995) and HIT (Richardson et al., 1990) are mediated by V_{1b} receptors. Generally, AVP binds to V_1 receptors coupled to a pertussis toxin (PTX)-insensitive G-protein, probably G_q, which activates phospholipase C-β (PLC-β)

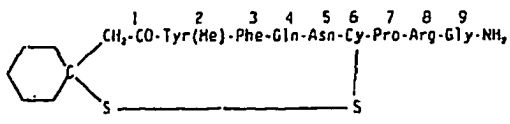
(Thibonnier et al., 1993). Activation of PLC is responsible for the hydrolysis of phosphatidylinositol-4, 5-bisphosphate (PIP_2), resulting in the generation of IP_3 and DAG. IP_3 stimulates the release of Ca^{2+} from the ER into the cytosol via activation of IP_3 receptor (IP_3R)/ Ca^{2+} channel (Berridge, 1993). Molecular structure of the IP_3R shows that this receptor consists of IP_3 binding, coupling and Ca^{2+} channel domains. When IP_3 binds to its binding domain, leading to a conformational change of the coupling domain, which, in turn, induces Ca^{2+} channel opening (Mignery and Sudhof, 1990). The mechanisms underlying the receptor-activated sustained Ca^{2+} influx are not yet clear, however, there is a well-established hypothesis indicating that Ca^{2+} release activates Ca^{2+} influx through Ca^{2+} channels on the plasma membrane (Petersen and Maruyama, 1983; Putney, 1990; von Tscharner et al., 1986). Ca^{2+} binds to and activates a number of intracellular proteins, including calmodulin that contribute to the ultimate cellular responses, including insulin and/or glucagon release. DAG activates PKC, leading to phosphorylation of key proteins that contribute to the cellular response (Fig. 10). In addition, V_1 receptors can also couple to other effectors, such as phospholipase D (PLD) and phospholipase A_2 (PLA_2). Activation of PLD evokes the hydrolysis of phospholipid to form phosphatidic acid (PA), which is further metabolized to DAG that activates PKC. Activation of PLA_2 generates arachidonic acid (AA) from the membrane phospholipid, and AA is metabolized to prostaglandin and epoxyeicosatrienoic acids, which modulate various cellular responses (Jackson, 1996).

AVP stimulates insulin release via the activation of both PLC-dependent and -independent pathways (Chen et al., 1994; Li et al., 1992; Richardson et al., 1990). However, in the pancreatic α cells, the mechanism underlying AVP-induced glucagon release has never been established. Therefore, this leads us to study the mechanisms involving the effects of AVP on both the $[Ca^{2+}]_i$ increase and glucagon release.

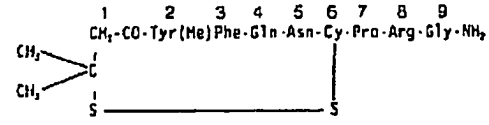
G-protein

G-proteins are located predominantly at the intracellular face of the plasma membrane, where they can interact with both receptors (upstream components) and effectors (downstream components) of the different signaling systems. They are trimeric molecules, consisting of α, β and γ subunits and they accomplish their job by a cycle of nucleotide exchange and GTP hydrolysis upon subunit dissociation and

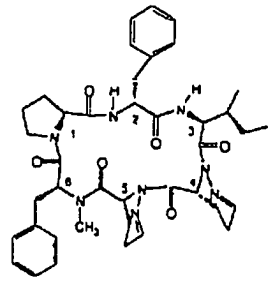
reassociation. The β- and γ-subunits associate tightly as a complex. The α-subunit has a high affinity biding-site for guanine nucleotides (GDP and GTP). The Gα-subunit serves as a regulator of effector proteins by which its inactive form (GDP-bound) binds tightly to the βγ complex, whereas the active form of the Gα-subunit (GTP-bound) dissociates from the βγ. The Gα-subunit, itself is an enzyme that possesses intrinsic GTPase activity and hydrolyzes GTP to form GDP (Fig. 11).



A) $d(CH_2)_5[Tyr(Me)^2]AVP$



B) $dP[Tyr(Me)^2]AVP$



C) L-366,948; position: 1) L-pro; 2) D-2-Napthylalanine
3) L-Ile; 4) D-pipecolic acid
5) L- pipecolic acid; 6) D-His

Fig. 9. Structures of AVP receptor antagonists A) $d(CH_2)_5[Tyr(Me)^2]AVP$; b) $dP[Tyr(Me)^2]AVP$ (modified from Manning and Sawyer, 1989) and that of OT receptor antagonist C) L-366,948 (modified from Pettibone et al., 1991).

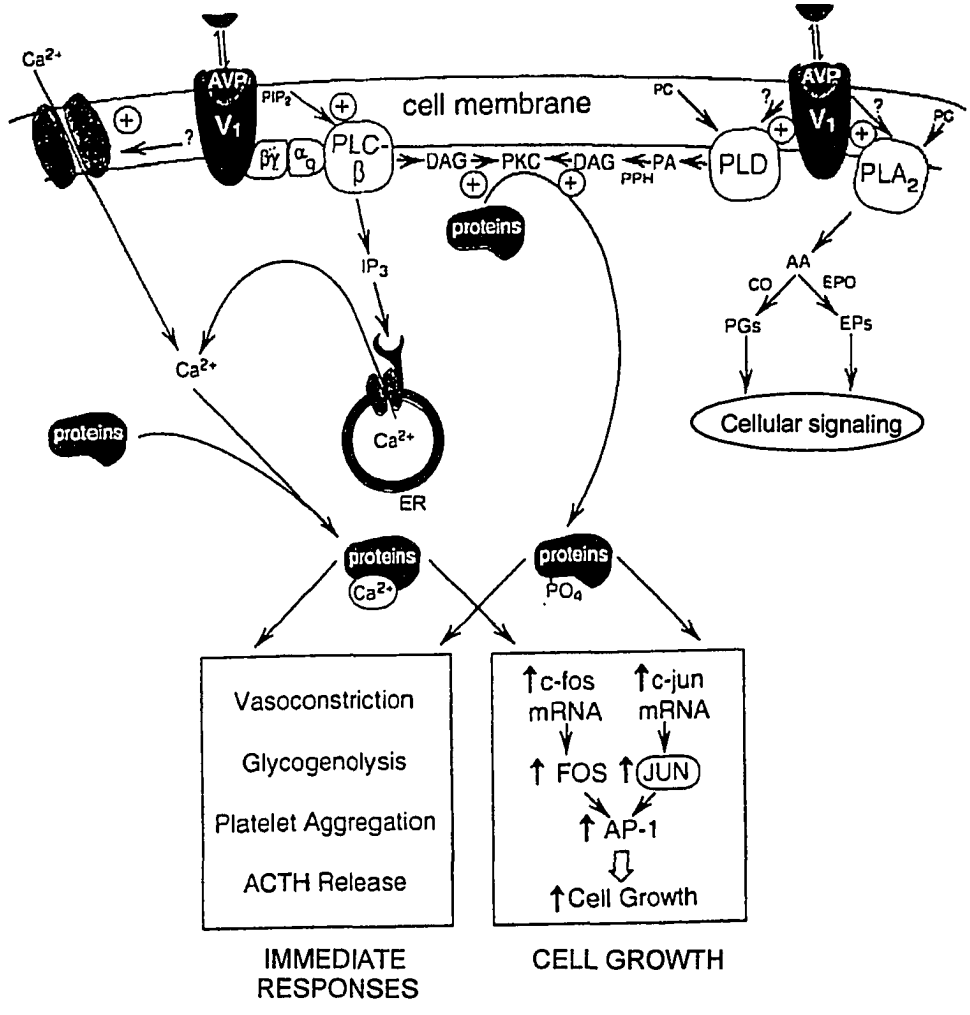


Fig. 10. Mechanisms of V_1 receptor-effector coupling. V_1, V_1 vasopressin receptor; AVP, arginine vasopressin; α_q, β, γ, subuits of G protein; PLD, phospholipase D; PLC-β, phospholipase C-β; PLA_2, phospholipase A_2; DAG, 1,2-diacylglycerol; ER, endoplasmic reticulum; PKC, protein kinase C; PIP_2, phosphatidylinositol-4,5-bisphosphate; IP_3, inositol-1,4,5-trisphosphate; PA, phosphatidic acid; PPH, phosphatidate phosphohydrolase; PC, phosphatidylcholine; AA, arachidonic acid; PGs, prostaglandins; EPs, epoxyeicosatrienoic acids; CO, cyclooxygenase; EPO, epoxygenase; AP-1, transcription factor consisting of heterodimer of *FOS* and *JUN*; c-*fos* and c-*jun* are proto-oncogenes; FOS and JUN are products of the c-fos and c-jun gene expressions, respectively (modified from Jackson, 1996).

The G-proteins are classified based on the identity of Gα subunits into four major subfamilies; G_s, $G_{i/o}$, G_q, and G_{12}. Chorela toxin (CTX) from *Vibrio cholerae* catalyzes the ADP-ribosylation of a conserved arginine residue at position 202 of the Gα subunit in the G_s family, which results in the inhibition of GTPase activity and the interaction with the βγ subunit (Serventi et al., 1992). PTX from *Bordetella pertussis* catalyzes the ADP-ribosylation of a cystein residue at position 4 from the C-terminus of Gα subunits in the $G_{i/o}$ family, resulting in the inhibition of receptor-G protein coupling. G_q and G_{12} lack of the cystein residue that can undergo ADP-ribosylation by PTX, and thus are referred to as PTX-insensitive G-proteins (Strathmann and Simon, 1990). Generally, G_q couples to PLC-β (Taylor et al., 1991).

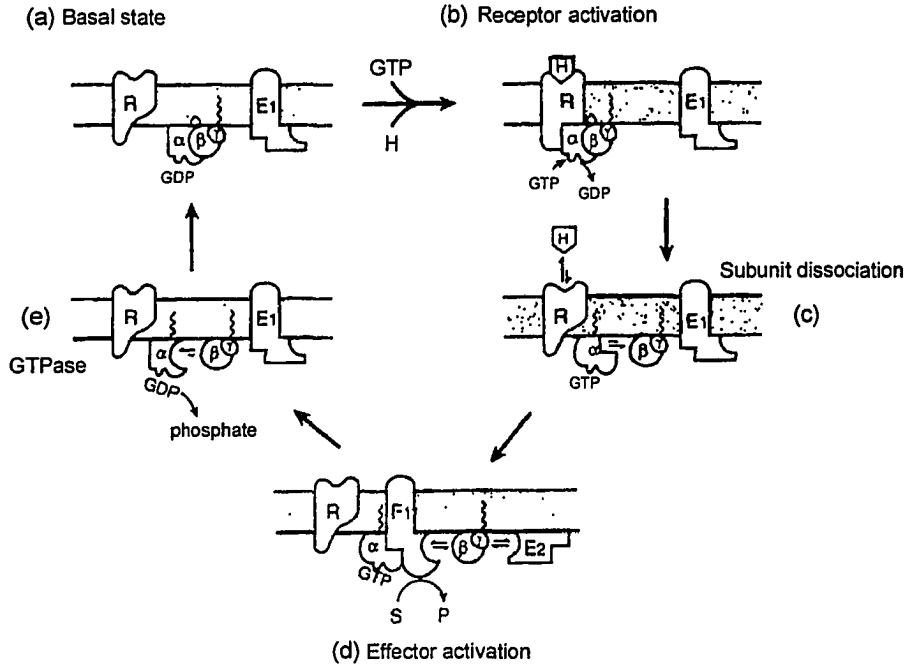


Fig. 11. The G-protein-mediated transmembrane signaling (modified from Hepler and Gilman, 1992).

Involvement of phospholipases in the mechanism of AVP

Phospholipase C

Phosphoinositides (PPI) or inositol lipids consist of a glycerol backbone containing 2 fatty acyl groups (at 1- and 2-positions) and a phosphate group coupled to the sugar *myo*-inositol at the 3-position. Phosphorylation of phosphatidylinositol (PI) to phosphatidylinositol 4-mono phosphate (PIP) and then to phosphatidylinositol 4,5-bisphosphate (PIP_2) occurs predominantly in the plasma membrane. The PIP_2 is the primary hydrolyzed product of PPI, that usually serves as a primary substrate in cells for the second messenger generation. PLC is a family of isozymes that hydrolyzes phospholipids at the 3-position of phosphodiester bond of glycerol backbone. PLC hydrolyzes PIP_2 to generate IP_3 and DAG.

PPI-specific PLC (PPI-PLC) is a subfamily of PLC that specifically hydrolyzes only inositol-containing lipids, but not other phospholipids, such as phosphatidylcholine (PC). PPI-PLC has been classified into three classes; PPI-PLC-β, -γ, and -δ. They are distinct proteins that contain only a small amount of sequence identity (Rhee and Choi, 1992). PPI-PLC-β has been further classified into two subtypes; βI and βII, which exhibit a high degree of sequence identity.

All PPI-PLC isozymes catalyze the hydrolysis of PI, PIP, and PIP_2 in vitro; however, only PIP_2 is the substrate in vivo. Both PPI-PLC-β and PPI-PLC-γ isozymes are involved in receptor-activated PIP_2 hydrolysis in the cells; however, the mechanisms of their activations are different due to a difference in the primary amino acid sequence. PPI-PLC-γ contains src homology (SH2 and SH3) domains, which are found to mediate the binding to other proteins that contain phosphorylated tyrosine residues such as growth factor receptors, but PPI-PLC-β does not. Activation of PPI-PLC-β is mediated by the G_q-coupled signal transduction (Taylor et al., 1991), whereas PPI-PLC-γ is activated by the agonist-occupied receptors that possess intrinsic tyrosine kinase activity (Ullrich and Schlessinger, 1990).

Stimulation of PIP_2 hydrolysis leads to IP_3 formation that causes an elevation of $[Ca^{2+}]_i$. In β-cells, AVP increases insulin release by promoting the formation of IP_3 (Li et

al., 1992); however, whether AVP induces glucagon release through the same mechanism as that of insulin release remains to be determined.

Calcium signaling

Regulated in $[Ca^{2+}]_i$ have been found to mediate exocytosis in both pancreatic α and β cells (Bode et al, 1994; Li et al., 1992 and Wollheim and Pozzan, 1984). The $[Ca^{2+}]_i$ in unstimulated cells is about 100 nM, which is approximately 10,000 fold lower than the extracellular Ca^{2+} concentration. The low $[Ca^{2+}]_i$ is maintained by a variety of cellular processes. The major barrier is the surface membrane which is highly impermeable to Ca^{2+}. In unstimulated cells, Ca^{2+} channels in the plasma membrane are inactive or closed.

In a glucagon secreting cell line, In-R1-G9, the Ca^{2+} channels can exhibit spontaneously brief activation (Ca^{2+} spikes) that permits significant basal Ca^{2+} influx and leads to glucagon release (Bode et al., 1994). In the meantime, these Ca^{2+} channels can be activated or opened to allow a large Ca^{2+} influx. In addition to the prevention of the Ca^{2+} influx, the plasma membrane exhibits two energy-dependent processes of extrusion of Ca^{2+} from the cells to limit elevations of $[Ca^{2+}]_i$. These are the Ca^{2+}-Mg^{2+} ATPase or plasmalemmal Ca^{2+} pump and the Na^+-Ca^{2+} exchange mechanism, which depends on the Na^+ gradient established by Na^+-K^+ ATPase. Within the cell, the ER and mitochondria are the major organelles that contribute to the maintenance of a low $[Ca^{2+}]_i$. The ER has a high affinity, but low capacity for Ca^{2+} sequestering that plays a role in maintaining $[Ca^{2+}]_i$ in the nanomolar range, whereas the mitochondria are low-affinity, but high-capacity Ca^{2+}-sequestering organelles that seem to serve as a protective function against large increases in $[Ca^{2+}]_i$. Ca^{2+} is pumped into the ER by the action of membrane-bound Ca^{2+}-Mg^{2+} ATPase, which works differently from the one in the plasma membrane. In mitochondria, Ca^{2+} is sequestered based on the mitochondrial proton gradient (Fig. 12).

Generally, receptor-activated Ca^{2+} mobilization via the PPI cascade can involve two phases: 1) Ca^{2+} release from the ER, and 2) a more prolonged phase of extracellular Ca^{2+} entry (Putney, 1987). Therefore, $[Ca^{2+}]_i$ can rise either through the release of Ca^{2+} from the ER or through the entry of extracellular Ca^{2+} via the plasma

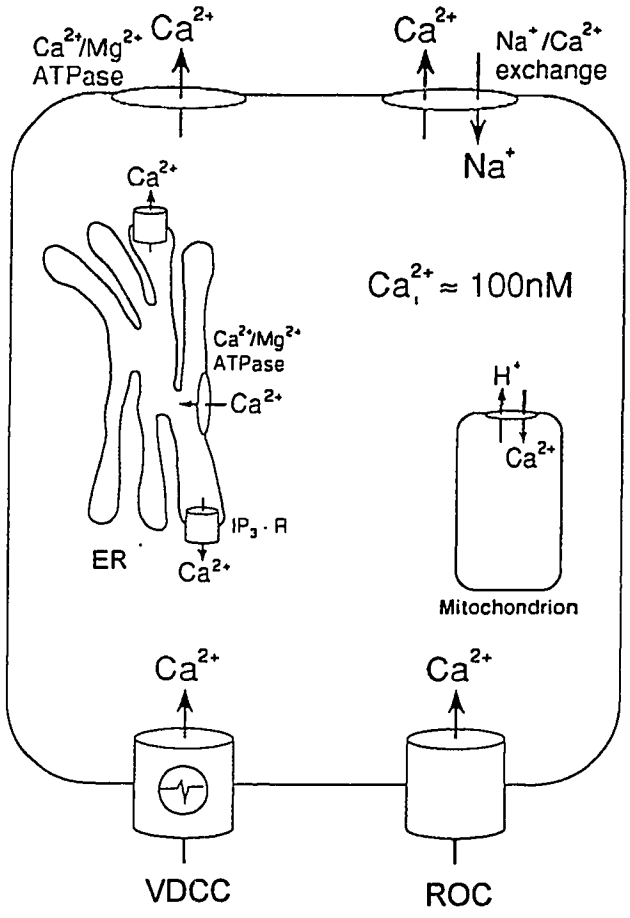


Fig. 12. Schematic depiction of a cell illustrating mechanisms of regulation of intracellular ionized calcium. ER, endoplasmic reticulum; IP_3; inositol 1,4,5 trisphosphate; VDCC, voltage-dependent Ca^{2+} channel; ROC, receptor-operated ca^{2+} channel (modified from Gershengorn and Perlman, 1995).

membrane. The regulation of Ca^{2+} influx is unclear; however, there is evidence demonstrating the existence of Ca^{2+} release-activated Ca^{2+} influx or so called capacitative Ca^{2+} entry mechanism (Petersen and Maruyama, 1983; Putney, 1990; von Tscharner et al., 1986). This mechanism indicates that the depletion of intracellular Ca^{2+} store, resulting from IP_3-induced Ca^{2+} release, evokes Ca^{2+} influx through the

opening of Ca^{2+} channels on the plasma membrane (Ca^{2+}-release activated channels; Leung et al., 1996). The most popular strategy to prove this hypothesis is the use of drugs such as thapsigargin (TG) (Jackson et al., 1988; Thastrup, 1990), which inhibits microsomal (but not plasmalemmal) Ca^{2+}-ATPase, thereby depleting intracellular Ca^{2+} stores. TG also activates sustained Ca^{2+} entry to the same or greater extent as PLC-linked agonist. However, subsequent addition of any agonists in the presence of TG does not further increase the rate of Ca^{2+} influx (Takemura et al., 1989). Thus, this indicates that depletion of the agonist-sensitive intracellular Ca^{2+} stores regulates Ca^{2+} influx at the plasma membrane.

The influx of extracellular Ca^{2+} appears to be mediated by either the voltage-dependent Ca^{2+} channel (VDCC) or receptor-operated Ca^{2+} channel (ROC). The three major subtypes of VDCCs have been designated as L-type (long-lasting), T-type (transient) and N-type (neither long-lasting nor transient) Ca^{2+} channels. The L- and T-types VDCCs and ROCs have been reported in the endocrine α cells (Bode et al., 1994; Rorsman, 1988). The elevation of $[Ca^{2+}]_i$ leads to an increase in the binding of Ca^{2+} to a specific regulatory protein, calmodulin (Klee et al., 1980), which stimulates cellular responses and secretion.

The activation of VDCC is controlled by the change of the plasma membrane potential, which can initiate a number of cellular responses, including muscle contraction and exocytotic secretion in endocrine and nerve cells. The L-type VDCC is characterized by its sensitivity to dihydropyridines (DHP). It is inhibited by the DHP antagonist nimodipine and is activated by the DHP agonist Bay K 8644 (Smith et al., 1993). DHP has a higher affinity for the channels in inactive states than active or resting states. The T-type VDCC, activated at the membrane potential of ~ -50 mV, may be important for the pacemaker activity in several tissues. There are no specific blockers for the T-type VDCC; however, Ni^{2+}, tetrandine and felodine partially block this type of channel.

The ROCs are activated by agonists that bind to the plasma membrane receptor. Their structures and the mechanisms of their activations are not yet well-established. The ROCs provide a number of pathways by which Ca^{2+}can be delivered to the cytosol and the ER. Store-operated Ca^{2+} channels (SOCs) are a major subfamily of ROCs, which are activated by a decrease in Ca^{2+} in the ER. In order to initiate or maintain a

specific type of Ca^{2+} signal, ROCs, which are non-selective cation channels, can deliver Ca^{2+} directly to specific regions of the cytosol. In addition, the opening of ROCs increase Na^{+} influx, which induces depolarization of the plasma membrane, leading to the opening of VDCCs and the subsequent inflow of Ca^{2+}. SOCs deliver Ca^{2+} specifically to the ER, thus maintaining oscillating Ca^{2+} signals (Barritt, 1999). ROCs can be blocked by an antagonist, SK&F 96365 (1-(beta-[3-(4 methoxyphenyl) propoxyl]-4-methoxyphenethyl)-1H-imidazole) (Cabello and Schilling, 1993).

Phospholipase A_2

PLA_2 is a group of ubiquitous enzymes that exist in many cells and tissues. The hydrolysis of cellular phospholipids, such as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) by PLA_2 causes the release of free fatty acids (FFAs), arachidonic acid (AA), and lysophospholipids (Fig. 13 A). It hydrolyzes the ester linkage of membrane glycerophospholipids at the sn-2 position of glycerol moiety. The released AA may affect a number of cellular functions, because it activates PKC (Nishizuka, 1992) and MAP kinase (Rao et al., 1994), and the concomitant formation of lysophosphatidylcholine (lysoPC) may cause damages to cellular membranes (Weltzien, 1979). AA and lysoPC are metabolized via cyclooxygenase to various protaglandins, thromboxanes, and leukotrienes that are called eicosanoids, and to epoxyeicosatrienoic acids (EPs) via epoxygenase (EPO) (Needleman et al., 1986). The eicosanoids are pro-inflammatory mediators, whereas the EPs are the metabolites that mediate vasodilation, mitogenesis, platelet aggregation, Ca^{2+} signaling and steroidogenesis (Medhora and Harder, 1998). In general, the agonists that hydrolyze PC also promote PI hydrolysis in their target cells, leading to a biphasic increase in DAG, with a rapid and transient peak followed by a prolonged accumulation (Exton, 1990). The first peak is due to PI hydrolysis and is associated with increases in IP_3 and $[Ca^{2+}]_i$. The second peak is due to PC hydrolysis and is associated with an increase in choline and choline phosphate (P-choline) (Fig. 13 B).

The PLA_2 enzymes are classified by the use of functional activities into at least four different subfamilies (Kramer and Sharp, 1997). First, secretory PLA_2 ($sPLA_2$), with a low molecular weight of 14 kDa, is characterized by a catalytic requirement of Ca^{2+} and a structure that is maintained by disulfide bridges. Three $sPLA_2$ proteins have been

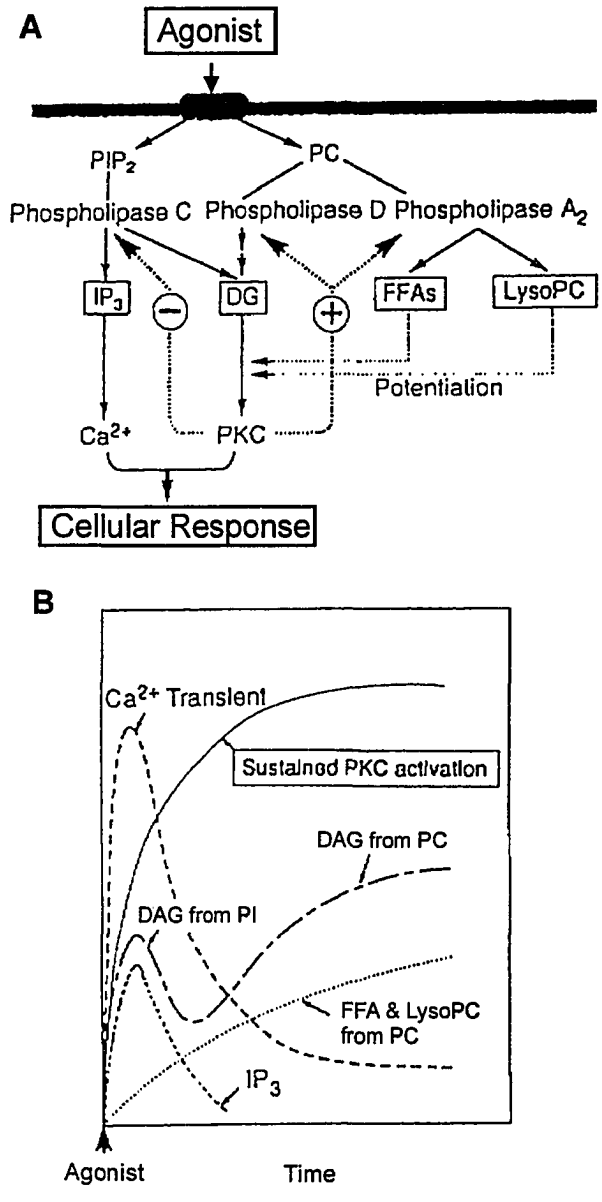


Fig. 13. Schematic representation of agonist-induced membrane phospholipid degradation (A). Time course of the generation of various signaling molecules (B). PIP_2, phosphatidylinositol 4,5 bisphosphate; PC, phosphatidylcholine; DAG, diacyl glycerol; FFAs, free fatty acids; IP_3, inositol 1,4,5 trisphosphate; LysoPC, Lysophosphatidylcholine; PKC, protein kinase C; PI, phosphoinositol (modified from Nishizuka, 1992)

purified and characterized as group I, II and III (Glaser et al., 1993; Dennis, 1994). $sPLA_2$ enzymes are usually secreted from cells and are found in snake and bee venoms, synovial fluid and pancreatic secretion. They have been associated with several toxics (e.g. neurotoxicity, myotoxicity, etc.). pathological (e.g. inflammation, hypersensitivity), or physiological (e.g. contraction, proliferation) processes (Lambeau et al., 1994). However, they are believed not to be involved in cell signaling. Second, Ca^{2+}-sensitive cytosolic PLA_2 ($cPLA_2$) with a molecular weight of 85 kDa has been purified, cloned and biochemically characterized (Clark et al., 1995). $cPLA_2$ is the only known PLA_2 that is involved in the receptor-mediated eicosanoid production and intracellular signal transduction processes (Kramer et al., 1997). It is activated by an increase in $[Ca^{2+}]_i$ and phosphorylation of MAP kinase. Increased $[Ca^{2+}]_i$ causes translocation of $cPLA_2$ from the cytosol to the plasma membrane (Clark et al., 1995). Third, a variety of ATP-sensitive Ca^{2+}-independent cytosolic PLA_2 (ASCI-PLA_2), with molecular weights ranging from 29 to 85 kDa have been purified from different tissues. Based on the primary structure of the ASCI-PLA_2 derived from Chinese hamster ovary cell (CHO), it is noteworthy that it has no structural relationship to $cPLA_2$ (Tung et al., 1997). Fourth, the platelet-activating factor acetylhydrolase or lipoprotein-associated PLA_2 is bound to lipoproteins in the plasma membrane and it is independent of Ca^{2+} (Stafforini et al., 1996). The remarkable difference of this PLA_2 from others is its specificity for short and/or oxidized acyl groups at *sn*-2 position of phospholipids.

At least three different kinds of PLA_2 are expressed in the pancreatic β-cells, such as low molecular weight $sPLA_2$, $cPLA_2$, and ASCI-PLA_2 (Ma et al., 1998). PLA_2, such as ASCI-PLA_2 and $cPLA_2$ are found to mediate the insulin secretion in pancreatic islets and clonal β-cells (Parker et al., 1996; Ramanadham et al., 1994). In addition, the PLA_2 enzymes are found to be involved in AVP-induced signal transduction (Spatz et al., 1994; Loxley, et al., 1993); however, whether the AVP-activated PLA_2 pathway is involved in the regulation of glucagon release from the pancreatic α-cells remains to be determined.

Phospholipase D

PLD is an enzyme that was first discovered in plants by Hanahan and Chaikoff in 1948. In 1975, Saito and Kanfer reported the appearance of PLD in mammals, and since that time, this enzyme has been shown to exist in most mammalian tissues. It catalyzes the hydrolysis of glycerophospholipids at their distal phosphodiester bond to generate phosphatidic acid (PA) and the corresponding free polar head group. Although, PE (Kiss and Anderson, 1989) and PI (Balsinde et al., 1989) have also been reported to be substrates for PLD in some systems, PC is the preferred substrate in most systems (Lambeth, 1993).

PLD activation can be initiated by a number of stimuli, such as hormones, peptides, cytokines, oxidants, neurotransmitters, and growth factors in a variety of mammalian systems (Boarder, 1994). Activation of PLD results in the transient generation of PA, which is further metabolized to lysophosphatidic acid (lyso PA) and DAG. The PA, lyso PA, and DAG have been recognized as the intracellular second messengers and have been found to activate PKC (Fig. 14) (Ando et al., 1989; Dunlop and Larkins, 1985). PLD also catalyzes a transphosphatidylation reaction, in which a primary alcohol serves as the phosphatidyl group acceptor. This reaction exchanges the polar head group of phospholipid substrate with the primary alcohol (e.g. ethanol and propanol), yielding the phosphatidylalcohol, which has been considered as a specific marker for PLD activity, a valuable tool for PLD research. In fact, phosphatidylalcohol is a more quantitative and less ambiguous marker for PLD activity than PA, which can be rapidly metabolized to other products (Fig. 15; Olson and Lambeth, 1996).

Activation of PLD has been suggested to be either dependent on, or independent of Ca^{2+}. The signal-activated PLD is regulated by several different pathways involving G-proteins, hydrolysis of PIP_2, DAG, activation of PKC (especially PKC-α and -β) and protein tyrosine kinase, stimulation of calmodulin/myosin light chain kinase, and changes in $[Ca^{2+}]_i$ (Boarder, 1994). In addition, the small molecular weight G-proteins, such as ADP-ribosylation factor (ARF), rho and ras can activate PLD (Fig. 14) (Olson and Lambeth, 1996).

Several forms of PLD exist in mammalian cells, such as membrane-associated PLD and cytosolic PLD. The cell membranes contain at least two types of PLD: one

that acts as an integral membrane protein and is activated by sodium oleate, an unsaturated fatty acid, but not by ARF, and a second type that acts as a peripheral membrane protein and is activated by ARF, but not by sodium oleate. The sodium oleate-activated PLD enzyme exhibits an acidic optimal pH (6.0-6.5) and does not require Ca^{2+} for activation (Balsinde et al., 1989).

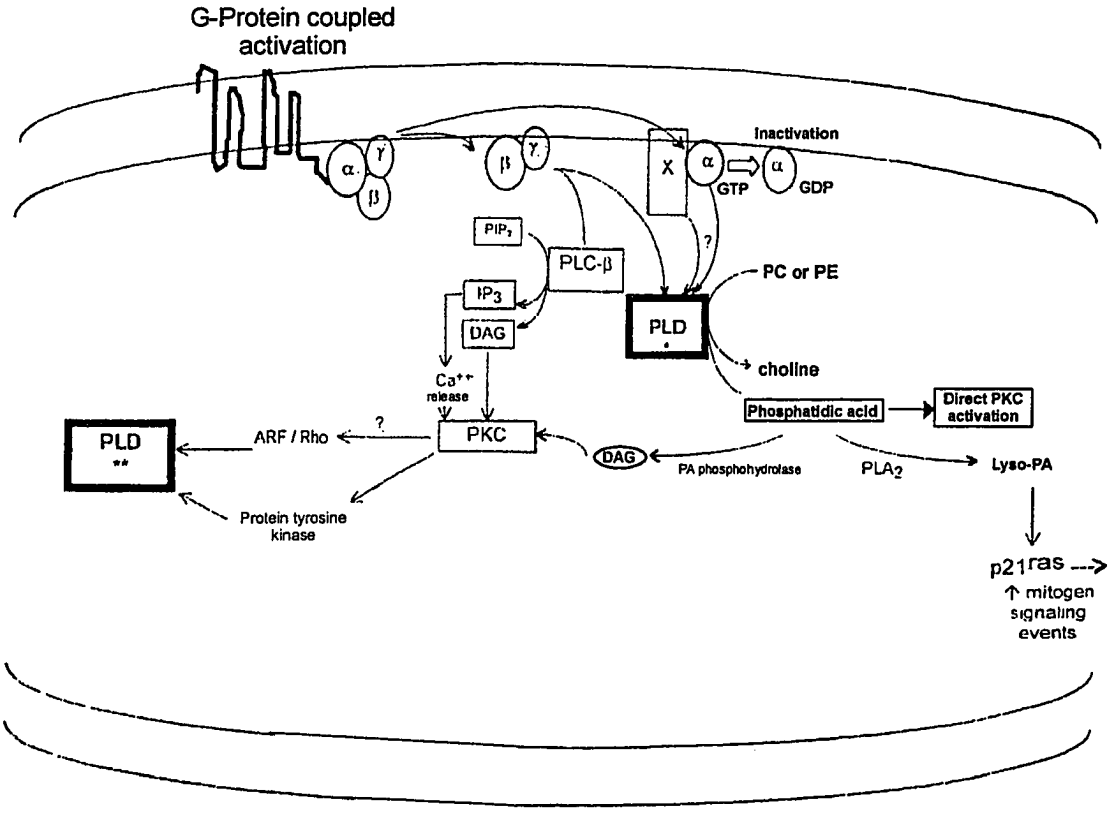
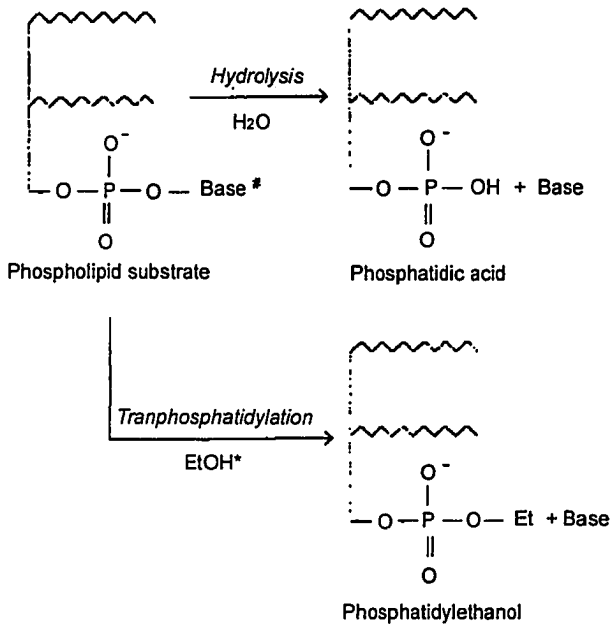


Fig. 14. Different agonists and pathways that activate phospholipase D signaling in mammalian cells. *, Short-term activation initiated by α subunits by either direct or co-factor-mediated (denoted by "X") activation; **, sustained activation initiated by β/γ subunits acting through PLCβ and followed by the release of downstream signaling molecules; IP_3, inositol 1,4,5-trisphosphate; PIP_2, phosphatidylinositol (4,5)-bisphosphate; ARF, ADP-ribosylation factor; PKC, protein kinase C; DAG, diacyl glycerol; PLA_2, phospholipase A_2 (modified from Gomez-Cambronero and Keire, 1998)



#Base = choline, ethanolamine, etc.

*Other short-chain alcohols can substitute for ethanol

Fig. 15. A schematic representation of phospholipase D activity (modified from Shukla and Halenda, 1991)

The cytosolic PLD is Ca^{2+} dependent (Balsinde et al., 1989; Huang et al., 1992), which has been identified in many different tissues, such as human neutrophils (Balsinde et al., 1989), bovine lung, brain, kidney, spleen, heart and liver (Wang et al, 1991) and Madin-Darby canine kidney (MDCK) cells (Huang et al., 1992).

Specific and potent inhibitors for PLD have not yet been identified. A number of compounds, for example the PI 3-kinase inhibitor wortmannin and the protease inhibitors carbobenzyloxy-leucine-tyrosine-chloromethylketone (zLYCK) and leupeptin, have been reported to block PLD activation. The phosphatase inhibitor, 2,3-diphosphoglycerate appears to be a direct, but not a very potent inhibitor for PLD (K_i = 9 mM; Kanaho et al., 1993). Another compound that may directly inhibit PLD is 1,2-

bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid (BAPTA), which is used as an intracellular Ca^{2+} chelator (Coorsen and Haslam, 1993). In addition, choline, and some cation amines, e.g. *N*-methylglucamine, ethanolamine and $Tris^{+}$, inhibit PLD activation in permeabilized macrophages (El-Moatassim and Dubyak, 1993). However, the mode of action of these compounds remain unclear; it is likely that some of them block PLD-upstream events (Liscovitch and Chalifa-Caspi, 1996).

In 1987, Bocckino et al. demonstrated a direct relationship between PLD and AVP, in which AVP increased PA formation that preceded a secondary increase in DAG levels, suggesting that AVP can activate PLD. In addition, AVP activates PLD in the rat Leydig (Vinggaard and Hansen, 1991) and glomerular mesangial cells (Kusaka, et al., 1996). Activation of PLD stimulates insulin release from the pancreatic islets (Metz and Dunnlop, 1990). However, the role of PLD in AVP-induced glucagon release has never been established.

Involvement of protein kinase C in the mechanism of AVP

PKC was first discovered and identified by Nishizuka and coworker (1977) as a cytosolic, calcium-activated, phospholipid-dependent kinase. It is the enzyme that takes part in cellular responses to various agonists, including hormones, neurotransmitters and some growth factors. It is activated by an increase in DAG in the plasma membrane that results from agonist-induced hydrolysis of PI, PC and PE.

PKC is a family of serine/threonine-specific protein kinases, consisting of at least ten isozymes, that can be classified into three groups: 1) the conventional (cPKCs) α, βI, βII, and γ (require negatively charged phospholipids e.g. phosphatidylserine (PS), DAG or a phorbol ester, and Ca^{2+} for optimal activation); 2) the novel (nPKCs) δ, ε, θ, η, and μ (require negatively charged phospholipids, DAG or a phorbol ester, but no Ca^{2+} for optimal activation); 3) the atypical (aPKCs) λ/ι (mouse/human) and ζ (require only negatively charged phospholipids for activation) (Table 3).

PKC contains regulatory and catalytic domains, which consist of 4 conserved regions (C1-C4) and 5 variable regions (V1-V5). The regulatory domain at the NH_2 terminal, which is involved in the binding of substrates and activators, consists of a phorbol ester binding domain (C1), a Ca^{2+} binding domain (C2), and a pseudosubstrate-

binding site. The catalytic domain at carboxyl-terminal, which is involved in catalytic activity and phosphorylation of substrates, consists of an ATP-binding site (C3), and a protein kinase domain (C4), which contains both substrate binding site and the phosphoryl transfer region) (Nishizuka, 1988) (Fig. 16). The pseudosubstrate binding site in the regulatory domain will bind to a substrate binding site in the catalytic domain during the inactive state of the enzyme. Activation of PKC causes a conformational change in the enzyme, releasing the pseudosubstrate from the substrate binding site, allowing the substrate to bind to the enzyme, which is phosphorylated on serine and threonine residues (Fig. 17).

The isozymes are characterized by differences in the C1-C4 domains (Stabel, 1994 and Nishizuka, 1995). The Ca^{2+} binding region (C2) is missing in nPKCs and aPKCs. cPKCs and nPKCs consist of two zinc fingers in the phorbol ester binding site (C1), whereas aPKCs contains only a single zinc finger in C1. PKCμ is classified as an nPKC, because it is phospholipid-dependent, Ca^{2+}-insensitive, and activated by phorbol esters. However, it does not have a pseudosubstrate domain (Hofmann, 1997).

Table 3. PKC subspecies in mammalian tissues. (Modified from Nishizuka, 1992)

Sub-species	Amino acid residues	Molecular size (kDa)	Activators
	Group A: conventional PKCs (cPKCs)		
α	672	76.79	PS, Ca^{2+}, DG, FFA, LysoPC
βI	671	76.79	PS, Ca^{2+}, DG, FFA, LysoPC
βII	673	76.93	PS, Ca^{2+}, DG, FFA, LysoPC
γ	697	78.37	PS, Ca^{2+}, DG, FFA, LysoPC
	Group B: novel PKCs (nPKCs)		
δ	673	77.52	PS, DG
ε	737	83.47	PS, DG, FFA
η	683	77.97	PS, DG
μ	912	115	PS, DG
	Group C: atypical PKCs (aPKCs)		
θ	707	81.57	PS, DG
ζ	592	67.74	PS, FFA
λ	586	67.2	PS, FFA

DG, diacyl glycerol; PS, phosphatidylserine; FFA, cis unsaturated fatty acid; Lyso PC, lysophosphatidylcholine.

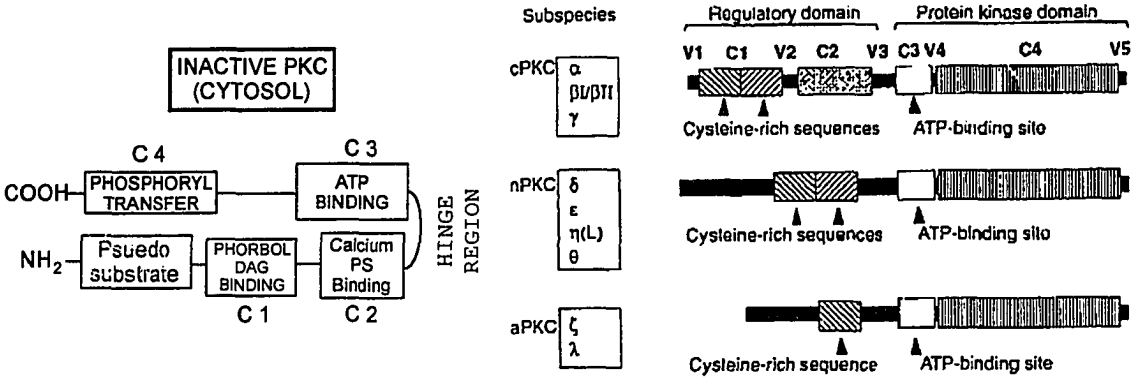


Fig. 16. Structure of PKC subspecies. Four conserved (C1-C4) and five variable (V1 to V5) regions of the cPKC group are indicated (modified from Mochly-Rosen and Gordon, 1998; Nishizuka, 1992).

Several PKC isozymes are usually found within one cell. In general, most inactive PKCs are localized in the cytosol and, upon activation, they become more hydrophobic and translocate to the plasma membrane (Mochly-Rosen and Gordon, 1998). However, recent evidence demonstrates that both inactive and active PKC isozymes can be localized in specific intracellular sites, depending on their bindings to specific anchoring molecules and, upon stimulation, translocated to new distinct intracellular sites. For instance, inactive PKC-βII are associated with the fibrillar structures of cardiac myocytes, and translocate to the perinucleus and the cell periphery upon activation (Disatnik et al., 1994). The anchoring proteins for activated PKC isozymes are called "receptors for activated C-kinase" (RACKs), and there is another set of proteins that anchor inactive PKC isozymes, which are called "receptors for inactive C-kinase" (RICKs; Mochly-Rosen et al., 1991). It is likely that the specific and unique cellular functions of PKCs are determined by the binding of isozymes to specific anchoring proteins in close proximity to particular groups of substrates and are different from others. RICKs, such as PS binding proteins/substrates, are proteins that bind PKC in an isozyme-specific and saturable manner, because they are localized

differently in each inactive PKC isozymes. PMA and other PKC activators induce the release of RICK from PKC, resulting in the binding of PKC to its RACK (Mochly-rosen and Gordon, 1998). The RACK-bound PKC is active and the binding site for substrate is available, leading to the induction of various cellular responses (Fig. 17).

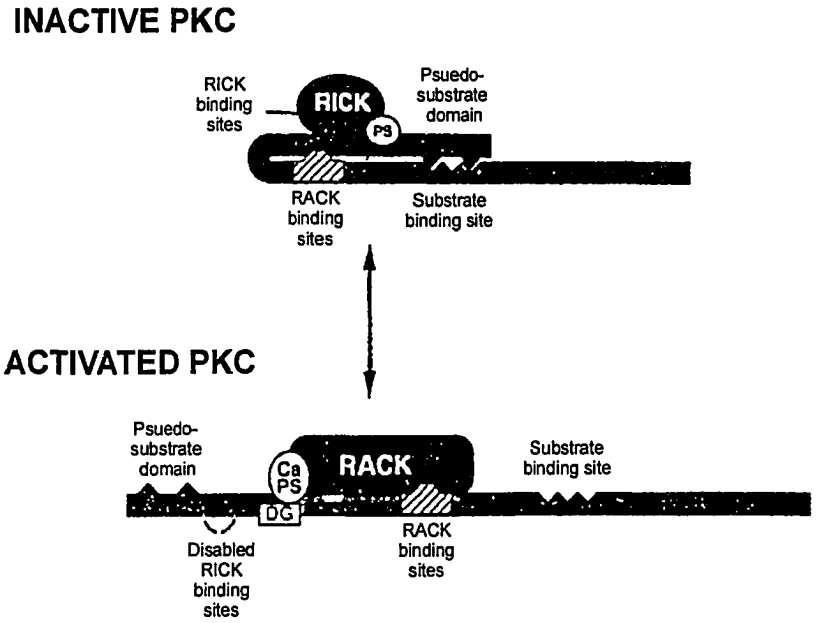


Fig. 17. A model for interaction of PKC with anchoring proteins. The binding sites for RICKs and RACKs are located in the regulatory domain of PKC. RICKs, receptors for inactive C-kinase; RACKs, receptors for activated C-kinase; PS, phosphatidylserine; Ca, calcium; DG, diacylglycerol (modified from Mochly-Rosen and Gordon, 1998).

Various PKC isozymes are identified in pancreatic islets (e.g. α, β, δ, ε, ζ, and ι), and in β-cell lines (e.g. α, β, δ, ε, ζ, μ and ι) (Jones and Persaud, 1998), however, the identification of PKC isozymes presented in α-cells has never been established. Activation of PKC by a phorbol ester PMA increases glucagon release from the pancreas (Hii et al., 1986; Niki et al., 1986). In addition, the down-regulation of PKC by pretreating rat islets with PMA for 24 h leads to a decrease in arginine-induced glucagon release (Bjaaland et al., 1988). Together, these findings suggest the

participation of PKC in the regulation of glucagon release. Although, PKC seems to play a positive role in the regulation of glucagon release from the pancreas, PKC negatively regulates AVP-induced IP_3 production and $[Ca^{2+}]_i$ increases in many different systems, such as in the rat glomerulosa (Gallo-Payet et al., 1991), vascular smooth muscle (Stassen et al., 1989) and pancreatic β cells (Gao et al., 1994). Therefore, it is imperative to characterize the role of PKC on AVP-induced glucagon release, since AVP may physiologically induce glucagon release.

PMA has been used as a substitute of DAG in vitro to activate PKC and it is a useful tool that has been used to study the functions of PKC in many different cell systems. However, PMA also exerts additional effects that are not related to PKC activation; for example, it increases insulin release by evoking membrane depolarization and $[Ca^{2+}]_i$ increase in RINm5F cells (Yada et al., 1989). Since DAG is rapidly metabolized, OAG, a DAG analog, also has been used to mimic endogenous PKC activation to study the role of PKC in ligand-induced cellular responses (Willkinson and Hallam, 1994).

In addition to the use of endogenous and exogenous PKC activators, several specific PKC inhibitors have also been used to investigate the involvement of PKC on agonist-induced cellular responses. A number of specific PKC inhibitors are currently available for the inhibition of a number of PKC isozymes, including staurosporine, Ro 31-8220, Gö 6976, CGP 54345, CGP 53506 (Hofmann, 1997) and LY379196 (Dr. James R. Gillig, personal communication). They all bind to the ATP binding site (C3) of the catalytic domain to inhibit protein kinase activity.

Staurosporine, a bisindolylmaleimide compound, is a potent, but not a specific PKC inhibitor, because it also inhibits protein kinase A (PKA), protein kinase G (PKG), myosin light chain kinase (MLCK) and tyrosine kinase. Ro 31-8820, a derivative of staurosporine, is much more specific for inhibition of PKC than staurosporine. It can inhibit most of different PKC isozymes, including cPKCs, nPKCs and aPKCs (Wilkinson et al., 1993). Gö 6976, an indolocarbazole compound, is a potent and specific inhibitor for PKC-α, -βI (Hofmann, 1997) and -μ (Gschwendt et al., 1996). CGP 54345, a phenylamino-pyridine compound, is the most selective PKC-α inhibitor, which has no inhibitory effect on any other PKC isozymes (Hofmann, 1997). CGP 53506, a phenylamino-pyridine derivative, is a preferential inhibitor for all cPKC isozymes

(Hofmann, 1997). LY379196 is the inhibitor specific for only PKC-βI and -βII (Dr. James R. Gillig, personal communication) (Table 4).

Activation of PKC by DAG has been demonstrated to inhibit the formation of IP_3 by inhibiting the receptor-G protein coupling, or the activation of PPI-PLC in many systems. The negative feed back regulation of PKC may cause phosphorylation of the receptor, G protein or PPI-PLC, interference with the coupling of α and βγ subunit, or inactivation of PLC-β, thus leading to the attenuation of IP_3 formation and $[Ca^{2+}]_i$ (Fig. 18) (Babich et al., 1993; Mangoura et al, 1995). Furthermore, activation of PKC has been found to inhibit Ca^{2+} influx through Ca^{2+} channels (Ashcroft, 1994; Drummond, 1985; Sena et al., 1995). In the present study, therefore, it is important to investigate the role of PKC on AVP-induced increases in IP_3 production, $[Ca^{2+}]_i$ and glucagon release in α-cells.

Table 4. IC_{50} values of isozyme-selective PKC inhibitors (modified from Hofmann, 1997; Wilkinson et al., 1993; Dr. James R. Gillig, personal communication).

	α	βI	βII	γ	δ	ε	η	ζ	μ
Staurosporine (nM)	2.5	2.7	9.2	3.6	27	49	9.2	1300	n.d.
Ro 31-8220 (nM)	5	24	14	27	-	24	-	-	-
Gö 6976 (nM)	2.3	6.2	n.d.	n.d.	no inh.	no inh.	n.d.	no inh.	20
CGP 53506 (μM)	.79	4.8	3.3	3.0	>500	>500	9.2	>500	-
CGP 54345 (μM)	5.8	100	100	100	>100	>100	100	>100	-
LY379196 (μM)	0.6	.05	.03	0.6	0.7	5	0.3	48	-

n.d., not determined; no inh., no inhibition

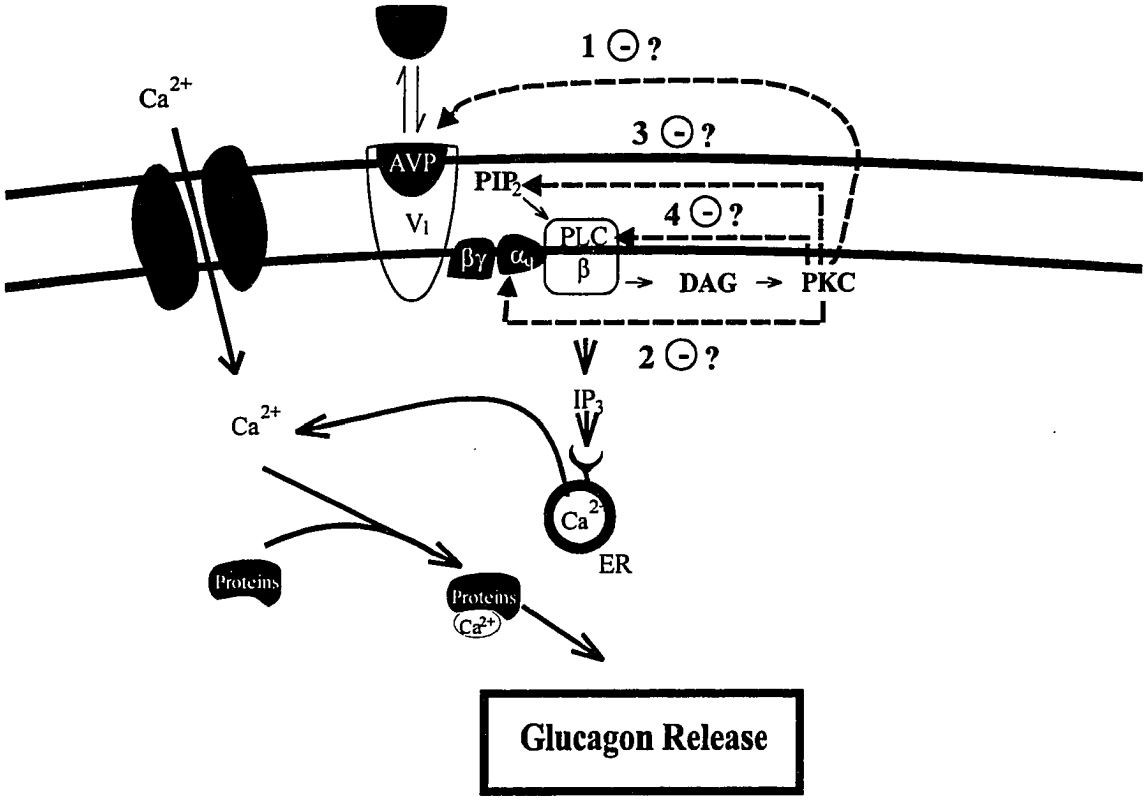


Fig. 18. A model of the negative feed back regulation of PKC on AVP-induced glucagon release. 1) PKC may exert the negative feedback role to inhibit the receptor-G protein coupling; 2) PKC may exert the negative feedback role to inhibit the coupling between α and βγ subunit; 3) PKC may exert the negative feedback role to inhibit the formation of PIP_2; 4) PKC may exert the negative feedback role to inhibit the activation of PLCβ.

CHAPTER II CHARACTERIZATION OF RECEPTORS MEDIATING AVP- AND OT-INDUCED GLUCAGON RELEASE FROM THE RAT PANCREAS

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ABSTRACT

We characterized the receptors that mediate arginine vasopressin (AVP)- and oxytocin (OT)-induced glucagon release by use of a number of antagonists in the perfused rat pancreas and fluorescence imaging of the receptors. AVP and OT (3 pM-3 nM) increased glucagon release in a concentration-dependent manner. The antagonist with potent V_{1b} receptor-blocking activity, CL-4-84 (10 nM) abolished AVP (30 pM)-induced glucagon release, but did not alter OT (30 pM)-induced glucagon release. $d(CH_2)_5[Tyr(Me)^2]AVP$ (10 nM), a V_{1a} receptor antagonist, and L-366,948 (10 nM), a highly specific OT receptor antagonist, failed to inhibit AVP-induced glucagon release. In contrast, L-366,948 (10 nM) abolished OT (30 pM)-induced glucagon release, but did not change the effect of AVP. Fluorescent microscopy of rat pancreatic sections showed that fluorescence-labeled AVP and OT bound to their receptors in the islets of Langerhans and that the bindings were inhibited by 1 μM of CI-4-84 and L-366,948, respectively. Because AVP and OT at physiological concentrations (3-30 pM) increased glucagon release, we conclude that AVP and OT increase glucagon release under the physiologic condition through the activation of V_{1b} and OT receptors, respectively.

INTRODUCTION

ARGININE VASOPRESSIN (AVP) and oxytocin (OT) are synthesized in the hypothalamus and secreted from the posterior pituitary gland. AVP and OT are also found in various tissues, including ovary, oviduct, follicular fluid (22), adrenal (3), testis (14), thymus (11) and pancreas (2). In addition to the regulation of fluid homeostasis, AVP induces glycogenolysis (15), proliferation of the pituitary gland (20) and vascular smooth muscle cells (24), vasoconstriction (8) and secretion of catecholamine (10), glucagon and insulin (6). The major physiological functions of OT are to regulate milk ejection and uterine contractions, but it also increases adrenocorticotropic hormone (ACTH) (23), glucagon and insulin release (6). A small concentration of 20 pg/ml of AVP and OT increased glucagon release, but not insulin release from the perfused rat pancreas (6). Moreover, both AVP and OT elicited a concentration-dependent stimulation of glucagon release but failed to influence insulin release from rat islets (5). A high density of [^{3}H]oxytocin binding was present in the periphery of the islets of Langerhans that corresponded to the location of pancreatic α-cells (25). Together, these findings suggest that AVP and OT may play a physiological role in increasing glucagon release.

AVP receptors have been classified into V_{1a}, V_{1b} and V_2 receptors. V_{1a} receptors mediate glycogenolysis (15) and vasoconstriction (8); V_{1b} receptors mediate the release of ACTH (4), catecholamines (10), insulin (17) and glucagon (29) and V_2 receptors mediate antidiuresis (13).

A number of receptor antagonists have been used to pharmacologically characterize the receptors that mediate the effects of AVP and OT in many cells and tissues (18), including insulin and glucagon-secreting cells. AVP and OT can cross-react with each other's receptors; for example, both AVP and OT induce insulin release through V_{1b} receptors in the perfused rat pancreas and the clonal β-cell line RINm5F (17). In addition, both hormones induce ACTH release through V_{1b} receptors in the rat adenohypophysis (4, 23), and lysine vasopressin stimulates porcine myometrial contractions through OT receptors (30). Similarly, in our previous study, AVP and OT induced glucagon release by activating V_{1b} receptors in clonal α-cells In-R1-G9 (29).

In this study, we characterized the receptors that mediate AVP- and OT-induced glucagon release by using the antagonists that block V_{1a}, V_{1b}, and OT receptors, respectively, from the perfused rat pancreas. In addition, we used fluorescence-labeled vasopressin (VP) and OT as ligands to detect AVP and OT receptors in the rat islets. Fluorescence-labeled peptides have been used to study AVP receptors; for example, fluorescence-labeled AVP analogues have been used to study V_{1a} receptors (12) and V_2 receptors (21). From the results of the present study, we conclude that AVP and OT play a physiological role in increasing glucagon release through V_{1b} and OT receptors, respectively.

MATERIALS AND METHODS

Test agents

AVP, OT, $d(CH_2)_5[Tyr(Me)]^2$AVP and Phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma Chemical (St. Louis, MO). Phenylac^1,D-Tyr(Me)^2,Arg^{6,8},Lys^9-amide]-vasopressin (Fluo-VP) and fluo-Lys^8-oxytocin (Fluo-OT) were purchased from Advanced Bioconcept (Quebec, Canada). Pentobarbital sodium was purchased from Fort Dodge Laboratories (Fort Dodge, IA). 4-OH-phenacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (CL-4-84) was donated by Dr. Maurice Manning of Medical College of Ohio (Toledo, OH). Cyclo-(L-Pro-D-2-naphthyl-Ala-L-Ile-D-pipecolic acid-L-pipecolic acid-D-His) (L-366,948) was donated by Merck Research Laboratories (West Point, PA). ^{125}I-glucagon was purchased from Linco Research Inc. (St. Charles, MO). Glucagon antibody was donated by Dr. Joseph Dunbar of Wayne State University (Detroit, MI), and glucagon standard was donated by Eli Lilly Laboratories (Indianapolis, IN).

Pancreatic perfusion

Male Sprague-Dawley rats weighing 500-650 g were used in the dose-response experiments, and rats weighing 220-350 g were used in the receptor-antagonism experiments. All the rats were born and grown in our facilities (Laboratory Animal Resource). They were maintained at 22°C, 40-60% humidity and a 12:12-h light-dark cycle. The rats were fed ad libitum with Purina chow. The in situ rat pancreatic

perfusion with an open system was performed during the daytime as previously described (27). Briefly, the rats were anesthetized with pentobarbital sodium (60 mg/kg ip) and were maintained at 37°C on a hot plate during the experiment. The celiac arteries were cannulated with a polyvinyl tubing (0.625 mm ID), then the pancreata were immediately perfused with the Krebs-Ringer bicarbonate buffer (KRB) supplemented with 20 mM HEPES, 5.5 mM glucose, 1% dextran and 0.2% BSA as a basal medium. The KRB was continuously aerated with 95% O_2-5% CO_2 at pH 7.4. The perfusion rate was 1 ml/min, and the effluent fluid from the portal vein, which was cannulated with a vinyl tubing (1.12 mm ID), was ~1 ml/min. The rats were euthanized immediately after the placement of cannulas and the beginning of the flow. After an equilibration period of 20 min, the effluent fluid was collected every minute. For the dose-response experiments, after the baseline period of 10 min, the perfusate containing AVP or OT (3 pM-3 nM) was administered for 10 min followed by a washout period with the basal medium for 10 min. For the antagonism experiments, after the baseline period of 5 min, the pancreas was pretreated for 10 min with the medium containing one of the three antagonists: CL-4-84 (1, 3, 10 nM), an antagonist with potent V_{1b} blocking activity (26), d(CH_2)$_5$[Tyr(Me)]^{2}AVP (10 nM), a V_{1a} receptor antagonist (18), and L-366,948 (1, 3, 10 nM), a highly selective OT receptor antagonist (27). This was followed by the medium containing AVP or OT (30 pM) and an antagonist for 10 min, and the basal medium for another 10 min for the washout period. The perfusate containing arginine (1 mM) was administered as a positive control for 5 min at the end of all experiments. The effluent fractions were kept at 4°C and subsequently assayed for glucagon by use of radioimmunoassay, following the procedures provided by Linco Research (St. Charles, MO).

Fluorescence imaging of AVP and OT receptors in pancreatic islet

The rat pancreas was perfused with KRB, as described in *Pancreatic perfusion*, for 5 min to eliminate the blood inside the pancreas. The perfusion rate was set at 3 ml/min. The pancreas was then collected and cut into small pieces (~3 x 3 mm^2) and frozen in −80°C isopentane. The frozen tissue was sliced into 17- to 20-μM thickness, mounted on poly-L-lysine-coated slides and stored at −20°C until use. The tissue

sections were processed following a protocol provided by the manufacturer (Advanced Bioconcept). Briefly, the frozen tissue sections were preincubated in an incubation buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1% BSA, 1 mg/ml bacitracin and 0.5 mM PMSF, pH 7.4) containing CL-4-84 (1 or 10 μM), L-366,948 (1 or 10 μM), $d(CH_2)_5[Tyr(Me)]^2AVP$ (10 μM), AVP (10 μM) or OT (10 μM) at 4°C for overnight and incubated with the incubation buffer containing 30 nM of Fluo-VP or Fluo-OT in the absence or presence of an antagonist or unlabeled AVP or OT as indicated in *Pancreatic perfusion*, at room temperature for 1 h. After incubation, the sections were washed 4 times for 60 s in a cold rinsing buffer (50 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.4) at 4°C and air-dried in the dark under a cool stream of air. The fluorescence bindings were visualized using a fluorescent microscope (Leica DMLB; Leica Microscopy Systems, Heerbrugg, Switzerland) and photographs were taken with a 20X lens using the Leica MPS 60-MPS 30 photographic system.

Data expression and statistical analysis

The effluent concentrations of glucagon were expressed as a percentage of the baseline level (mean of last 5 baseline values) in mean ± SE. The area under the curve (AUC) for the 10-min treatment period was calculated using Transforms and Regressions (SigmaPlot 4.0; SPSS Inc., Chicago, IL). In dose-response experiments, the AUC was expressed as a percentage of the area of the basal control group. In antagonism experiments, the AUC was expressed as a percentage of the area of AVP or OT control group. Data were analyzed using analysis of variance (ANOVA) to determine the effect of treatment. The Fisher least significant difference test was used to determine the difference between means for which the ANOVA indicated a significant ($P<0.05$) F ratio.

RESULTS

The results in Figs. 1 and 2 show the profile of glucagon release to AVP and OT (3 pM-3 nM), respectively, together with the basal control profile, which was obtained by perfusion with KRB alone for 40 min. AVP and OT (3 pM-3 nM) increased glucagon

release from the perfused rat pancreas in a concentration-dependent manner. Both peptides increased glucagon release in a biphasic pattern: a peak followed by a sustained phase or a second peak (for 3 nM AVP and OT), in which the peak initiated in <1 min and reached the maximum within 2 min. AVP (3 pM-3 nM) induced a maximum increase in glucagon release by 2.5, 8, 12 and 10-fold, respectively, over the basal control group. The sustained glucagon release induced by AVP (3-300 pM) was ~2- to 3-fold that of the basal control group, and the second peak of glucagon release induced by 3 nM AVP was 9-fold over that of the basal control group (Fig. 1). At the highest concentration of AVP studied (3 nM), the flow rate in the portal vein was decreased by ~20%, presumably because of vasoconstriction, but the glucagon response was not delayed or reduced to any extent. The OT (3 pM-3 nM)-induced maximum increase in glucagon release were 3, 7, 14 and 11-fold, respectively, over that of the basal control group. The sustained glucagon release by 3-300 pM OT was ~2-fold and the second peak induced by 3 nM OT was 4-fold that of the basal control group (Fig. 2). The effluent glucagon concentrations returned to the baseline on removal of AVP and OT (during the washing period) and increased to ~5- to 14-fold of the baseline value on the administration of 1 mM arginine. By comparison of the AUCs, there were no significant differences between AVP and OT (3-300 pM)-induced glucagon release. At 3 nM, AVP-induced glucagon release was significantly different from that of OT. However, the difference was only in the sustained phase (Fig. 3). The EC_{50} of OT was 8.9 ± 2.9 pM and the EC_{50} of AVP was estimated to be 25.1 ± 11.3 pM, because the maximum glucagon release was not acquired in the AVP dose-response experiment.

AVP and OT at 30 pM were used in the antagonism experiments because of the submaximal increase in glucagon release by the two peptides. At 30 pM, AVP and OT induced about four- and twofold increases in the peak and the sustained phase, respectively, compared with the basal control group. CL-4-84 (1, 3, and 10 nM), an antagonist with V_{1a}/V_{1b} blocking activity, inhibited AVP (30 pM)-induced glucagon release in a concentration-dependent manner (Fig. 4). By comparison of the AUCs, CL-4-84 (3 and 10 nM) significantly reduced AVP-induced glucagon release with an IC_{50} of 2.2 ± 0.1 nM. Pretreatment with CL-4-84 (10 nM) abolished AVP-induced glucagon release and even lowered glucagon to the levels below the baseline. However,

$d(CH_2)_5[Tyr(Me)^2]AVP$ (10 nM), a V_{1a} receptor antagonist, and L-366,948 (10 nM), a highly specific OT receptor antagonist, failed to inhibit AVP-induced glucagon release (Fig. 5). In contrast, L-366,948 (1, 3, and 10 nM) inhibited OT (30 pM)-induced glucagon release in a concentration-dependent manner (Fig. 6). By comparison of the AUCs, 3 nM L-366,948 significantly lowered and 10 nM L-366,948 abolished OT-induced glucagon release. The IC_{50} of L-366,948 was 3 ± 0.3 nM. CL-4-84 (10 nM), the receptor antagonist with V_{1a}/V_{1b} blocking activity, did not significantly reduce OT (30 pM)-induced glucagon release (Fig. 7). None of the receptor antagonists alone significantly changed glucagon release.

The results in figs. 8 and 9 show the fluorescence imaging of AVP and OT receptors in the rat islets. Fluorescent microscopic examination of the pancreatic sections incubated with either Fluo-VP or Fluo-OT revealed selective fluorescence labeling of AVP and OT receptors expressed in the rat pancreatic islets (Figs. 8*B* and 9*B*) compared with the negative control (Figs. 8*A* and 9*A*). The binding was specific because the fluorescence was no longer detectable when the incubation was performed in the presence of 10 μM VP or OT (Figs. 8*C* and 9*C*). The fluorescence labeling of Fluo-VP was selective for V_{1b} receptors because it was blocked by preincubation of the tissue sections with 1 μM CL-4-84 (Fig. 8*D*), but not by 10 μM L-366,948 (Fig. 8*E*) or 10 μM $d(CH_2)_5[Tyr(Me)^2]AVP$ (Fig. 8*F*). The fluorescence labeling of Fluo-OT was selective for OT receptors expressed in the rat pancreatic islets, because it was blocked by preincubation of the tissue sections with 1 μM L-366,948 (Fig. 9*D*), but not by 10 μM CL-4-84 (Fig. 9*E*).

DISCUSSION

In the present study, AVP and OT (3 pM-3 nM) evoked glucagon release from the perfused rat pancreas in a concentration-dependent manner, in which AVP and OT at 3 and 30 pM increased glucagon release by about three- and eightfold, respectively. These findings indicated that AVP and OT may physiologically have increased glucagon release, because the concentrations of AVP and OT studied (3 and 30 pM) are similar

to the plasma concentrations of AVP (3-20 pM) (9) and OT (8-25 pM) in the rat (16). This statement is supported by the findings that a neural lobe extract evoked glucagon release (5) and a rise in plasma glucagon concentrations of the rats subjected to hemorrhage that was found to be mediated by an increase in the release of AVP and OT (7). In addition, AVP and OT are present in human and rat pancreatic extracts, suggesting that both peptides are synthesized in the pancreas and thus could exert a paracrine function on pancreatic hormone release (2).

The increase in glucagon release mediated by AVP and OT (30 pM) was higher in the larger rats (500-650 g, ~1 year old, used in the dose-response experiments) than in the smaller rats (220-350 g, 2-3 mo old, used in the antagonism experiments). We speculate that the pancreata of the larger (or older) rats express more V_{1b} and OT receptors or have a more active signal transduction system for these receptors than the smaller (or younger) rats. More work is needed to find out why these peptides evoke more glucagon release in larger (older) rats than smaller (younger) rats.

CL-4-84 is an antagonist with high affinity for both V_{1a} [inhibitory constant (K_i) = 0.45 ± 0.04 nM] and V_{1b} (K_i = 2.2 ± 0.1 nM) receptors (26). It is also a weak OT antagonist [antagonistic affinity (pA_2) = 7.38 ± 0.06] (19). $d(CH_2)_5[Tyr(Me)^2]AVP$ is a potent and selective V_{1a} receptor antagonist (pA_2 = 8.62) (18). L-366,948 is a highly selective OT receptor antagonist which is >400 times more selective for OT receptors than for V_{1a} and V_2 receptors (27). We also confirmed the results from pancreatic perfusion by detecting these receptors by use of fluorescence labeling VP and OT. We found that Fluo-VP and Fluo-OT selectively bound to V_{1b} and OT receptors, respectively, in the rat islets. The labels of Fluo-VP and Fluo-OT were seen in the entire islets, an observation suggesting that both V_{1b} and OT receptors are expressed in pancreatic α- and β-cells, among others. In addition, in perfused rat pancreata, we found that 0.3 nM OT increased insulin release about three-fold over the basal insulin level and this increase was antagonized by 3 nM L-366,948 (unpublished data). The present finding is different from the previous one from our laboratory, in which 100 nM OT induced insulin release from rat perfused pancreas by activating V_{1b} receptors (17). Moreover, in an autoradiographic binding study of the rat pancreas, a high density of [^{3}H]oxytocin binding was found in the periphery of the islets, which corresponded to the localization of α-cells (25). Thus, the lower concentration of OT (0.3 nM) may

induce insulin release by activating OT receptors, whereas the higher concentration of OT (100 nM) may induce insulin release by activating V_{1b} receptors (17).

Our present findings suggest that AVP evokes glucagon release by activating V_{1b} receptors in α-cells of the rat pancreas, which is similar to AVP-induced ACTH release from the rat adenohypophysis (4), catecholamine release from the rat adrenal medulla (10) and glucagon release from the hamster glucagonoma cells In-R1-G9 (29). However, in the perfused rat pancreas, OT evoked glucagon release by activating OT, but not AVP receptors. In the dog, OT has been shown to increase plasma levels of glucose, insulin and glucagon and to increase the rate of glucose production and uptake by activating OT receptors (1). *These results differ from those of ours in* In-R1-G9 cells, in which OT increased glucagon release through V_{1b} receptors (29). In addition, AVP and OT increased ACTH release through V_{1b} receptors (4, 23). Although AVP and OT induced glucagon release by activating different receptors, our preliminary data showed that there was no synergism between these two peptides (unpublished data). The action of AVP on glucagon release exerted an inverse relationship with glucose concentrations; in the presence of 1.4 mM glucose, AVP (3 pM)-induced glucagon release was significantly higher than that in the presence of 5.5 mM glucose (unpublished data).

By comparison of the responses at the same concentration of AVP and OT, the potencies of both peptides were similar in the perfused rat pancreas, with exception that 3 nM of AVP evoked a significantly higher increase in glucagon release than 3 nM of OT. This finding differs from that of our previous study, in which OT-induced glucagon release in In-R1-G9 cells was ~30-fold less potent than AVP (29). Apparently, OT receptors are not expressed in clonal In-R1-G9 α-cells. We also confirmed these findings by detecting AVP and OT receptors in In-R1-G9 cells by use of fluorescence-labeled VP and OT. We found that 30 nM of both Fluo-VP and Fluo-OT bound to V_{1b} receptors on the cell membrane since the bindings were blocked by 30 nM CL-4-84, but not by 300 nM L-366,948 or $d(CH_2)_5[Tyr(Me)^2]AVP$ (unpublished data). OT, therefore, increases glucagon release from In-R1-G9 cells by activating V_{1b} receptors (29). Based on these findings, we conclude that In-R1-G9 cells are not an adequate model for the study of OT-induced glucagon release.

Our present findings suggest that AVP and OT increase glucagon release under the physiological condition by activating V_{1b} and OT receptors, respectively. Because specific V_{1b} receptor antagonists are currently unavailable for the characterization of these receptors, further studies utilizing molecular approaches are warranted to confirm our present findings.

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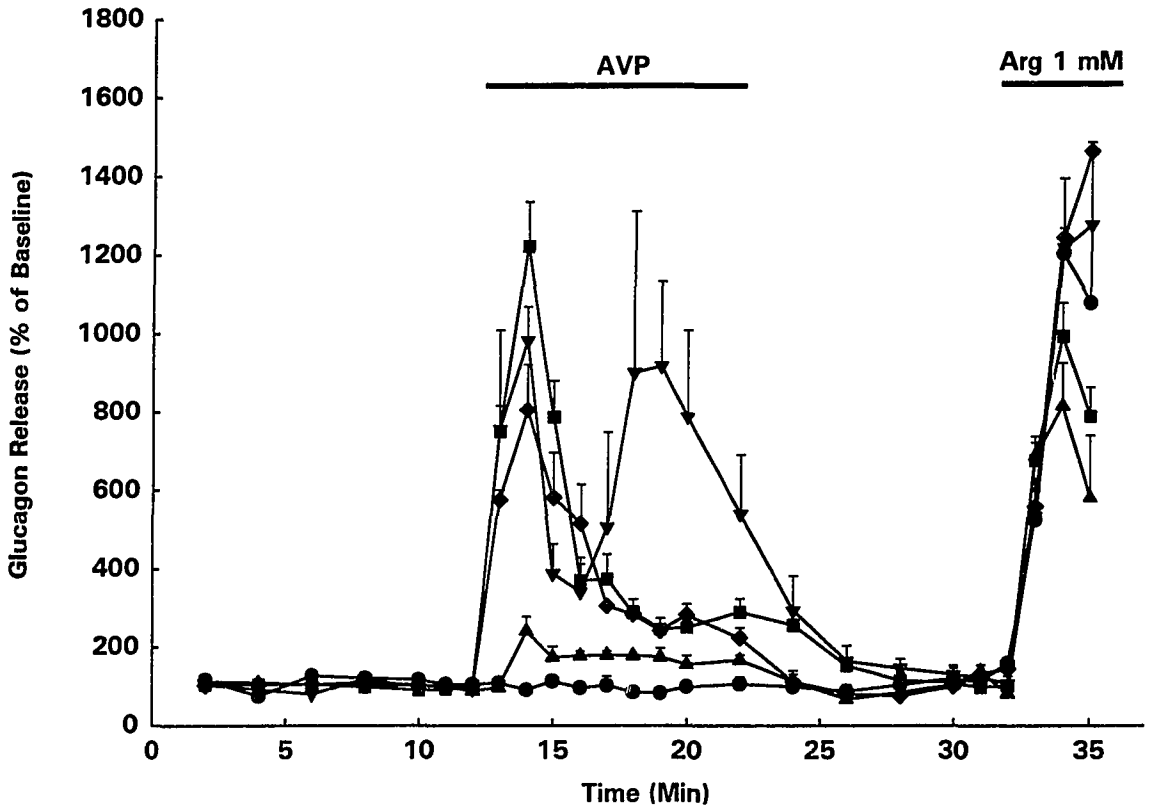

Fig. 1. Effect of arginine vasopressin (AVP, 3 pM-3 nM) on glucagon release from perfused rat pancreas. In these experiments, a 20-min equilibration period preceded *time 0*. AVP was administered for 10 min (heavy line). Values are mean ± SE, n = 3. ●, Basal control; ▲, AVP 3 pM; ◆, AVP 30 pM ; ■, AVP 300 pM; ▼, AVP 3 nM. Range of baseline glucagon concentrations of effluents was 24-106 pg/ml.

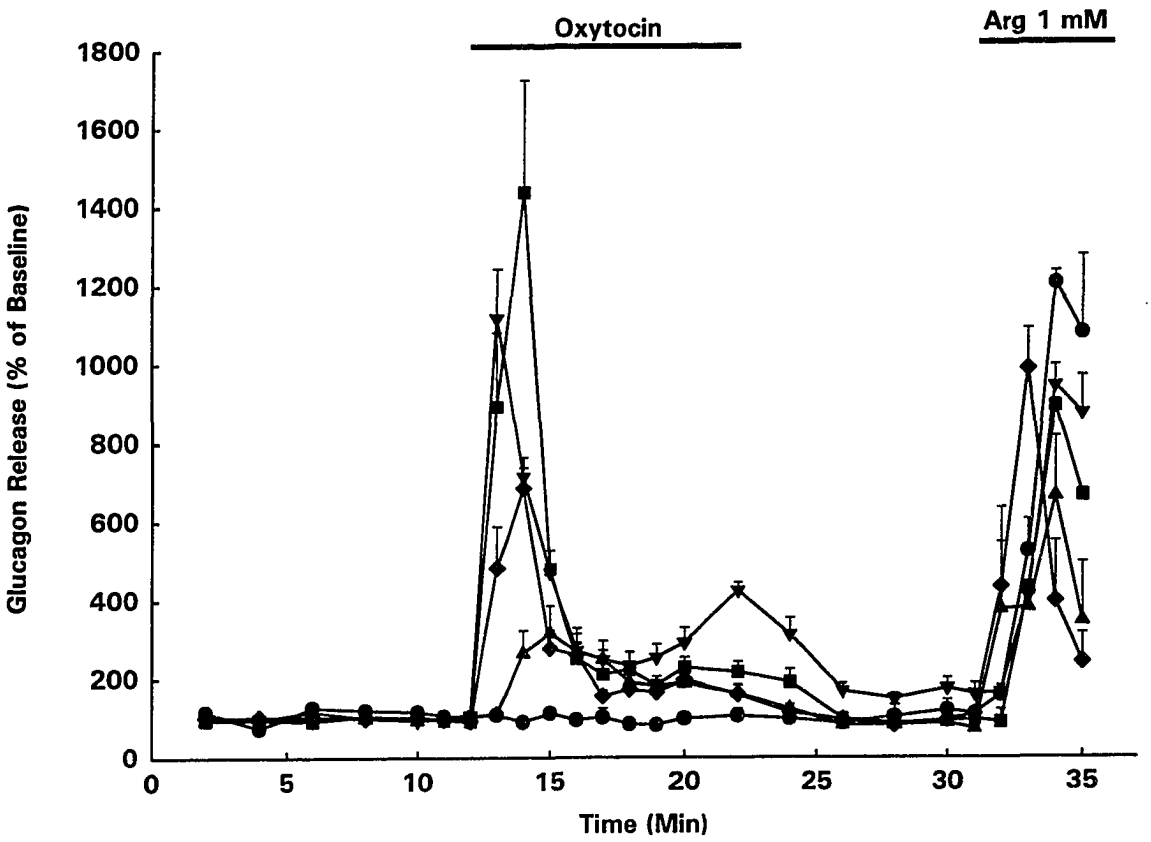

Fig. 2. Effect of oxytocin (OT, 3 pM-3 nM) on glucagon release from the perfused rat pancreas. In these experiments, a 20-min equilibration period preceded *time 0*. OT was administered for 10 min (heavy line). Values are mean ± SE, n = 3. ●, Basal control; ▲, OT 3 pM ; ◆, OT 30 pM; ■, OT 300 pM; ▼, OT 3 nM. Range of baseline glucagon concentrations of effluents was 35-182 pg/ml.

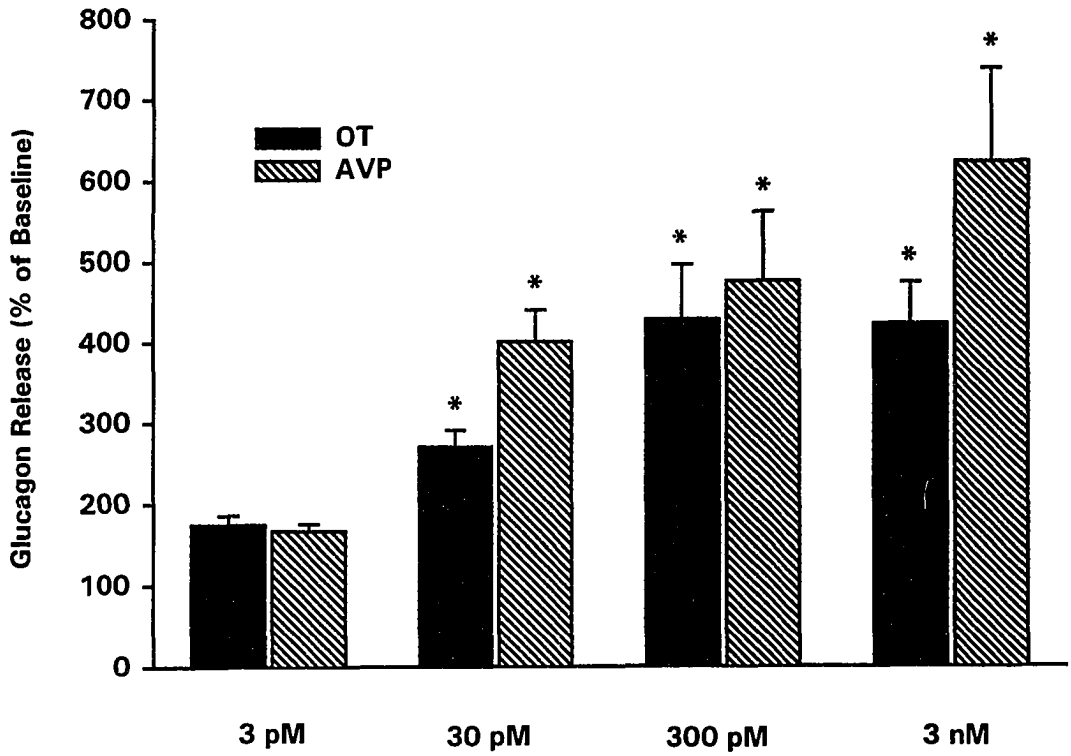

Fig. 3. Effects of AVP and OT-induced glucagon release from perfused rat pancreas. Values are mean ± SE (n = 3), obtained by calculating areas under 10-min glucagon release curve and expressed as a percentage of the control group. * $P < 0.05$ compared to the control group

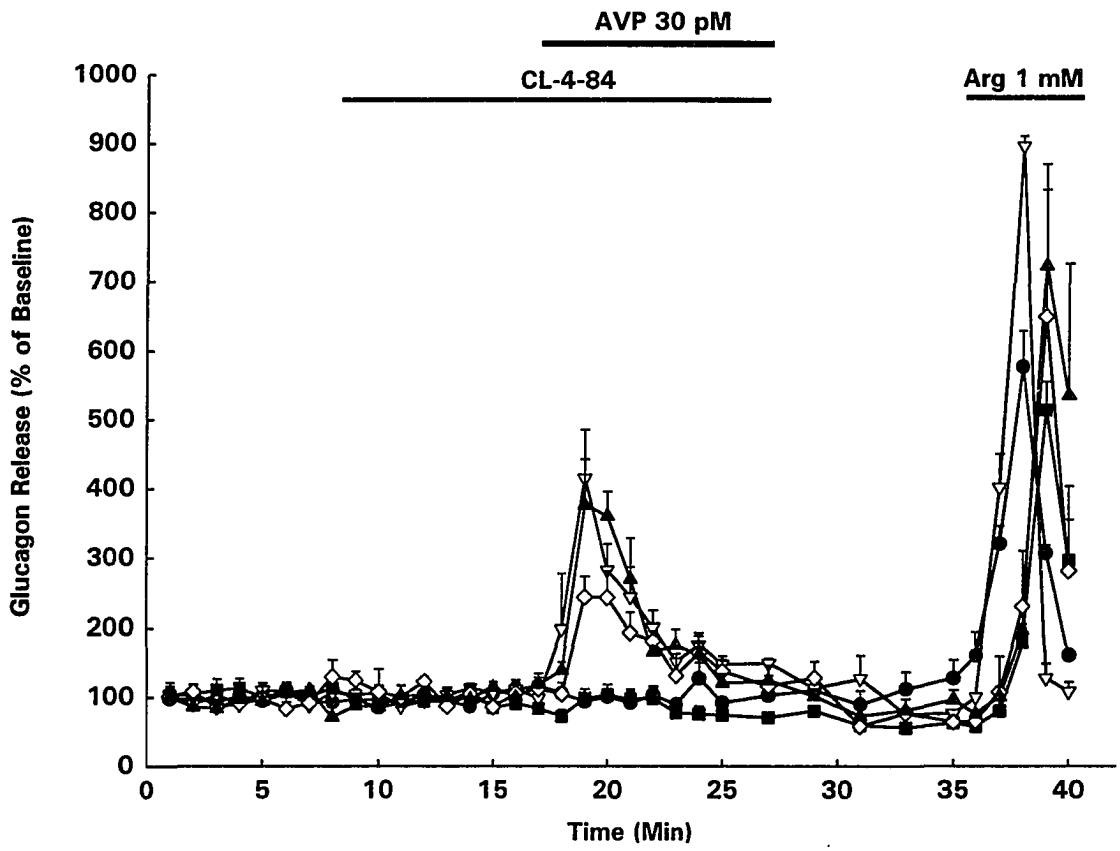

Fig. 4. Effect of CL-4-84 (1, 3 and 10 nM) on AVP-induced glucagon release from perfused rat pancreas. After baseline period of 5 min, CL-4-84 was administered for 10 min followed by AVP (30 pM) in the presence of CL-4-84 for another 10 min. Heavy lines show treatments as indicated above them. Values are mean ± SE, n = 3. ●, Basal control; ▽, AVP 30 pM; ▲, CL-4-84 1 nM + AVP 30 pM;, ◇ CL-4-84 3 nM + AVP 30 pM; ■, CL-4-84 10 nM + AVP 30 pM. Range of baseline glucagon concentrations of effluents was 32-225 pg/ml.

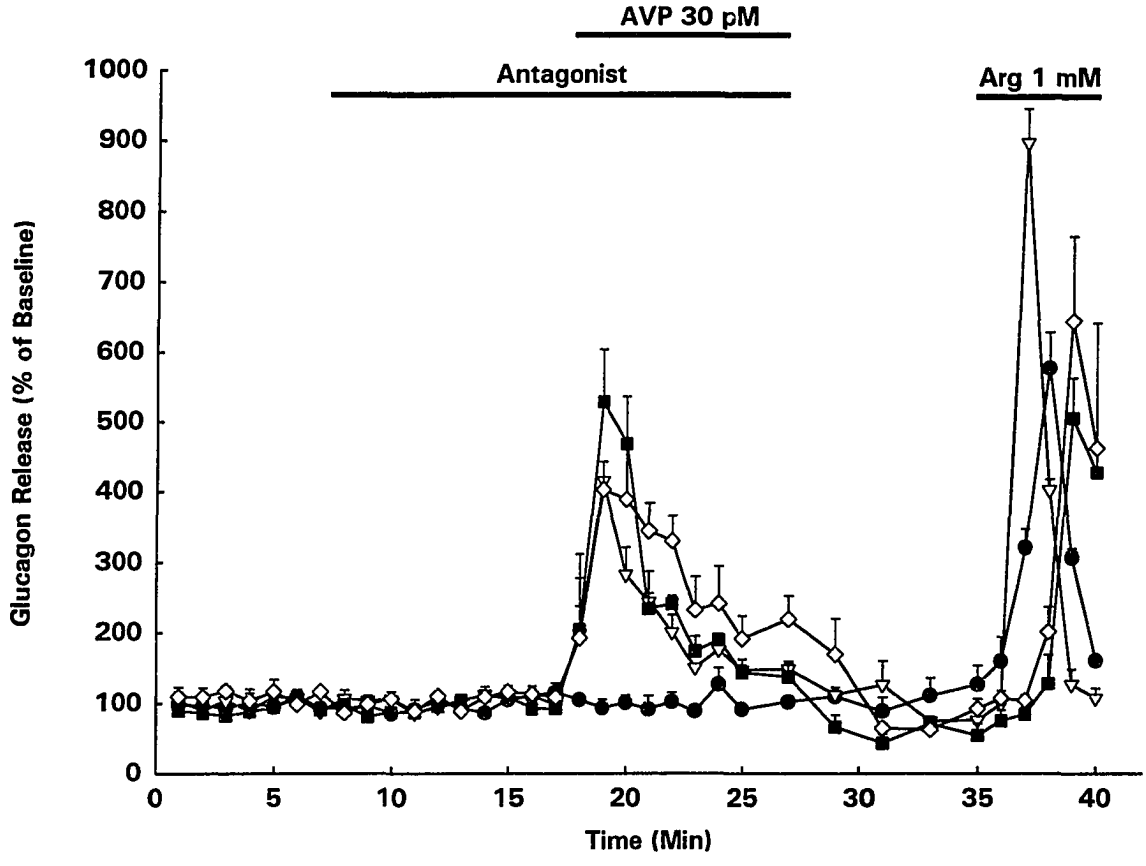

Fig. 5. Effects of $d(CH_2)_5[Tyr(Me)^2]AVP$ and L-366,948 (10 nM) on AVP-induced glucagon release from perfused rat pancreas. After baseline period of 5 min, antagonist was administered for 10 min followed by AVP (30 pM) in the presence of the antagonist for another 10 min. Heavy lines show the treatments as indicated. Values are mean ± SE, n = 3. ●, Basal control; ▽, AVP 30 pM; ■, $d(CH_2)_5[Tyr(Me)^2]AVP$ 10 nM + AVP 30 pM; ✧, L-366,948 10nM + AVP 30 pM. Range of baseline glucagon concentrations of effluents was 32-139 pg/ml.

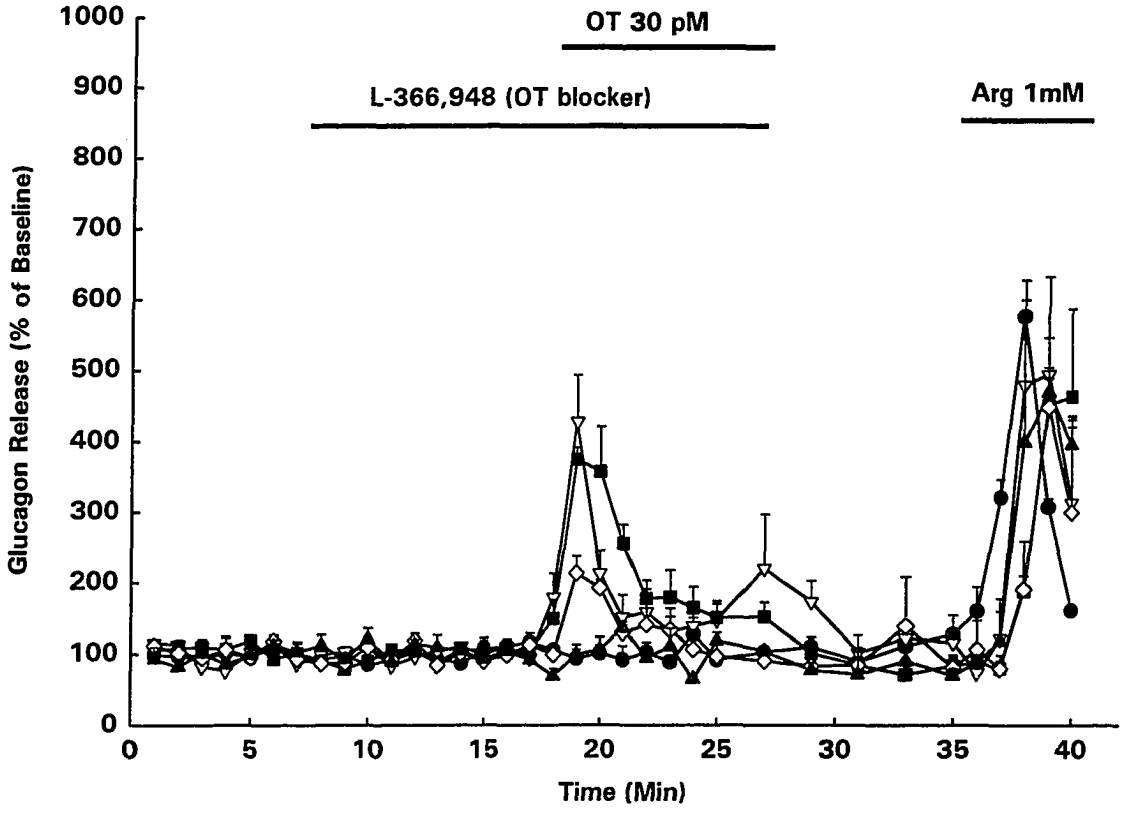

Fig. 6. Effects of L-366,948 (1, 3 and 10 nM) on OT-induced glucagon release from perfused rat pancreas. After the baseline period of 5 min, L-366,948 was administered for 10 min, followed by OT (30 pM) in the presence of L-366,948 for another 10 min. Heavy lines show the treatments as indicated. Values are mean ± SE, n = 3. ●, Basal control; ▽, OT 30 pM; ■, L-366,948 1 nM + OT 30 pM; ◇, L-366,948 3 nM + OT 30 pM; ▲, L-366,948 10 nM + OT 30 pM. Range of baseline glucagon concentrations of effluents was 32-175 pg/ml.

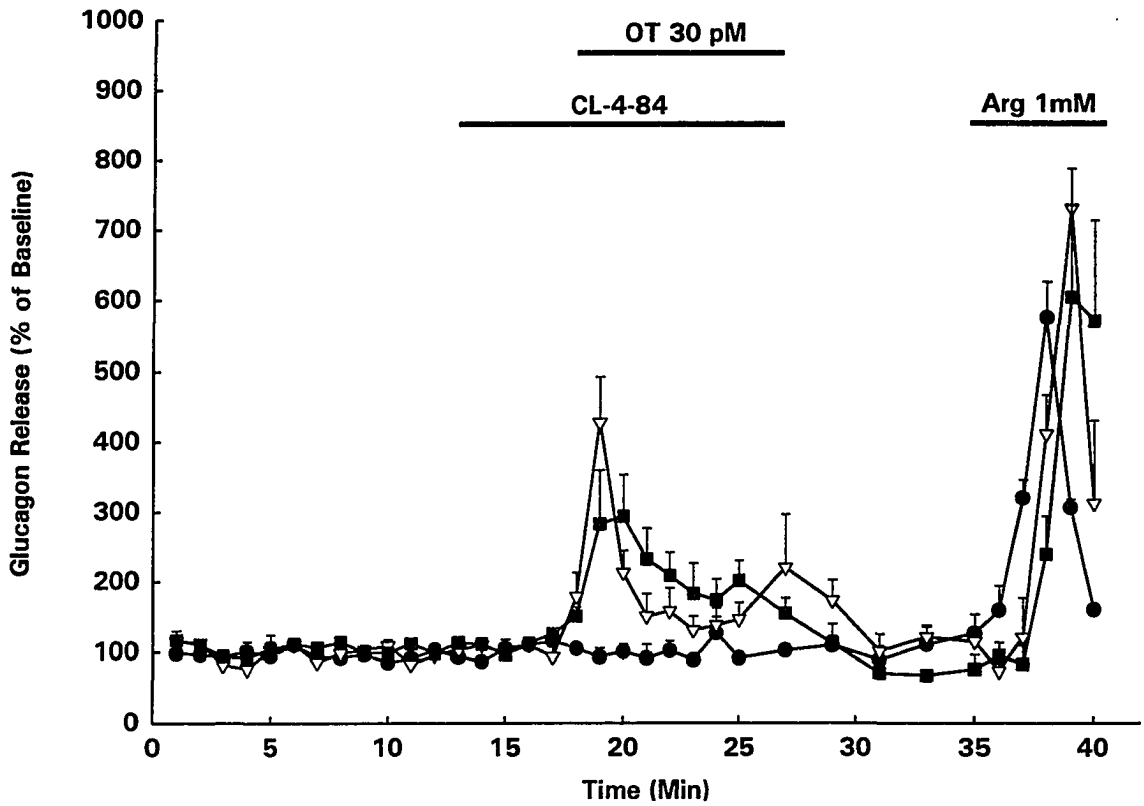

Fig. 7. Effect of CL-4-84 (10 nM) on OT-induced glucagon release from the perfused rat pancreas. After baseline period of 5 min, CL-4-84 was administered for 10 min followed by OT (30 pM) in the presence of CL-4-84 for another 10 min. Heavy lines show treatments as indicated above. Values are mean ± SE, n = 3. .●, Basal control; ▽, OT 30 pM; ■, CL-4-84 10 nM + OT 30 pM. Range of baseline glucagon concentrations of effluents was 32-139 pg/ml.

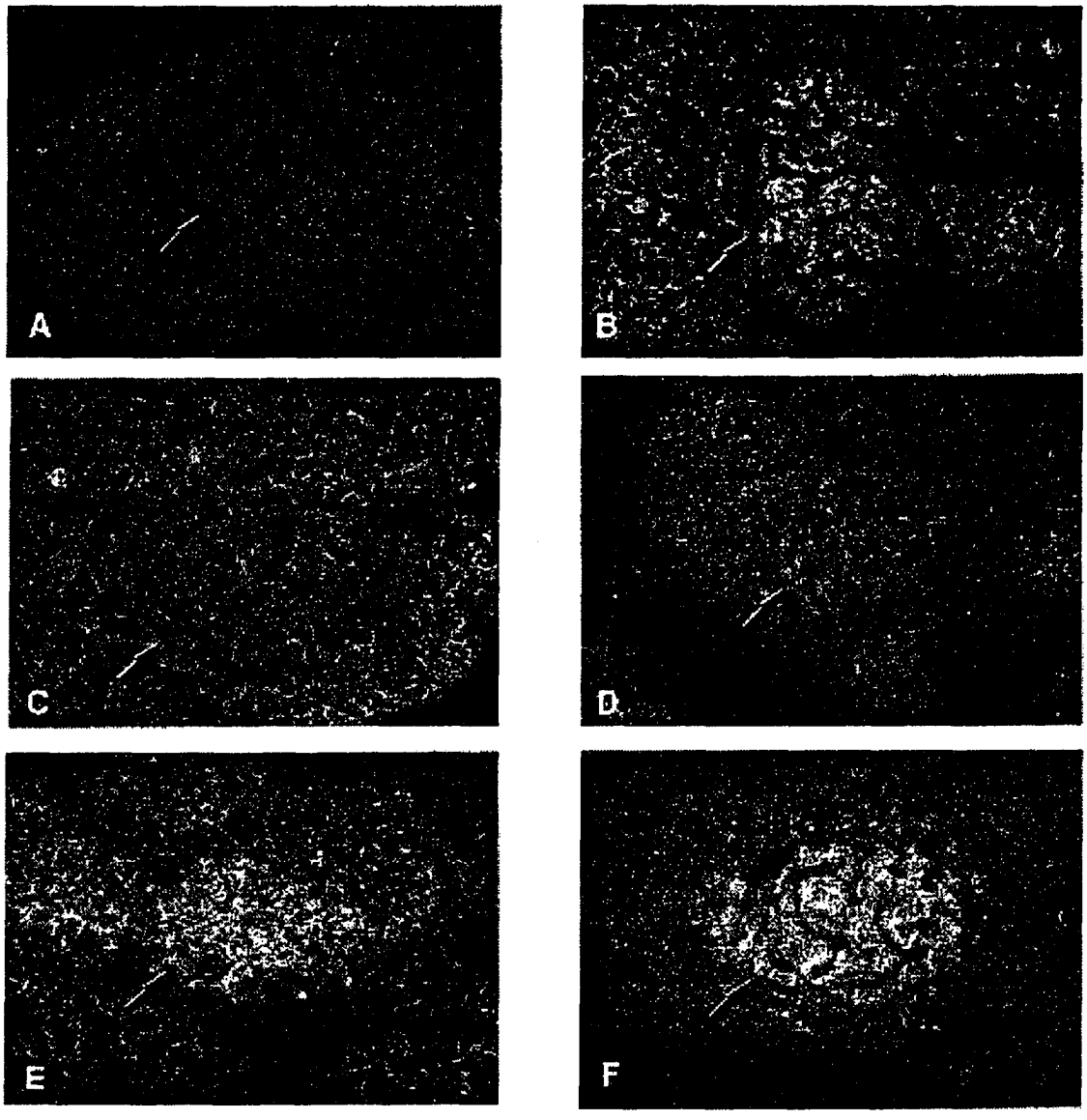

Fig. 8. Florescent microscopic images of the Islets of Langerhan in rat pancreatic sections labeled with Fluo-VP. Photograghs were taken under a 20X lens. Sections were incubated with an incubation buffer as the negative control (*A*), 30 nM Fluo-VP (*B*), 10 μM unlabeled AVP + 30 nM Fluo-VP (*C*), 1 μM CL-4-84 + 30 nM Fluo-VP (*D*), 10 μM L-366,948 + 30 nM Fluo-VP (*E*) and 10 μM $d(CH_2)_5[Tyr(Me)^2]AVP$ + 30 nM Fluo-VP (*F*) for 1 h at room temperature. Section was pre-incubated with the buffer, AVP, or an antagonist at 4°C for overnight. Data shown are representative of 3 rat pancreata.

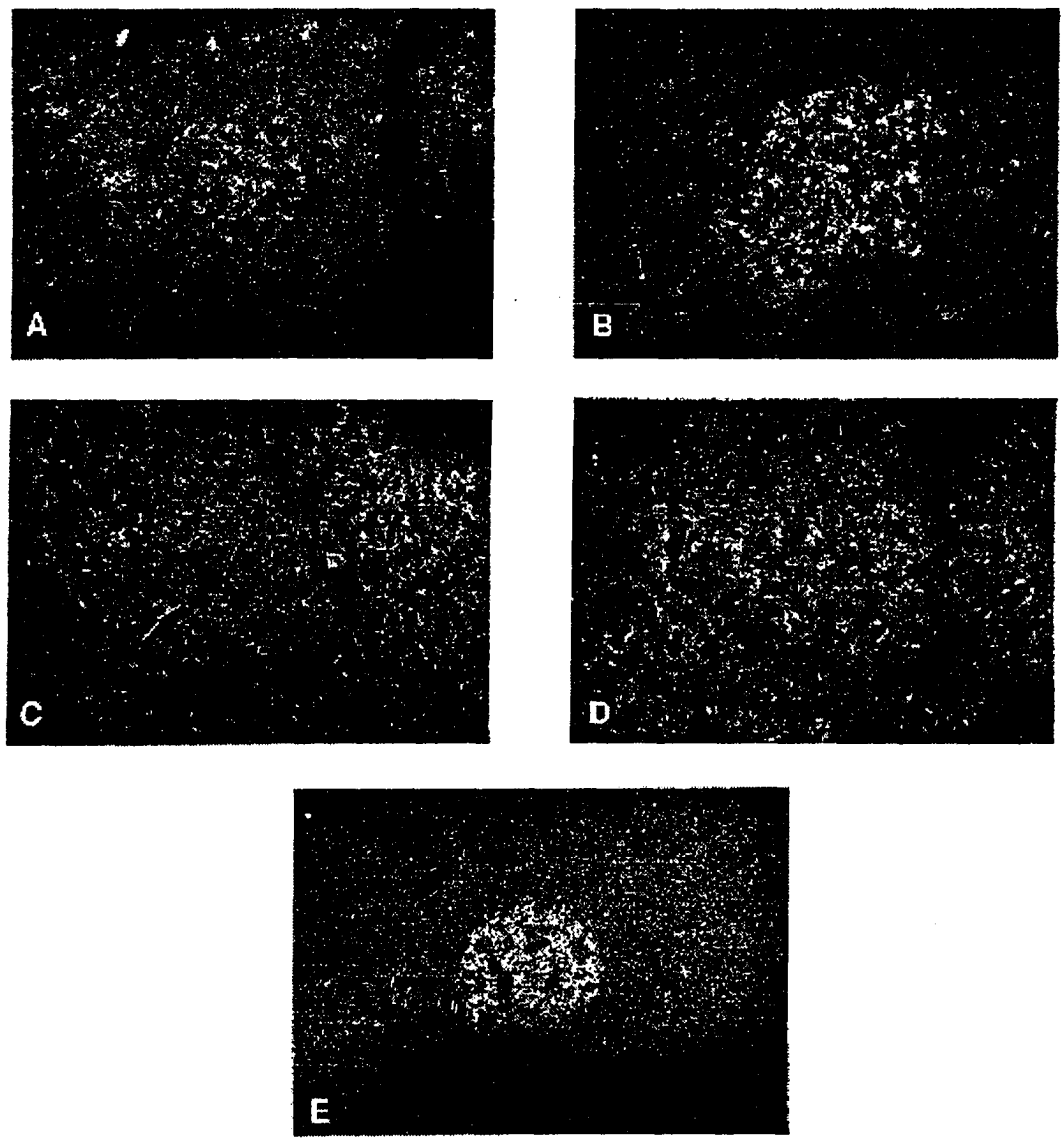

Fig. 9 Florescent microscopic images of Islets of Langerhan in rat pancreatic sections labeled with Fluo-OT. Photograghs were taken under a 20X lens. Sections were incubated with an incubation buffer as the negative control (*A*), 30 nM Fluo-OT (*B*), 10 μM unlabeled OT + 30 nM Fluo-OT (*C*), 1 μM L-366,948 + 30 nM Fluo-OT (*D*) and 10 μM CL-4-84 + 30 nM Fluo-OT (*E*) for 1 h at room temperature. Section was pre-incubated with buffer, OT, or an antagonist at 4°C for overnight. Data shown are representative of 3 rat pancreata.

CHAPTER III EFFECTS OF ARGININE VASOPRESSIN AND OXYTOCIN ON GLUCAGON RELEASE FROM CLONAL α-CELL LINE IN-R1-G9: INVOLVEMENT OF V_{1b} RECEPTORS

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ABSTRACT

Receptor antagonists were used to determine which receptor mediates the effect of arginine vasopressin (AVP) and oxytocin (OT) on glucagon release from hamster glucagonoma In-R1-G9 cells. Both AVP (10^{-9}-10^{-6} M) and OT (10^{-8}-10^{-5} M) increased glucagon release from In-R1-G9 cells in a concentration-dependent manner and AVP was ~30-fold more potent than OT in this aspect. The antagonists with potent V_{1b} receptor blocking activity, CL-4-84 (10^{-9}-10^{-6} M), dP[Tyr(Me)^2]AVP and AO-2-44 (10^{-8}-10^{-6} M), antagonized the effect of both AVP and OT in a concentration-dependent manner. Other receptor antagonists at 10^{-6} M failed to block the effect of AVP and OT; these included a highly selective OT-receptor antagonist, L-366,948 and a V_{1a} /V_2 receptor antagonist WK-3-6. However, these antagonists at higher concentrations (10^{-5} and 10^{-4} M) caused inhibition of AVP- and OT-induced glucagon release. The order of antagonistic potency was estimated as CL-4-84 ≈ dP[Tyr(Me)^2]AVP ≈ AO-2-44 > WK 3-6 > L366,948. d[D-3-Pal]VP (10^{-8}-10^{-5} M), a V_{1b} receptor agonist, also increased glucagon release in a concentration-dependent manner, which was antagonized by dP[Tyr(Me)^2]AVP (10^{-8}-10^{-6} M) and CL-4-84 (10^{-9}-10^{-6} M), but not by WK-3-6 (10^{-6} M) or L-366,948 (10^{-6} M). Therefore, the stimulatory effects of both OT and AVP on glucagon release may be mediated by V_{1b} receptors, but not by V_{1a}, V_2, or OT receptors.

INTRODUCTION

The neurohypophysial hormones arginine vasopressin (AVP) and oxytocin (OT), the nonapeptides synthesized in hypothalamus and released by posterior pituitary gland, exert different biological effects in mammals. The major physiological roles of AVP and OT are to regulate water and solute excretion by the kidney and blood pressure, as well as to regulate uterine contractions and milk ejection, respectively. These two hormones and their receptors, however, are found in the pancreas and may physiologically stimulate the release of pancreatic hormones, glucagon and insulin (1-4). AVP and OT have a greater impact on glucagon release than insulin release since a low concentration of these agonists (20 pg/ml) caused an increase in glucagon release, but not insulin release, from the perfused rat pancreas (3). Moreover, they elicited a concentration-dependent stimulation of glucagon release but failed to influence insulin release from rat islets incubated in Medium 199 containing 5.6 mM glucose (5).

AVP and OT exert their effects through at least four subtypes of receptors (V_{1a}, V_{1b}, V_2, and OT receptors). These receptor subtypes have been distinguished on a functional, pharmacological, and/or molecular biological basis. V_{1a} receptors mediate glycogenolysis (6, 7 [review]) and vasoconstriction (8, 9 [review]), V_{1b} (or V_3) receptors mediate the release of ACTH (10, 11), catecholamine (12) and insulin (13), and V_2 receptors mediate antidiuresis (14, 15 [review]). Vasopressin and OT may cross-react with each other's receptors; for instance, lysine vasopressin stimulates porcine myometrial contraction through OT receptors (16) and OT stimulates the release of ACTH (17) and insulin (13) through V_{1b} receptors, but not OT receptors in the rat.

In the present study, we investigated the functional receptors that mediated AVP- and OT-induced glucagon release from clonal α-cell line In-R1-G9 by using static incubation. We studied the effect of the antagonists for V_{1a}, V_{1b}, V_2, and OT receptors on AVP- and OT- induced glucagon release. In addition, we investigated the effect of deamino[D-3-(3'-pyridyl)-Ala^2, Arg^8]VP (d[D-3-Pal]VP), an agonist with potent V_{1b} activity (19), on glucagon release. We have concluded that the stimulatory effects of AVP- and OT-induced glucagon release from In-R1-G9 cells were mediated through V_{1b} receptors, but not V_{1a}, V_2, or OT receptors.

MATERIALS AND METHODS

Cell culture

The hamster glucagonoma In-R1-G9 cells were maintained in RPMI-1640 medium with 10% fetal bovine serum and aerated with 5% CO_2-95% air at 37°C. All experiments were performed using cells from passages 24 to 34.

Static incubation

The cells were plated onto 24-well plates (Corning, Oneonta, NY) at 10^5 cells/well and were grown for 3-4 days. The growth medium was then removed and replaced with Krebs-Ringer bicarbonate buffer (KRB) for the experiment. Glucagon released from In-R1-G9 cells was determined using static incubation in response to agonists and antagonists. For determination of the dose responses to AVP, OT, and d[3-D-Pal]VP (Sigma Chemical, St. Louis, MO), the cells were incubated at 37°C with an agonist for 15 min after preincubation with KRB for 15 min. For the antagonism study, dP[Tyr(Me)^2]AVP (Sigma Chemical, St. Louis, MO), 4-OH-phenacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (CL-4-84), desGly^{9}d$(CH_2)_5$[Tyr(Et)^2]AVP (WK-3-6), d$(CH_2)_5$[D-Phe^2, Ile^4]-AVP (AO-2-44) (Dr. M. Manning, Toledo, OH) or cyclo-(L-Pro-D-2-naphthyl-Ala-L-Ile-D-pipecolic acid-L-pipecolic acid-D-His) (L-366,948) (Merck Research Laboratories, West Point, PA) was administered for 10 min before the administration of the agonist to ensure blockade of receptors. The concentration of glucagon in the media was measured by radioimmunoassay, following the procedures provided by Linco Research (St. Charles, MO). ^{125}I- glucagon was purchased from Linco Research.

To calculate half-maximal effective concentration (EC_{50}) values of AVP and OT on glucagon release, near saturating concentrations of AVP at 10^{-6} M, OT at 10^{-5} M and d[3-D-Pal]VP at 10^{-5} M were set at 100%. The glucagon released by AVP (10^{-10}-10^{-6} M), OT (10^{-9}-10^{-5} M) or d[3-D-Pal]VP (10^{-9}-10^{-5} M) was expressed as a percentage of the value of AVP (10^{-6} M), OT (10^{-5} M) or d[3-D-Pal]VP (10^{-5} M) group, respectively.

To calculate half-maximal inhibitory concentration (IC_{50}) values of the receptor antagonists on glucagon release, the concentration of AVP at 10^{-8} M, OT at 3×10^{-7} M or d[3-D-Pal]VP at 10^{-7} M on glucagon release was set at 100%. The inhibitory effects of antagonists were expressed as the percentage of the control value of each agonist.

EC_{50} and IC_{50} values were calculated using Pharmcals.bas software (Springer-Verlag, Berlin, NY).

Statistical analyses

All values were expressed as mean ± SE. Results were analyzed using ANOVA and Tukey's test was used for mean comparisons. The significance level was set at $P < 0.05$.

RESULTS

Effects of AVP, OT and d[D-3-Pal]VP on glucagon release

AVP (10^{-9}-10^{-6} M), OT (10^{-8}-10^{-5} M) and d[3-D-Pal]VP (10^{-8}-10^{-5} M) increased glucagon release from In-R1-G9 cells in a concentration-dependent manner (Fig.1). AVP was ~30-fold more potent than OT when ED_{50} values were compared (AVP: 5.0 ± 0.6 nM vs. OT: 190.0 ± 20.0 nM). d[3-D-Pal]VP was ~10-fold less potent than AVP and ~4-fold more potent than OT when EC_{50} values were compared (AVP: 5.0 ± 0.6 nM, OT: 190.0 ± 20.0 nM and d[3-D-Pal]VP: 52.0 ± 7.1 nM). AVP (10^{-8} M), OT (3 x 10^{-7} M) and d[3-D-Pal]VP (10^{-7} M) were used in the antagonism study because these concentrations of the peptides caused submaximal increases in glucagon release.

Effects of AVP and OT receptor antagonists on AVP- and OT-induced glucagon release

L-366,984 (10^{-6} M), a highly selective OT receptor antagonist (20), failed to block AVP (10^{-8} M)- and OT (3 x 10^{-7} M)-induced glucagon release, which were 98.2 ± 1.1 %, and 99.3 ± 0.9 %, respectively (n = 3). This receptor antagonist, however, at higher concentrations (10^{-5} and 10^{-4} M) inhibited AVP (10^{-8} M)- and OT (3 x 10^{-7} M)-induced glucagon release by 14.1 ± 8.7 %, 72.8 ± 8.7 % and 12.2 ± 11.6 %, 63.1 ± 8.9 %, respectively (n = 2) (Fig. 2A). WK 3-6 (10^{-6} M), a highly potent V_{1a} and V_2 receptor antagonist (21), failed to inhibit the AVP- and OT-induced glucagon release, which were 97.0 ± 1.7 % and 96.2 ± 2.7 %, respectively (n = 3). The higher

concentrations of WK 3-6 (10^{-5} and 10^{-4} M) caused 26.1 ± 3.0 % and 70.5 ± 2.6 % inhibition of AVP (10^{-8} M)-induced glucagon release (n = 2) (Fig. 2B). The 10^{-5} and 10^{-4} M of WK 3-6 caused 35.0 ± 4.6 % and 82.1 ± 7.1 % inhibition of OT (3 x 10^{-7} M)-induced glucagon release (n =2) (Fig. 2B). AO-2-44 (10^{-8}-10^{-6} M), a $V_2/V_{1a}/V_{1b}$ *receptor antagonist (21), inhibited* AVP (10^{-8} M)- and OT (3 x 10^{-7} M)-induced glucagon release in a concentration-dependent manner (n = 3) (Fig. 2 C). dP[Tyr(Me)^2]AVP (10^{-8}-10^{-6} M), an antagonist with potent V_{1b} and V_{1a} receptor blocking activities (18), caused a concentration-dependent inhibition of AVP (10^{-8} M) and OT (3 x 10^{-7} M)-induced glucagon release (n = 3) (Fig. 2D). In addition, CL-4-84 (10^{-9}-10^{-6} M), a highly potent V_{1a} and V_{1b} *receptor antagonist (22) also dose-dependently inhibited* AVP- and OT-induced glucagon release (n = 3) (Fig. 2E). The order of antagonistic potency on AVP- and OT-induced glucagon release was: CL-4-84 ≈ dP[Tyr(Me)^2]AVP ≈ AO-2-44 > WK 3-6 > L366,948, when IC_{50} values were compared (Table 1). None of the antagonists at the concentrations studied changed glucagon release by themselves (data not shown).

Effects of AVP and OT receptor antagonists on d[3-D-Pal]VP-induced glucagon release

dP[Tyr(Me)^2]AVP (10^{-8}-10^{-6} M) and CL-4-84 (10^{-9}-10^{-6} M) caused a concentration-dependent inhibition of d[3-D-Pal]VP (10^{-7} M)-induced glucagon release (Fig. 3A and Fig. 3B), whereas WK 3-6 (10^{-6} M) and L-366,948 (10^{-6} M) failed to do so (n =3, data not shown).

DISCUSSION

V_{1b} receptors, as we conclude, most likely mediate AVP and OT-evoked glucagon release from In-R1-G9 cells and are similar to those mediating ACTH release from rat adenohypophysis (10) and catecholamine release from rat adrenal medulla (12). We have demonstrated that: 1) AVP was ~30-fold more potent than OT in increasing glucagon release, and 2) the order of potency of antagonists on AVP- and OT-induced glucagon release was estimated as: CL-4-84 ≈ dP[Tyr(Me)^2]AVP ≈ AO-2-44 > WK 3-6 > L366,948.

L-366,948 is a highly specific antagonist for OT receptors. It is >400 times more selective for OT receptors than V_{1a} and V_2 receptors (20). WK 3-6 is a highly potent V_{1a} (pA_2 = 8.17) and V_2 receptor (pA_2 = 7.75) antagonist (21). AO-2-44 is a potent $V_2/V_{1a}/V_b$ receptor antagonist, and is ~35 times less potent for binding V_{1a} receptors than WK 3-6, but is ~350 times more potent for binding V_{1b} receptors than WK3-6 (21). CL-4-84 has a high antivasopressor potency (anti-V_{1a}, pA_2 = 8.74) (24), which is similar to WK-3-6 (21). However, it also has high affinity for V_{1b} receptors (K_i = 2.2 ± 0.1 nM) (22). dP[Tyr(Me)^2]AVP is a highly potent V_{1b} receptor antagonist, but it also blocks V_{1a} receptors. However, dP[Tyr(Me)^2]AVP has the highest V_{1b} receptor blocking activity among a group of receptor antagonists studied on AVP- and OT-induced ACTH release (10, 17, 18) and insulin release (13, 18). Therefore, the rank order of potency for these receptor antagonists on V_{1b} receptors is estimated as CL-4-84 ≈ dP[Tyr(Me)^2]AVP ≈ AO-2-44 > WK 3-6 > L366,948, which is consistent with our data on AVP- and OT-induced glucagon release. Although, we stated that WK 3-6 and L366,948 are specific antagonists for V_{1a}/V_2 receptors and OT receptors, respectively, extremely high concentrations of these antagonists (10^{-5} and 10^{-4} M) may antagonize other receptor subtypes, including V_{1b}.

d[D-3-Pal]VP, a synthetic analog of vasopressin, is a highly specific agonist for V_{1b} receptors. In the pituitary gland, the relative agonistic potency of this analog to AVP on V_{1b} receptor is 1/36 (19). It is also a weak agonist at V_2 receptors in the kidney, with a relative potency 1/381 that of AVP and a weak V_{1a} receptor antagonist for the vasoconstrictor response (pA_2 = 6.22) (19). In the present study, we found that d[D-3-Pal]VP caused an increase in glucagon release in a concentration-dependent manner and this effect was antagonized by dP[Tyr(Me)^2]AVP, an antagonist with V_{1b} receptor blocking activity. Other antagonists were not effective.

We conclude that: 1) AVP and OT induce glucagon release from In-R1-G9 cells by activating the same receptor subtype, probably V_{1b} receptors, and 2) OT interacts with these receptors with a lower affinity or intrinsic activity. Because AVP and OT have similar structures, it is conceivable that OT may act on AVP receptors (V_{1b}) to induce glucagon release from this clonal α-cell line. In addition, OT increases ACTH release from rat pituitary cells by activating V_{1b} receptors, but not OT receptors (17). This is consistent with the previous work from our laboratory regarding OT-and AVP-

induced insulin release (13). However, the OT-induced glucagon release is less than that of OT-induced insulin release. OT-induced insulin release is only 9-fold less potent than AVP (13), whereas it is 30-fold less potent than AVP-induced glucagon release. Since the AVP/OT analogs used in the present study have not been extensively characterized in the hamster cells, these conclusions can be drawn only if it is assumed that the behavior of receptors and these peptides in the hamster is similar to their behavior in other species (e.g., rats) which they have been fully characterized.

The V_{1b} receptors have been found in both pituitary (10, 11) and extrapituitary tissues (25), such as rat adrenal medulla (12) and pancreatic β-cells (13, 25). In fact, the mRNA for these receptors has been detected in both human and rat pancreata (25). We have evidence that V_{1b} receptors may mediate the effect of AVP- and OT-induced glucagon release from the hamster α-cell line. We will further characterize these receptors in the pancreatic α-cells. Moreover, because a highly specific V_{1b} receptor antagonist is still not available to characterize these receptors, a molecular biological approach is necessary to confirm the characterization of these receptors in α-cells.

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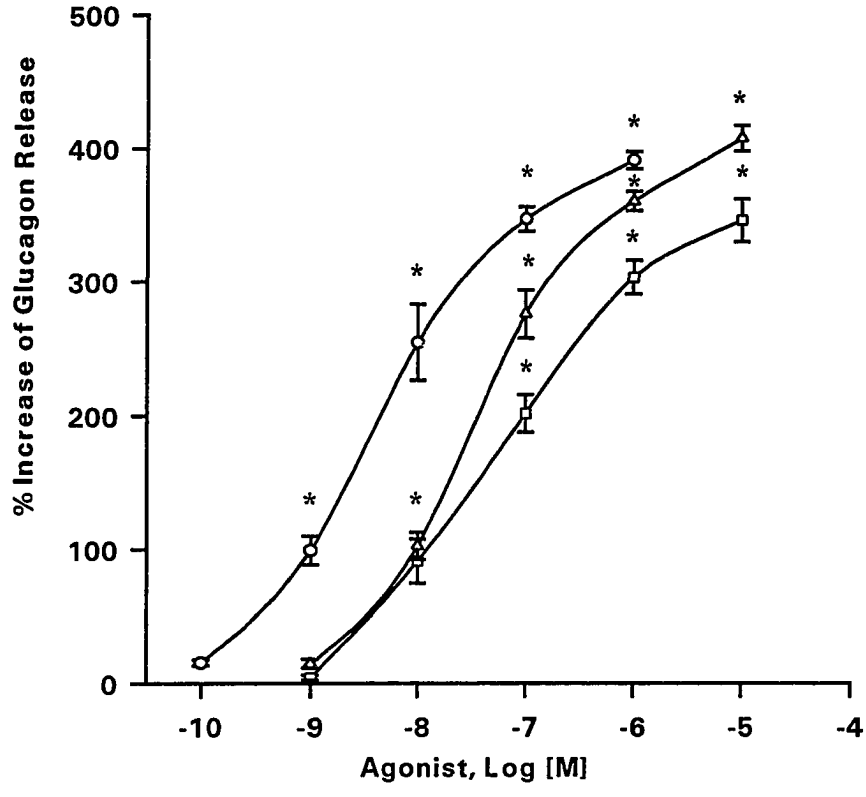

FIG. 1. Effect of AVP (O), d[D-3-Pal]VP (Δ) and OT (❑) on glucagon release. Values are mean ± SE; n = 3 cultures with quadruplicates. Static incubation was performed for 15 min to determine glucagon release. The glucagon release of the control group in AVP, d[D-3-Pal]VP and OT experiments was 197.0 ± 14.7, 221.4 ± 11.3 and 212.6 ± 11.0 pg/well/15 min, respectively.

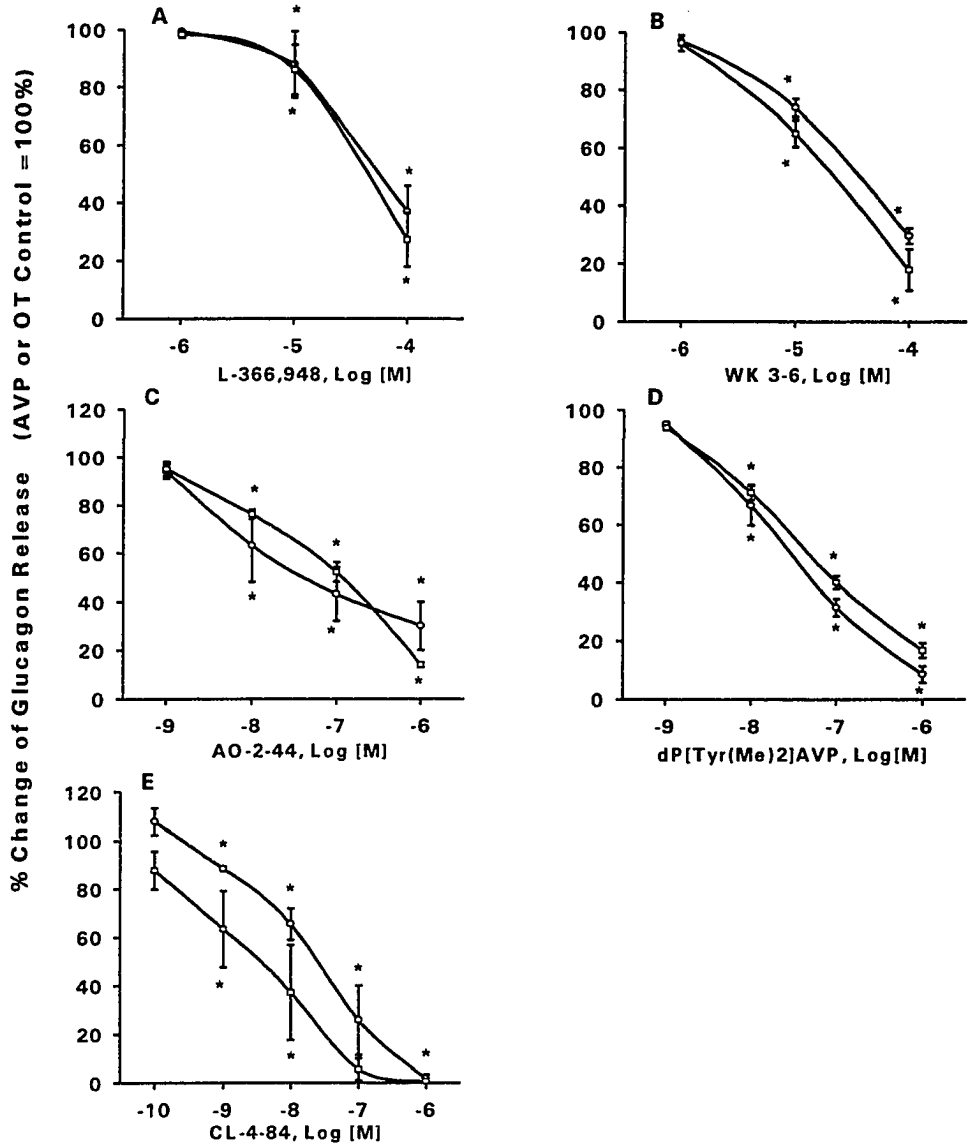

FIG. 2. Effect of L-366,948 (A), WK-3-6 (B), AO-2-44 (C), dP[Tyr(Me)^2]AVP (D) and CL-4-84 (E) on AVP- and OT-induced glucagon release. AVP 10 nM (o) and OT 300 nM (□). Static incubation was performed with AVP or OT for 15 min. The potential antagonist was given 10 min before and in the presence of an agonist. Values are mean ± SE; n = 3 cultures with quadruplicates, except for 10^{-5} and 10^{-4} M which were n = 2. *$P < 0.05$ vs. agonist control group (100%) at the corresponding concentration of the antagonist.

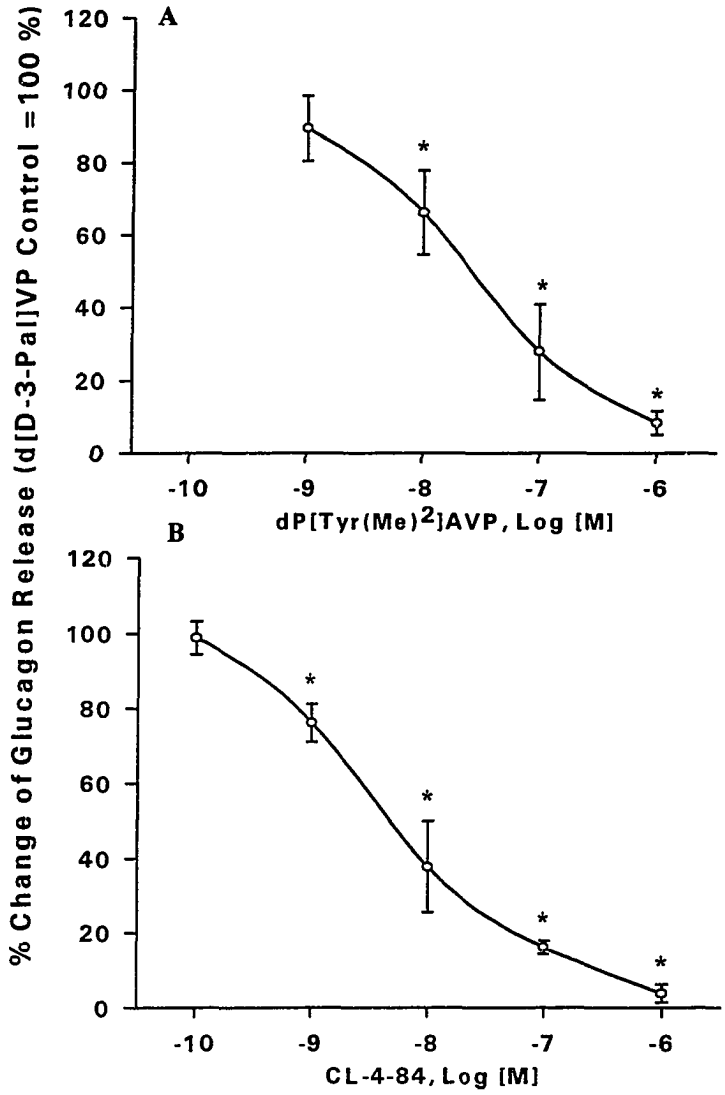

FIG. 3. Effect of dP[Tyr(Me)^2]AVP (A) and CL-4-84 (B) on d[D-3-Pal]VP-induced glucagon release. The monolayer cells were treated with the agonist (100 nM) for 15 min to determine glucagon release, which for the basal control group was 230.8 ± 14.2 pg/well/15 min and d[D-3-Pal]VP treated group was 797.6 ± 17.9 pg/well/15 min. Values are mean ± SE; n = 3 cultures with quadruplicates. The antagonist was given 10 min before and in the presence of the agonist. Values are mean ± SE; n = 3 cultures with quadruplicates. *$P < 0.05$ vs. the agonist control group (100%) at the corresponding concentration of the antagonist.

Table 1

IC_{50} of AVP and OT receptor antagonists on AVP- and OT-induced glucagon release.

Antagonist	AVP	OT
cyclo-(L-Pro-D-2-naphthyl-Ala-L-Ile-D-pipecolic acid-L-pipecolic acid-D-His) (L-366,948)	55,053 ± 27,382	61,763 ± 26,241
desGly^{9}d$(CH_2)_5$[Tyr(Et)^2]AVP (WK-3-6)	35,526 ± 6,139	21,255 ± 5,246
d$(CH_2)_5$[D-Phe^2, Ile^4]-AVP (AO-2-44)	42 ± 28	67 ± 11
4-OH-Phenylacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (CL-4-84)	20 ± 9	5 ± 4
dP[Tyr(Me)^2]AVP	29 ± 4	62 ± 6

Values are mean ± SE; n = 3 cultures with quadruplicates, except for 10^{-5} and 10^{-4} M which were n = 2. IC_{50}, half-maximal inhibitory concentration (nM); AVP, arginine vasopressin; OT, oxytocin.

CHAPTER IV MECHANISMS OF AVP-INDUCED GLUCAGON RELEASE IN CLONAL α-CELLS IN-R1-G9: INVOLVEMENT OF CA^{2+}-DEPENDENT AND -INDEPENDENT PATHWAYS

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ABSTRACT

1 The mechanisms underlying AVP-induced increase in $[Ca^{2+}]_i$ and glucagon release in clonal α-cells In-R1-G9 were investigated.

2 AVP increased $[Ca^{2+}]_i$ and glucagon release in a concentration-dependent manner. In Ca^{2+}-containing medium, AVP increased $[Ca^{2+}]_i$ in a biphasic pattern; a peak followed by a sustained plateau. In Ca^{2+}-free medium, the Ca^{2+} response to AVP became monophasic with a lower amplitude and no plateau. Both the basal and AVP-induced glucagon releases were lower in the absence than in the presence of extracellular Ca^{2+}. When $[Ca^{2+}]_i$ was stringently deprived by BAPTA, a Ca^{2+} chelator, AVP still significantly increased glucagon release.

3 Pretreatment with pertussis toxin failed to alter the AVP-induced glucagon release or increase in $[Ca^{2+}]_i$. Thapsigargin, a microsomal Ca^{2+} ATPase inhibitor, abolished both the Ca^{2+} peak and sustained plateau.

4 AVP increased intracellular concentration of IP_3.

5 U-73122 (8 μM), a phospholipase C inhibitor, abolished AVP-induced increases in $[Ca^{2+}]_i$, but only reduced AVP-induced glucagon release by 39 %.

6 Pretreatment with PD 98,059, a MAPK kinase inhibitor, ACA, a phospholipaseA_2 inhibitor, or nimodipine, an L-type Ca^{2+} channel blocker failed to alter AVP-induced glucagon release or increase in $[Ca^{2+}]_i$.

7 The results suggest that AVP causes glucagon release through both Ca^{2+}-dependent and -independent pathways. For the Ca^{2+}-dependent pathway, the G_q protein activates phospholipase C, which catalyzes the formation of IP_3. IP_3 induces Ca^{2+} release from

the endoplasmic reticulum, which, in turn, triggers Ca^{2+} influx. Both Ca^{2+} release and Ca^{2+} influx contribute to AVP-induced glucagon release.

INTRODUCTION

Arginine vasopressin (AVP), a neurohypophysial nonapeptide hormone, is synthesized in supraoptic and paraventricular nuclei of the hypothalamus. After being synthesized, it is stored in neurosecretory granules and is released from the posterior pituitary gland (Russel *et al.*, 1990). AVP exerts a number of physiological roles in mammals; it plays a major role in regulating body fluid volume, osmolality and contributes to the maintenance of blood pressure. In addition, AVP induces glycogenolysis (Kirk *et al.*, 1979), proliferation of the pituitary gland (McNichol *et al.*, 1990) and vascular smooth muscle cells (Sperti & Colucci, 1991), vasoconstriction (Fox *et al.*, 1987) and secretion of glucagon and insulin (Dunning *et al.*, 1984).

AVP induces glucagon release from clonal α-cells (Yibchok-anun & Hsu, 1998) and rat pancreas (Yibchok-anun *et al.,* 1999) through V_{1B} receptors in a concentration-dependent manner. AVP at the concentrations existing in the plasma (3-30 pM) increases glucagon release from perfused rat pancreas, which suggests that AVP may physiologically regulate glucagon release (Yibchok-anun *et al.,* 1999). However, the mechanisms underlying AVP-induced glucagon release remain unknown. Typically, AVP activates V_1 receptors, which couple to G_q, and thus activate phospholipase C-β (PLC-β), which in turn hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) to DAG and IP_3 (Thibonnier, 1992). DAG activates PKC, whereas IP_3 promotes Ca^{2+} release from endoplasmic reticulum (ER), leading to an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ induces Ca^{2+} influx through voltage-dependent (VDCC) and -independent Ca^{2+} channels (VICC) (Chen *et al.*, 1994; Li *et al.*, 1992; Thorn *et al.*, 1991).

In-R1-G9 cells are clonal glucagon-secreting cells derived from the hamster pancreatic islet (Takaki *et al.*, 1986). The synthesis and secretion of glucagon by In-R1-G9 cells share the basic characteristics of α-cells of the endocrine pancreas (Rorsman *et al.*, 1991); for instance, glucagon secretion from these cells is stimulated by forskolin, arginine, and theophylline and is inhibited by somatostatin (Fehmann *et al.*,

1995). We therefore used this cell line as a model for the α-cell of the pancreatic islet to study the mechanisms underlying AVP-induced glucagon release.

In this study, we found that AVP induced glucagon release via both Ca^{2+}-dependent and -independent pathways. For Ca^{2+}-dependent pathway, we used an aminosteroid 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole,2,5-dione (U-73122), a PLC inhibitor, to determine whether AVP induces glucagon release through a PLC-dependent pathway. In addition, we determined if the VDCC mediated AVP-induced Ca^{2+} influx by treating the cells with nimodipine, an L-type VDCC inhibitor.

MATERIALS AND METHODS

Cell culture

The hamster glucagonoma In-R1-G9 cells were maintained in RPMI 1640 with 10 % fetal bovine serum and aerated with 5% CO_2-95% air at 37°C. All experiments were performed using cells from passages 24-30.

Glucagon release

In-R1-G9 cells were plated into Corning 24-well plates at 10^5 cells/well and were grown for 3-4 days. The culture medium was then removed and replaced with modified Krebs-Ringer bicarbonate buffer (KRB) containing (in mM): 136 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5 $NaHCO_3$, 10 HEPES, 1.67 glucose and 0.1% BSA, pH 7.4. For determination of the dose response to AVP, cells were incubated at 37°C with AVP for 15 min after preincubation with KRB for 15 min. For Ca^{2+}-free experiments, cells were incubated at 37°C with AVP in Ca^{2+}-free KRB containing 10 μM EGTA for 15 min after preincubation with Ca^{2+}-containing KRB for 15 min. The following drugs were used in the study: U-73122 was given 100 sec; N-(p-amylcinnamoyl) anthranilic acid (ACA), a PLA_2 inhibitor, or nimodipine was given 5 min; PD 98,059, a MAPK kinase inhibitor, was given 30 min prior to the administration of AVP. 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester

(BAPTA-AM), an intracelluar Ca^{2+} chelator, was given 30 min in Ca^{2+}-free KRB before the administration of AVP. Pertussis toxin (PTX) (100 ng/ml) was given 16 h before the experiment. The cells were then treated with AVP in KRB containing one of the antagonists, BAPTA-AM or PTX. The concentration of glucagon in the media was measured by radioimmunoassay (RIA), following the procedures provided by Linco Research Inc.

Measurement of $[Ca^{2+}]_i$ in cell suspension

20 x 10^6 cells were loaded with 2 μM fura-2 acetoxymethyl ester (fura-2AM) in KRB for 30 min at 37°C. The loaded cells were centrifuged (300 x *g*, 2 min), then resuspended at a concentration of 2 x 10^6 cells/ml with KRB containing (in mM): 136 NaCl, 4.8 KCl, 1.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 Hepes, 1.67 glucose and 0.1% BSA and kept at 24°C until use. The 340/380 nm fluorescence ratios were monitored by a SLM-8000 spectrofluorometer (SLM instruments, Urbana, IL). The Ca^{2+}-free environment was created by centrifugation (300 x *g*, 30 sec) and the cells were resuspended in the Ca^{2+}-free KRB. When needed, cells were pretreated with U-73122, 4-OH-phenacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (CL-4-84), an antagonist with potent V_{1B} blocking activity (Thibonnier *et al.*, 1997), desGly^{9}d$(CH_2)_5$[Tyr(Et)^2]AVP (WK-3-6), a V_{1A}/V_2 antagonist (Jard *et al.*, 1986), ACA or nimodipine for 100 sec before the AVP application. The cells were pretreated with thapsigargin (TG) and PTX for 5 min, 30 min and 2 h, respectively before $[Ca^{2+}]_i$ measurement. The $[Ca^{2+}]_i$ was calibrated as previously described (Hsu *et. al.*, 1991).

Measurement of IP_3

Intracellular IP_3 was measured using a competitive radioreceptor-binding assay kit purchased from Dupont Co., Boston, MA. 2 x 10^6 cells in 1 ml of KRB were placed in polypropylene tubes and equilibrated in a shaking water bath at 37°C for 15 min. Incubation with AVP was terminated by adding ice-cold 20% (w/v) trichloroacetic acid in 15 sec. The concentration of IP_3 was determined by following the instructions provided by the manufacturer.

Cyclic AMP (cAMP) measurement

cAMP measurements were studied in the cultured cell monolayer under conditions similar to the glucagon release experiments. After treating the cells with AVP (1 μM) for 15 min, the cells were scraped from the plates in 0.01 N HCl and incubated in a water bath at 75 °C for 20 min to heat-inactivate phosphodiesterase (PDE). After centrifugation, the cell extracts were neutralized by 0.01 N NaOH and resuspended in the assay buffer. The cAMP levels were determined using RIA as previously described (Richards *et. al.,* 1979).

Drugs

All reagents were from Sigma Chemical (St. Louis, MO), except the fura-2AM was from Molecular Probes (Eugene, OR), U-73122, PD 98,059 and BAPTA-AM were from Biomol Research Laboratory (Plymouth Meeting, PA) and ^{125}I-glucagon was from Linco Research Inc. (St. Charles, MO).

Data and Statistics

All values were presented as mean ± s.e. Results were analyzed using the SAS PROC MIXED procedure and a randomized block design. There were two factors, treatment and block. Individual mean comparisons were performed using the F test. The significance level was set at $P < 0.05$.

RESULTS

Effect of AVP on glucagon release and $[Ca^{2+}]_i$ increase in normal and Ca^{2+}-deprived conditions

AVP (1-1000 nM) increased glucagon release (Fig. 1A) and $[Ca^{2+}]_i$ (Fig. 1B) in a concentration-dependent manner. Because AVP at 100 nM caused submaximal increases in glucagon release and $[Ca^{2+}]_i$, we chose this concentration to study the mechanisms underlying AVP-induced glucagon release and increases in $[Ca^{2+}]_i$. AVP (100 nM) significantly increased glucagon release to ~3 times of the basal control level in Ca^{2+}-containing medium. The concentration of glucagon release in the basal control

group was 505 ± 61 pg/well/15 min (n = 6 cultures with triplicates). The basal glucagon release was not significantly lower in Ca^{2+}-free medium (444 ± 114 pg/well/15 min, n = 6 cultures with triplicates) than in Ca^{2+}-containing medium. In Ca^{2+}-free medium, AVP (100 nM) increased glucagon release to 2.1 times of the basal control level (control = 444 ± 114 pg/well/15 min; AVP = 938 ± 180 pg/well/15 min, n = 6 cultures with triplicates). In addition, although $[Ca^{2+}]_i$ was deprived by preincubating the cells in Ca^{2+}-free medium containing 50 μM BAPTA-AM for 30 min, AVP still increased glucagon release to 1.6 times of the basal control level (control = 866 ± 205 pg/well/ 15 min; BAPTA-AM + AVP = 1419 ± 45 pg/well/15 min, n = 6 cultures with triplicates). BAPTA-AM alone did not significantly change glucagon release (control = 505 ± 61 pg/well/ 15 min; BAPTA-AM = 866 ± 205 pg/well/15 min, n = 6 cultures with triplicates) (Fig. 2).

To confirm whether the $[Ca^{2+}]_i$ was deprived after pretreating the cells with 50 μM BAPTA-AM in Ca^{2+}-free medium for 30 min, we investigated its effect on AVP-, bradykinin-, ionomycin- and TG-induced $[Ca^{2+}]_i$ increases. After the basal $[Ca^{2+}]_i$ was lowered by BAPTA to ≤ 25 nM, none of the above agonists increased $[Ca^{2+}]_i$ under this condition (data not shown).

The basal $[Ca^{2+}]_i$ in In-R1-G9 cells was 97 ± 4 nM (n = 20). AVP (1-1000 nM) increased $[Ca^{2+}]_i$ in a concentration-dependent manner and in a biphasic pattern; a peak followed by a sustained plateau (Fig. 3B). The peak usually reached within 30 sec and gradually decreased toward the baseline for over 4 min (the sustained plateau). To determine whether the Ca^{2+} peak was due to the release of Ca^{2+} from the intracellular Ca^{2+} stores and the sustained plateau was due to Ca^{2+} influx, we measured $[Ca^{2+}]_i$ in Ca^{2+}-free KRB supplemented with 10 μM EGTA. The basal $[Ca^{2+}]_i$ in Ca^{2+}-free/EGTA KRB (75 ± 3 nM, n = 20) was lower than that in Ca^{2+}-containing KRB (97 ± 4 nM, n = 20). In the absence of extracellular Ca^{2+}, AVP (1-1000 nM) evoked only a Ca^{2+} peak (without the sustained plateau) in a concentration-dependent manner as well; however the amplitude was smaller than that induced by AVP in the presence of extracellular Ca^{2+} (Fig. 3).

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

AVP increases glucagon release from In-R1-G9 cells (Yibchok-anun & Hsu, 1998) and perfused rat pancreas (Yibchok-anun *et al.*, 1999) by activating V_{1B} receptors. To study if AVP-induced $[Ca^{2+}]_i$ increase is also mediated by V_{1B} receptors, we pretreated the cells with different AVP receptor antagonists for 100 sec before the application of AVP (100 nM). CL-4-84 (0.1 nM-100 nM), a V_{1A}/V_{1B} receptor antagonist (Thibonnier *et al.*, 1997), inhibited AVP-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner (Fig. 4). CL-4-84 at the highest concentration studied (100 nM) abolished the AVP-induced rise in $[Ca^{2+}]_i$. IC_{50} of CL-4-84 was 2 ± 0.5 nM. WK-3-6 (1 μM), a potent V_{1A}/V_2 receptor antagonist (Jard *et al.*, 1986), failed to block AVP-induced $[Ca^{2+}]_i$ increase (data not shown). Neither CL-4-84 nor WK-3-6 alone significantly changed basal $[Ca^{2+}]_i$ (data not shown).

Effects of PTX and U-73122 on AVP-induced glucagon release and $[Ca^{2+}]_i$ increase

To determine whether AVP-induced increases in glucagon release and $[Ca^{2+}]_i$ are mediated through PTX-sensitive G protein and PLC, PTX and U-73122 were used to antagonize the effect of AVP. Pretreatment with PTX (100 ng/ml) for 16 and 2 h failed to inhibit either AVP (100 nM)-induced glucagon release (AVP = 3,515 ± 290 pg/well/15 min; PTX + AVP = 3,446 ± 341 pg/well/15min, n = 3 cultures with quadruplicates; $P > 0.05$) or an increase in $[Ca^{2+}]_i$ (AVP = 194 ± 22 nM; PTX + AVP = 260 ± 39 nM, n = 4; $P > 0.05$) (Fig. 5A), respectively. PTX (100 ng/ml) alone did not significantly change glucagon release (control = 1,681 ± 182 pg/well/15 min; PTX = 1,276 ± 139 pg/well/15 min, n = 3 cultures with quadruplicates) or $[Ca^{2+}]_i$ (control = 126 ± 15 nM; PTX = 167 ± 27 nM, n = 4). U-73122 (2, 4 and 8 μM) inhibited AVP-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner (Fig. 6A). At the highest concentration studied (8 μM), U-73122 abolished AVP-induced the rise in $[Ca^{2+}]_i$, but only reduced AVP-induced glucagon release by 39 % (Fig. 6B). The lower concentrations of U-73122 (2 and 4 μM) partially inhibited AVP-induced $[Ca^{2+}]_i$ increase, but did not reduce AVP-induced glucagon release. U-73122 alone did not change $[Ca^{2+}]_i$ until 8 μM was applied, which increased $[Ca^{2+}]_i$ by ~ 15 %, and then gradually returned to the basal level within 100 sec. To determine if U-73122 was

specific to AVP-induced $[Ca^{2+}]_i$ increase, ionomycin (300 nM), a Ca^{2+} ionophore, was used for this purpose. Ionomycin elicited a biphasic rise in $[Ca^{2+}]_i$ with a pattern similar to that induced by AVP (100 nM). U-73122 (8 μM) failed to inhibit ionomycin-induced rise in $[Ca^{2+}]_i$ (ionomycin = 244 ± 41 nM; U73122 + ionomycin = 300 ± 34 nM, n = 4; $P > 0.05$) (Fig. 5B).

Effects of thapsigargin (TG) and nimodipine on AVP-induced glucagon release and $[Ca^{2+}]_i$ increase

We next hypothesized that AVP increases $[Ca^{2+}]_i$ by inducing Ca^{2+} release from ER, which in turn increases Ca^{2+} influx. We depleted intracellular Ca^{2+} stores of In-R1-G9 cells by pretreating the cells with 1 μM TG, a microsomal Ca^{2+}-ATPase inhibitor (Thastrup *et. al.,* 1990), for 30 min, which abolished both AVP-induced Ca^{2+} release and influx (Fig. 5C). The $[Ca^{2+}]_i$ after TG treatment was 129 ± 19 nM (n = 4). To investigate if the AVP-induced Ca^{2+} influx was attributable to the opening of VDCCs, we pretreated In-R1-G9 cells with 1 μM nimodipine, an L-type VDCC blocker, for 100 sec. Nimodipine failed to inhibit AVP-induced glucagon release (basal = 916 ± 182 pg/well/15 min; AVP = 2,080 ± 535 pg/well/15 min; nimodipine = 914 ± 215 pg/well/15 min; nimodipine + AVP = 2,333 ± 806 pg/well/15 min, n = 3 cultures with quadruplicates) or $[Ca^{2+}]_i$ increase (Fig. 5D).

Failure of ACA and PD 98059 to affect AVP-induced glucagon release

Since AVP was found to increase glucagon release via a PLC-independent pathway, we determined whether PLA_2 and/or MAPK were involved in this pathway by pretreating the cells with 1 μM ACA, a PLA_2 inhibitor, or 30 μM PD 98,059, a MAPK kinase inhibitor. ACA failed to alter the effect of AVP-induced glucagon release (basal = 311 ± 68 pg/well/15 min; AVP = 1,125 ± 26 pg/well/15 min; ACA = 292 ± 55 pg/well/15 min; ACA + AVP = 1,173 ± 83 pg/well/15 min, n = 3 cultures with quadruplicates). PD 98,059 also failed to inhibit AVP-induced glucagon release (basal = 490 ± 153 pg/well/15 min; AVP = 1,538 ± 298 pg/well/15 min; PD = 570 ± 157 pg/well/15 min; PD 98,059 + AVP = 1,632 ± 353 pg/well/15 min, n = 3 cultures with quadruplicates).

Effects of AVP on intracellular IP_3 and cyclic AMP concentrations

To confirm whether AVP induces glucagon release and $[Ca^{2+}]_i$ increase through PLC-IP_3 system, but not adenylyl cyclase-cAMP system, we measured the AVP-induced changes in the intracellular concentration of IP_3 and cyclic-AMP. AVP significantly increased intracellular IP_3 concentration by 147 % (control = 16 ± 4 pmol/2 million cells; AVP (100 nM) = 39 ± 4 pmol/2 million cells, n = 5; $P < 0.05$). AVP failed to change intracellular cyclic-AMP concentrations (control = 3.6 ± 0.2 pmol/well/15 min; AVP (1 μM) = 3.8 ± 0.2 pmol/well, n = 4; $P > 0.05$).

DISCUSSION

Results from the present study suggest that AVP induces increases in $[Ca^{2+}]_i$ in In-R1-G9 by activating V_{1B} receptors, because this effect of AVP was inhibited by CL-4-84, a V_{1A}/V_{1B} receptor antagonist (Thibonnier *et al.*, 1997), but not by WK 3-6, a highly potent V_{1A} and V_2 receptor antagonist (Jard *et al.*, 1986). This finding is consistent with our previous ones in which AVP induced glucagon release from In-R1-G9 (Yibchok-anun & Hsu, 1998) and perfused rat pancreas (Yibchok-anun *et al.*, 1999) by activating V_{1B} receptors. Unfortunately, no specific V_{1B} receptor antagonists are available yet for the characterization of these receptors. A molecular biological approach is warranted to confirm the characterization of V_{1B} receptors in α-cells.

The V_{1B} receptor has seven transmembrane-binding domains, and is coupled to a PTX-insensitive G-protein, probably G_q (Thibonnier *et al.*, 1993). In this study, we found that PTX (100 ng ml^{-1}) failed to inhibit either AVP-induced glucagon release or $[Ca^{2+}]_i$ increase. These results suggest that in In-R1-G9 cells, V_{1B} receptors are coupled to G_q, which is a PTX-insensitive G-protein, thereby increasing $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ contributes to glucagon release.

The activation of PLC via G_q is responsible for the hydrolysis of PIP_2 into DAG and IP_3, which causes Ca^{2+} release from the ER. In this study, since AVP increased IP_3 formation and it had no effect on cAMP production, the activation of PLC-β-IP_3 system should be one of the signal transduction pathways through which AVP induces

glucagon release. In addition, we found that 8 μM U-73122, the specific PLC inhibitor, abolished AVP-induced $[Ca^{2+}]_i$ increase and reduced AVP-induced glucagon release. These findings confirm that AVP activates PLC-β to increase IP_3 production, leading to the elevations of $[Ca^{2+}]_i$ and glucagon release.

An increase of $[Ca^{2+}]_i$ frequently triggers exocytosis (Wollheim & Pozzan, 1984; Li *et al.*, 1992; Tse *et al.*, 1993; Gromada *et al.*, 1997). Ca^{2+} oscillation is the pacemaker of pulsatile glucagon release in α-cells (Bode *et al.*, 1994). Thus, Ca^{2+} is hypothesized to be a major signal for glucagon release in In-R1-G9 cells. AVP induced $[Ca^{2+}]_i$ increase in In-R1-G9 cells in a biphasic pattern; a peak followed by a sustained plateau. In Ca^{2+}-free experiments, AVP only evoked a Ca^{2+} peak without a sustained phase. The Ca^{2+} peak evoked by AVP in the absence of extracellular Ca^{2+} was lower than in the presence of extracellular Ca^{2+}. These results suggest that the Ca^{2+} peak evoked by AVP is partly due to the release from intracellular stores and to the influx, whereas the sustained plateau is attributed to an increase in Ca^{2+} influx. When the intracellular Ca^{2+} stores were depleted by TG, a microsomal Ca^{2+}-ATPase inhibitor, the AVP-induced $[Ca^{2+}]_i$ increase including the sustained phase was totally abolished. This finding suggests that the AVP-induced Ca^{2+} influx depends on AVP-induced Ca^{2+} release. The involvement of Ca^{2+}-channels was further studied. Nimodipine did not alter AVP-induced glucagon release or $[Ca^{2+}]_i$ increase. Thus, the AVP-induced Ca^{2+} influx was not mediated through L-type VDCCs. These results are consistent with those of Bode *et al.* (1994) in which TG and U-73122 inhibited the spontaneous Ca^{2+} oscillation in single In-R1-G9 cells, but the L-type VDCC antagonists verapamil and nifedipine did not. In contrast to α-cells, AVP induces Ca^{2+} influx in clonal β-cells RINm5F partly by the opening of L-types VDCCs (Chen *et al.*, 1994; Li *et al.*, 1992).

The increase in $[Ca^{2+}]_i$ from both Ca^{2+} release and influx may contribute to glucagon release, because AVP was still able to increase glucagon release in the absence of extracellular Ca^{2+}. However, both basal and AVP-induced glucagon releases were lower in the absence than in the presence of extracellular Ca^{2+}. More interestingly, AVP increased glucagon release under stringent Ca^{2+} deprivation, which was obtained by pretreatment of the cells with 50 μM BAPTA in Ca^{2+}-free KRB. This

result suggests the existence of a Ca^{2+}-independent pathway of AVP-induced glucagon release.

U-73122 has been shown to inhibit PLC-mediated events in a variety of cells, for example, human neuroblastoma cells (Thompson *et al.*, 1991) and erythroleukemia cells (Wu *et al.*, 1992), human neutrophils and platelets (Bleasdale *et al.*, 1990), GH_3 rat pituitary cells (Smallridge *et al.*, 1992), rat gonadotropic cells (Hawes *et al.*, 1992), rat hepatocytes (Kimura & Ogihara, 1997), porcine kidney cells (Dibas *et al.*, 1997), rat pancreatic acinar cells (Yule & Williams, 1992) and a clonal β-cell line, RINm5F (Chen *et al.*, 1994; Yang *et al.*, 1997). In addition, U-73122 was used to block the formation of IP_3 and DAG in many different cell preparations (Bleasdale & Fisher, 1993). In In-R1-G9 cells, U-73122 inhibited AVP-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner, but had a much smaller impact on AVP-induced glucagon release. For instance, U-73122 at 8 μM abolished the effect of AVP-induced $[Ca^{2+}]_i$ increase, but only reduced AVP-induced glucagon release by 39 %. U-73122 at 4 μM inhibited AVP-induced $[Ca^{2+}]_i$ increase by 50 %, but had no effect on AVP-induced glucagon release. This finding is consistent with that of Chen *et al.* (1994) in which U-73122 inhibited AVP-induced increase in $[Ca^{2+}]_i$ much greater than the increase in insulin secretion. The effect of U-73122 was highly specific to PLC because it failed to alter ionomycin-induced increase in $[Ca^{2+}]_i$. Ionomycin, a Ca^{2+} ionophore, increases $[Ca^{2+}]_i$ by promoting Ca^{2+} release from intracellular stores and Ca^{2+} influx in HIT cells (Swope & Schonbrunn, 1988) and oocytes of *Xenopus laevis* (Yoshida & Plant, 1992) without the involvement of a G protein. In addition, a remarkable phenomenon observed in the present study was that AVP still stimulated glucagon release after AVP-induced $[Ca^{2+}]_i$ increase was abolished. Together, these results strongly suggest that AVP-induced glucagon release involves a mechanism that is independent of an elevation of $[Ca^{2+}]_i$ or an activation of PLC-β. This may be due to multiple signal transduction pathways involved the V_{1B} receptor-mediated hormone secretion (Thibonnier *et al.*, 1997).

In a smooth muscle cell line A_7r_5, V_1 receptors are coupled to several signaling pathways including PLC, PLA_2 and PLD (Thibonnier *et al.*, 1991). AVP stimulates secretion of endothelin-1 and prostanoids from human brain endothelial cells by a receptor-mediated activation of PLC and PLA_2 (Spatz *et al.*, 1994). However, in our study, ACA (1 μM), a PLA_2 inhibitor (Konrad *et al.*, 1992), failed to affect AVP-induced

glucagon release in In-R1-G9 cells, which suggests that PLA_2 is not involved in AVP-induced glucagon release. In CHO-V_3 cells, V_{1B} receptors couple to p44/p42 MAPKs (Thibonnier *et al.*, 1997). However, AVP-induced glucagon release should not involve the activation of p44/p42 MAPKs, because PD 98,059 failed to prevent AVP-induced glucagon release.

AVP activates phospolipase D (PLD) in rat Leydig (Vinggaard & Hansen, 1991) and glomerular mesangial cells (Kusaka, *et al.,* 1996). Activation of PLD leads to phosphatidic acid (PA) formation, stimulating insulin release (Metz & Dunlop, 1990). We found that PLD from *Streptomyces sp.* increased glucagon release under both *normal and* Ca^{2+}*-deprived condition* (unpublished data). Carbobenzyloxy-leucine-tyrosine-chloromethylketone (zLYCK), an inhibitor of PLD (Kusaka, *et al.,* 1996), significantly inhibited AVP-induced glucagon release (unpublished data). In addition, a pseudosubstrate peptide inhibitor specific for PKC-ζ and PKC-λ inhibited the AVP-induced glucagon release (unpublished results). PA and DAG (derived from PA via PLD) can activate PKC (Ando *et al.,* 1989; Dunlop & Larkins, 1985). It is likely that activation of PLD, leading to activation of PKC-ζ and/or PKC-λ, may be involved in the PLC-independent mechanism of AVP-induced glucagon release. Activation of PLC requires Ca^{2+} (Chaudry & Rubin, 1990; Gardner, 1989); thus the PLC-independent pathway may be one of the Ca^{2+}-independent pathways. The G protein-coupled PLD pathway in rat myocardium is both Ca^{2+}-dependent and -independent (Lindmar & Loffelholz, 1998). However, whether PLD plays a role in the Ca^{2+}-independent pathway of AVP-induced glucagon release remains to be determined.

Another possibility is that AVP acts independently of Ca^{2+} at a distal site to trigger exocytosis. This would be similar to carbachol-induced insulin release in RINm5F cells, in which carbachol may stimulate insulin release by acting at a site beyond the point of increased $[Ca^{2+}]_i$ (Tang *et. al,* 1995). Although U-73122 (8 μM) abolished AVP-induced $[Ca^{2+}]_i$ increase, small increases in $[Ca^{2+}]_i$ at the level close to secretory granules might not have been detected by our method. These small increases in $[Ca^{2+}]_i$ might have triggered glucagon release. Further work is needed to identify the other pathways, particularly the Ca^{2+}-independent ones, which are coupled to AVP-induced glucagon release.

In summary, AVP increased $[Ca^{2+}]_i$ probably through G_q, which is a PTX-insensitive G protein. U-73122 inhibited AVP-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner, but only partially antagonized AVP-induced glucagon release. We conclude that, in In-R1-G9 cells, AVP induces glucagon release through multiple signaling pathways that are both Ca^{2+}-dependent and -independent. For the Ca^{2+}- dependent pathway, G_q activates PLC, which promotes the formation of IP_3 and DAG. IP_3 stimulates Ca^{2+} release from the ER, which in turn triggers Ca^{2+} influx via non-L-type Ca^{2+} channels. Both Ca^{2+} release and Ca^{2+} influx contribute to AVP-induced glucagon release. The Ca^{2+}-independent pathway for AVP-induced glucagon release is not well-understood and needs further investigation.

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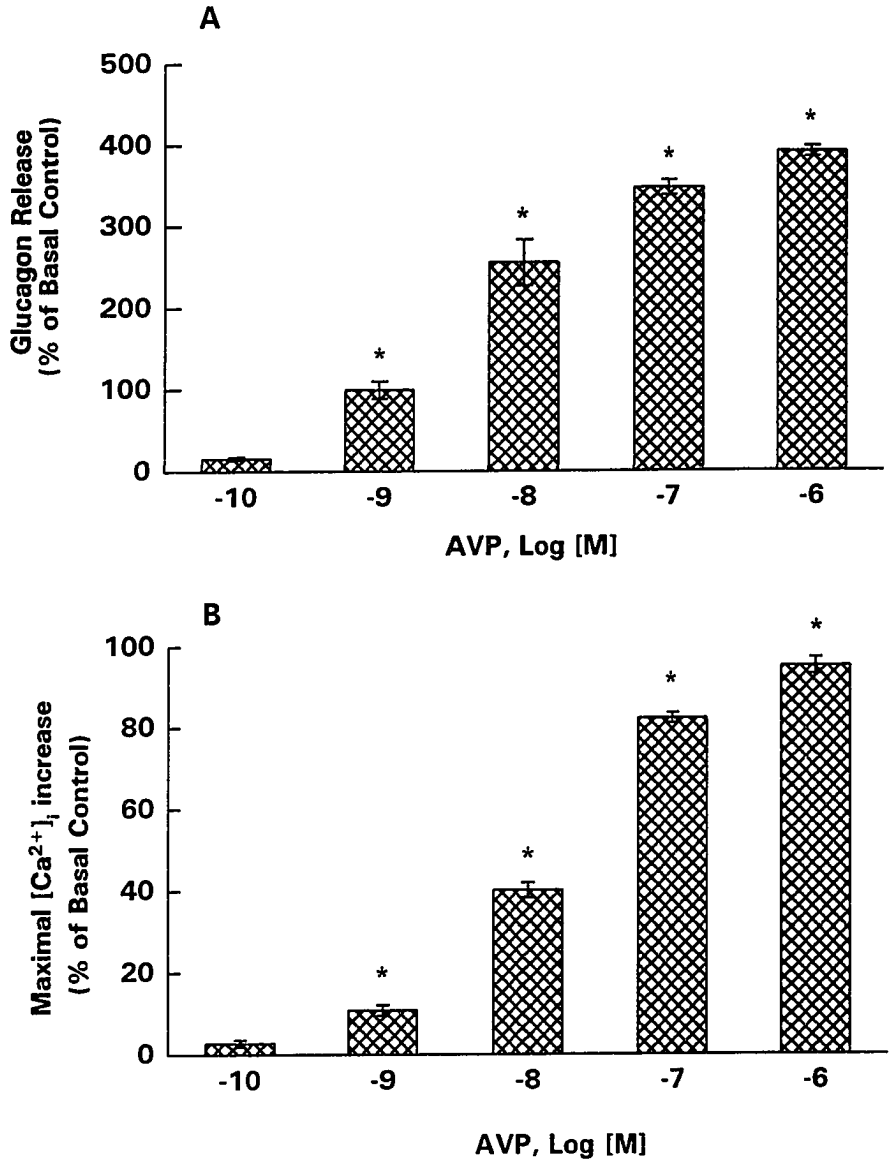

Fig. 1. Effects of AVP on glucagon release (A) and $[Ca^{2+}]_i$ increase (B) in In-R1-G9 cells. In A), the basal $[Ca^{2+}]_i$ was 97 ± 4 nM. Values are mean ± s.e. (n = 4). In B), static incubation was performed for 15 min to determine glucagon release. The concentration of glucagon release in the basal control group was 1,184 ± 84 pg/well/15 min. Values are mean ± s.e. (n = 3 cultures with quadruplicates). * $P < 0.05$, compared with the control group.

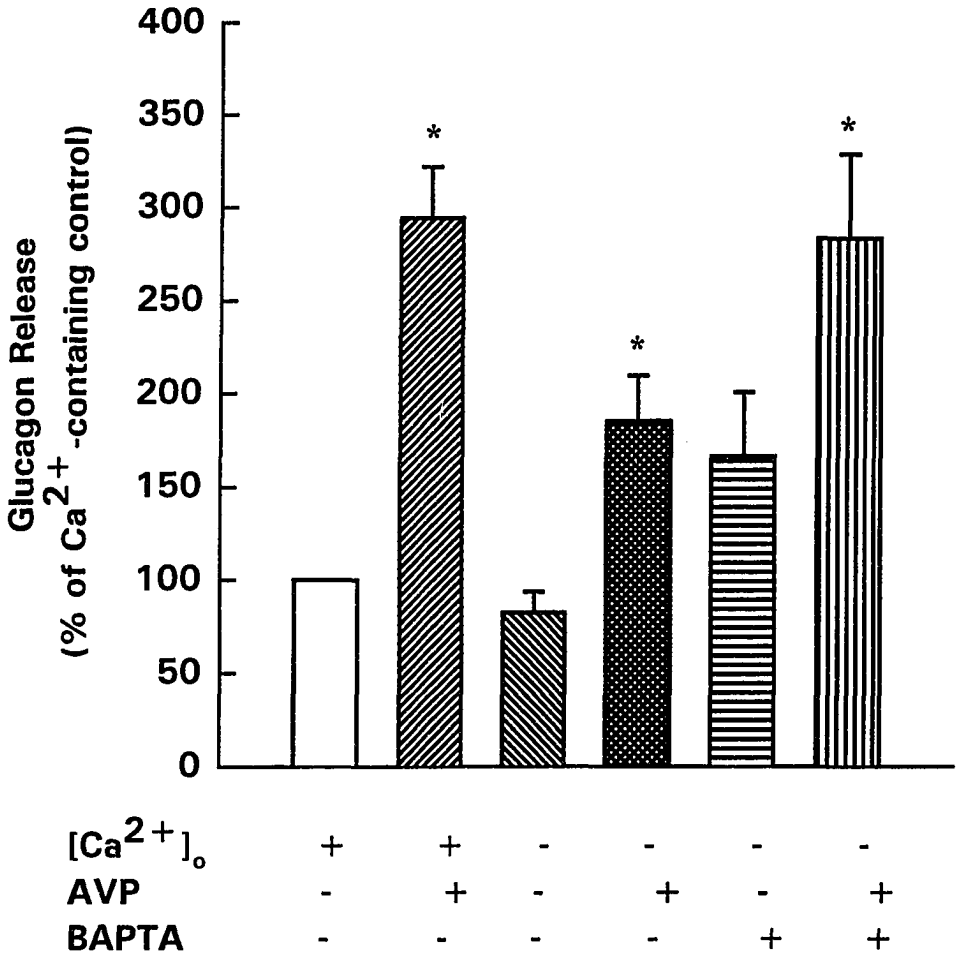

Fig. 2. Effects of AVP (100 nM) on glucagon release in Ca^{2+}-containing, Ca^{2+}-free media and 50 μM BAPTA-AM in Ca^{2+}-free media. BAPTA-AM in Ca^{2+}-free media was given 30 min before the administration of AVP. Static incubation was performed for 15 min to determine the glucagon release. The concentration of glucagon release in the basal control group was 505 ± 61 pg/well/15 min. Values are mean ± SE (n = 6 cultures with quadruplicates). * $P < 0.05$, compared with the basal control group at the $[Ca^{2+}]_o$ of 2.5 mM.

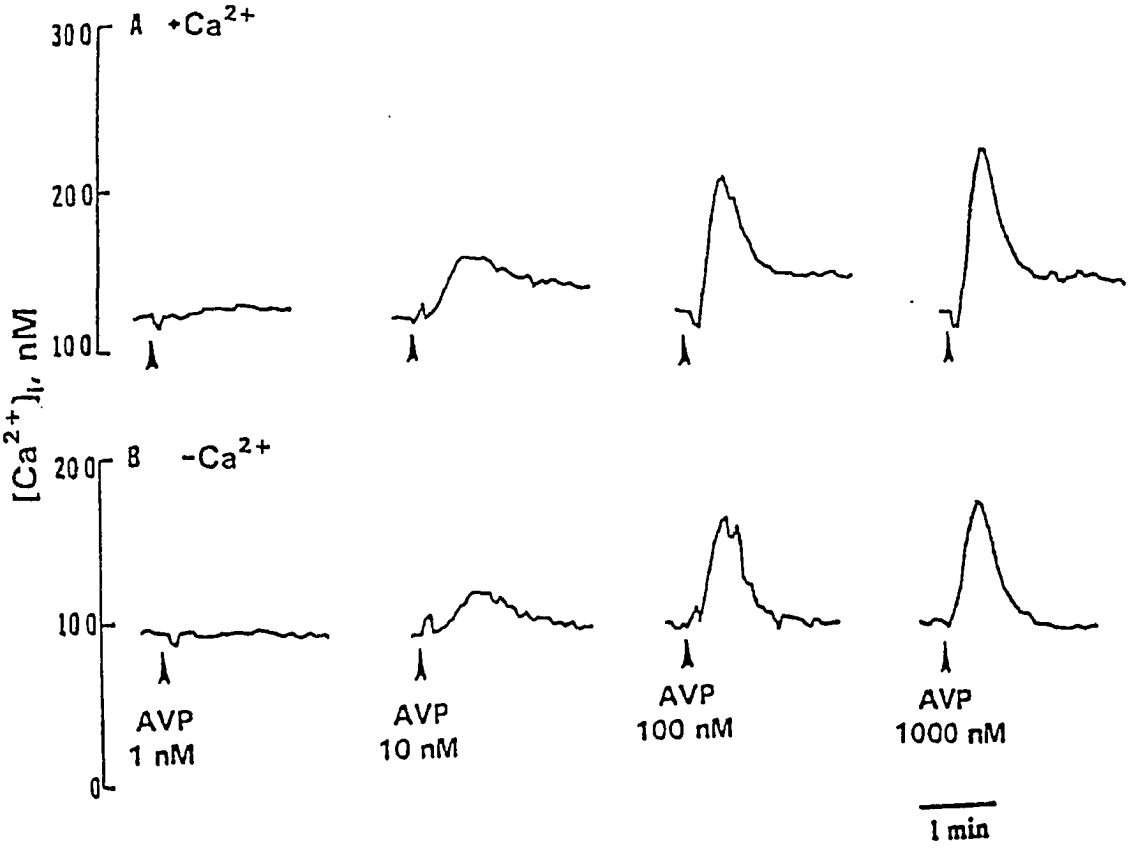

Fig. 3. Effects of AVP on $[Ca^{2+}]_i$ in Ca^{2+}-containing (A) and Ca^{2+}-free (B) media. Data shown are representative of 4 experiments.

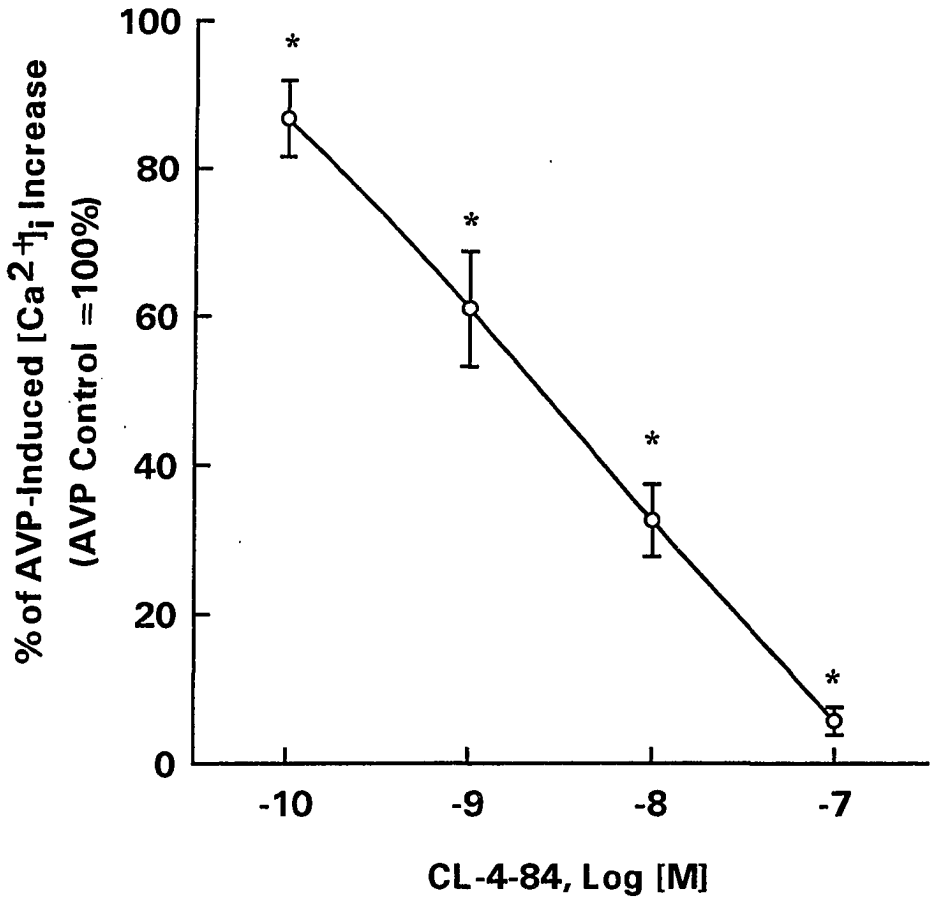

Fig. 4. Effects of CL-4-84 on AVP (100 nM)-induced maximal increase in $[Ca^{2+}]_i$. CL-4-84 was given 100 sec before the administration of AVP. Values are mean ± SE (n = 4). * $P < 0.05$, compared with the AVP control group, which had a maximal $[Ca^{2+}]_i$ increase of 188 ± 11 nM

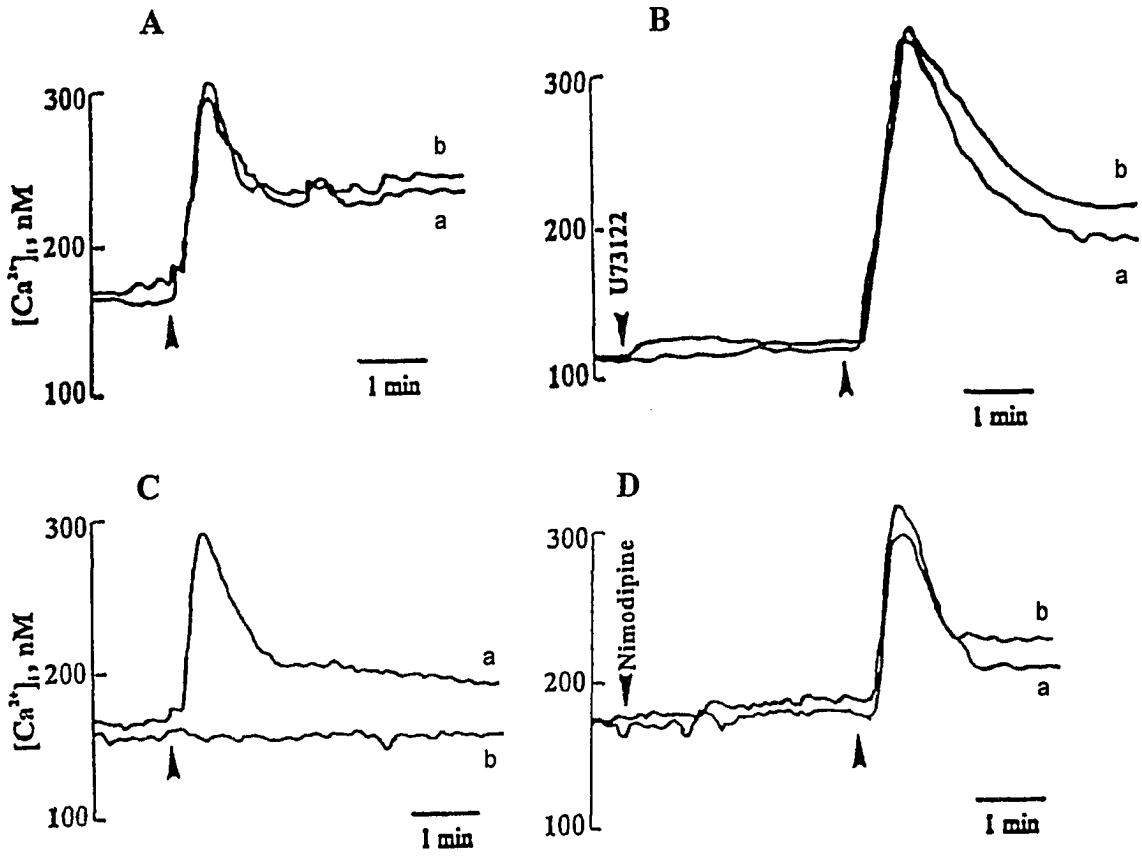

Fig. 5. Effects of AVP and ionomycin on $[Ca^{2+}]_i$ increase in In-R1-G9 cells. A) Effect of PTX on AVP-induced $[Ca^{2+}]_i$ increase. Curve a shows data of AVP (100 nM) alone as a control; curve b shows the effect of PTX pretreatment (100 ng ml^{-1}) for 2 h before AVP administration. B) Effects of U-73122 on ionomycin-induced $[Ca^{2+}]_i$ increase. Curve a shows data of ionomycin (300 nM) alone as a control; curve b shows the effect of U-73122 (8 μM) pretreatment for 100 sec before ionomycin administration. C) Effect of TG on AVP-induced $[Ca^{2+}]_i$ increase. Curve a shows data of AVP (100 nM) alone as a control; curve b shows the effect of TG pretreatment (1 μM) for 30 min before AVP administration. D) Nimodipine did not affect AVP-induced $[Ca^{2+}]_i$ increase. Curve a shows data of AVP (100 nM) alone as a control; curve b shows the effect of nimodipine pretreatment (1 μM) for 100 sec before AVP administration. Arrow indicates AVP or ionomycin administration. Data shown are representative of 4 experiments.

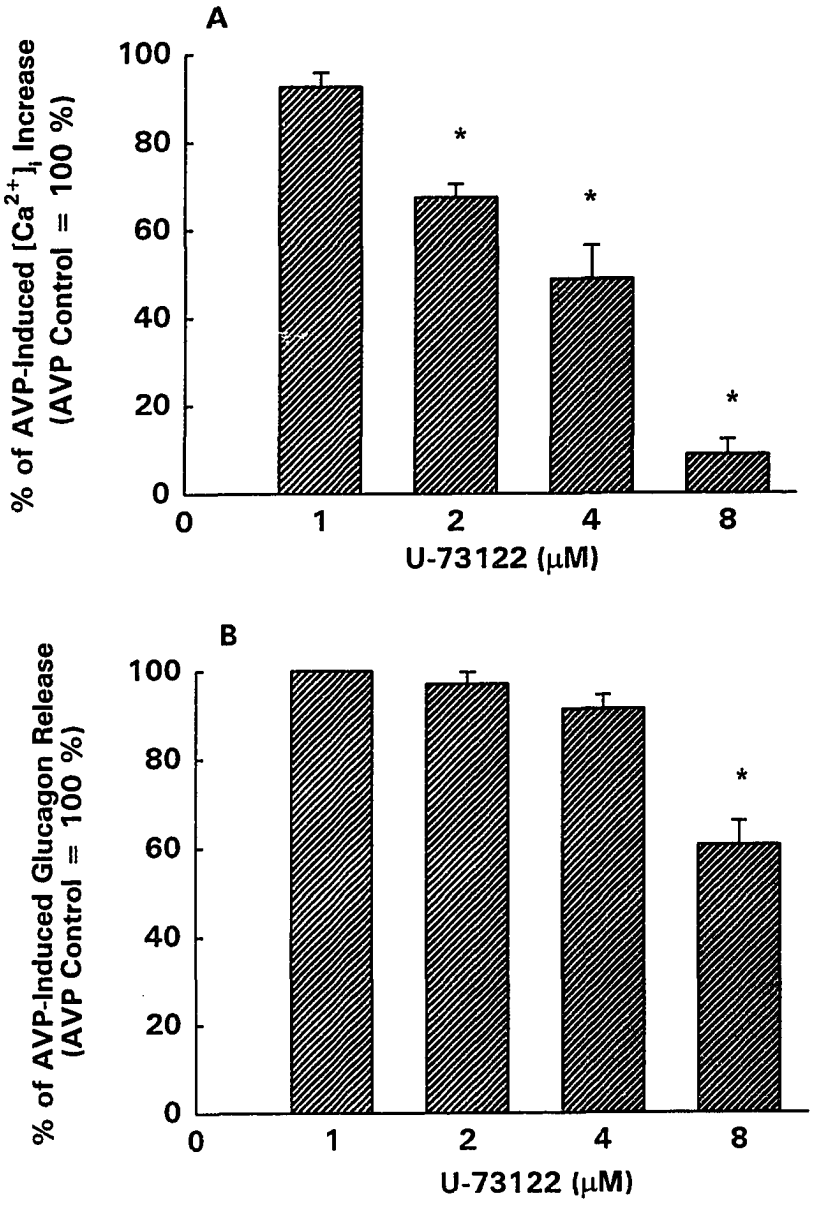

Fig. 6. Effects of U-73122 on AVP-induced maximal $[Ca^{2+}]_i$ increase (A) and glucagon release (B) in In-R1-G9 cells. U-73122 was given 100 sec before AVP (100 nM). Values are mean ± SE (n = 4 for $[Ca^{2+}]_i$ experiments and n = 3 cultures with quadruplicates for secretion). * $P < 0.05$, compared with AVP (100 nM) alone as the control group.

CHAPTER V PROTEIN KINASE C ATTENUATES ARGININE VASOPRESSIN-INDUCED INCREASES IN IP_3 AND $[CA^{2+}]_i$ IN CLONAL α-CELLS IN-R1-G9

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ABSTRACT

AVP (100 nM) increased $[Ca^{2+}]_i$ and inositol 1,4,5 trisphosphate (IP_3) production in clonal α-cell line In-R1-G9. PKC activators phorbol 12-myristate 13-acetate (PMA) and 1-oleoyl-2-acetyl-sn-glycerol (OAG) antagonized, whereas Ro 31-8220, a PKC inhibitor, potentiated AVP-induced increase in IP_3 production and $[Ca^{2+}]_i$. The potentiation by Ro 31-8220 was decreased by SKF 96365, a receptor-operated Ca^{2+} channel (ROC) inhibitor, but not by nimodipine, a voltage-dependent Ca^{2+} channel inhibitor. In addition, Ro 31-8220 blocked the effects of PMA and OAG on AVP-induced increases in IP_3 and $[Ca^{2+}]_i$. Down regulation of PKC by pretreatment with PMA for 72 h enhanced AVP-induced $[Ca^{2+}]_i$ increase. We conclude that in In-R1-G9 cells, PKC exerts a negative feedback on AVP-induced increase in IP_3 production, causing a decrease in Ca^{2+} release and influx; the latter is mediated by ROC.

INTRODUCTION

PKC is a family of Ca^{2+}- and phospholipid-dependent enzymes that mediates a wide range of signal transduction processes. At least 11 PKC isozymes have been identified and divided into three classes based on different requirements for their activations (Nishizuka, 1986; Nishizuka, 1988; Housey et al., 1988; Persons et al., 1988). The conventional isozymes (cPKCs) α, βI, βII, γ are activated by Ca^{2+}, negatively charged phospholipids, diacyl glycerol (DAG) or phorbol esters, whereas the novel isozymes (nPKCs) δ, ε, η, θ, μ are Ca^{2+}-independent (Hug and Sarre, 1993; Jaken, 1996; Johannes et al., 1994). The atypical isozymes (aPKCs) λ/ι and ζ are

insensitive to stimulation by Ca^{2+}, DAG or phorbol esters; however, they are activated by phospholipid-derived second messengers, such as IP_3 (Jaken, 1996; Weinstein, 1990), phosphatidylserine (Nishizuka, 1992) and phosphatidic acid (Limatola et al., 1994). Numerous PKC isozymes have been detected in pancreatic islets (Knutson and Hoenig, 1994). In addition, down-regulation of PKC by pretreating rat islets with PMA for 24 h leads to impairment of arginine-induced glucagon release, which suggests that PKC is present in the α-cells of the pancreatic islets (Bjaaland et al., 1988).

PKC is activated by DAG, which is generated via breakdown of phosphoinositides by Ca^{2+}-mobilizing neurotransmitters and hormones such as acetylcholine, vasopressin, oxytocin and bombesin (Ashcroft, 1994). There is evidence that Ca^{2+} (Pipeleers et al., 1985; Charles et al., 1987; Niki et al., 1986) and activation of PKC (Hii et al., 1986; Bjaaland et al., 1988) are important in the regulation of glucagon release from α-cells. PMA, substituting in vitro for DAG that is one of the physiological activators for PKC, has been found to increase glucagon release (Hii et al., 1986; Niki et al., 1986), which suggests the participation of PKC in the regulation of glucagon release.

AVP physiologically regulates glucagon release from the rat pancreas by activating V_{1b} recepotrs (Yibchok-anun et al., 1999). The existence of high concentration of AVP in the pancreas (Amico et al., 1988) suggests a possible local action of this hormone. In addition, AVP induces an increase in $[Ca^{2+}]_i$ in hamster glucagonoma In-R1-G9 cells in a biphasic pattern; a peak followed by a sustained phase (Yibchok-anun and Hsu, 1998). This effect is mediated by V_{1b} receptors that couple to PTX-insensitive G protein, probably G_q. Activation of G_q catalyzes PLC-β to increase the formation of DAG and IP_3. DAG activates PKC and IP_3 promotes calcium release from the endoplasmic reticulum (ER), leading to an increase in $[Ca^{2+}]_i$, which stimulates glucagon release (Yibchok-anun and Hsu, 1998). However, it is not known if PKC has an impact on AVP-induced increases in the production of IP_3 and $[Ca^{2+}]_i$ that finally lead to glucagon release.

It is a common practice to study the effect of enzymes by inhibiting or activating their activities. In the present study, we used Ro 31-8220, a bisindoylmaleimide PKC inhibitor, that is selective and specific for PKC isozymes (Davis et al., 1989) to elucidate the effect of PKC on AVP-induced increase in the production of IP_3 and $[Ca^{2+}]_i$ from In-R1-G9 cells. We also used PMA and OAG, a DAG analog, to activate PKC. In

addition, we induced down-regulation of PKC by a long-term PMA treatment to probe for the role of PKC on AVP-induced increases in $[Ca^{2+}]_i$.

AVP increases $[Ca^{2+}]_i$ in a biphasic pattern, a peak followed by a sustained phase, and the latter suggests the involvement of Ca^{2+} influx (Yibchok-anun and Hsu, 1998). Thus, we used SKF 96365, a receptor-operated Ca^{2+} channel (ROC) blocker (Li et al., 1997) and nimodipine, a voltage-dependent Ca^{2+} channel (VDCC) blocker, to determine which type of Ca^{2+} channels contributes to the influx. Results of the present study suggest that PKC may exert a negative feedback on AVP-induced increase in IP_3 production, leading to an attenuation of Ca^{2+} release and influx; the latter is mediated by ROC.

MATERIALS AND METHODS

Cell culture

The hamster glucagonoma In-R1-G9 cells were maintained in RPMI 1640 (Sigma, St. Louis, MO) with 10 % fetal bovine serum (Sigma, St. Louis, MO) and aerated with 5% CO_2/95% air at 37°C.

Measurement of $[Ca^{2+}]_i$ in cell suspension

20 x 10^6 cells were loaded with 2 μM fura-2 acetoxymethyl ester (fura-2AM; Molecular probes, Eugene, OR) in KRB containing (in mM): 136 NaCl, 4.8 KCl, 1.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 Hepes, 1.67 glucose and 0.1% BSA for 30 min at 37°C. The cells were then centrifuged (300 x *g*, 2 min) and resuspended at a concentration of 2 x 10^6 cells/ml with KRB and kept at 24°C until use. Fluorescence ratios of 340/380 nm were monitored by a SLM-8000 spectrofluorometer (SLM instruments, Urbana, IL). The Ca^{2+}-free environment was created by centrifugation (300 x *g*, 30 sec) and cell resuspension in Ca^{2+}-free KRB supplemented with 10 μM EGTA. Cells were pretreated with Ro 31-8220 (Roche Products Ltd., Hertfordshire, UK) for 30 min, PMA or OAG (Sigma Chemical, St. Louis, MO) for 10 min before the AVP or ionomycin (Sigma Chemical, St. Louis, MO) application. Cells also were

pretreated with SKF 96365 (Biomol Research, Plymouth Meeting, PA) for 10 min or nimodipine (Research Biochemicals International, Natick, MA) for 5 min in the presence of Ro 31-8220 prior to the AVP application. For down regulation of PKC, cells were treated with 200 nM PMA in culture media for 72 h, and resuspended in KRB containing 200 nM PMA during the experiment. The $[Ca^{2+}]_i$ was calibrated as previously described (Hsu et. al., 1991).

Measurement of IP_3

Intracellular IP_3 was measured using a competitive radioreceptor-binding assay kit (Dupont Co., Boston, MA). 2×10^6 cells in 1 ml of KRB were placed in polypropylene tubes and equilibrated in a shaking water bath at 37°C for 15 min. The cells were pretreated with Ro 31-8220, OAG or PMA for 30, 10 and 10 min, respectively, before the application of AVP. Incubation with AVP was terminated by adding ice-cold 20% (w/v) trichloroacetic acid at 15 sec. The concentration of IP_3 was determined according to the manufacturer instructions.

Statistical analysis

All values were presented as mean ± S.E.M. Results were analyzed using ANOVA and individual mean comparisons were made using Least Significant Difference test. The significance level was set at $P < 0.05$.

RESULTS

Effects of Ro 31-8220, OAG or PMA on AVP-induced IP_3 production

AVP (100 nM) increased IP_3 production ~ 2 fold over the basal level. This concentration of AVP was used throughout the study. Ro 31-8220 (10 μM), a selective PKC inhibitor, significantly enhanced AVP-induced IP_3 production. A membrane-permeable DAG analog, OAG (30 μM) totally abolished AVP-induced IP_3 production. In addition, the phorbol ester PMA (100 nM) significantly reduced AVP-induced IP_3 production. Ro 31-8220, OAG or PMA alone did not significantly change the IP_3 content (Fig. 1).

Effects of Ro 31-8220 on AVP-induced $[Ca^{2+}]_i$ increase

AVP increased $[Ca^{2+}]_i$ in a biphasic pattern; a peak followed by a sustained phase in Ca^{2+}-containing medium. Ro 31-8220 (10 μM) alone did not change the basal $[Ca^{2+}]_i$ (control = 108.3 ± 10.1 nM; Ro 31-8220 = 105.7 ± 7.7 nM, n = 11). Pretreatment of the cells with Ro 31-8220 potentiated AVP-induced increases in both the maximal and sustained $[Ca^{2+}]_i$ (Fig. 2A).

In the absence of extracellular Ca^{2+}, AVP evoked only a peak increase of $[Ca^{2+}]_i$ without the sustained phase which is due to the Ca^{2+} release from the ER (Yibchok-anun and Hsu, 1998). Pretreatment with Ro 31-8220 (10 μM) failed to enhance AVP-induced $[Ca^{2+}]_i$ increase (Fig. 2B).

In our previous report, the Ca^{2+} peak induced by AVP in In-R1-G9 cells was attributable to the release from ER and to the influx, whereas the sustained phase was attributable to Ca^{2+} influx through Ca^{2+} channels (Yibchok-anun and Hsu, 1998). To determine whether the potentiation of AVP-induced $[Ca^{2+}]_i$ increase by Ro 31-8220 was due to the sustained opening of Ca^{2+} channels and to determine which type of Ca^{2+} channels was involved, SKF 96365 (0.3-30 μM) or nimodipine (1 μM) was applied. SKF 96365, an ROC blocker, inhibited Ca^{2+} peak potentiated by Ro 31-8220 in a concentration-dependent manner, and the highest concentration studied (30 μM) significantly inhibited sustained Ca^{2+} phase (Table 1). Nimodipine, a VDCC blocker, did not significantly inhibit the Ro 31-8220-elicited potentiation (Table 1).

Effects of OAG on AVP-induced $[Ca^{2+}]_i$ increase

OAG (30 μM) alone did not change the basal $[Ca^{2+}]_i$ (control = 115.2 ± 21.4 nM; OAG = 113.1 ± 22.7 nM, n = 4). OAG pretreatment for 10 min significantly decreased AVP-induced $[Ca^{2+}]_i$ increase. In addition, pretreatment with Ro 31-8220 (10 μM) for 30 min abolished the inhibitory effect of OAG on AVP-induced $[Ca^{2+}]_i$ increase and further enhanced the AVP-induced sustained phase of $[Ca^{2+}]_i$ increase (Fig. 3A).

In the Ca^{2+}-free medium, OAG significantly decreased AVP-induced $[Ca^{2+}]_i$ increase (Fig. 3B). Although, Ro 31-8220 failed to enhance the effect of AVP in the

absence of extracellular Ca^{2+} (Fig. 2B), it was still able to reverse the inhibitory effect of OAG on AVP-induced $[Ca^{2+}]_i$ increase (Fig. 3B).

Effects of PMA on AVP-induced $[Ca^{2+}]_i$ increase

AVP-induced $[Ca^{2+}]_i$ increase was inhibited by a 10-min treatment with PMA (0.1-100 nM) in a concentration-dependent manner (Table 2). At the highest concentration studied (100 nM), PMA abolished AVP-induced $[Ca^{2+}]_i$ increase both in the peak and sustained phase and this effect was blocked by the pretreatment with Ro-31-8220 (10 μM) for 30 min (Fig. 4A).

In the Ca^{2+}-free medium, PMA (100 nM) still abolished the AVP-induced $[Ca^{2+}]_i$ increase. Again, Ro 31-8220 attenuated the inhibitory effect of PMA on AVP (Fig. 4B).

Effects of AVP, Ro 31-8220 and OAG on $[Ca^{2+}]_i$ in cells pretreated with PMA for 72 h

After a 72-h PMA (200 nM) treatment, both the peak and sustained intracellular Ca^{2+} increases induced by AVP became larger and lasted longer compared to those in PMA-untreated cells (Fig. 5A). The AVP-induced $[Ca^{2+}]_i$ increases in PMA-pretreated cells were not altered by pretreating the cells with 10 μM Ro 31-8220 (Fig. 5B) or 30 μM OAG (Fig. 5C). The basal $[Ca^{2+}]_i$ were not different between the control and PMA-treated cells (control = 144.5 ± 30.4 nM; PMA-treated cells = 148.1 ± 32.4 nM, n = 4).

Effects of Ro 31-8220, OAG and PMA on ionomycin-induced $[Ca^{2+}]_i$ increase

To determine whether the effects of Ro 31-8220, OAG and PMA on PKC were specific to AVP-induced $[Ca^{2+}]_i$ increase, we investigated their effects on ionomycin (300 nM)-induced $[Ca^{2+}]_i$ increase. In Ca^{2+}-containing medium, ionomycin induced a biphasic elevation of $[Ca^{2+}]_i$ with a pattern similar to that induced by AVP, but the sustained phase was greater than that induced by AVP. In addition, the sustained increase in $[Ca^{2+}]_i$ induced by ionomycin disappeared in the absence of extracellular Ca^{2+} (data not shown). The pretreatment with 10 μM Ro 31-8220 (Fig. 6A), 30 μM OAG (Fig 6B) or 100 nM PMA (Fig. 6C) did not affect ionomycin-induced $[Ca^{2+}]_i$ increase. The basal $[Ca^{2+}]_i$ after the pretreatments with the PKC inhibitor or activators

were not different from those of untreated cells (control = 107.6 ± 11.4 nM; Ro 31-8220 = 101.8 ± 9.9 nM; OAG = 104.5 ± 12 nM; PMA = 118 ± 16.9 nM, n = 5).

DISCUSSION

The present results showed that AVP increased IP_3 production and $[Ca^{2+}]_i$ in a clonal α-cell line In-R1-G9. This study explored the role of PKC on AVP increased IP_3 production and $[Ca^{2+}]_i$, and the results suggested that AVP activates PKC via DAG, which attenuates the AVP-induced increases in IP_3 production and $[Ca^{2+}]_i$. AVP induced a translocation of various PKC isozymes from cytosol to membrane in In-R1-G9 cells in the Western blot analysis (our unpublished data), which supports the notion that the AVP treatment activates PKC. In the present study, we demonstrated that the selective PKC inhibitor Ro 31-8220 potentiated AVP- induced IP_3 production and $[Ca^{2+}]_i$ increases. Ro 31-8220, which is a derivative of a non-specific PKC inhibitor staurosporine, has been developed as a potent and selective PKC inhibitor (Davis et al., 1989), but much less potent inhibitor for cyclic AMP-dependent kinase (PKA) or Ca^{2+}/calmodulin-dependent kinase (Minichiello et al., 1999). This compound has been widely used to inhibit a number of PKC isozymes, including conventional, novel and atypical in different systems (Minichiello et al, 1999; Ison et al., 1993; Limatola et al., 1994). Phorbol esters and DAG analogs have been used as tools to mimic the responses stimulated by physiological ligands, providing evidence to support the role of PKC in ligands-induced cellular responses (Wilkinson and Hallam, 1994). Our results showed that both PMA and OAG, which activate cPKCs and nPKCs, attenuated AVP-induced IP_3 production and $[Ca^{2+}]_i$ increases. The effects of PMA and OAG were specific for PKC activation because they were blocked by the pretreatment with Ro 31-8220.

Since PMA may exert some effects which are not pertinent to PKC activation, such as the stimulation of insulin release via membrane depolarization and $[Ca^{2+}]_i$ increase in RINm5F cells (Yada et al., 1989) and inhibition of phosphoinositide hydrolysis via targets other than PKC in the peripheral tissue of rat and chicken (Bhave, et al., 1990), we investigated the role of PKC on AVP-induced $[Ca^{2+}]_i$ increase after the

down-regulation of PKC. A long-term treatment with PMA, leading to PKC down regulation, is mostly due to the degradation of membrane-bound activated PKC by the proteolytic enzymes, for example, calpain (Kikkawa et al., 1989; Kishimoto et al., 1989). In this study, the intracellular Ca^{2+} response to AVP was clearly enhanced after the PKC down-regulation, which is consistent with the result of the Ro 31-8220 experiment. This result can be explained by the elimination of the inhibitory effect of PKC on control cells. Ro 31-8220 and OAG failed to alter AVP-induced $[Ca^{2+}]_i$ increase after the down-regulation of PKC, suggesting a deficiency of PKC in these cells after a long-term treatment with PMA.

The effects of Ro 31-8220, OAG and PMA were highly specific for the PLC-IP_3 system because they did not alter the ionomycin-induced $[Ca^{2+}]_i$ increase. Ionomycin is a Ca^{2+}-ionophore that increases $[Ca^{2+}]_i$ by promoting Ca^{2+} release and Ca^{2+} influx without coupling to a G protein or generating IP_3 production (Swope and Schonbrunn, 1988; Yoshida and Plant, 1992; Teitelbaum and Berl, 1994). Our present findings strongly suggested that PKC negatively regulates AVP- induced IP_3 production and $[Ca^{2+}]_i$ increases. Based on the specificity of OAG and PMA that can activate only cPKCs and nPKCs, it is likely that either or both groups of PKC isozymes may be involved in this aspect. Further studies are needed to determine which PKC isozymes are involved in the attenuation of these effects of AVP.

Our findings are consistent with those in rat glomerulosa cells (Gallo-Payet et al., 1991) and vascular smooth muscle cells (Stassen et al., 1989), in which PKC plays a negative role in AVP-induced formation of inositol phosphate and $[Ca^{2+}]_i$ increase. Activation of PKC blocks the AVP-induced formation of inositol phosphate in rat pancreatic β-cells (Gao et al., 1994) and $[Ca^{2+}]_i$ increase in clonal β-cells HIT-T15 (Hughes et al., 1992). PKC blocks the accumulation of inositol phosphate induced by high Ca^{2+} (3 mM) in bovine parathyroid cells (Kifor et al., 1990) and inhibits PTH-induced IP_3 production and $[Ca^{2+}]_i$ increase in rat osteoblastic cells (Babich et al., 1997). In addition, PMA completely inhibits PIP_2 hydrolysis activated by carbachol in astrocytes from chicken embryo (Mangoura et al., 1995). These results suggest that PKC inhibits the activation of PLC-β. In addition, PKC might inhibit the substrate supply of PIP_2 to decrease IP_3 formation, but there is no evidence to support this

hypothesis. Further work is needed to determine if PKC indeed inhibits the supply of PIP_2.

Although Ro 31-8220 failed to potentiate AVP-induced $[Ca^{2+}]_i$ increase in the absence of extracellular $[Ca^{2+}]_i$, it was still able to counteract the inhibitory effect of OAG and PMA on AVP-induced changes. However, Ro 31-8220 no longer enhanced AVP-induced sustained phase seen in the presence of extracellular $[Ca^{2+}]_i$. These results suggest that PKC reduces AVP-induced $[Ca^{2+}]_i$ increase partly via an inhibition of Ca^{2+} influx. Activation of PKC also inhibits Ca^{2+} signaling by reducing influx of Ca^{2+} into the β-cells (Ashcroft, 1994). SKF 96365, an ROC inhibitor, but not nimodipine, a VDCC inhibitor, inhibited Ro 31-8220-potentiated AVP-induced Ca^{2+} influx in a concentration-dependent manner. SKF 96365 (30 μM) did not affect AVP-induced $[Ca^{2+}]_i$ increase in the absence of Ro 31-8220 (data not shown). These results suggest that the PKC-attenuation of AVP-induced Ca^{2+}-influx is mediated by the inactivation of ROCs, but not of VDCCs. This finding is different from what has been found in the mouse pancreatic β-cells, in which PKC inactivates VDCCs (Arkhammar et al., 1994).

Taken together, we conclude that PKC exhibited a negative feedback control via V_{1b} receptors that couple to PLC-β to inhibit IP_3 production induced by AVP, attenuating AVP-induced increase in Ca^{2+} release and influx. The attenuation of Ca^{2+} influx is mediated by the closure of ROC. Since AVP physiologically regulates glucagon release from the rat pancreas (Yibchok-anun et al., 1999), this phenomenon might occur in α-cells of the pancreatic islet. Further work in pancreatic islet is needed to prove or disprove this hypothesis.

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TABLE 1

Effects of SKF 96365 and nimodipine on Ro 31-8220-elicited potentiation of AVP-induced $[Ca^{2+}]_i$ increase

Treatment	$[Ca^{2+}]_i$, nM				
	Basal	Peak	Δ_1	Sustained phase	Δ_2
1. AVP	96 ± 1.7	183.2 ± 9.2	89.4 ± 10.2	97.6 ± 5.7	3.7 ± 4.8
2. Ro 31-8220 + AVP	105.4 ± 3.5	245 ± 16.3	139.6 ± 21.3[a]	162.8 ± 2.5	57.3 ± 4.5[c]
3. Ro 31-8220 + 0.3 μM SKF 96365 + AVP	100.6 ± 7.2	205.3 ± 26.9	104.7 ± 22	155.5 ± 12.2	55 ± 6
4. Ro 31-8220 + 3 μM SKF 96365 + AVP	93.5 ± 6.5	184.1 ± 18.3	90.5 ± 15.3[b]	142 ± 10.6	48.4 ± 7.1
5. Ro 31-8220 + 30 μM SKF 96365 + AVP	114.2 ± 7.4	191.2 ± 17.1	77 ± 10.8[b]	132.8 ± 12.7	18.6 ±7[d]
6. Ro 31-8220 + 1 μM nimodipine + AVP	97.4 ± 6.7	205.4 ± 13.3	108 ± 8.4	154 ± 6.8	46.6 ± 5.3

The values are means ± S.E.M. (n = 4). The concentrations of AVP and Ro 31-8220 used were 100 nM and 10 μM, respectively. Ro 31-8220, SKF 96365 or nimodipine was given 30, 10 and 5 min, respectively before AVP administration. The sustained phase of $[Ca^{2+}]_i$ increase was measured at 120s post-AVP administration.

Δ_1: Peak – Basal; Δ_2: Sustained phase – Basal

[a] $P < 0.05$, compare with treatment 1 in the same column.

[b] $P < 0.05$, compare with treatment 2 in the same column.

[c] $P < 0.05$, compare with treatment 1 in the same column.

[d] $P < 0.05$, compare with treatment 2 in the same column.

TABLE 2

Effects of PMA on AVP-induced $[Ca^{2+}]_i$ increase

Treatment	$[Ca^{2+}]_i$, nM				
	Basal	Peak	Δ_1	Sustained phase	Δ_2
1. AVP	100.4 ± 2.4	203.1 ± 24	102.7 ± 23	119.2 ± 3.7	18.8 ± 1.8
2. PMA (0.1 nM) + AVP	106.4 ± 8.2	211 ± 24.5	104.6 ± 19.7	125.2 ± 13.6	18.8 ± 4.8
3. PMA (1 nM) + AVP	98.8 ± 2.4	144.5 ± 6.5	45.7 ± 6.4[a]	104.6 ± 4.4	5.8 ± 3[b]
4. PMA (10 nM) + AVP	109.3 ± 14.6	129.2 ± 19.5	19.9 ± 6.1[a]	116.3 ± 18.4	7.1 ± 4[b]
5. PMA (100 nM) + AVP	109 ± 5.9	110.8 ± 5.3	1.9 ± 0.6[a]	112 ± 5.2	3.1 ± 1[b]

The values are means ± S.E.M. (n = 4). The concentration of AVP used was 100 nM. PMA was given 10 min before AVP administration. The sustained phase of $[Ca^{2+}]_i$ increase was measured at 120s post-AVP administration.

Δ_1: Peak – Basal

Δ_2: Sustained phase – Basal

[a] $P < 0.05$, compare with treatment 1 in the same column.

[b] $P < 0.05$, compare with treatment 1 in the same column.

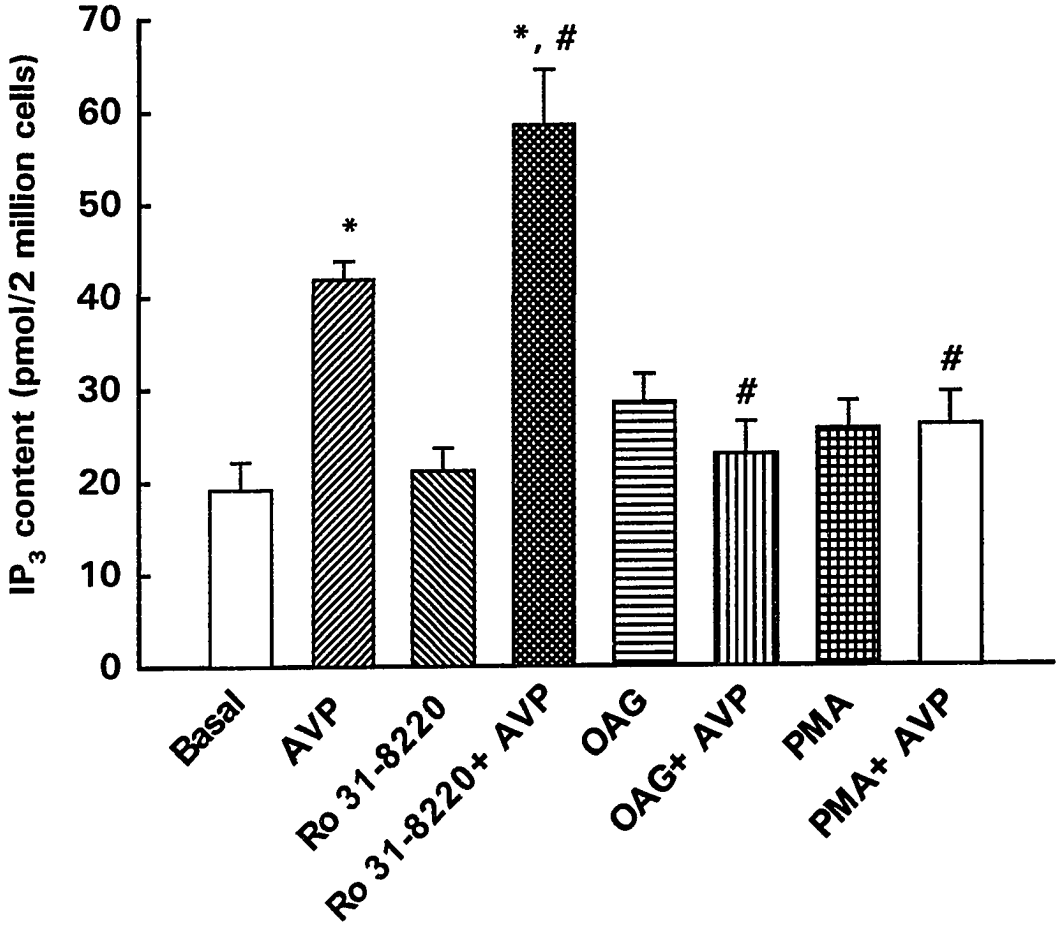

Fig.1. Effects of Ro 31-8220, OAG and PMA on AVP-induced IP_3 production. Ro 31-8220 (10 μM) was given 30 min and OAG (30 μM) or PMA (0.1 μM) was given 10 min before the administration of AVP (100 nM). Values are mean ± S.E.M. (n = 5). $^{*}P < 0.05$, compared to the basal control group; $^{\#}P < 0.05$, compared to the AVP alone group.

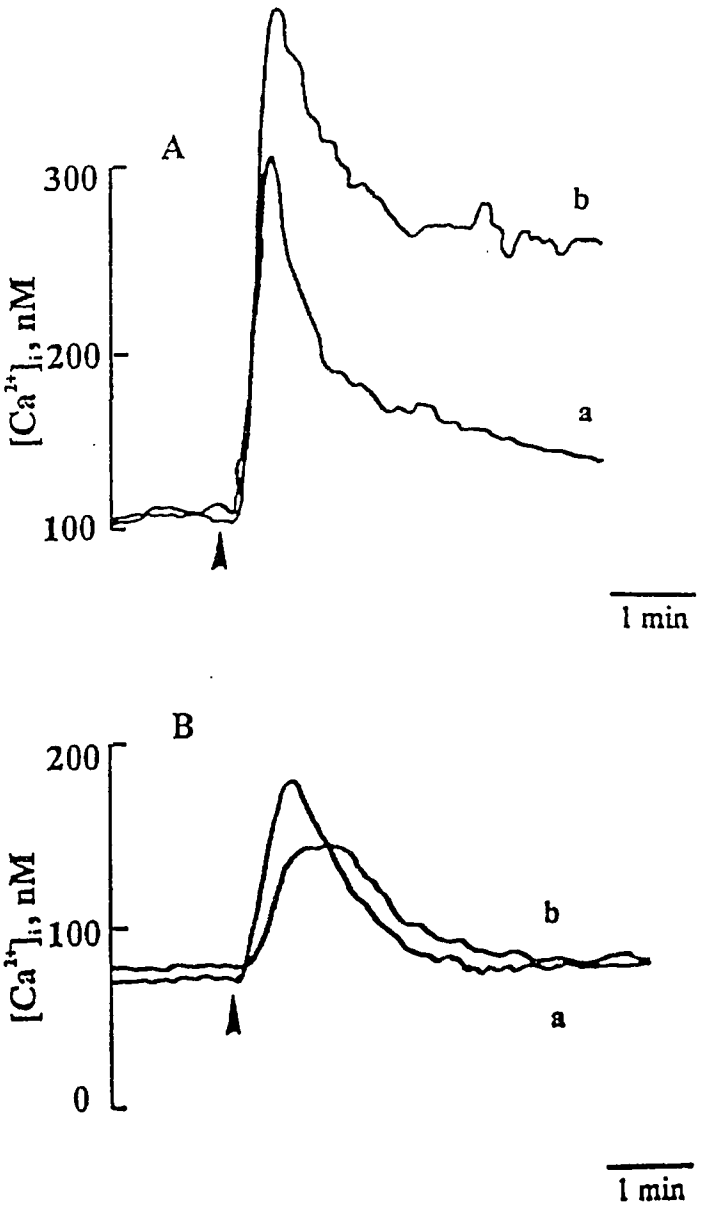

Fig. 2. Effect of Ro 31-8220 on AVP-induced $[Ca^{2+}]_i$ increase in the presence (A) and absence (B) of extracellular Ca^{2+}. Curve a shows the data of AVP (100 nM) alone as a control; curve b shows the effect of Ro 31-8220 (10 μM) pretreatment for 30 min before the AVP administration. Arrow indicates the AVP administration. Data shown are representative of 11 and 5 experiments for A and B, respectively.

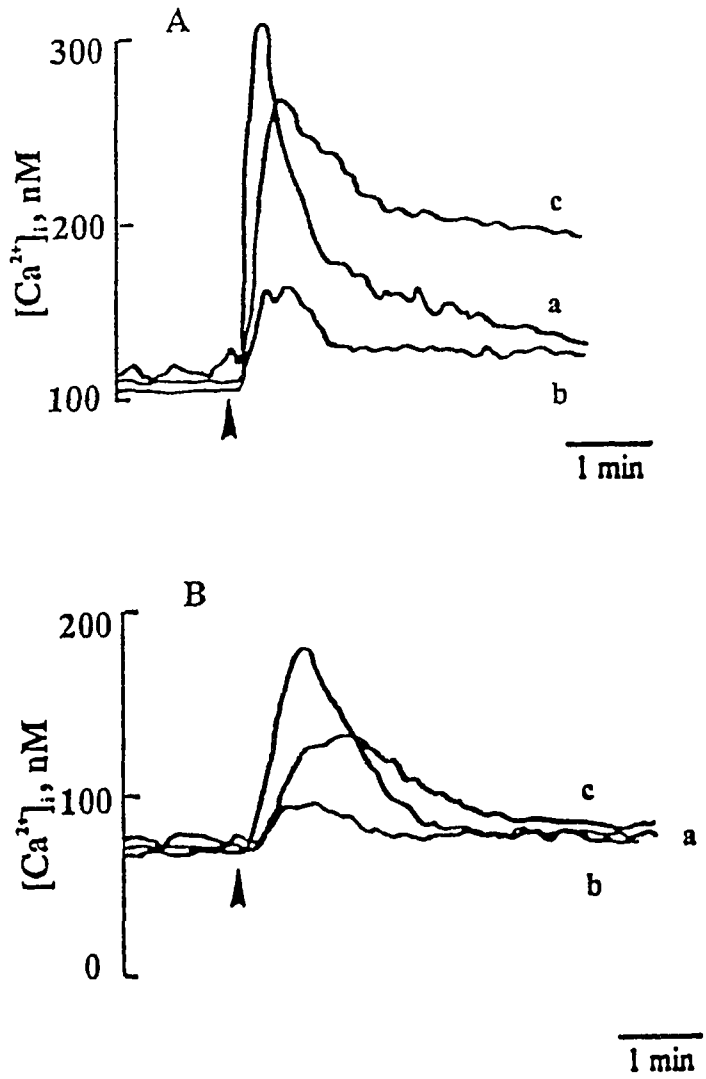

Fig.3. Effect of OAG on AVP-induced $[Ca^{2+}]_i$ increase in the presence (A) and absence (B) of extracellular Ca^{2+}. Curve a shows the data of AVP (100 nM) alone as a control; curve b shows the effect of the OAG (30 μM) pretreatment for 10 min before the AVP administration, and curve c shows the effect of Ro 31-8220 (10 μM) pretreatment for 30 min before the AVP administration on OAG-treated cells. Arrow indicates the AVP administration. Data shown are representative of 5 experiments.

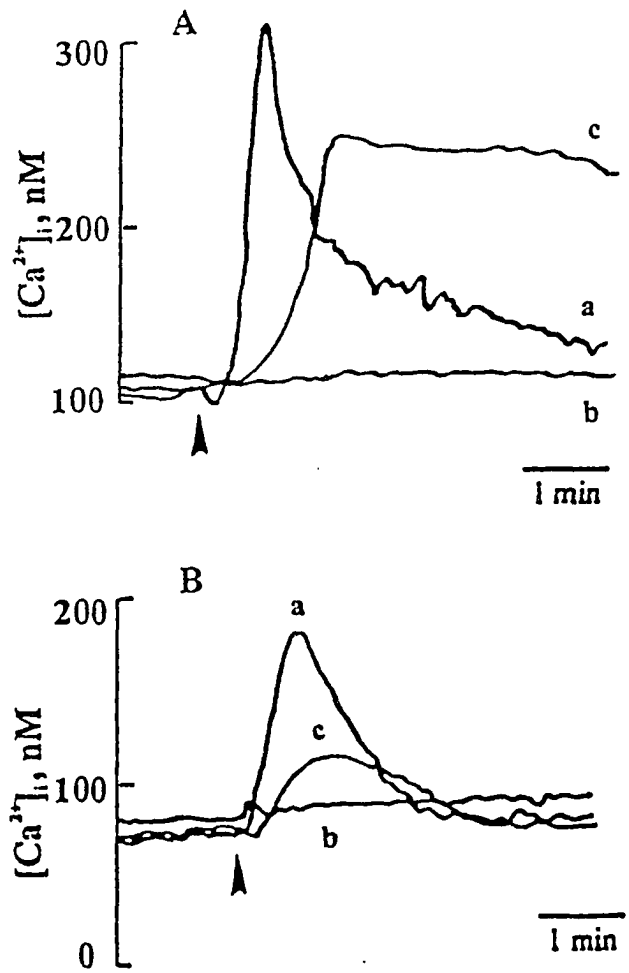

Fig. 4. Effect of PMA on AVP-induced $[Ca^{2+}]_i$ increase in the presence (A) and absence (B) of extracellular Ca^{2+}. Curve a shows the data of AVP (100 nM) alone as a control; curve b shows the effect of PMA (100 nM) pretreatment for 10 min before the AVP administration and curve c shows the effect of Ro 31-8220 (10 μM) pretreatment for 30 min before the AVP administration on PMA-treated cells. Arrow indicates the AVP administration. Data shown are representative of 4 experiments.

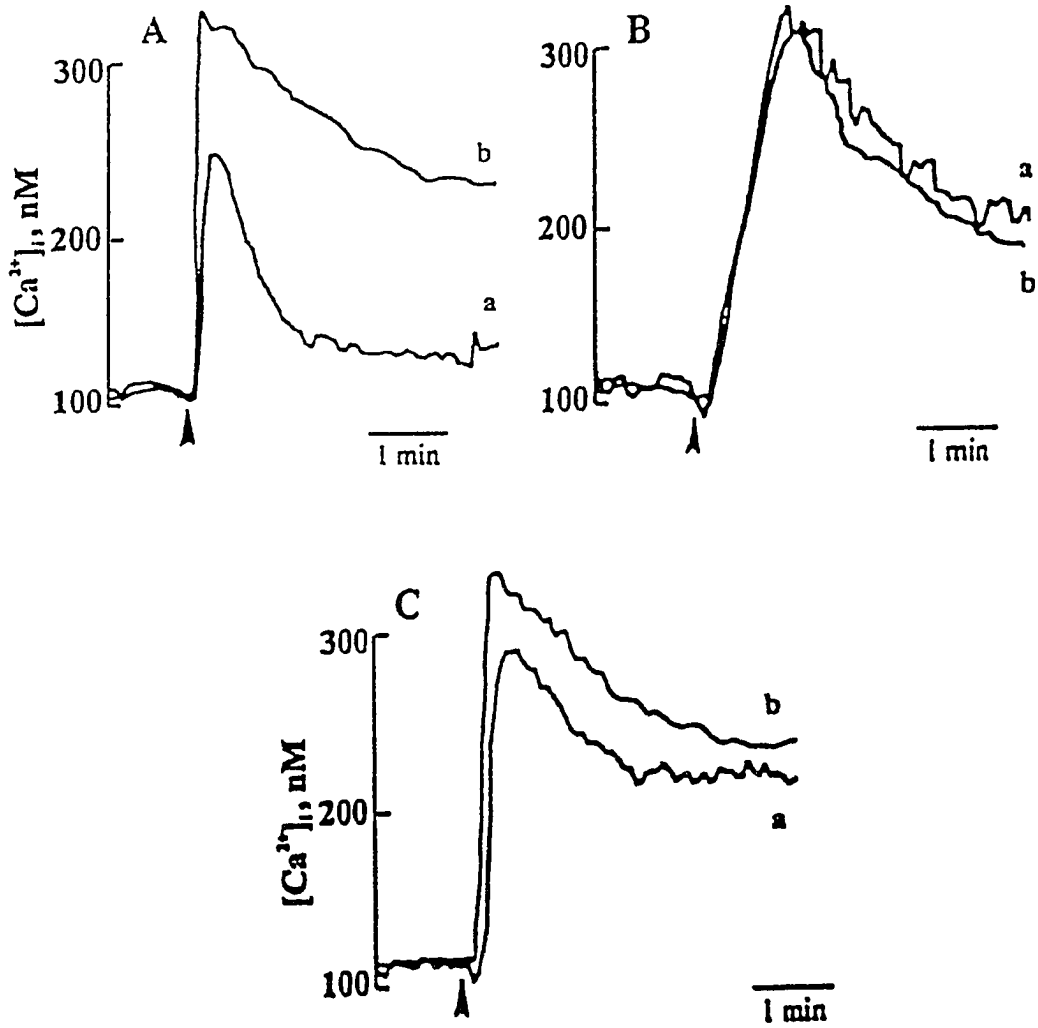

Fig. 5. Effect of PMA (200 nM) pretreatment for 72 h on AVP-induced $[Ca^{2+}]_i$ increase (A). Curve a shows the data of AVP (100 nM) in normal cells as a control; curve b shows the data of AVP (100 nM) in the PMA-pretreated cells. Effects of Ro 31-8220 (B) and OAG (C) on AVP-induced $[Ca^{2+}]_i$ increase in PMA-pretreated cells for 72 h. In panels B and C, curve a shows the data of AVP alone in PMA-pretreated cells as a control; curve b shows the effect of Ro 31-8220 (10 μM) (B) or OAG (30 μM) (C) pretreatment for 30 and 10 min, respectively before the AVP administration in PMA-pretreated cells. Arrow indicates the AVP administration. Data shown are representative of 4 experiments.

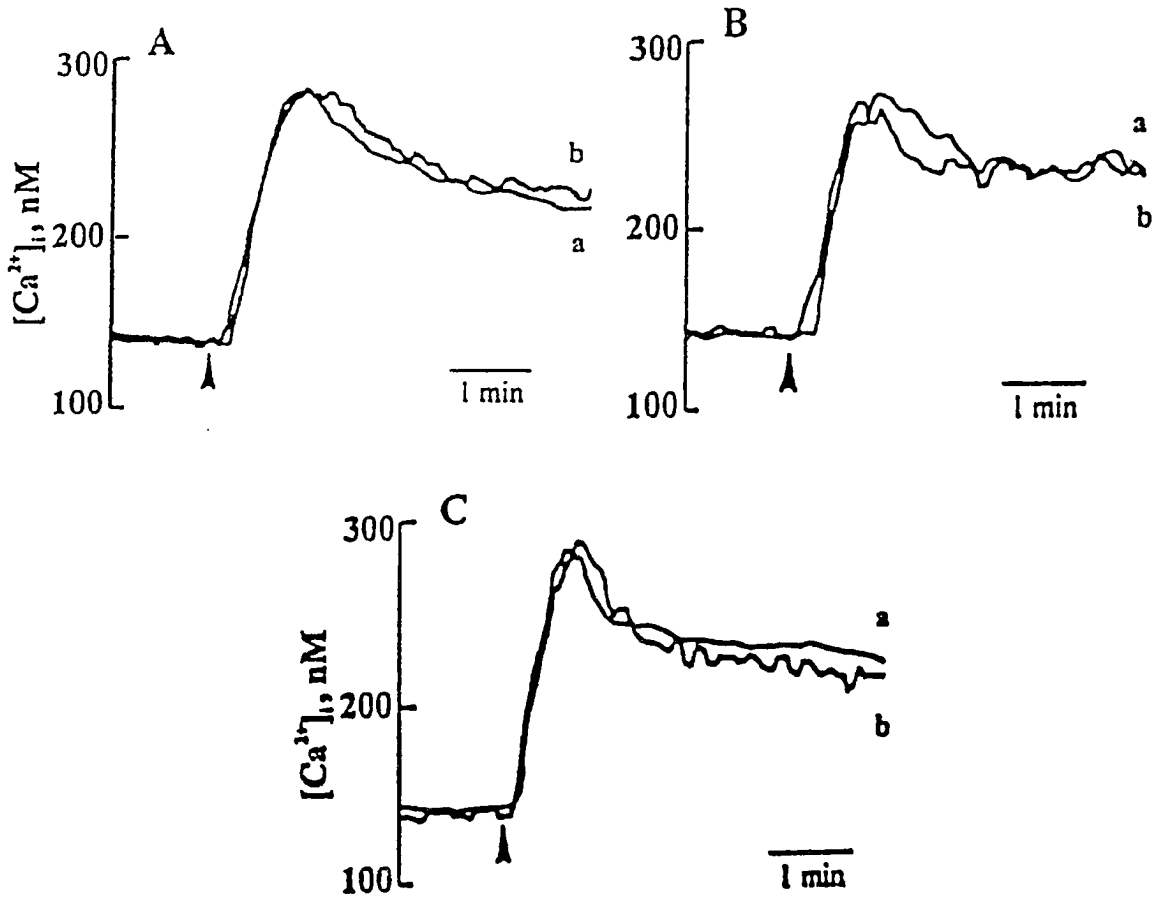

Fig. 6. Effects of Ro 31-8220 (A), OAG (B) and PMA (C) on ionomycin-induced $[Ca^{2+}]_i$ increase. Curve a shows the data of AVP (100 nM) alone as a control; curve b shows the effect of Ro 31-8220 (10 μM), OAG (30 μM) or PMA (100 nM) pretreatment for 30 and 10 min, respectively before the AVP administration. Arrow indicates the AVP administration. Data shown are representative of 4 experiments.

CHAPTER VI NOVEL PROTEIN KINASE C ISOZYMES INHIBIT AND ATYPICAL PROTEIN KINASE C ISOZYMES STIMULATE ARGININE VASOPRESSIN-INDUCED GLUCAGON RELEASE IN CLONAL α-CELL LINE IN-R1-G9

A paper to be submitted to *Diabetes*

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ABSTRACT

We examined the role of PKC isozymes on AVP-induced glucagon release. PMA and OAG, activators of cPKCs and nPKCs, antagonized AVP-induced glucagon release, whereas Ro 31-8220, a drug that inhibits all PKC isozymes, potentiated AVP-induced glucagon release. In addition, Ro 31-8220 blocked the effect of OAG and PMA on AVP-induced glucagon release. AVP-induced glucagon release was enhanced when cPKC and nPKC isozymes were down-regulated by pretreatment of the cells with PMA for 72 h. However, the cPKC inhibitors, CGP 53506, CGP 54345, LY 379196 and Gö 6976, as well as PKC 20-28 (Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln), a pseudosubstrate peptide for PKC-α and -β, failed to affect the AVP-induced glucagon release, suggesting that cPKCs may not be involved in this release. In contrast, a pseudosubstrate peptide inhibitor specific for PKC-ζ and -λ (Myr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys) significantly reduced the AVP-induced glucagon release. The present findings suggest that nPKCs and aPKCs play inhibitory and stimulatory roles, respectively, on AVP-induced glucagon release from In-R1-G9 cells.

INTRODUCTION

PKC is a family of phospholipid dependent serine/threonine-specific protein kinases (1) which are classified into three groups: 1) the conventional (cPKCs) α, βI, βII, γ; 2) the novel (nPKCs) δ, ε, η, θ, μ; and 3) the atypical (aPKCs) λ/ι and ζ. The cPKCs

are activated by Ca^{2+}, negatively charged phospholipids, e.g. phosphatidylserine (PS), diacyl glycerol (DAG), and phorbol esters (2, 3). The nPKCs are activated by negatively charged phospholipids, DAG, and phorbol esters, but not Ca^{2+} (2-5). The aPKCs are activated by negatively charged phospholipids, phosphatidic acid (PA) (6), and inositol 1,4,5-trisphosphate (IP_3) (3, 7), and are insensitive to Ca^{2+}, DAG or phorbol esters (5).

In pancreatic α-cells (8) and clonal α-cells In-R1-G9 (9), activation of V_{1b} receptors by AVP stimulates phospholipase C (PLC). PLC catalyzes the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) to IP_3, a second messenger that releases Ca^{2+} from the endoplasmic reticulum (ER), and DAG, which activates cPKCs and nPKCs (10).

It is well established that Ca^{2+} (11, 12) and PKC (13, 14) play an important role in the regulation of glucagon release from endocrine α-cells. Phorbol-12 myristate 13-acetate (PMA), which is known to activate cPKCs and nPKCs, has been found to stimulate glucagon release from isolated rat islets (13, 14). However, in clonal α-cell line In-R1-G9, activation of cPKCs and nPKCs by OAG, a DAG analog, and PMA attenuated AVP-induced increases in IP_3 production and $[Ca^{2+}]_i$ (15). Ro 31-8220, a specific PKC inhibitor for all PKC isozymes (16), enhanced AVP-induced increases in IP_3 production and $[Ca^{2+}]_i$ (15). In addition, down regulation of cPKCs and nPKCs by pretreating In-R1-G9 cells with PMA for 72 h potentiated AVP-induced increase in $[Ca^{2+}]_i$ (15). These findings suggest that, in In-R1-G9 cells, PKC exerts a negative feedback on AVP-induced increase in IP_3 production, causing an attenuation of the increase in $[Ca^{2+}]_i$. However, the role of PKC on AVP-induced glucagon release from this clonal cell line remains to be determined.

In the present study, we used a number of PKC inhibitors and PKC activators to determine the role of PKC isozymes on AVP-induced glucagon release from In-R1-G9 cells. These include Gö 6976, CGP 53506, CGP 54345, and LY 379196, which are specific for the inhibition of cPKC isozymes; Ro 31-8220, which inhibits all PKC isozymes (16); pseudosubstrate peptide specifics for cPKCs (Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln, PKC 20-28), and aPKCs (Myr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys, peptide Z) to determine which PKC isozymes are involved in AVP-induced glucagon release. In addition, we investigated the effects of OAG and PMA, the activators of cPKCs and nPKCs, on AVP-induced glucagon release. We further down-regulated

cPKCs and nPKCs by treating the cells with PMA (200 nM) for 72 h and used it as a means to examine the role of PKC isozymes on AVP-induced glucagon release.

MATERIALS AND METHODS

Cell culture

The hamster glucagonoma In-R1-G9 cells were maintained in RPMI-1640 medium with 10% fetal bovine serum and aerated with 5% CO_2-95% air at 37°C. All experiments were performed using cells from passages 24 to 30.

Glucagon release

In-R1-G9 cells were plated into Corning 24-well plates at 10^5 cells/well and were grown for 3-4 days. The culture medium was then removed and replaced with modified Krebs-Ringer bicarbonate buffer (KRB) containing (in mM): 136 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 5 $NaHCO_3$, 10 HEPES, 1.67 glucose and 0.1% BSA, pH 7.4. The cells were then preincubated at 37°C with KRB for 15 min before administration of AVP (100 nM) for 15 min as a positive control. The following drugs were used in the study: PMA, OAG, CGP 53506, CGP 54345, LY 379196, and Gö 6976 were given 10 min, a pseudosubstrate peptide inhibitor specific for cPKCs (PKC 19-31), and for aPKCs (peptide Z) were given 45 min, and Ro 31-8220 was given 30 min prior to the administration of AVP. For down regulation of PKC, the cells were treated with 200 nM PMA in culture media for 72 h, and resuspended in KRB during the experiment. The cells were then treated with AVP alone or AVP in KRB containing one of the antagonists for 15 min. After the treatments, the supernatant was collected and the concentration of glucagon was measured by radioimmunoassay, following the procedures provided by Linco Research.

Statistical Analysis

All values were normalized as percentage of basal control group and presented as mean ± S.E.M. Because most of the stimulators and inhibitors used in this study themselves significantly increased glucagon release, the absolute increases of glucagon

release obtained from the treated groups minus the stimulator/inhibitor alone groups were analyzed using ANOVA and mean comparisons were made using the Fisher least significant difference test. The significance level was set at $P < 0.05$.

Reagents

RPMI 1640 medium, fetal bovine serum, AVP, PMA, and OAG were purchased from Sigma Chemical (St. Louis, MO). Gö 6976 was purchased from Biomol (Plymouth Meeting, PA). ^{125}I-glucagon was purchased from Linco Research (St. Charles, MO). CGP 54345 and CGP 53506 were donated by Novartis Pharma (Basel, Switzerland). LY 379196 was donated by Eli Lilly (Indianapolis, IN), and Ro 31-8220 was donated by Roche (Hertfordshire, UK). Peptide Z and PKC (20-28) were synthesized by the Protein Facility, Iowa State University (Ames, IA).

RESULTS

Effects of Ro 31-8220, OAG, or PMA on AVP-induced glucagon release

AVP (100 nM) significantly increased glucagon release ~ 3-6 fold over the basal level. This concentration of AVP was used throughout the study. Ro 31-8220 (10 μM), a PKC inhibitor that inhibits all PKC isozymes (16), alone significantly increased glucagon release and it markedly enhanced AVP-induced glucagon release (Fig. 1A).

OAG (30 μM), a DAG analog that is resistant to metabolism, and PMA (100 nM) also significantly increased glucagon release. However, they both totally inhibited AVP-induced glucagon release (Fig. 1B and 1C). In addition, pretreatment with Ro 31-8220 (10 μM) for 30 min abolished the inhibitory effects of OAG (Fig. 1B) and PMA (Fig. 1C) on AVP-induced glucagon release. The glucagon release in the presence of Ro 31-8220 and OAG or PMA was additive to that of Ro 31-8220, OAG or PMA alone.

In contrast to the acute effect of PMA, pretreatment of the cells with PMA (200 nM) for 72 h, which down-regulates cPKCs and nPKCs, enhanced AVP-induced glucagon release (Fig. 2).

Effects of inhibitors of cPKCs on AVP-induced glucagon release

Gö 6976 (0.5 μM), a specific inhibitor for PKC-α, -βI, and -μ, failed to alter AVP-induced glucagon release (Fig. 3). CGP 53506 (50 μM; Fig. 4), an inhibitor specific for all cPKCs isozymes, CGP 54345 (100 μM; Fig. 5), an inhibitor specific for only PKC-α, and LY 379196 (0.3 μM; Fig. 6), an inhibitor specific for only PKC-β, did not alter AVP-induced glucagon release. All of the antagonists, except LY 379196, alone that have been used in this study significantly increased glucagon releases (basal = 834 ± 172 pg/well/15 min; Gö 6976 = 1,370 ± 192 pg/well/15 min; CGP 53506 = 2,555 ± 363 pg/well/15 min; CGP 54345 = 1,516 ± 128 pg/well/15 min; LY 379196 = 1,006 ± 222 pg/well/15 min; n = 4 cultures with triplicates).

Effects of PKC pseudosubstrate peptides on AVP-induced glucagon release

Two PKC psuedosubstrates peptides, PKC 20-28 and peptide Z, were used in this study. PKC 20-28 is a myristoylated peptide, Myr-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln, which is similar to the pseudosubstrate region of PKC-α and PKC-β. The pseudosubstrates occupy the active binding site of PKC, thus preventing the transformation of PKC from inactive to active state (17). The addition of myristic acid to this peptide is to increase its permeability to the cell membrane, thus penetrating into the cells and acting as an inhibitor for PKC (18, 19). Pretreatment of the In-R1-G9 cells with 10 μM PKC 20-28 for 45 min did not affect AVP-induced glucagon release (Fig. 7). Peptide Z (Myr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys), is a myristoylated peptide with a sequence based on the pseudosubstrate regions of PKC-ζ and PKC-λ. Peptide Z has been shown to specifically inhibit PKC-ζ in clonal β-cells RINm5F (20), and *Xenopus laevis*

oocytes (21). In the present study, peptide Z (10 μM) significantly inhibited AVP-induced glucagon release by 40% (Fig. 8). Both PKC 20-28 (basal = 914 ± 181 pg/well/15 min; PKC 18-28 = 1,365 ± 332 pg/well/15 min; n = 4 cultures with triplicates) and peptide Z (basal = 638 ± 153 pg/well/15 min; peptide Z = 808 ± 138 pg/well/15 min; n = 5 cultures with triplicates) alone significantly increased glucagon release.

DISCUSSION

This study explored the role of PKC on AVP-induced glucagon release and the results suggested that activation of PKC exerted both inhibitory and stimulatory roles on AVP-induced glucagon release. Ro 31-8220, a derivative of staurosporine that can inhibit all PKC isozymes (16), enhanced AVP-induced glucagon release. In addition, PMA and OAG, which have been known to activate cPKCs and nPKCs, but not aPKCs, attenuated AVP-induced glucagon release. The inhibitory effects of PMA and OAG were antagonized by Ro 31-8220, suggesting their specificity for PKC activation. Down regulation of cPKCs and nPKCs by treating the cells with PMA for 72 h enhanced AVP-induced glucagon release. These findings suggest that either cPKCs and/or nPKCs play an inhibitory role on AVP-induced glucagon release. Our previous report indicated that AVP activates PKC via DAG, which attenuates the AVP-induced increases in IP_3 production and $[Ca^{2+}]_i$ (10). Together, it is likely that cPKCs and/or nPKCs exerted a negative feedback to inhibit IP_3 production induced by AVP, leading to an attenuation of AVP-induced $[Ca^{2+}]_i$ increase, thus decreasing glucagon release.

In an attempt to discriminate between cPKCs and nPKCs as the ones that play the negative role on AVP-induced glucagon release, we utilized a number of PKC antagonists specific for cPKC isozymes, as well as a synthetic pseudosubstrate peptide specific for PKC-α and -β to inhibit AVP-induced

glucagon release. Unfortunately, specific inhibitors for nPKCs are not yet available for such a study. First, Gö 6976, an indolocarbazole compound, which is a potent and specific inhibitor for PKC-α, -βI (22) and -μ (23) failed to inhibit AVP-induced glucagon release. Second, CGP 54345, a selective inhibitor for PKC-α, and CGP 53506, a selective inhibitor for cPKCs (i.e., α, β, and γ) (22) also failed to alter AVP-induced glucagon release. They both are derivatives of phenylamino-pyridine compound. Third, LY 379196, which is the inhibitor specific for only PKC-βI and -βII (24, 25) with IC_{50} values of 0.05 and 0.03 μM, respectively, did not affect AVP-induced glucagon release. The IC_{50} values of LY 379196 against other PKC isozymes are as follows (in μM): 0.6 for PKC-α and -γ; 0.7 for PKC-δ, 5 for PKC-ε, 48 for PKC-ζ, and 0.3 for PKC-η. At a concentration of 0.6 μM, LY 379196 will show non-specific PKC inhibition (Dr. James R. Gillig, personal communication). Fourth, PKC 20-28, a pseudosubstrate peptide inhibitor of PKC-α and -β, did not significantly inhibit AVP-induced glucagon release. These findings led to the conclusion that cPKCs are not involved, but nPKCs; including δ, ε, η,and θ, may exert an inhibitory effect on AVP-induced glucagon release. This is consistent with our unpublished data from Western blot experiments, in which cPKCs appear not to be present in this clonal cell line (In-R1-G9). nPKCs have been reported to be involved in the negative regulation of HCl secretion from the parietal cells (26). However, this finding is different from the ones in human insulinoma cells and rat isolated islets, in which cPKCs stimulated insulin release (27, 28).

When the effect of peptide Z, a pseudosubstrate peptide specific for aPKCs, was examined, it appeared that PKC-ζ and/or -λ may be involved in the stimulatory effect of AVP on glucagon release, because peptide Z reduced AVP-induced glucagon release by 40%. Although Ro 31-8220 can inhibit all PKC isozymes in other cells, we hypothesize that it may not inhibit aPKCs in In-R1-G9 cells, thus pretreatment of these cells with Ro 31-8220 enhanced AVP-induced glucagon release. Further work is needed to determine if Ro 31-8220

inhibits aPKCs in these cells. Since the present findings were obtained using pharmacologic tools, which have shown some non-specific effects of these drugs, further work using Western blot in the presence and absence of these drugs is warranted to confirm and extend these findings.

Activation of PKC increases glucagon release from the rat islets (13, 14). In the present study, we found that treatment of the cells with PMA and OAG significantly increased glucagon release. However, after down-regulation of PKC by a 72-h PMA treatment, PMA and OAG were still able to increase glucagon release, suggesting that both activators may increase glucagon release through the mechanism that is independent of PKC activation (unpublished data). This finding is consistent with some previous reports, in which PMA exhibited some effects that are not pertinent to PKC activation. For instance, PMA stimulates acetylcholine synthesis in cultured endothelial cells through a PKC-independent mechanism (29). PMA also inhibits phosphoinositide hydrolysis via targets other than PKC in the chicken sympathetic neurons and rat chromaffin cells (30). However, the mechanisms, by which most of the antagonists used in this study, including OAG increased glucagon release, remain unknown.

In conclusion, the results of the present study suggested that nPKC isozymes may play an inhibitory role on AVP-induced $[Ca^{2+}]_i$ increase, which is due to a negative feedback on AVP-induced increase in IP_3 production. The attenuation of the increase in $[Ca^{2+}]_i$ contributes to attenuation in AVP-induced glucagon release. On the other hand, atypical PKC isozyme ζ and/or -λ may at least partially mediate AVP-induced glucagon release from In-R1-G9 cells.

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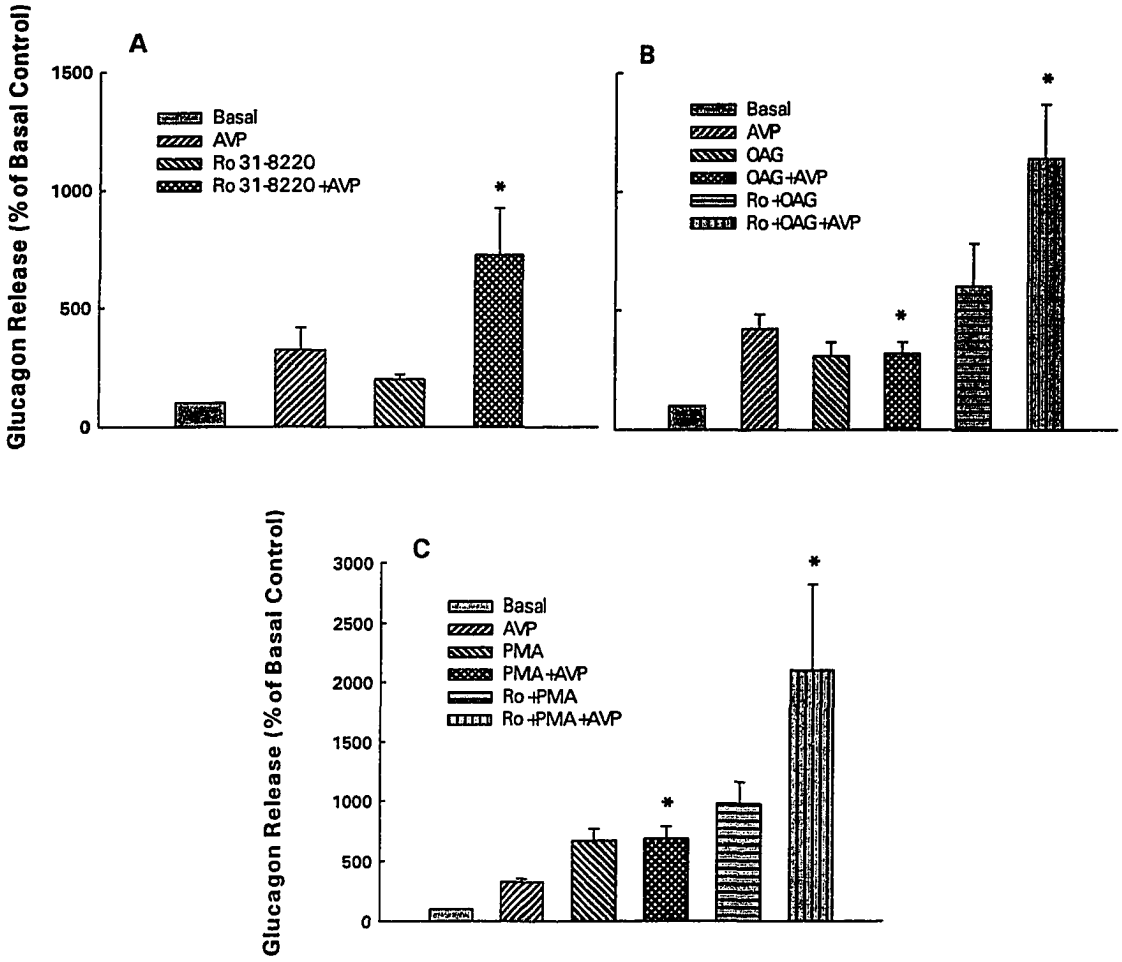

Fig. 1. Effects of Ro-31-8220 (A), OAG, Ro-31-8220 + OAG; (B), PMA and Ro 31-8220+ PMA (C) on AVP-induced glucagon release. Ro 31-8220 (10 μM) was given 30 min and OAG (30 μM) or PMA (0.1 μM) was given 10 min before the administration of AVP (100 nM). Static incubation was performed for 15 min to determine the glucagon release. The glucagon release in the basal control group was 958 ± 375 pg/well/15 min for panel A, 974 ± 236 pg/well/15 min for panel B, and 1184 ± 719 pg/well/15 min for panel C. Values are mean ± SE (n = 3 cultures with quadruplicates). * $P < 0.05$, compared the delta increase (the absolute increase of glucagon level obtained from the treated groups minus the antagonist alone groups) of the antagonist-treated group with the AVP-treated groups.

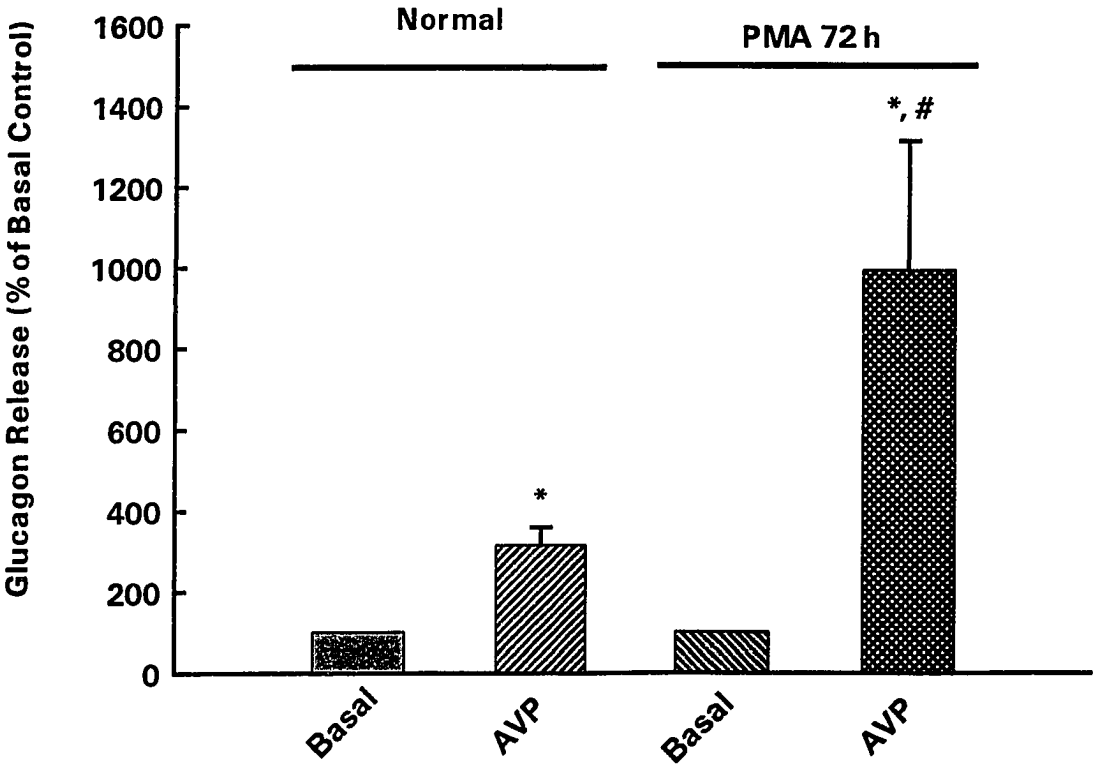

Fig. 2. . Effect of PMA (200 nM) pretreatment for 72 h on AVP (100 nM)-induced glucagon release. Static incubation was performed for 15 min to determine the glucagon release. The glucagon release in the basal control group was 579 ± 118 pg/well/15 min for normal cells and 570 ± 208 pg/well/15 min for PMA treated cells. Values are mean ± SE (n = 3 cultures with quadruplicates). * $P < 0.05$, in comparison of delta increase of each treatment with the basal control group. # $P < 0.05$, compared the normal to PMA-treated group with regard to the delta increases after AVP treatment.

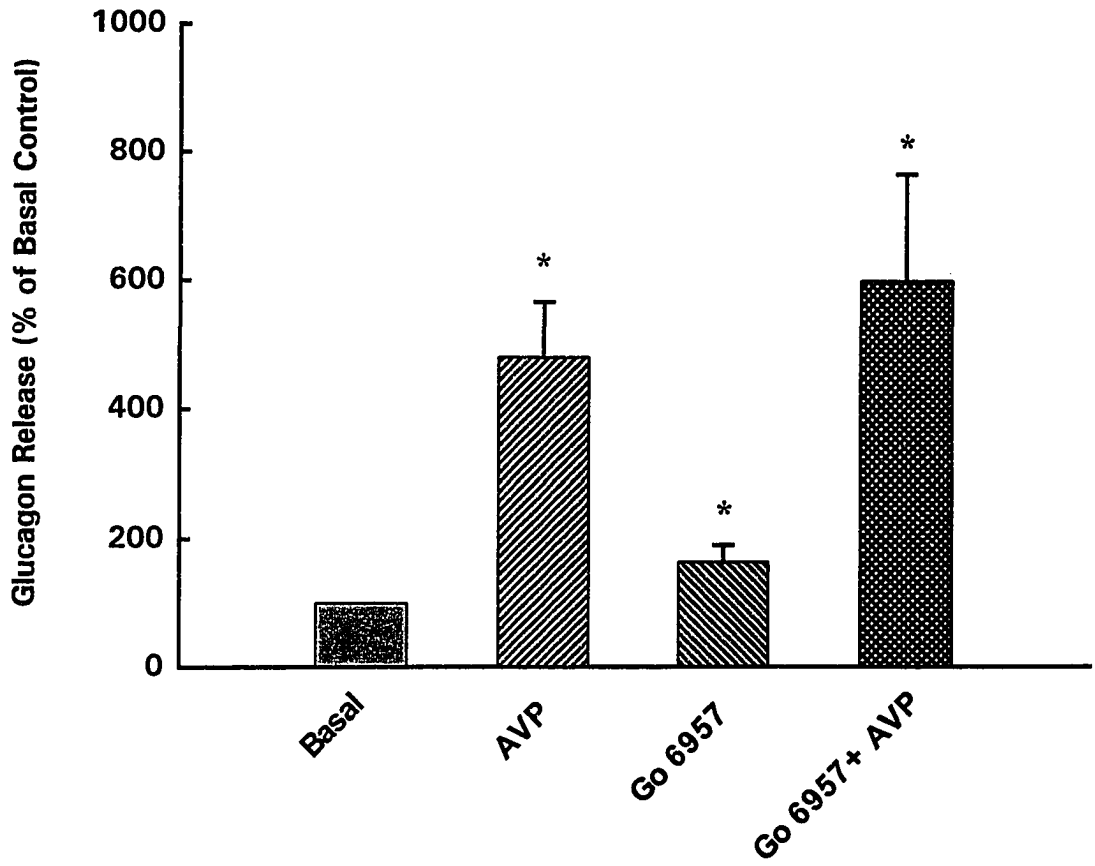

Fig. 3. Effect of Gö 6976 on AVP-induced glucagon release. Gö 6976 (O.5 μM) was given 10 min before the administration of AVP (100 nM). Static incubation was performed for 15 min to determine the glucagon release, which was 579 ± 118 pg/well/15 min in the basal control group. Values are mean ± SE (n = 3 cultures with quadruplicates). * $P < 0.05$, compared with the basal control group.

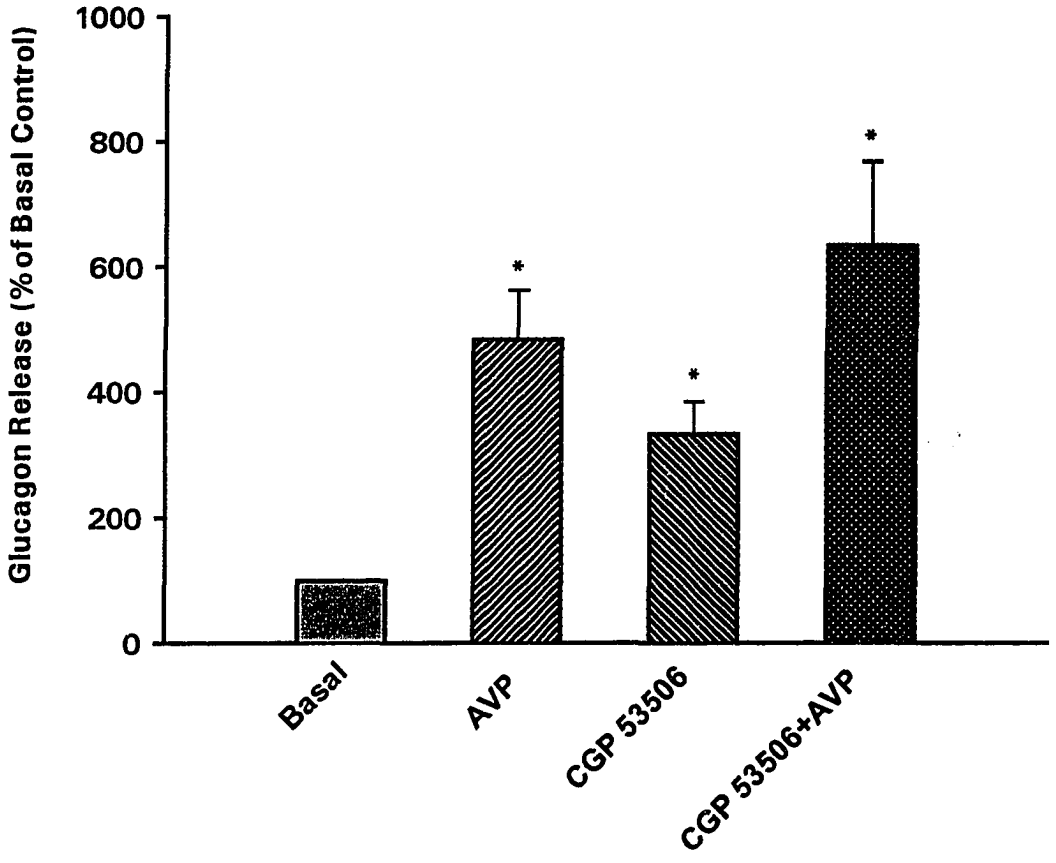

Fig. 4. Effect of CGP 53506 on AVP-induced glucagon release. CGP 53506 (50 μM) was given 10 min before the administration of AVP (100 nM). Static incubation was performed for 15 min to determine the glucagon release, which was 834 ± 172 pg/well/15 min in the basal control group. Values are mean ± SE (n = 4 cultures with quadruplicates). * $P < 0.05$, compared with the basal control group.

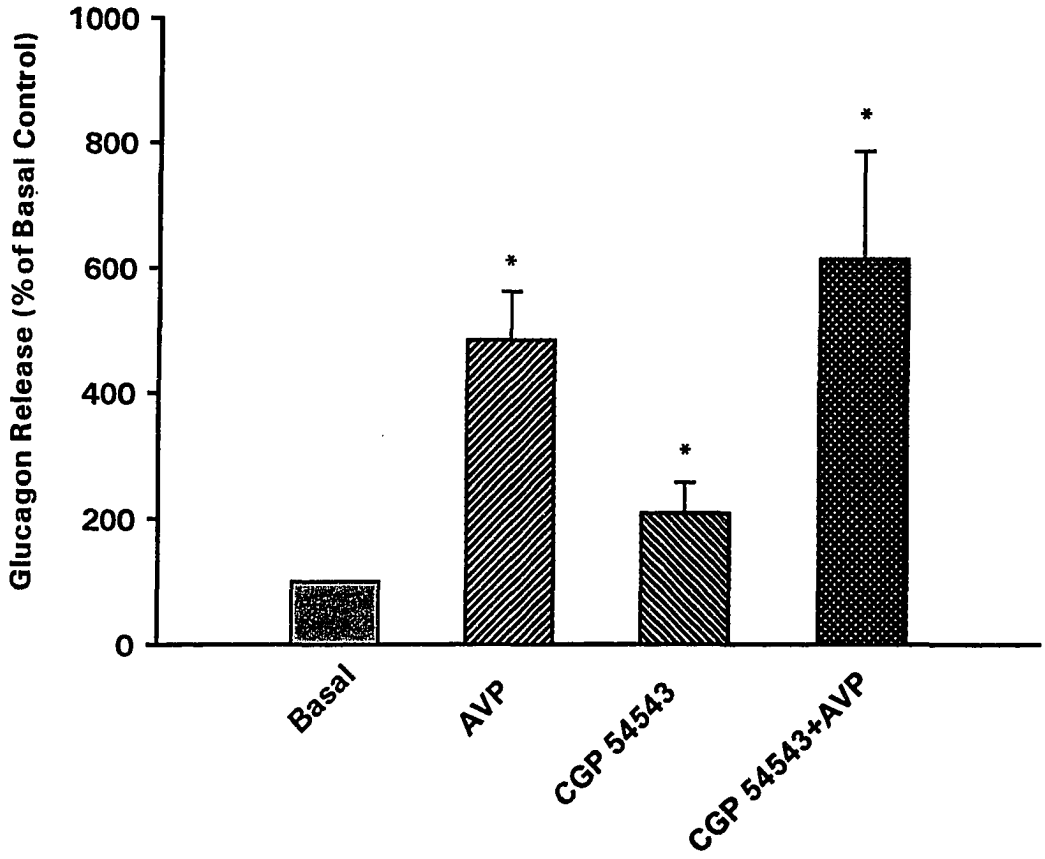

Fig. 5. Effect of CGP 54345 on AVP-induced glucagon release. CGP 54345 (100 μM) was given 10 min before the administration of AVP (100 nM). Static incubation was performed for 15 min to determine the glucagon release, which was 834 ± 172 pg/well/15 min in the basal control group. Values are mean ± SE (n = 4 cultures with quadruplicates). * $P < 0.05$, compared with the basal control group.

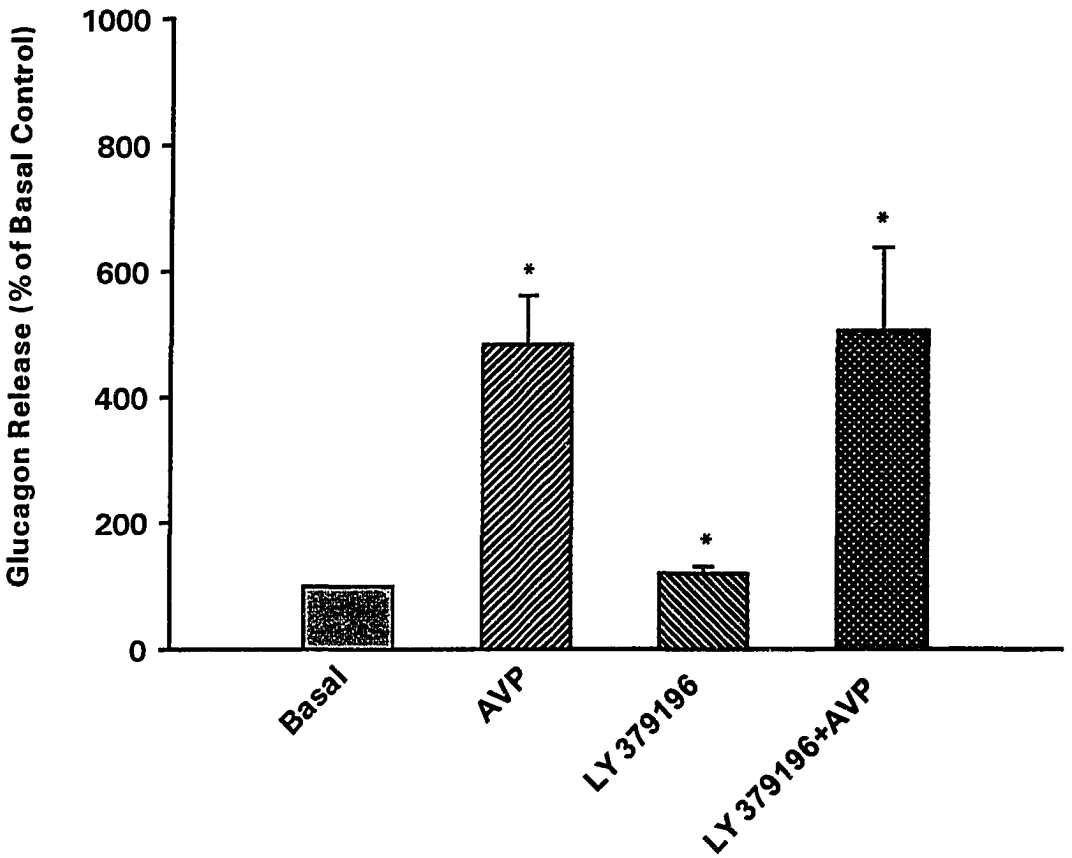

Fig. 6. Effect of LY 39196 on AVP-induced glucagon release. LY 39196 (300 nM) was given 10 min before the administration of AVP (100 nM). Static incubation was performed for 15 min to determine the glucagon release, which was 834 ± 172 pg/well/15 min in the basal control group. Values are mean ± SE (n = 4 cultures with quadruplicates). * $P < 0.05$, compared with the basal control group.

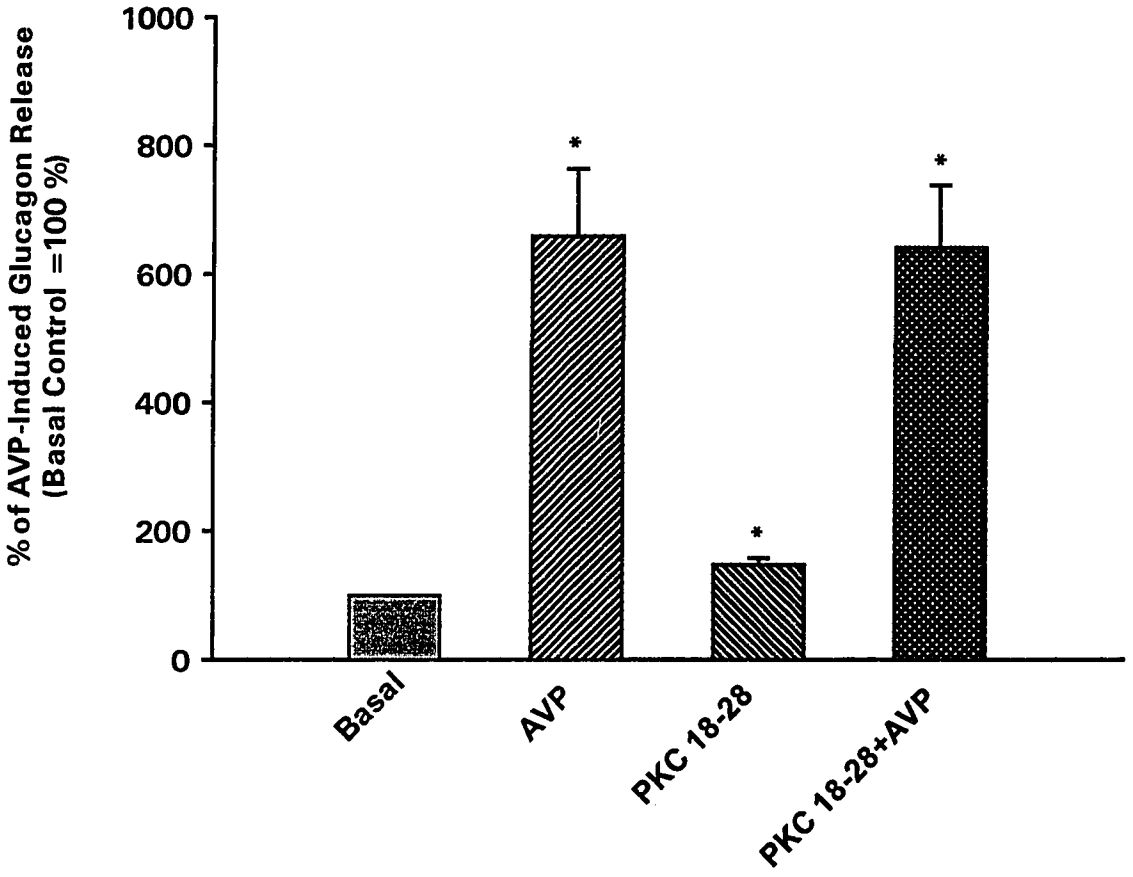

Fig. 7. Effect of pseudosubstrate peptide specific for cPKCs (PKC 18-28) on AVP-induced glucagon release. PKC 18-28 (10 μM) was given 45 min before the administration of AVP (100 nM). Static incubation was performed for 15 min to determine the glucagon release, which was 914 ± 181 pg/well/15 min in the basal control group. Values are mean ± SE (n = 4 cultures with quadruplicates). * $P <$ 0.05, compared with the basal control group.

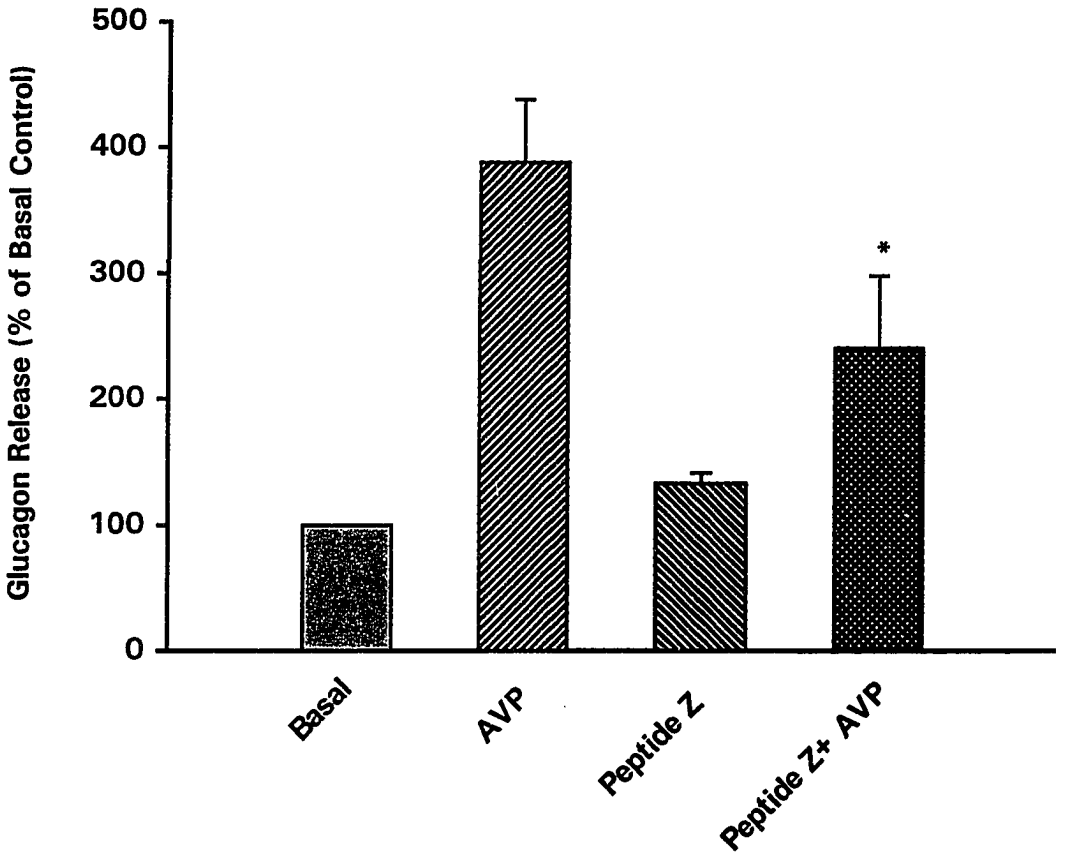

Fig. 8. Effect of pseudosubstrate peptide specific for aPKCs (Peptide Z) on AVP-induced glucagon release. Peptide Z (10 μM) was given 45 min before the administration of AVP (100 nM). Static incubation was performed for 15 min to determine the glucagon release, which was 914 ± 181 pg/well/15 min in the basal control group. Values are mean ± SE (n = 4 cultures with quadruplicates). * $P <$ 0.05, compared between delta increases of peptide Z + AVP group and the AVP group.

CHAPTER VII GENERAL CONCLUSIONS

General Discussion

This chapter contains the discussion of all the major conclusions obtained from the present study that may be found in the Discussion section of each chapter. In addition, it consists of the discussion of possible physiological and clinical implications, as well as the suggestion for further study that is related to our experimental findings.

Receptors mediating AVP- and OT-induced glucagon release

AVP and OT influence a number of biological activities. Both peptides and their receptors are found in the pancreas and stimulate glucagon and insulin release (Altszuler and Hamshire, 1981; Amico et al., 1988, Dunning et al., 1988). However, they exhibit a greater impact on glucagon release than insulin release because a low concentration of AVP and OT (20 pg/ml) caused an increase in glucagon release, but not insulin release from the perfused rat pancreas (Dunning et al., 1984). In addition, they elicited a concentration-dependent stimulation of glucagon release, but not insulin release from rat islets (Dunning et al., 1984).

According to the data of the present study obtained from *in situ* pancreatic perfusions, AVP and OT at 3-30 pM concentrations, similar to those existing in plasma (Franchini and Cowley, 1996; Kjaer et al., 1995), significantly increase glucagon release. These results are consistent to those of Dunning et al. (1984) who reported that a small amount of neurohypophysial lobe extract (0.025 NL eq/ml) stimulates an increase in glucagon release from rat pancreatic islets. In addition, Dunning et al. (1985) found that plasma glucagon level in the rat is elevated in response to the increases in AVP and OT concentrations associated with hemorrhage. AVP and OT may physiologically regulate glucagon release from the rat pancreas, but whether these two peptides exert peripheral or paracrine effects on glucagon release is unclear.

High concentrations of AVP and OT are found in the pancreas and these could exert a paracrine function on pancreatic hormone release (Amico et al., 1988). Diabetes mellitus is one of the most serious metabolic diseases and insulin release is decreased or abolished in diabetic patients, leading to hyperglycemia. In addition, most

of these patients have excessive glucagon release, which further aggravates hyperglycemia in diabetes (Unger and Orci, 1995). The plasma concentrations of AVP and OT are also higher in the diabetic patients than normal persons (Tallroth et al., 1992; Volpi et al., 1998). A morphometric study showed that both AVP- and OT-immunoreactive neuronal somata of the supraoptic nucleus (SON) and the paraventricular nucleus (PVN) in the diabetic rats underwent marked hypertrophy, representing hyperactivity of both peptides' immunoreactive neurons (Dheen et al., 1994). Furthermore, there is evidence showing that elevated plasma concentration of OT, e.g., as seen in response to suckling in lactating rats, are accompanied by a rise of glucagon release which is blocked by an OT receptor antagonist (Björkstrand et al., 1996). In conclusion, AVP and OT seem to exert both paracrine and peripheral functions to physiologically increase glucagon release. Moreover, they may play a major pathological role in the hypersecretion of glucagon in diabetic subjects.

AVP and OT exert their effects through at least 4 different types of receptors, including V_{1a}, V_{1b}, V_2, and OT receptors. The V_{1b} receptor mRNA has been detected in the pancreas (Saito et al., 1995) and the high density of ^{3}H-labeled OT binding has been demonstrated in the periphery of islets that corresponds to the location of pancreatic α-cells (Stock et al., 1990). Since CL-4-84, an antagonist with potent V_{1b} blocking activity, dose-dependently inhibits AVP-induced glucagon release from the perfused rat pancreas and inhibits the binding of fluorescence-labeled VP to its receptor in the rat islets, it appears that AVP induces glucagon release by activating V_{1b} receptors in the rat pancreas. In addition, the potent and selective OT receptor antagonist, L 366,948, dose-dependently inhibits OT-induced glucagon release from the perfused rat pancreas and inhibits the binding of fluorescence-labeled OT to its receptor in the rat islets. These results suggest that OT induces glucagon release by activating OT receptors in the rat pancreas. However, in clonal α-cell line In-R1-G9, both AVP and OT may induce glucagon release by acting through V_{1b} receptors, because CL-4-84 and other antagonists with V_{1b} blocking activity, but not L 366,948, dose-dependently inhibit the effect of OT-induced glucagon release in In-R1-G9 cells. This cell line appears not to express OT receptors, and thus is not a good model to study the mechanism underlying OT-induced glucagon release. Further studies utilizing molecular biology are warranted to confirm the present findings.

Since AVP and OT may aggravate hyperglycemia in diabetic subjects by increasing glucagon secretion, it is likely that the use of V_{1b} and OT receptor antagonists may help decrease glucagon release in these patients. Further work is needed to prove or disprove this hypothesis.

Mechanisms underlying AVP-induced glucagon release in In-R1-G9 cells

The information that many hormones and neurotransmitters transmitted to the cells is transduced by a membranous signaling system of G-protein-coupled receptors. The activated receptors mediate the processes that increase the concentration of *second messengers in the cytosol, leading to the activation of downstream effectors* (Offermanns and Schultz, 1994). The signaling pathway of AVP-coupled V_1 receptor is generally through the activation of $G_{q/11}$, which exerts multiple signal transduction pathways, such as PLC, PLD, and PLA_2 in smooth muscle cells and CHO cells with V_{1a} receptor expression (Briley et al., 1994; Thibonnier et al., 1993). In clonal β cells RINm5F, AVP also activates multiple signal transductions, including PLC and PLD pathways (Chen et al., 1994).

Although Ca^{2+} is hypothesized to be a major signal for glucagon release, the results obtained from the present study showed that AVP stimulated glucagon release through both Ca^{2+}-dependent and -independent pathways in In-R1-G9 cells. Activation of PLC by AVP is considered as the major Ca^{2+}-dependent pathway. When AVP binds to V_{1b} receptor, it activates a G-protein, probably G_q, which in turn activates PLC. Activation of PLC catalyzes the hydrolysis of PIP_2 to generate IP_3 and DAG. IP_3 releases Ca^{2+} from the ER, which in turn induces Ca^{2+} influx. This mechanism is so called the "capacitative mechanism" (Putney, 1990); however, the mechanisms underlying Ca^{2+} influx after rapid release of Ca^{2+} from the ER are still not well-understood. The Ca^{2+} influx induced by the capacitative mechanism may be mediated through ROCs in In-R1-G9 cells. A similar phenomenon has been observed in the CHO cells with stable expression of cloned V_{1a} receptors (Briley et al., 1994). Both Ca^{2+} release and Ca^{2+} influx contribute to glucagon release, because AVP-induced glucagon release was dramatically lower in the absence of extracellular Ca^{2+} than in the presence of extracellular Ca^{2+}.

Even though PLC is the major mechanism for AVP-induced glucagon release, U-73122, a PLC inhibitor, had much less impact on AVP-induced glucagon release than $[Ca^{2+}]_i$ increase, suggesting a PLC-independent pathway is involved. PLD seems to be the one that is involved in PLC-independent pathway of AVP-induced glucagon release, because zLYCK, an inhibitor of PLD (Kusaka et al., 1996), markedly inhibited AVP-induced glucagon release in the present study. In addition, bacterial PLD from *Streptomyces sp.* increased glucagon release in a concentration-dependent manner under both normal and Ca^{2+}-deprived condition (unpublished data). However, further investigations such as the measurement of PLD activity before and after AVP administration is needed to test this hypothesis. In addition, it would be worthwhile to study the effect of simultaneous pretreatment of In-R1-G9 cells with PLC and PLD inhibitors on AVP-induced glucagon release to answer the question whether AVP induces glucagon release through both of these pathways. Based on our findings, we also conclude that PLA_2 may not be involved in AVP-induced glucagon release. This is similar to what happens with the clonal β cells RINm5F, in which PLA_2 does not mediate AVP-induced insulin release (Chen et al., 1994).

In addition to the Ca^{2+}-dependent pathway, AVP stimulates glucagon release under Ca^{2+}-deprived condition, which suggests that the Ca^{2+}-independent pathway is involved in AVP-induced glucagon release. This mechanism is still not well-defined in In-R1-G9 cells, because it is possible that the changes of $[Ca^{2+}]_i$ are significant, but too small to be detected by the technique used in this study. Further investigations are needed to study AVP-induced $[Ca^{2+}]_i$ increase by using more specific and sophisticated tools that can determine the local changes of the $[Ca^{2+}]_i$; for example simultaneously loading the cells with two different Ca^{2+} indicators, aequorin and fura-2. Aequorin can measure the focal increase in $[Ca^{2+}]_i$ in a small region of cytoplasm, and Fura-2 is predominantly a measure of mean cytoplasmic $[Ca^{2+}]$ (Rembold et al., 1995). Dual use of these two indicators may help to determine the local small changes of $[Ca^{2+}]_i$. In addition, the use of a confocal laser scanning microscope to determine the localized intracellular Ca^{2+} gradients in the cells loaded with the Ca^{2+}-sensitive dye Fura-2 may be useful to investigate the spatial and temporal heterogeneity of $[Ca^{2+}]_i$ signals in the cytosol in response to the agonist-evoked stimulation (Nitschke et al., 1997). These

advanced tools will provide precise measurements of $[Ca^{2+}]_i$ changes in specific regions of the cells.

Nevertheless, a Ca^{2+}-independent pathway may indeed exist in the AVP-induced glucagon release. One possibility is that AVP acts at a distal site beyond the point of increased $[Ca^{2+}]_i$ to trigger exocytosis. This would be similar to what has been found in RINm5F cells, in which carbachol stimulates insulin release by a direct action at the exocytotic apparatus to trigger exocytosis (Tang et. al, 1995). Further work is needed to identify this pathway by investigating the effect of AVP-induced glucagon release in the cells pretreated with tetanus and/or botulinum toxins under a stringent Ca^{2+} deprivation (Fassio et al., 1999). Tetanus toxin is known to block exocytosis by cleaving the complex of vesicle-associated membrane protein (VAMP or synaptobrevin), and botulinum toxin is known to block exocytosis by cleaving syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa). The VAMP, syntaxin and SNAP-25 are essential components of the exocytotic apparatus.

Another possibility is that PLD may play a role in the Ca^{2+}-independent pathway of AVP mechanisms because the data from our preliminary experiment shows that a bacterial PLD significantly increased glucagon release under a stringent Ca^{2+}-deprivation. Activation of PLD leads to the generation of PA that is converted to DAG by the action of phosphohydrolase. Atypical PKCs, PKC-ζ and PKC-λ, are activated by PA, but not by DAG (Dimitrijevic et al., 1995). In a Western blot study, we found that both PKC-ζ and PKC-λ were present in In-R1-G9 cells; however, AVP activated only PKC-ζ, but not PKC-λ (unpublished data). In addition, a pseudosubstrate peptide inhibitor specific for PKC-ζ and PKC-λ inhibited the AVP-induced glucagon release (unpublished results). It is likely that PLD activation, leading to the activation of PKC-ζ, may be involved in the Ca^{2+}-independent pathway of AVP mechanisms. Activation of PLD is either dependent or independent of Ca^{2+} (Lindmar and Loffelholz, 1998) and measurement of PLD activity induced by AVP under a stringent Ca^{2+} deprivation and the immunoblotting analysis of PLD isozymes in In-R1-G9 cells may provide clues to this question.

The roles of PKC isozyme in the regulation of AVP-induced $[Ca^{2+}]_i$ increase and glucagon release

PKC has been established for over ten years as a family of kinases that is responsible for many diverse and critical cellular functions; for example, it plays a role in cell-cell contact (Liosas et al., 1996), suppression of apoptosis (Romanova et al., 1996) and the growth of cancer cells (Choi et al., 1990). Different PKC isozymes modulate exocytosis in various systems. cPKCs have been found to play a role in stimulation of gut hormones (e.g. gastrin, serotonin and somatostatin), including their synthesis and/or secretion (Kawakita et al., 1995), as well as stimulation of insulin release from human insulinoma cells (Miura et al., 1998). cPKC-α and nPKC-ε, but not -δ, increase the secretion of an N-terminal fragment of the amyloid precursor protein involved in Alzheimer's disease (Kinouchi et al., 1995). nPKC-ε plays a stimulatory role in thyrotropin-releasing hormone-stimulated prolactin secretion (Akita et al., 1994), but in parietal cells, nPKCs may be involved in the inhibition of HCl secretion (Chew et al., 1997). aPKC isozyme-ζ stimulates carbachol-induced insulin secretion in RINm5F cells (Tang and Sharp, 1998) and aPKCs also stimulate glucose-induced insulin release from pancreatic β-cells (Harris et al., 1996). The activation of PLC promotes the formation of DAG, which in turn activates cPKCs and nPKCs (Regazzi et al., 1990). The physiological function of PKC in glucagon release is unclear. PMA, an activator of cPKCs and nPKCs, has been found to increase glucagon release from rat islets (Hii et al., 1986; Niki et al., 1986). In addition, down regulation of PKC by pretreating the rat islets with 200 nM PMA for 18-24 h causes the impairment of arginine-induced glucagon release (Bjaaland et al., 1988).

A number of highly specific PKC inhibitors have been developed in recent years (Hofmann, 1997). In this study, Ro 31-8220, an inhibitor for most of the PKC isozymes, enhanced AVP-induced IP_3 formation, $[Ca^{2+}]_i$ increase and glucagon release. In addition, PMA and OAG, activators of cPKCs and nPKCs, inhibit AVP-induced IP_3 formation, $[Ca^{2+}]_i$ increase and glucagon release. These results suggest that cPKCs and/or nPKCs play a negative role in the regulation of AVP-induced glucagon release in In-R1-G9 cells. Similar results are seen in clonal β cells (Yang et al., 1997), rat

glomerulosa (Gallo-Payet et al., 1991), and vascular smooth muscle cells (Stassen et al., 1989).

In the present study, Gö 6976, CGP 54345, CGP 53506, LY 379196, inhibitors of cPKCs, and a pseudosubstrate peptide specific for cPKCs, all failed to inhibit AVP-induced glucagon release. These results suggested that cPKCs are not involved in AVP-induced glucagon release. In addition, the results from the Western blot experiments showed that PKC-α and -β were not detectable in In-R1-G9 cells (unpublished data). A pseudosubstrate for aPKCs inhibited AVP-induced glucagon release. Taken together, our findings suggest that nPKCs play an inhibitory role and aPKCs play a stimulatory role on AVP-induced glucagon release in In-R1-G9 cells. We further suggest that activation of nPKCs exhibit a negative feedback control on V_{1b} receptor-$G_{q/11}$-PLC-β pathway to inhibit IP_3 production, leading to an attenuation of AVP-induced $[Ca^{2+}]_i$ increase and glucagon release. Further work using immunoblotting is needed to identify which isozymes indeed regulate the action of AVP in pancreatic α cells.

It is likely that different PKC isozymes balance their functions in regulating AVP-induced glucagon release by exerting both positive and negative impacts on AVP's actions. However, as we discussed earlier, AVP may play a role in the hypersecretion of glucagon in diabetic subjects, and specific inhibitor or antisenses against aPKCs may be useful in the control of diabetes mellitus.

In summary, the results from the present study suggest that in α cells, AVP induces glucagon release through multiple signal transduction pathways that are both Ca^{2+}-dependent and Ca^{2+}-independent. For the Ca^{2+}-dependent pathway, AVP activates V_{1b} receptors, which in turn, activates G_q. The G_q protein activates PLC, causing increases in the formations of IP_3 and DAG. IP_3 releases Ca^{2+} from the ER and triggers Ca^{2+} influx through ROCs, leading to glucagon release. DAG activates PKC, which in turn exerts both inhibitory and stimulatory influences on AVP-induced glucagon release. nPKCs may inhibit AVP-induced IP_3 production, attenuating AVP-induced increase in Ca^{2+} release and Ca^{2+} influx via the closure of ROCs, leading to a decrease in glucagon release. aPKCs may stimulate glucagon release through an unknown pathway.

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