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Arginine vasopressin-induced glucagon release: Interaction with glucose and cyclic AMP-dependent protein kinase

by

Ehab A. H. Abu-Basha

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Program of Study Committee:
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2002

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Major Professor

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For the Major Program

DEDICATION

THIS DISSERTATION IS HEREBY DEDICATED TO

THE MEMORY OF MY FATHER

AND

MY MOST BELOVED MOTHER AND WIFE

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LIST OF ABBREVIATIONS

AC	adenylyl cyclase
AVP	arginine vasopressin
AUC	area under curve
BAPTA-AM	1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester
BSA	bovine serum albumin
CaMK II	Ca^{2+}/calmodulin kinase II
cAMP	cyclic adenosine monophosphate
$[Ca^{2+}]_i$	intracellular calcium concentration
DOPE	dioleoyl phosphatidylethanolamine
EGTA	[ethylenebis (oxyethylenenitrilo)] tetraacetic acid
ER	endoplasmic reticulum
Fura-2AM	fura-2 acetoxymethyl ester
G-protein	guanine nucleotide-binding protein
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
IBMX	3-isobutyl-1-methylxanthine
IP_3	inositol 1,4,5-trisphosphate
KRB	Krebs-Ringer bicarbonate buffer
PDE	phosphodiesterase
PIP_2	phosphatidylinositide 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLC	phospholipase C
RP	reserve pool
RRP	readily releasable pool
VDCC	voltage-dependent Ca^{2+} channel
SNAREs	soluble N-ethylmaleimide-sensitive factor (NSF) attachment protein receptor

SNAP-25	synaptosomal-associated protein of 25 kDa
VAMP	vesicle-associated membrane protein
SVs	synaptic vesicles
TFA-DODAPL	trifluoroacetylated lipopolyamine

ABSTRACT

We studied the glucose dependency of arginine vasopressin (AVP)-induced insulin and glucagon release from the perfused rat pancreas and the intracellular mechanism of cAMP/PKA-dependent enhancement of AVP-induced glucagon release.

The study purpose was to investigate the glucose dependency of AVP-induced insulin, glucagon and somatostatin release from the perfused rat pancreas. AVP (30 or 300 pmol/L) was tested in the presence of glucose concentrations of 0, 1.4, 5.5 (basal level), or 20 mmol/L. The rates of insulin release at 0 and 1.4 mmol/L glucose were ~70-80% and ~60-70% less, respectively, than that at the baseline level. AVP (30 or 300 pmol/L) failed to change insulin release at 0 and 1.4 mmol/L glucose. At the basal glucose level, AVP (300 pmol/L) induced a biphasic insulin release, a peak followed by a sustained phase. In addition, the combination of glucose (20 mmol/L) and AVP (300 pmol/L) induced a higher insulin peak and sustained phase than 20 mmol/L glucose alone. The rates of glucagon release at 0 and 1.4 mmol/L glucose were ~3- and ~2-fold more, respectively, than that at the baseline glucose level. At 0 and 1.4 mmol/L glucose, both 30 and 300 pmol/L AVP caused a higher glucagon peak and sustained phase than 0 and 1.4 mmol/L glucose alone. At the basal glucose level, AVP (30 or 300 pmol/L) induced a biphasic glucagon release, a peak followed by a sustained phase. The rate of glucagon release at 20 mmol/L glucose was ~60-70% less than that at the baseline level. When AVP (300 pmol/L) was administered in 20 mmol/L glucose, it induced a transient glucagon peak, which was 2.4-fold of the baseline level. These findings suggested that AVP may increase insulin and glucagon release by a direct action on β- and α-cells, respectively. These increases are glucose-dependent; the higher the glucose concentration, the greater the enhancement of AVP induced insulin release. In contrast, the lower the glucose concentration, the greater the enhancement of AVP-induced glucagon release. AVP not only can enhance glucose-induced insulin release, but also can initiate insulin release. α-cells are much more sensitive to AVP than β-cells in hormone release. Furthermore, our results confirmed the previous findings that hypoglycemia directly increases glucagon and decreases insulin release.

Increasing intracellular cAMP levels, by using forskolin or by preventing cAMP degradation with the phosphodiestrease inhibitor IBMX, enhanced AVP-induced glucagon release from the perfused rat pancreas and the clonal α-cells InR1G9. Pre-treatment with SQ-22536, an adenylyl cyclase inhibitor, abolished both the effect of forskolin on glucagon release and the enhancement effect on AVP-induced glucagon release. cAMP produced its effects through the stimulation of PKA, as a selective PKA inhibitor H-89 reduced the effect of forskolin on AVP-induced glucagon release and abolished the effect of forskolin on glucagon release. cAMP/PKA did not increase $[Ca^{2+}]_i$ nor did it enhance AVP-induced $[Ca^{2+}]_i$ increase. Forskolin and IBMX enhanced AVP-induced glucagon release in Ca^{2+}-containing medium but not in Ca^{2+}-free medium. CaMK II plays a partial role in AVP-induced glucagon release, since the selective inhibitor, KN93, partially blocked AVP-induced glucagon release and forskolin/AVP-induced glucagon release. The fact that glucagon release was prolonged with forskolin treatment in the perfused rat pancreas and from InR1G9 cells led us to hypothesize that PKA promotes the mobilization of secretory granules from the reserve pool (RP) to the readily releasable pool (RRP). To test this hypothesis, InR1G9 cells were loaded with styryl dye FM1-43. The combination of AVP and forskolin induced more increase in fluorescence intensity (~ 4.8 fold of the control group) than AVP or forskolin alone (~ 2.1 and ~ 1.5 fold of the control group, respectively), which reflects an increase in the number of secretory granule membranes fused with the plasma membrane and in the size of the RRP. Secretory granules in the RP are thought to be reversibly connected to the actin-based cytoskeleton by synapsin I that have been proposed to play a major structural role in the assembly and maintenance of the RP secretory granules as well as a dynamic role in controlling secretory granules transitions from RP to RRP. Pretreatment with antisynapsin I antibody abolished the effect of forskolin/AVP-induced glucagon release but not glucagon release in the control, AVP, or forskolin groups. In addition, FM1-43 loading experiments showed that synapsin I is involved in recruitment of secretory granules from RP to RRP. Our results suggested that cAMP, acting through PKA, increases the number of secretory granules in the RRP by mobilization of granules from the RP, an action mediated by synapsin I.

CHAPTER I. GENERAL INTRODUCTION

Dissertation Organization

This dissertation is written in an alternative thesis format. It contains a general introduction, two research papers, a general discussion, and 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, "Glucose-Dependency of Arginine Vasopressin-Induced Insulin and Glucagon Release from the Perfused Rat Pancreas ", accepted for publication in Metabolism, Chapter III, "Cyclic AMP-Dependent Protein Kinase Enhances Arginine Vasopressin-Induced Glucagon Release by Increasing the Readily Releasable Pool: Involvement of Synapsin I", to be submitted to Journal of Biological Chemistry, and "Chapter IV, "General Discussion".

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

Research Objectives

Glucagon is synthesized and secreted from the pancreatic α-cells in response to low blood glucose levels, and it plays a key role in maintaining glucose homeostasis. Understanding the regulation of glucagon release may contribute to a better control of type 2 diabetes because excessive glucagon release in diabetic patients further aggravates the hyperglycemia caused by low insulin release (Unger and Oric, 1995). Glucagon can be inhibited or stimulated by a variety of nutrients and hormones. In our laboratory, we demonstrated the ability of AVP, a hormone found in the posterior pituitary gland as well as

in the pancreas, to stimulate glucagon (Yibchok-anun and Hsu, 1998) and insulin release (Lee et al., 1995) in rat pancreas, α-cell line InR1G9 and β-cell line RINm5F, respectively. AVP may regulate glucose metabolism by its direct glycogenolytic and gluconeogenic effects on the liver (Kirk et al., 1979), and by increasing insulin and glucagon release from the endocrine pancreas. The fact that AVP is present in the pancreas (Amico et al., 1988) suggests that AVP may exert a local control on the endocrine pancreas.

The effects of AVP on the endocrine pancreas, insulin release in particular, are controversial. AVP (18.5-185 pmol/L) caused a concentration-dependent stimulation of glucagon release but failed to influence insulin release from isolated rat islets in medium containing 5.6 mmol/L glucose (Dunning et al., 1984a). In isolated mouse islets, AVP failed to change insulin release at ≤ 7 mmol/L glucose but increased insulin at 10-30 mmol/L glucose (Gao et al., 1990; 1992). This implies that AVP is not a primary insulin secretagogue, but is an enhancer of glucose-induced insulin secretion. In the perfused rat pancreas, AVP (185 pmol/L) in 5.5 mmol/L glucose caused a small insulin release (Dunning et al., 1984b). In contradiction, AVP inhibited glucose-induced insulin release in rat islets (Khalaf and Taylor, 1988) and in hamster beta cell line (HIT) (Richardson et al., 1990). Since there are discrepancies in the effects of AVP on the endocrine pancreas, insulin release in particular, we investigated the direct effect of AVP (30 or 300 pmol/L) on insulin, glucagon and somatostatin release from the perfused rat pancreas in the presence of various glucose concentrations (0, 1.4, 5.5, or 20 mmol/L).

During hypoglycemic stress, glucagon release increases and insulin release decreases. Increased glucagon release is the main counterregulatory factor in the recovery of hypoglycemia (Gerich et al., 1979; Cryer PE, 1981). However, the mechanism underlying

hypoglycemia-induced glucagon release is uncertain. A number of studies suggest that the increase in release is due to activation of the autonomic nervous system (Havel and Taborsky, 1989; Havel and Valverde, 1996; Havel et al., 1996) while, others suggest that hypoglycemia directly stimulates glucagon release from the perfused rat pancreas and the perfused rat pancreatic islets (Weir et al., 1974; Oliver et al., 1976, respectively). Weir et al. (1974) showed that when perfusate was decreased from 5.5 mmol/L to 1.4 mmol/L glucose, glucagon was released in a biphasic pattern. While, Oliver et al. (1976) showed that low glucose concentrations of glucose per se failed to increase glucagon release, but arginine and epinephrine increased glucagon release at low glucose concentrations. We investigated the direct effects of various glucose concentrations (0, 1.4, 5.5, or 20 mmol/L) on insulin and glucagon release using the perfused rat pancreas.

Ca^{2+} plays a central role in secretagogue-induced glucagon secretion (Pipeleers et al., 1985; Hii and Howell, 1987). Stimulation of pancreatic α-cells with secretagogues such as AVP results in a rise in $[Ca^{2+}]_i$ due to Ca^{2+} release from endoplasmic reticulum and Ca^{2+} influx from the extracellular space (Yibchok-anun et al., 2000). AVP-induced glucagon release is largely mediated by a Ca^{2+}-dependent pathway (Yibchok-anun et al., 2000). Many effects of Ca^{2+} are mediated through Ca^{2+} -binding proteins such as calmodulin (CaM) (Colbran et al., 1989). The effects of Ca^{2+} /CaM may be mediated by Ca^{2+} /CaM-dependent protein kinase II (CaMK II) in neurons as well as in pancreatic β-cells (Hanson and Schulman, 1992; Easom, 1999). cAMP enhances glucagon and insulin release from pancreatic α- and β-cells, respectively, an action mediated by the activation of cAMP-dependent protein kinase (PKA) (Wollheim et al., 1987; Gromada et al., 1997). We studied the interaction between the G protein-PLC pathway and PKA, in which cAMP/PKA

enhances AVP-induced glucagon release from the perfused rat pancreas and α-cell line InR1G9. The activation of specific effectors upstream and downstream from the Ca^{2+} signal remain largely unknown; for instance cAMP stimulates exocytosis even when $[Ca^{2+}]_i$ is clamped at fixed concentrations and when the membrane potential is held too negative for Ca^{2+} channels to open (Renstrom et al., 1997). Also elevation of intracellular cAMP concentration by forskolin, an adenylyl cyclase activator, evoked a PKA-dependent enhancement of exocytosis without increasing the $[Ca^{2+}]_i$ (Renstrom et al., 1997). We investigated the mechanisms by which cAMP/PKA enhances AVP-induced glucagon release. The fact that glucagon release was prolonged with forskolin treatment in the perfused rat pancreas and from InR1G9 cells had led us to hypothesize that cAMP/ PKA promotes the mobilization of secretory granules from the reserve pool (RP) to the readily releasable pool (RRP).

One possible mechanism for the recruitment of the secretory granules from RP is the release of granules from the actin-based cytoskeleton induced by phosphorylation of synapsin I (Greengard et al., 1993; Brodin et al., 1997; Benfenati et al., 1999; Hosaka et al., 1999). Synapsin I has been proposed to play a major structural role in the assembly and maintenance of the RP of synaptic vesicles as well as a dynamic role in controlling synaptic vesicle transitions from the RP to the RRP (Greengard et al., 1993; Brodin et al., 1997; Benfenati et al., 1999). By analogy with neuronal cells, synapsin I in α-cells cells may function as a linker of glucagon secretory granules and the cytoskeletal network. Therefore, we hypothesized that synapsin I is involved in mobilization of glucagon granules from RP to RRP. A summary of possible interactions between PLC and PKA are shown in Fig. 1.

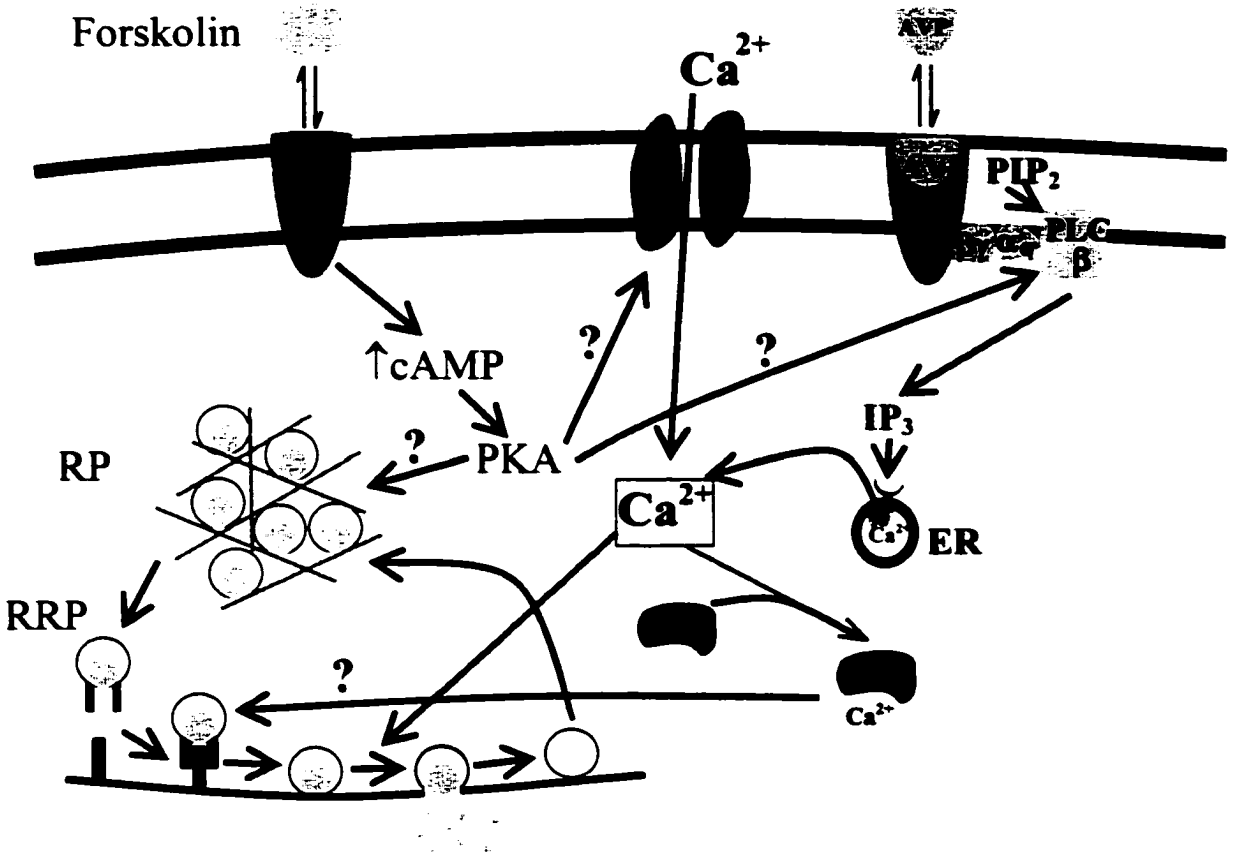

Fig 1. Summary of possible interactions between Phospholipase C-β (PLC-β) and cAMP-dependent protein kinase A (PKA). PKA may enhance arginine vasopressin (AVP)-induced IP_3 formation, which in turn further increases the Ca^{2+} release. PKA may enhance AVP-induced glucagon release by enhancement of Ca^{2+} influx or by mobilization of secretory granules from the reserve pool (RP) to the readily releasable pool (RRP).

In summary, the objectives of this study are: 1) to investigate the glucose-dependency of arginine vasopressin-induced insulin and glucagon release from the perfused rat pancreas, and 2) to characterize the mechanisms by which cAMP/PKA enhances AVP-induced glucagon release and provide further details in the intracellular molecular components involved in this enhancement, particularly at the level of exocytosis.

Background and Literature Review

This section provides background information related to the studies that are presented in the dissertation: 1) The Pancreas; 2) Glucagon; 3) Events of exocytosis; 4) Regulation of hormone release by arginine vasopressin (AVP); 5) Role of cAMP-dependent protein kinase (PKA) in exocytosis; 6) The interaction between PKA and Phospholipase C (PLC); 7) Exocytotic proteins activation/deactivation and modulation of exocytosis; 8) Study of single cell exocytosis using fluorescent dye FM1-43.

The Pancreas

The pancreas is a mixed gland with both exocrine and endocrine activities. It lies in the abdominopelvic cavity in the dorsal part of both the epigastric and the mesogastric abdominal segments, caudal to the liver. The pancreas 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 component is larger than the endocrine component and represents ~97-99% of the pancreatic volume (Hsu and Crump, 1989). It secretes digestive enzyme-rich fluid that enters into the proximal

part of the duodenum through one or two ducts. The digestive juice contains principally proteases, amylase and lipase the breakdown of proteins, carbohydrates and fats, respectively. The endocrine pancreas comprises the islets of Langerhans that are dispersed within the pancreas and represent ~1-3% of the pancreatic volume (Hsu and Crump, 1989). The pancreatic islets contain at least four endocrine cells: β (or B) cells, α (or A) cells, δ (or D) cells and F (or PP) cells, which secrete insulin, glucagon, somatostatin, and pancreatic polypeptide, respectively (Pelletier, 1977). The β cells form the central core and represent ~75% of the islet cells, whereas α cells are generally located in the periphery and represent ~20% of the islet cells (Karam, 1995). The δ cells are located between α and β cells and represent ~3-5% of the islet cells, while F cells are located near the α cells and represent <2% of the islet cells (Karam, 1995).

The pancreatic islets are surrounded by an extensive, fenestrated capillary network that carries its hormones into the circulation. In addition to the pancreatic branches of the splenic artery, the cranial and caudal pancreaticoduodenal arteries are the major arteries supply blood to the pancreas, the former branching from the celiac artery, and the latter branching from the cranial mesenteric artery (Evans, 1993; Dyce et al., 1996). Venous blood returns to the portal vein. The pancreas is innervated by sympathetic and parasympathetic fibers (Hadley, 1992). The sympathetic fibers that innervate the pancreas arise from the celiac plexus and the parasympathetic fibers arise from the vagus nerve (Evans, 1993; Dyce et al., 1996).

The islets of Langerhans play a central role in the hormonal control of fuel metabolism and glucose homeostasis. Maintenance of a normal plasma glucose

concentration during periods of food consumption and fasting requires a delicate balance between glucose production and utilization. Although control of the glucose homeostasis implicates many hormonal, neural, and autoregulatory factors, insulin and glucagon are the major determinants of this control.

Glucagon

Glucagon is a polypeptide hormone consisting of a single chain of 29 amino acids. Glucagon plays an important role in maintaining glucose homeostasis. It is secreted from the pancreatic α-cells in response to low blood glucose levels and stimulates hepatic glucose output by increasing glycogenolysis and gluconeogenesis, while at the same time inhibiting glycolysis (Burcelin et al., 1996). In addition to its metabolic actions in the liver, glucagon is also involved in the regulation of adipose, cardiac, renal, gastrointestinal, and pancreatic functions, including the potentiation of glucose-induced insulin release (Burcelin et al., 1996).

Glucagon can be inhibited or stimulated by a variety of nutrients and hormones. Glucose, somatostatin, insulin, free fatty acid, ketones, and γ-aminobutyric acid are examples for nutrients and hormones that inhibit glucagon secretion (Ganong, 1995). α_2-adrenergic agonists, β-adrenergic agonists, acetylcholine, phosphodiestrase inhibitors, cortisol, cholecystokinin, gastrin, secretin, and amino acids such as arginine, alanine, serine, glycine, cysteine and threonine are stimulators for glucagon secretion (Ganong, 1995). Understanding the regulation of glucagon release may contribute to better control of type 2

diabetes because excessive glucagon release in diabetic patients further aggravates the hyperglycemia caused by low insulin release (Unger and Oric, 1995).

Events of Exocytosis

Neurotransmitters and hormones are packaged in distinct vesicles, namely synaptic vesicles (SVs) and secretory granules or large dense core vesicles (LDVs), respectively. The key event in the SVs or LDVs cycle is exocytosis by membrane fusion. Membrane fusion occurs by a similar mechanism in organisms from yeast to man, and in organelles from the nuclear envelope to SVs (Bennett and Scheller, 1993, 1994; Ferro-Novick and Jahn, 1994). Synaptic transmission requires a localized and a rapid signal that can be repeated at high frequencies and speeds (Sudhof, 1995). This is accomplished by three unique events in the cycle: a) before exocytosis, SVs are predocked at a specialized area called the active zone, b) the probability that a single docked SV will undergo exocytosis in response to Ca^{2+} is low and can be modulated over a wide range, c) SVs quickly undergo endocytosis and recycle locally close to the site of exocytosis (Sudhof, 1995).

Synaptic vesicle cycle

Exocytosis has been extensively studied in neurons and thus has been used as a model to describe neuroendocrine cells. The synaptic vesicle (SV) cycle can be divided into 9 steps as shown in Fig. 2: 1) Docking. The initial contact between presynaptic plasma membrane and SV membrane that occurs only at the active zone. The SVs that are filled with neurotransmitter dock at the active zone. 2) Priming. The maturation process of SVs that makes them ready for fusion in response to the Ca^{2+} signal. 3) Fusion/ exocytosis. The

primed SVs go through fusion/exocytosis in response to a Ca^{2+} spike. Only one of many docked SVs fuses. 4) Endocytosis. After fusion/exocytosis, the empty SV membranes are rapidly internalized and become coated vesicle. 5) Translocation. Coated vesicles sheds their coats, acidify and translocate into the interior, becoming recycling SVs. 6) Endosome fusion. Recycling SVs fuse with early endosomes. 7) Budding. Budding from endosomes regenerates the SVs. 8) Neurotransmitter uptake. SVs are filled with neurotransmitters by the electrochemical gradient created by a proton pump (active transport). 9) Translocation. The filled SVs translocate back to the active zone by diffusion or cytoskeleton-based transport process. The SV cycle takes ~1 minute (Sudhof, 1995; Pyle et al., 2000), of which the exocytosis/fusion occurs in less than 1 ms, while endocytosis occurs in less than 5 s, and the other steps of the cycle occur in the remaining 55 s.

Three functional pools of synaptic vesicles have been defined (Murthy and Stevens, 1999). These are: a) the readily releasable pool (RRP) of synaptic vesicles: the most important vesicle pool for neurotransmitter release and constitutes vesicles that are immediately ready for release, b) the reserve pool (RP): when the RRP is depleted, further stimulation leads to recruitment of additional vesicles for exocytosis from RP. The RRP and the RP constitute the recycling pool, c) the resting pool: resting pool vesicles undergoes exocytosis only under extensive stimulation.

SNAREs and SNARE-interacting protein

Exocytosis requires proteins known as SNAREs, a set of three synaptic proteins, two from the plasma membrane (syntaxin and SNAP-25 (synaptosome-associated protein of 25 kDa) and one from synaptic vesicle protein (synaptobrevin, also known as VAMP, vesicle-

associated membrane protein) (Sudhof, 1995; Mochida, 2000). During the priming step, SNAREs assemble a stable ternary complex (core complex) (Sudhof, 1995). The core complex forms the anchor for a cascade of protein-protein interactions required for exocytosis (Sudhof, 1995). SNAREs were originally identified as membrane receptors for NSF (N-ethylmaleimide-sensitive ATPase) and SNAPs (soluble NSF attachment proteins) and designated as SNAREs (SNAP receptor) (Sollner et al., 1993). NSF and SNAPs are soluble proteins that are essential for many intracellular vesicle fusion reactions (Mochida, 2000). The discovery that botulinum and tetanus toxins block exocytosis by selectively proteolyzing the individual SNARE proteins provides evidence of the importance of these proteins in exocytosis (Schiavo et al., 1992; Sollner et al., 1993; Regazzi et al., 1995; Sadoul et al., 1995; Wheeler et al., 1996). The functions of SNAREs and SNARE-interacting proteins are summarized in Table 1. The protein-protein interaction is complicated (Sollner et al., 1993); nevertheless, SNAREs are essential for SV fusion machinery (Sollner et al., 1993; Sudhof, 1995). SNARE-interacting proteins may regulate the efficiency and the strength of synaptic transmission underlying the synaptic plasticity (Sollner et al., 1993).

Molecular events of exocytosis

Sudhof (1995) proposed a model for the molecular events in exocytosis (Fig. 3). According to this model, when docking occurs, synaptobrevin (also known as vesicle-associated membrane protein (VAMP)) and syntaxin are complexed to synaptophysin and munc18 (nSec1) (synaptic vesicle proteins), respectively. These complexes dissociate during or after docking by unknown mechanisms. Syntaxin and SNAP-25 bind tightly to each other

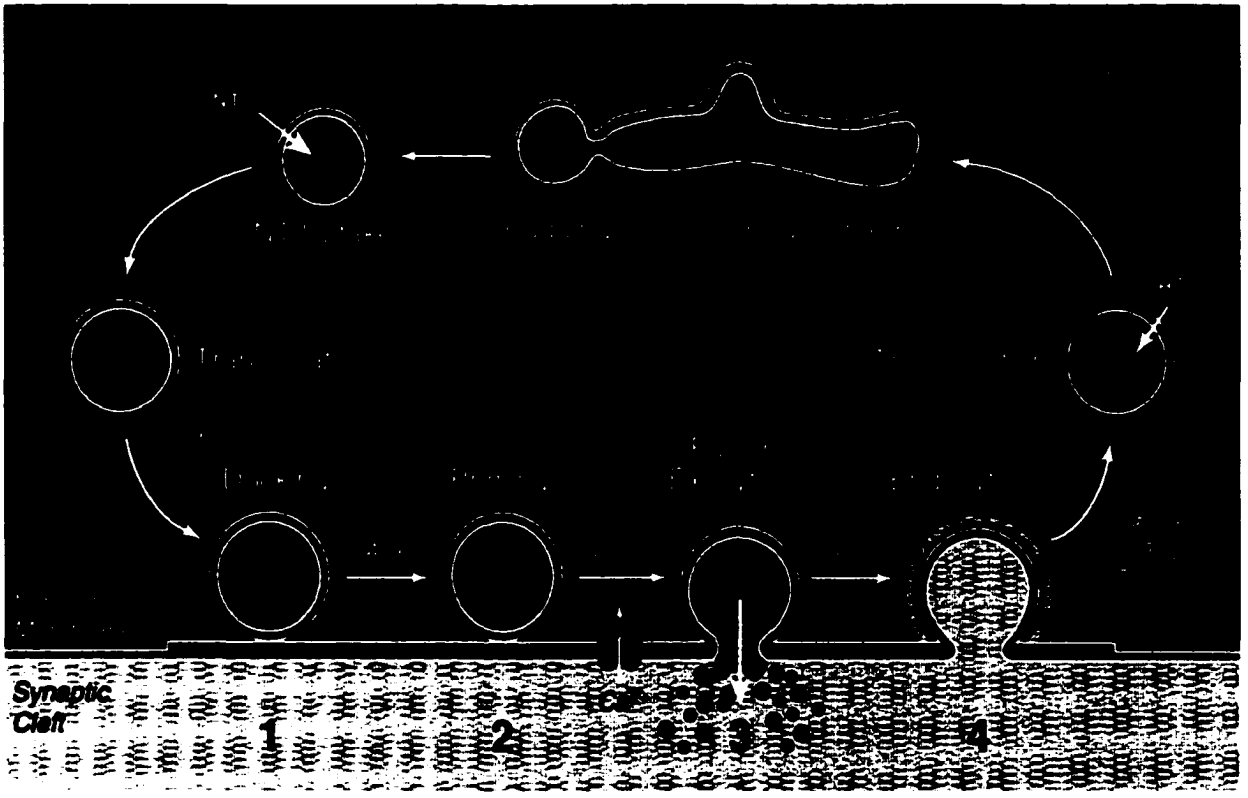

Fig 2. The nine steps of the synaptic vesicle cycle in the presynaptic nerve terminal (Reprinted with permission from Nature, Sudhof TC, The synaptic vesicle cycle: a cascade of protein-protein interactions, 375: 645-653, 1995).

Table 1: SNARES and SNARE-associated proteins implicated in exocytosis.**

Classification	Proteins	Localization	Speculated Functions
SNARES (SNARE core complex)	Synaptobrevin/VAMP	Synaptic vesicles	Fusion machinary
	Syntaxin	Plasma membranes	
	SNAP-25	Plasma membranes	
SNARE core complex-interacting	NSF (an ATPase)	Cytoplasm	SNAREs disassembly
Proteins	α-SNAP	Cytoplasm	SNAREs disassembly
	Complexin	Cytoplasm	Modulation of SNAREs assembly
	Snapsin	Synaptic vesicles	Modulation of SNAREs-synaptotagmin Interaction
	N-(and P/Q-type) Channels	Plasma membranes	Synchronous NT release Transmission of voltage signal to SNARE Complexes
Syntaxin-interacting proteins	Tomosyn	Cytoplasm and plasma membranes	Stimulation of SNAREs assembly
	Munc-18	Cytoplasmic face of plasma membranes	Inhibition of SNAREs assembly Essential for synaptic vesicle fusion
	Syntaphilin	Plasma membranes	Inhibition of SNAREs assembly
	Munc-13	Cytoplasmic face of plasma membranes	Enhancement of SNAREs assembly by DAG Promotion of synaptic vesicle trafficking by interaction with Doc2

Table 1 (Continued)

GTP-binding proteins and associated proteins	Rab3A (a GTPase)	Synaptic vesicles	Limiting fusion machinery Generation of LTP Recruitment of synaptic vesicle to active zone
	Rabphilin-3A	Synaptic vesicles	Modulation of synaptic vesicle fusion Modulation of synaptic vesicle trafficking
	Rim	Active zone	Promotion of synaptic vesicle fusion
Ca^{2+} -binding proteins containing two C2 domains	Synaptotagmin I	Synaptic vesicles	Trigger of synaptic vesicle fusion
	Doc 2	Synaptic vesicles	Promotion of synaptic vesicle trafficking by interaction with Munc-13
	Munc-13		
	Rabphilin-3A		
	Rim		

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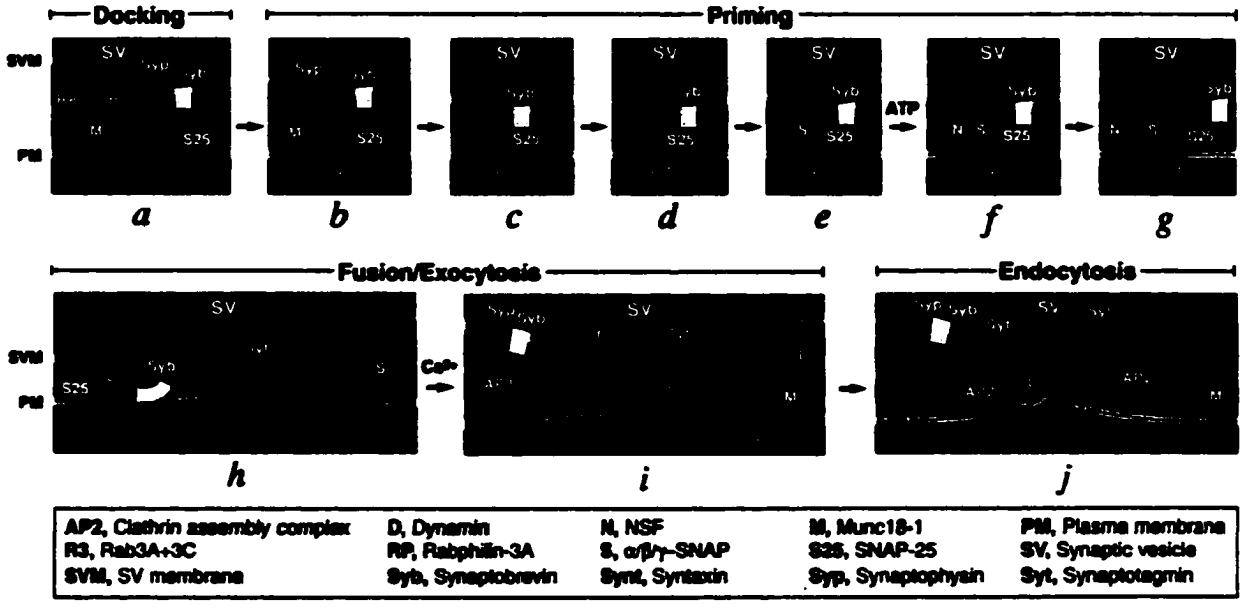

Fig 3. Protein-protein interaction cascade that mediate synaptic vesicle exo- and - endocytosis (Reprinted with permission from Nature, Sudhof TC, The synaptic vesicle cycle: a cascade of protein-protein interactions, 375: 645-653, 1995).

and form a high affinity site for synaptobrevin to form the core complex with 1:1:1 stoichiometry, which becomes sodium dodecyl sulfate resistant (Figs. 3 and 4). The core complex is composed of four parallel helices (four-helical bundle), with syntaxin contributing to its H3 domain, synaptobrevin contributing to its sole coiled-coil domain, and SNAP-25 contributing to both of its coiled-coils (Fig. 5) (Lin and Scheller, 2000). Sixteen layers, denoted from -7 to +8 mediate the interactions between the four helices (Fig. 5). The interactions are primarily hydrophobic, with the exception of layer 0 in the middle of the bundle where syntaxin and SNAP-25 possess glutamine residues that interact ionically with the arginine residue from synaptobrevin (Lin and Scheller. 2000). These residues are conserved among SNARE homologous and are responsible for the recent classification into Q-SNAREs and R-SNAREs (Fasshauer et. al, 1998). SNARE complexes are formed by coiled-coil interactions immediately before fusion (Sutton et al., 1998).

After the formation of the core complex, it serves as a receptor for SNAP and binds NSF where disassembly of the core complex by NSF ATPase activity occurs after the fusion of synaptic vesicles to the plasma membrane. Synaptotagmin then acts as a Ca^{2+} sensor in completing the fusion of secretory vesicles with the plasma membrane and followed by the release of their contents into the extracellular space. Subsequently, synaptotagmin acts as a nucleus for clathrin coat assembly by serving as an AP2 receptor (clathrin assembly complex) in which clathrin and/or dynamin bind to AP2 and this will trigger endocytosis, which completes the exocytosis cycle.

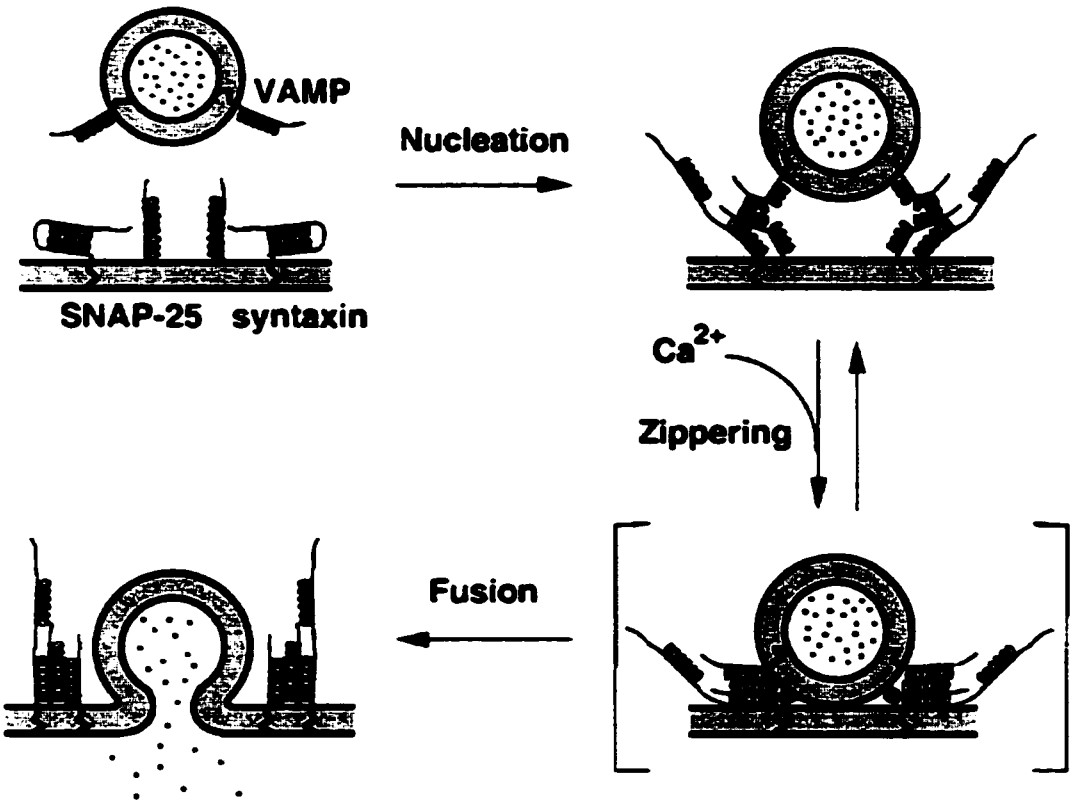

Fig 4. Model pathway of SNARE-mediated synaptic vesicle membrane fusion. Syntaxin is initially unbound to VAMP or SNAP-25. Nucleation of the ternary complex is the initial event in membrane fusion. Calcium induces further zippering of the parallel helices, bringing the vesicle and plasma membranes into contact. This transient intermediate state either reverses rapidly or proceeds forward to membrane fusion, with the energy for fusion provided by complex formation (Reprinted with permission from Annu Rev Cell Dev Biol, Lin RC, Scheller RH, Mechanisms of synaptic vesicle exocytosis. 16:19-49, 2000).

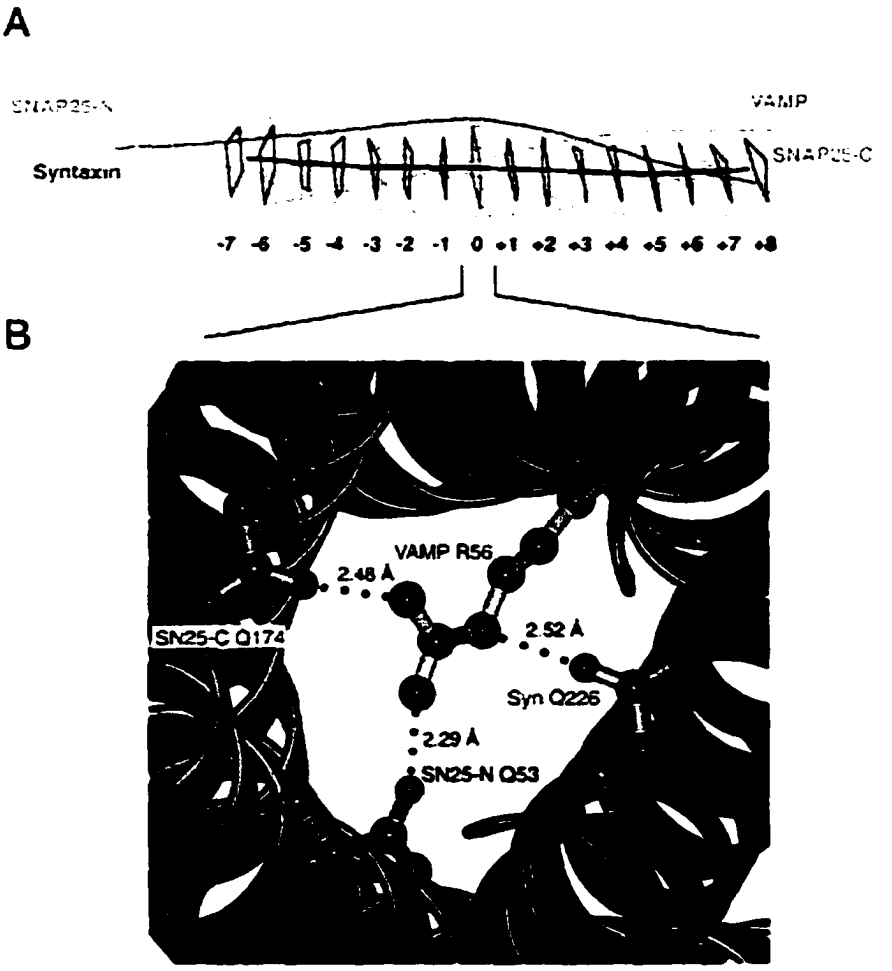

Fig 5. Structure of the synaptic SNARE core complex. (*a*) The complex is made up of four parallel helices: syntaxin H3 domain, VAMP coiled-coil domain, SNAP-25 amino-terminal helix, and SNAP-25 carboxy-terminal helix. Sixteen layers, from -7 to +8, mediate the interactions between the helices. (*b*) At layer 0, syntaxin and SNAP-25 contribute glutamine residues and VAMP contributes an arginine. Layer 0 possesses the greatest diameter of the layers and may be important in helix alignment or dissociation of the complex. Notice that because the coiled-coil domains of syntaxin and VAMP extend up to the transmembrane domains and align in a parallel fashion, the binding of syntaxin on the plasma membrane to VAMP on the vesicle would force the two membranes into apposition (Reprinted with permission from Annu Rev Cell Dev Biol, Lin RC, Scheller RH, Mechanisms of synaptic vesicle exocytosis. 16:19-49, 2000).

Regulation of hormone release by arginine vasopressin (AVP)

Biosynthesis and action of AVP

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 released from the posterior pituitary gland (Russell et al., 1990). AVP exerts a number of physiological roles in mammals; it plays a major role in regulating body fluid volume, osmolality and 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 and Colucci, 1991), vasoconstriction (Fox et al., 1987), and release of catecholamine (Grazzini et al., 1996), ACTH (Antoni et al., 1984; Laszlo et al., 2001), insulin (Dunning et al., 1984a,b; Chen et al., 1994) and glucagon (Yibchok-anun and Hsu, 1998; Yibchok-anun et al., 1999).

AVP receptors

The effects of AVP are mediated by two 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). V_{1a} receptor is the most widespread subtype of AVP receptors and has been found in vascular smooth muscle, myometrium, 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 and 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 rat pancreas (Saito et al., 1995). V_{1b} receptor 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). The V_2 receptor is found principally in cells of the renal collecting duct system.

Signal transduction of V_1 receptors

AVP receptors are G protein-coupled receptors (Mouillac et al., 1995). The V_1 receptors are coupled to G_q to activate phospholipase C (Michell et al., 1979; 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 signal transduction pathway of AVP has been investigated in various tissues, including smooth muscle, endothelium and endocrine cells (Spatz et al., 1994). V_{1b} receptors mediate AVP-induced ACTH release (Antoni et al., 1984 and Schosser et al., 1994), insulin release in the perfused rat pancreas and clonal β-cells (RINm5F) (Lee et al., 1995) and glucagon release in the perfused rat pancreas and clonal α-cells InR1G9 (Yibchok-anun and Hsu, 1998; Yibchok-anun et al., 1999). Generally, AVP binds to V_{1b} 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-β catalyzes the hydrolysis of phospatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol and inositol 1,4,5-trisphosphate (IP_3). Diacylglycerol (DAG) activates Protein kinase C (PKC) and IP_3 induces Ca^{2+} release from the endoplasmic reticulum, which leads to Ca^{2+} influx. Both Ca^{2+} release and Ca^{2+} influx contribute to AVP-induced insulin and glucagon release (Chen et al., 1994;

Yibchok-anun et al., 2000). AVP induces insulin (Chen et al., 1994) and glucagon (Yibchok-anun et al., 2000) release through Ca^{2+} –dependent and –independent pathways.

Effect of glucose on AVP-induced insulin and glucagon release

AVP may regulate glucose metabolism by its direct glycogenolytic and gluconeogenic effects on the liver (Kirk et al., 1979), and by increasing insulin and glucagon release from the endocrine pancreas. The fact that AVP is present in the pancreas (Amico et al., 1988) suggests that AVP may exert a local control on the endocrine pancreas. The effects of AVP on the endocrine pancreas, insulin release in particular, are controversial. AVP (18.5-185 pmol/L) caused a concentration-dependent stimulation of glucagon release but failed to influence insulin release from isolated rat islets in medium containing 5.6 mmol/L glucose (Dunning et al., 1984a). In isolated mouse islets, AVP failed to change insulin release at ≤ 7 mmol/L glucose but increased insulin at 10-30 mmol/L glucose (Gao et al., 1990; 1992). This implies that AVP is not a primary insulin secretagogue, but is an enhancer of glucose-induced insulin secretion. In the perfused rat pancreas, AVP (185 pmol/L) in 5.5 mmol/L glucose caused a small insulin release (Dunning et al., 1984b). In contrary, AVP inhibited glucose-induced insulin release in rat islets (Khalaf and Taylor, 1988) and in hamster beta cell line (HIT) (Richardson et al., 1990). Khalaf and Taylor (1988) showed that AVP (10 nmol/L) inhibited insulin release in the presence of 15 mmol/L glucose and was without effect in the presence of 5 mmol/L glucose. Richardson et al. (1990) showed that low doses of AVP (10 pmol/L) inhibited insulin release, while higher doses of AVP (100 pmol/L –100 nmol/L) increased insulin release. The inhibition of insulin release was not expected and was not explained. AVP (1-100 nmol/l) failed to stimulate

somatostatin release in the absence of extracellular glucose, however, AVP increased somatostatin release in the presence of glucose (3-30 mmol/L) (Gao et al., 1992). Since there are discrepancies in the effects of AVP on the endocrine pancreas, insulin release in particular, we investigated the direct effect of AVP (30 or 300 pmol/L) on insulin, glucagon and somatostatin release from the perfused rat pancreas in the presence of various glucose concentrations (0, 1.4, 5.5, or 20 mmol/L). We also investigated the direct effects of various glucose concentrations on insulin and glucagon release.

Role of cAMP-dependent protein kinase (PKA) in exocytosis

Changes in the intracellular cAMP concentration play an important role in the adjustment of cellular processes initiated by hormones, neurotransmitters and nutrients (Sutherland, 1972). The action of cAMP is mediated by the activation of cAMP-dependent protein kinase (PKA), which catalyzes the phosphorylation of a number of intracellular regulatory proteins including synapsin, rabphilin-3A, syntaxin and SNAP-25. In pancreatic β-cells, cAMP has been found to potentiate Ca^{2+} -dependent exocytosis (Wollheim et al., 1987; Gillis and Misler, 1993). This is likely to be the mechanism by which glucagon (Rasmussen et al., 1990), glucose-dependent insulinotropic polypeptide (Gremlich et al., 1995; Ding and Gromada, 1997) and glucagon-like peptide-1 (Thorens, 1995) enhance glucose-stimulated insulin release. The action of these hormones involves stimulation of adenylyl cyclase with resultant increase in intracellular cAMP concentration and activation of PKA. The ability of cAMP to stimulate insulin release can be prevented by Rp-cAMPS, a specific inhibitor of PKA (Wollheim et al., 1987). Experiments on permeabilized islets or β-cells have suggested that the principal action of cAMP is exerted at a site distal to the

elevation of $[Ca^{2+}]_i$, and using capacitance measurements to determine exocytosis showed that this effect accounted for up to 80% of the exocytosis (Ammala et al., 1993). cAMP stimulates exocytosis even when $[Ca^{2+}]_i$ is clamped at fixed concentrations and when the membrane potential is held too negative for voltage-dependent Ca^{2+} channels to open (Renstrom et al., 1997). Also elevation of intracellular cAMP concentration by forskolin, an adenylyl cyclase activator, evoked a PKA-dependent enhancement of exocytosis without increasing the $[Ca^{2+}]_i$ (Renstrom et al., 1997). Using capacitance measurements as a single-cell assay of exocytosis, cAMP –induced stimulation of exocytosis involves both increasing the release probability of secretory granules already in the RRP and by accelerating the refilling of this pool (Renstrom et al., 1997).

The ability of cAMP to stimulate exocytosis is not confined to the pancreatic β-cell, and it has been reported to enhance glucagon release from pancreatic α-cells (Gromada et al., 1997). Forskolin evoked PKA-dependent potentiation of exocytosis by: a) enhancement of Ca^{2+} influx through L-type channels, which accounted for less than 30% of total stimulatory effect, b) acceleration of granules mobilization resulting in increase in the number of RRP, which accounted for 70% of the effect (Gromada et al., 1997). In addition, cAMP enhances exocytosis in rat pituitary somatotrophs (Cuttler et al., 1993) and melanotrophs (Skider et al., 1990; Lee, 1996), bovine lactotrophs (Sikdar et al., 1990), dopamine-secreting PC –12 cells (Joseph and Kumar, 1995). In adrenal medulla, forskolin potentiates the secretory responses to nicotine, muscarine, bradykinin and histamine (Warashina, 1998). In these studies the stimulatory effect of cAMP/PKA was distal to further increase in $[Ca^{2+}]_i$. Skidar et al. (1998) investigated the putative modulation of unitary exocytosis events by cAMP in rat melanotrophs. They found an increase in the size of vesicles that fuse with plasma

membrane. Recently Machado et al. (2001) studied the role of cAMP/PKA on the late phase of exocytosis from single bovine chromaffin cells. They demonstrated that cAMP, probably acting on PKA, modulates the kinetics of exocytosis and increases the quantal size of secretory vesicles. Moreover, recruitment from RRP was depressed by inhibitors of cAMP/PKA and enhanced with their activator at Drosophila neuromuscular junctions (Kuromi and Kidokoro, 2000). The mobilization of vesicles was depressed with low cAMP, while it was enhanced with high cAMP (Kuromi and Kidokoro, 2000).

The interaction between PKA and Phospholipase C (PLC)

The heterotrimeric G proteins mediate a variety of cellular processes by coupling transmembrane receptors to different effector molecules, including adenylyl cyclase and phospholipase C-β (Simon et al., 1991; Neer, 1995). Activation of adenylyl cyclase results in the production of cAMP and activation of PKA. PLC catalyzes the hydrolysis of PIP_2 to generate DAG and IP_3, leading to activation of PKC and mobilization of $[Ca^{2+}]_i$, respectively. Interaction between the G protein-PLC pathway and PKA has been documented in numerous studies (Blackmore and Exton, 1986; Pittner and Fain, 1989a,b; Burgess et al., 1991; Rhee and Choi, 1992; Liu and Simon, 1996; Higashi et al., 1996; Rhee and Bae, 1997; Ali et al., 1998; Dodge and Sanborn, 1998; Yue et al., 1998). Although it is generally agreed that PKA can inhibit G protein-activated PLC activity (Rhee and Choi, 1992; Liu and Simon, 1996; Rhee and Bae, 1997; Ali et al., 1998; Dodge and Sanborn, 1998; Yue et al., 1998), it can enhance the G protein-PLC pathway in some cases (Blackmore and Exton, 1986; Pittner and Fain, 1989a,b; Burgess et al., 1991; Higashi et al., 1996).

Inhibition of PLC via PKA

The activation of PKA attenuates the PLC signaling pathway in a variety of cells. The proposed targets for phosphorylation includes cell surface receptors, G proteins, and PLC itself (Rhee and Bae, 1997). In human Jurkat T cells, activation of PKA results in an increase in phosphorylation of Ser^{1248} and a concomitant decrease in the tyrosine phosphorylation of PLCγ-1, the latter of which might be responsible for the decreased PLC activity apparent in Jurkat cells treated with PKA-stimulating agonists (Rhee and Choi, 1992). The interaction of PLC and PKA was studied in COS cells transfected with cDNAs encoding PLCβ-2, G protein subunits, and PKA (Liu and Simon, 1996). Expression of the catalytic subunit of PKA specifically inhibited $G_{\beta\gamma}$ stimulation of PLCβ-2 activity, without affecting G_q-induced activation. In addition, PKA directly phosphorylates serine residues of PLCβ-2 both in vivo and in vitro (Liu and Simon, 1996).

Phosphorylation of PLCβ-3 in response to a membrane-permeable cAMP analog 8-(4-chlorophenylthio)-cAMP (cpt-cAMP) treatment in rat basophilic leukemia (RBL-2H3) cells expressing only PLCβ-3 results in inhibition of $G_{\beta\gamma}$-stimulated PLCβ-3. PLCβ-3 activated by the $G_{\alpha i}$-coupled formylmethionylleucylphenylalanine (fMLP) receptor but had no effect on platelet-activating factor (PAF)-stimulated PLCβ-3 activity, presumably mediated by G_q (Ali et al., 1998). These studies led to the conclusions that phosphorylation of PLCβ-2 and PLCβ-3 by PKA could explain the inhibition of $G_{\beta\gamma}$-stimulated PI turnover by cAMP (Liu and Simon, 1996; Ali et al., 1998). In a human myometrial cell line (PHM1-41) and in COS-M6 cells overexpressing the oxytocin receptor, preincubation with cpt-cAMP (a cell permeable PKA activating agent), forskolin or relaxin inhibited oxytocin-stimulated

phosphatidylinositide turnover in PHM1-41 cells. The inhibition was reversed by H-89, a relatively specific protein kinase A inhibitor (Dodge and Sanborn, 1998). In PHM1-41 and COS-M6 cells, phosphorylation of PLCβ-3 Ser^{1105} by PKA results in a direct inhibition of G_q-stimulated PLCβ-3 activity, which can at least partially explain the inhibitory effect of PKA on $G\alpha_q$-coupled receptor-stimulated PI turnover observed in a variety of cells and tissues (Yue et al., 1998).

Stimulation of PLC via PKA

Although the activation of PKA attenuates the PLC signaling pathway in a variety of cells, PKA can stimulate the G protein-PLC pathway in some cases. Ethanol and AVP activate PLC in rat hepatocytes, generating IP_3 and causing an increase in $[Ca^{2+}]_i$ (Blackmore and Exton, 1986; Pittner and Fain, 1989a,b; Burgess et al., 1991; Higashi et al., 1996). The Ca^{2+} mobilization and IP_3 accumulation induced by ethanol and AVP are potentiated by cpt-cAMP (Higashi et al., 1996), although, agents that increase cAMP levels have no significant effects on basal inositol phosphate formation, but enhance AVP-induced inositol phosphate formation (Pittner and Fain, 1989a,b). Thus, at least two mechanisms might be involved: first, PKA potentiates the agonist-induced IP_3 formation (Blackmore and Exton, 1986; Pittner and Fain, 1989a,b; Higashi et al., 1996), second, PKA potentiates the Ca^{2+} release from IP_3-sensitive Ca^{2+} stores in the cell by making the receptor more responsive to lower levels of IP_3 (Burgess et al., 1991).

Exocytotic proteins activation/deactivation and modulation of exocytosis

Mechanism of granules mobilization

The individual vesicle cycles and the transitions of vesicles between cycles are subject to extensive regulation, and Ca^{2+} mediates most of this regulation (Pyle et al., 2000). The control of synaptic vesicle cycle by increasing the number of vesicles in RRP through mobilization of vesicles from the RP (regulation of the releasable pools), or by increasing the number of docked vesicles that are ready for fusion (release probability of docked vesicles) is one way to regulate neurotransmitter release (Turner et al., 1999). The filling of the RRP, measured as recovery from depletion, is enhanced by elevated $[Ca^{2+}]_i$ (Von Ruden and Neher, 1993). Protein Kinase C (PKC) (Gillis et al., 1996, Stevens and Sullivan, 1998;; Smith et al., 1998; Sudhof, 2000) and PKA (Renstrom et al., 1997) are also involved in the regulation of the size of RPP, which means the transfer of vesicles from the resting and reverse pools to reverse and RRP pool, respectively.

The RRP is thought to correspond to the number of SVs docked morphologically to the presynaptic membrane that can be released (Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997). Morphological data have demonstrated that SVs in the RP are organized in clusters in which the SVs are reversibly linked to the actin-based cytoskeleton, by a family of abundant SVs -associated phosphoproteins, the synapsins (Landis et al., 1988; Bernstein and Bamburg, 1989; Hirokawa et al., 1989; Benfenati et al., 1993; Kuromi and Kidokoro, 1998). Synapsins are a family of phosphoproteins of SVs (Greengard, 1987). Three genes for synapsins have been described. The synapsin I gene, which produces alternatively spliced transcripts encoding synapsins Ia and Ib, and the synapsin II gene encoding synapsins IIa and IIb (Sudhof et al., 1989). Sequence comparisons revealed that synapsins are composed of a

mosaic of domains. All synapsins share a short amino-terminal domain (A-domain), a linker sequence (B-domain) that is rich in short-chain amino acids (proline/alanine/ glycine/ serine), and a large central domain (C-domain) that comprises approximately one-half of the total synapsin sequences (Sudhof et al., 1989). After the C-domain, different combinations of domains are observed (the D-, E-, F-, and G-domains). At the very carboxyl terminus synapsins Ia and IIa contain an additional short common domain of 50 residues (the E-domain) (Sudhof et al., 1989). The E-domain is absent from synapsins Ib and IIb. Synapsin III, the newest member of the synapsins family was identified recently and is present in rat, mouse and human brains and predominantly expressed in neurons (Hosaka and Sudhof, 1998; Kao et al., 1998). Synapsin III is closely similar to synapsins Ia and IIa, with the highest similarity observed in the A-, C-, and E-domains (Hosaka and Sudhof, 1998). Therefore, synapsin III is called synapsin IIIa.

Granules mobilization due to synapsins activation

The synapsins are known to associate with the regulation of neurotransmitter release, synaptic plasticity and synaptogenesis (Llinas et al. 1985; Han et al., 1991; Ferreira et al., 1994; Rosahl et al., 1995). Synapsins have been proposed to play a major structural role in the assembly and maintenance of the RP of SVs as well as a dynamic role in controlling SVs transitions from the RP to the RRP (Greengard et al., 1993; Brodin et al., 1997; Benfenati et al., 1999). Synapsin I is associated with the regulation of neurotransmitter release, whereas synapsin II is known to be related not only to neurotransmitter release but also to synaptogenesis and synaptic plasticity (Ferreira et al., 1994; Rosahl et al., 1995). Synapsin I links SVs to the cytoskeleton, thus regulating the availability of SVs for exocytosis

(Benfenati et al., 1993, Ceccaldi et al., 1995). No study has yet examined whether synapsin II is associated with the SVs in the neurohypophysis, thus it remains unknown whether synapsin II plays a significant role in the regulation of SVs in the neurosecretory cells (Nomura et al., 2000). Recently, Sugiyama et al. (2000) showed that the number of releasable SVs were increased by synapsin II transfection into NG108-15 cells when compared to the control cells, which suggests that synapsin II may be involved in the regulation of SVs number in presynapsin-like structure NG108-15. However more studies are needed to elaborate the role of synapsin II in the regulation SVs.

Several studies have suggested distinct roles for synapsin I and synapsin II during neuronal development. In cultured hippocampal neurons, synapsin I plays a major role in axonal elongation and branching (Chin et al., 1995), whereas synapsin II plays a major role in the initial elongation of undifferentiated processes (Ferreira et al., 1994, 1998). A recent study demonstrates that synapsin III has a role during development distinct from those of synapsins I and II (Ferreira et al., 2000).

Protein kinases have an important role in mediating the release of SVs from the RP to RRP. Synapsin I and II are phosphorylated by both PKA and CaMK II (Nicol et al., 1997; Hosaka and Sudhof, 1999). Although PKA can phosphorylate synapsin I and II, the phosphorylation of synapsin I by CaMK II that is considerd to be the main way to mediate the release of synaptic vesicles (Turner et al., 1999). Therefore, the association of synapsin I with SVs has been characterized in detail.

The introduction of the dephosphorylated form of synapsin I into the squid giant axon (Llinas et al., 1985; Lin et al., 1990), the gold fish Mauthner neuron (Hackett et al., 1990), or nerve terminals isolated from mammalian brain (Nichols et al., 1992) binds SVs and inhibits

neurotransmitter release. In contrast, introduction of synapsin I after its phosphorylation has no effect on neurotransmitter release (Greengard et al., 1993). The phosphorylation of synapsin I by CaMK II results in removal of the inhibition of the interaction between SVs and plasma membranes and thereby induces concomitant changes in Ca^{2+}-dependent neurotransmitter release. (Matsumoto et al., 1990). These results indicate that the dephosphorylated form of synapsin I provides an inhibitory constraint for SVs exocytosis and that constraint is relieved upon phosphorylation of synapsin I (Greengard et al., 1993). Synapsin I is able to bind in vitro with various cytoskeletal proteins, including actin (Baines and Bennett, 1986; Bahler and Greengard, 1987). This indicates that synapsin I reversibly connects SVs to the actin-based cytoskeleton and that linkage is disrupted by phosphorylation (Benfenati et al., 1993, Ceccaldi et al., 1995; Hosaka et al., 1999). The binding of synapsin I to actin filaments leads to the formation of actin bundles in vitro and phosphorylation of synapsin I reduces actin binding and abolishes actin-bundling activity (Bahler and Greengard, 1987; Petrucci and Morrow, 1987). Moreover, microinjection of recombinant synapsin protein (domin E) inhibits exocytosis (Augustine et al., 1999; Ilrdi et al, 1999) and attenuates the supply of vesicles from the RP (Augustine et al., 1999). Thus, synapsin is involved in the release of SVs from the RP to refill the RRP pool thereby preventing its depletion by exocytosis, in addition to its role in vesicle fusion (Fig. 5) (Augustine et al., 1999). Synapsin I is phosphorylated at its N-terminus by CaMK II and PKA (Sudhof, 1995) and has been identified in adrenal medulla, adrenal chromaffin cells, rat islets, insulinoma cells including MIN6 and BTC3, and glucagon-secreting cells including α-TC and InR1G9 cells (Matsumoto et al., 1999). The role of synapsin I in pancreatic β-cells is controversial. While some studies suggested that synapsin I is co-localizes with insulin

secretory granules (Matsumoto et al., 1999; Tabuchi et al., 2000), other studies suggested that synapsin I is not co-localized with insulin secretory granules, and thus may not play a role in granule mobilization (Krueger et al., 1999).

In summary, under resting conditions, in which synapsin I is in the dephosphorylated state, most of the secretory vesicles are anchored to the cytoskeleton. Under neuroendocrine activity, in which synapsin I is in the phosphorylated state, the complex composed of SVs, synapsin I and actin dissociates and lead to liberation of more SVs from the RP to RRP (Greengard et al., 1993). Therefore, phosophorlation of synapsin I plays a key role in the regulation of the SVs and the transition from the RP to RRP.

Phosphorylation of exocytotic proteins

Several lines of evidence suggest that phosphorylation of exocytotic proteins plays a major role in modulating neuroendocrine release. However, the mechanisms by which protein kinases regulate neuroendocrine release are largely unknown. PKA, and CaMK II have been shown to phosphorylate a wide variety of vesicle proteins. Several protein kinase substrates have been characterized to function in the alteration of synaptic strength (synaptic plasticity) (see Sudhof, 1995 for review).

This review will focus on the proteins that are known to be phosphorylated by PKA and CaMK II. In addition to synapsin I, PKA and CaMK II phophorylate other exocytotic proteins including: **Rabphilin-3A**, a synaptic vesicle protein, involved in both the docking and endocytosis of synaptic protein (Burns et al., 1998; Augustine et al., 1999). It is an important target of CaMK II and PKA (Lonart et al., 1998; Fykse et al., 1995), which is phosphorylated at the NH_2-terminal (Burns et al., 1998). Rabphilin-3A binds to the vesicular

GTP-binding protein rab3A and maintains it in this state (Burns et al., 1998). Microinjection of recombinant rabphilin-3A protein in squid giant synapses inhibits exocytosis and causes a relative accumulation of vesicles that may be awaiting docking (Burns et al., 1998; Augustine et al., 1999). Thus Raphilin-3A has multiple functions in the synaptic vesicle trafficking; it plays a role in membrane budding during endocytosis and also is involved in vesicle docking (Fig. 6) (Augustine et al., 1999).

Syntaxin and SNAP-25 are plasma membrane proteins that bind tightly to each other and form a high-affinity site for synaptobrevin/VAMP to form a core complex (SNAREs) (Sudhof, 1995). Microinjection of clostridial toxin and several other reagents that prevent SNAREs from interacting inhibits neurotransmitter release (Augustine et al., 1999). Syntaxin and SNAP-25 are phosphorylated by CaMK II and PKA (Trudeau et al., 1998; Foster et al., 1998). SNAP-25 has been shown to be expressed in pancreatic islets and in pancreatic α- and β-cells (Sadoul et al., 1995). Botulinum neurotoxin (BoNT) A and E cleave SNAP-25 (Sadoul et al., 1995), while BoNT C1 cleaves syntaxin and leads to inhibition of Ca^{2+}-dependent insulin release from permeabilized HIT cells (Wheeler et al., 1996; Land et al., 1997). These findings suggest that SNAP-25 and syntaxin play a role in exocytosis of endocrine large-dense core granules.

Study of single cell exocytosis using fluorescent dye FM1-43

Several techniques have been developed to detect hormonal release. Radioimmunoassay is the mostly common used method to detect low concentrations of hormones such as insulin and glucagon. However, this method does not provide details with regards to the events of single cell exocytosis, endocytosis and synaptic vesicles recycling.

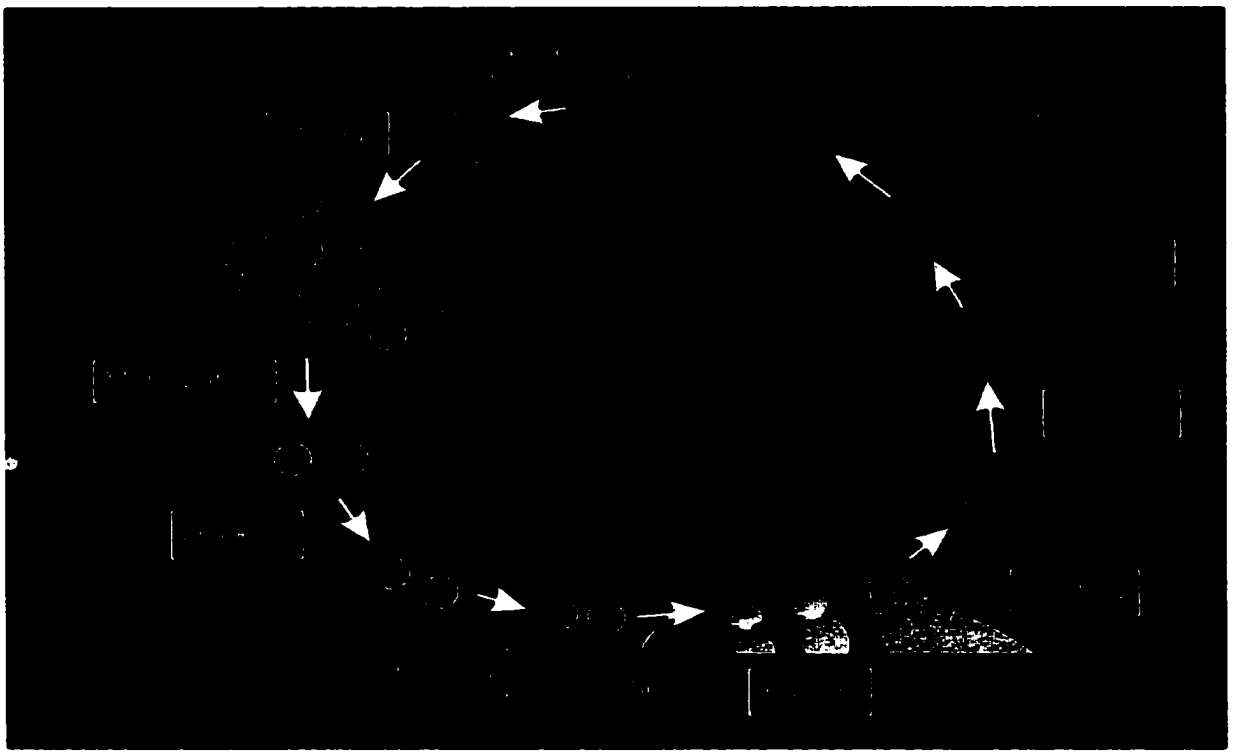

Fig 6. A model for the control of membrane trafficking reactions by presynaptic proteins (Reprinted with permission from J Physiol, Augustine GJ, Burns ME, DeBello WM, Hilfiker S, Morgan JR, Schweizer FE, Tokumaru H, Umayahara K, Proteins involved in synaptic vesicle trafficking. 520: 33-41, 1999).

Other methods have been developed to study single cell exocytosis including: electrophysiological measurements (cell membrane capacitance), amperometry and optical measurements using fluorescence imaging. Electrophysiological measurements of exocytosis using cell membrane capacitance measurements by the whole cell patch-clamp provides millisecond time resolution and measure the changes in the cell membrane area. This method has revealed three functionally distinct vesicle pools that give rise to multiple secretory phases (Murthy and Stevens, 1999). Amperometry is useful in monitoring secretion from single cells, even at the level of single exocytotic events, because it allows detection of attomole quantities with millisecond temporal resolution (Wightman et al., 1991; Chow et al., 1992). Amperometry has been applied to β-cells either by using a ruthenium oxide/cyanoruthenate-coated microelectrode to directly detect insulin (Huang et al., 1995; Aspinwall et al., 1997) or by using a carbon fiber microelectrode to detect 5 - hydroxytryptamine that had been allowed to accumulate in the secretory vesicles (Smith et al., 1995; Zhou and Misler, 1996). A limitation of amperometry is that a microelectrode is inherently a single-point sensor, meaning that measurements are made at one location on a cell with a detection area determined by the size of the electrode

Recent advances in fluorescence imaging have resulted in new approaches to monitor exocytosis with a non-invasive method. Styryl dyes, which were originally developed as membrane potential sensors, become a useful tool in the study of exocytosis, endocytosis and synaptic vesicle recycling (Betz et al, 1996; Cochilla et al, 1999). This is due to their ability to reversibly stain membranes, their inability to penetrate membranes, and their fluorescence (Betz et al, 1996; Cochilla et al, 1999). FM1-43 is one of the most extensively used styryl dyes to study secretory activity (Cochilla et al, 1999). It is non-fluorescent in aqueous

solution, but became fluorescent and its quantum yield increases by ~ 350 times when incorporated into the plasma membrane (Betz et al, 1992). Therefore, the fluorescence intensity is proportional to the amount of membrane exposed to FM1-43. Upon stimulation, exocytosis causes the vesicular membrane to fuse with the plasma membrane, and the dye binds to the exocytosing membrane resulting in an increase in the fluorescence intensity. The fluorescence intensity can be used as an index for RRP size (Sudhof, 2000).

We used the fluorescent styryl dye FM1-43 to investigate the mechanisms by which cAMP/PKA enhances AVP-induced glucagon release from clonal α-cells InR1G9 to determine if this enhancement is due to an increase in the number of secretory granules in RRP.

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CHAPTER II. GLUCOSE-DEPENDENCY OF ARGININE VASOPRESSIN-INDUCED INSULIN AND GLUCAGON RELEASE FROM THE PERFUSED RAT PANCREAS

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ABSTRACT

The study purpose was to investigate the glucose dependency of arginine-vasopressin (AVP)-induced insulin, glucagon and somatostatin release from the perfused rat pancreas. AVP (30 or 300 pmol/L) was tested in the presence of glucose concentrations of 0, 1.4, 5.5 (basal level), or 20 mmol/L. The rates of insulin release at 0 and 1.4 mmol/L glucose were ~70-80% and ~60-70% less, respectively, than that at the baseline level. AVP (30 or 300 pmol/L) failed to change insulin release at 0 and 1.4 mmol/L glucose. At the basal glucose level, AVP (300 pmol/L) induced a biphasic insulin release, a peak followed by a sustained phase. In addition, the combination of glucose (20 mmol/L) and AVP (300 pmol/L) induced a higher insulin peak and sustained phase than 20 mmol/L glucose alone. The rates of glucagon release at 0 and 1.4 mmol/L glucose were ~3- and ~2-fold more, respectively, than that at the baseline glucose level. At 0 and 1.4 mmol/L glucose, both 30 and 300 pmol/L AVP caused a higher glucagon peak and sustained phase than 0 and 1.4 mmol/L glucose alone. At the basal glucose level, AVP (30 or 300 pmol/L) induced a biphasic glucagon release, a peak followed by a sustained phase. The rate of glucagon release at 20 mmol/L glucose was ~60-70% less than that at the baseline level. When AVP (300 pmol/L) was administered in 20 mmol/L glucose, it induced a transient glucagon peak, which was 2.4-fold of the baseline level. At all glucose concentrations tested, AVP (30 or 300 pmol/L) failed to change somatostatin release. These results suggested that 1) hypoglycemia directly increases glucagon and decreases insulin release, 2) AVP-induces insulin and glucagon release by a direct action on β- and α-cells, respectively, 3) AVP- induces insulin and glucagon release in a glucose-dependent manner; the higher the glucose concentration, the greater the enhancement of AVP-induced insulin release. In contrast, the lower the glucose

concentration, the greater the enhancement of AVP-induced glucagon release, and 4) α-cells are more sensitive to AVP than β-cells in hormone release.

KEY WORDS

Arginine vasopressin; Insulin; Glucagon; Somatostatin; Perfused pancreas; Glucose-dependency.

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 released from the posterior pituitary gland.[1] AVP is also found in extrapituitary tissues including adrenal gland[2] and pancreas.[3] AVP exerts a number of physiological roles in mammals; it plays a major role in regulating body fluid volume, osmolality and maintenance of blood pressure. In addition, AVP induces glycogenolysis[4], proliferation of the pituitary gland [5] and vascular smooth muscle cells[6], vasoconstriction[7], release of catecholamine[9], insulin[10,11] and glucagon [12,13]

AVP stimulates insulin and glucagon release via the activation of both phospholipase C (PLC)-dependent and –independent pathways.[11-15] V_{1B} receptors mediate AVP-induced insulin release in the perfused rat pancreas and clonal β-cells RINm5F[16], and glucagon release in the perfused rat pancreas and clonal α-cell line InR1G9.[12,13] AVP induces insulin[11] and glucagon[17] release through Ca^{2+} –dependent and –independent pathways. For the Ca^{2+} –dependent pathway, G_q protein activates phospholipase C, which catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol and inositol 1,4,5-

triphosphate (IP_3). Diacylglycerol activates protein kinase C and IP_3 induces Ca^{2+} release from the endoplasmic reticulum, which leads to Ca^{2+} influx. Both Ca^{2+} release and Ca^{2+} influx contribute to AVP-induced insulin and glucagon release.[11,17]

AVP may regulates glucose metabolism by its direct glycogenolytic and gluconeogenic effects on the liver[4], and by modulating insulin and glucagon release from the endocrine pancreas. The fact that AVP is present in the pancreas[3] suggests that AVP may exert a local control on the endocrine pancreas. The effects of AVP on the endocrine pancreas, insulin release in particular, are controversial. AVP caused a concentration-dependent stimulation of glucagon release but failed to influence insulin release from isolated rat islets in medium containing 5.6 mmol/L glucose.[18] In isolated mouse islets, AVP failed to change insulin release at ≤ 7 mmol/L glucose.[19,20] This implies that AVP is not a primary insulin secretagogue, but is an enhancer of glucose-induced insulin secretion. In the perfused rat pancreas, AVP in 5.5 mmol/L glucose caused a small insulin release.[10] In contrast, AVP inhibited glucose-induced insulin release in rat islets[21] and in clonal β-cells HIT-T15.[22] AVP (1-100 nmol/l) failed to stimulate somatostatin release in the absence of extracellular glucose, however, AVP increased somatostatin release in the presence of glucose (3-30 mmol/L).[20] Since there are discrepancies in the effects of AVP on the endocrine pancreas, insulin release in particular, the present study was designed to study the direct effect of AVP (30 or 300 pmol/L) on insulin, glucagon and somatostatin release from the perfused rat pancreas in the presence of various glucose concentrations (0, 1.4, 5.5, or 20 mmol/L). We also investigated the direct effects of various glucose concentrations on insulin and glucagon release.

MATERIALS AND METHODS

Male Sprague-Dawley rats (350-450 g) were randomly used. The rats were anesthetized with pentobarbital sodium (60 mg/kg, intraperitoneally), and were maintained at 37°C on a hot plate during the experiment. Pancreatic perfusion was performed as previously described.[24] Briefly, after cannulation of the celiac artery, the rat pancreas was immediately perfused with Krebs-Ringer bicarbonate (KRB) solution supplemented with 20 mmol/L HEPES, 5.5 mmol/L glucose, 1% dextran, and 0.2% bovine serum albumin as a basal medium. The KRB was maintained at pH 7.4 and continuously aerated with 95% O_2 - 5% CO_2. The rats were euthanatized by the induction of pneumothorax immediately following the cannulation of the portal vein and the beginning of the flow.

Experimental Design

The first 20 minutes of perfusion was considered as an equilibration period. Subsequently, the effluent fluid was collected every minute from the cannula in the portal vein. The flow rate was set at 1 ml/minute. After a baseline period of 12 minutes, the medium containing glucose (0, 1.4, 5.5, or 20 mmol/L) was administered for 30 minutes with or without AVP (30 or 300 pmol/L). This was followed by a washout period during which the basal medium was administered for 10 minutes. AVP of 30 and 300 pmol/L were chosen because AVP (3 pmol/L) caused a small increase in glucagon release and failed to increase insulin release. The effluent fluids were kept at 4°C and assayed within 6 hours for insulin, glucagon and somatostatin using radioimmunoassys as previously described.[12,24,25]

Test Agents

AVP was purchased from Sigma Chemical Co. (St. Lous, MO), and dissolved in distilled water to make a stock solution (100 μmol/L). This solution was further diluted with KRB (basal medium) to attain appropriate concentrations. Glucagon and rat insulin standards were donated by Eli Lilly laboratories (Indianapolis, IN). Insulin antibody was donated by Dr. V. Leclercq-Meyer of the Free University of Brussels, Belgium. Glucagon antibody was donated by Dr. Joseph Dunbar of Wayne State University (Detroit, MI). Somatostatin antibody was donated by Dr. Y.N. Kenny Kwok of the University of British Columbia, Canada. ^{125}I-glucagon was purchased from Linco Research (St. Charles, MO). Somatostatin standard was purchased from Sigma Chemical Co. (St. Lous, MO).

Data Expression and Statistical Analysis

The effluent concentrations of insulin, glucagon, and somatostatin were expressed as a percentage of the baseline level (mean of last 5 baseline values) in mean ± SE. The area under the curves (AUCs) for the different concentrations of glucose with or without AVP treatment period of 30 minutes was calculated using the Transforms and Regressions (Sigma Plot 5.0; SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) was used to determine the effect of glucose concentrations on insulin and glucagon AUCs. Two-way ANOVA was used to determine the effect of AVP and glucose concentrations on insulin and glucagon AUCs. Individual mean comparisons were calculated using the *F* test. The significance level was set at $P < .05$.

RESULTS

Effects of glucose on Insulin and Glucagon Release

Insulin release remained constant in the basal glucose control group (5.5 mmol/L) throughout the perfusion period (Fig 1A). In the absence of extracellular glucose or the presence of low glucose (1.4 mmol/L), insulin secretion was ~ 70-80% and ~ 60-70% less than that at the baseline level, respectively (Fig 1A). By calculating the AUCs for 0 and 1.4 mmol/L glucose groups and comparing them with those of basal glucose group, both 0 and 1.4 mmol/L glucose had a significantly lower insulin release than the basal group. The higher concentration of glucose (20 mmol/L) induced a biphasic insulin release. The first transient insulin release peak was ~ 6-fold of the baseline level, followed by a sustained increase that was ~ 9-fold of the baseline level (Fig 2A). By calculating the AUCs for 20 mmol/L glucose group and comparing them with those of the basal glucose group, 20 mmol/L glucose significantly increased insulin release.

Glucagon release remained constant in the basal glucose control group throughout the perfusion period (Fig 1B). In the absence of extracellular glucose or the presence of 1.4 mmol/L glucose, glucagon release was ~ 3- and ~ 2-fold higher than that at the baseline level, respectively (Fig 1B). Relative to the level at the basal glucose concentration, glucagon release was significantly higher at the low glucose concentrations (0 and 1.4 mmol/L). The higher concentration of glucose (20 mmol/L) significantly inhibited glucagon release, which was ~60-70% less than that at the baseline level (Fig 1B). Relative to the level at the basal glucose concentration, glucagon release was significantly lower at the high glucose concentrations (20 mmol/L).

Effects of AVP on Insulin Release

At the basal glucose level AVP (30 pmol/L) failed to change insulin release, while AVP (300 pmol/L) induced a biphasic insulin release; a peak followed by a sustained phase (Fig 2C). By calculating the AUCs for the AVP (300 pmol/L) group and comparing them with those of the basal glucose group, AVP (300 pmol/L) significantly increased insulin release. Neither 30 nor 300 pmol/L AVP significantly increased insulin release at 0 and 1.4 mmol/L glucose (Fig 2A and B).

AVP (300 pmol/L) at 20 mmol/L glucose increased insulin release with a peak and a sustained phase of ~15- and ~25-fold of the baseline level, respectively (Fig 2D). By calculating the AUCs for AVP (300 pmol/L) in the 20 mmol/L glucose group and comparing them with those of the 20 mmol/L glucose alone group, AVP (300 pmol/L) in 20 mmol/L glucose significantly had a higher insulin release effect than did glucose alone. The insulin release induced by AVP (30 pmol/L) at 20 mmol/L glucose was not significantly different from that induced by 20 mmol/L glucose alone (Fig 2D). A summary of AVP (30 and 300 pmol/L)-induced insulin release in various glucose concentrations is shown in Fig 4A, which demonstrates that the insulinotropic effect of AVP is glucose-dependent.

Effects of AVP on Glucagon Release

At the basal glucose level, both 30 and 300 pmol/L AVP increased glucagon release; a peak followed by a sustained phase (Fig 3C). AVP 30 pmol/L increased glucagon release with a peak and a sustained phase of ~2-and ~0.5-fold of the baseline level, respectively, whereas AVP 300 pmol/L caused a peak of ~5- and a sustained phase of ~3-fold of the baseline level, respectively (Fig 3C). By calculating the AUCs for AVP (30 and 300 pmol/L)

groups and comparing them with those of basal glucose group, both 30 and 300 pmol/L AVP significantly increased glucagon release.

In the absence of extracellular glucose, AVP (30 pmol/L) increased glucagon release with a peak and a sustained phase of ~5- and ~10-fold of the baseline level, respectively, whereas AVP (300 pmol/L) caused a peak and a sustained phase of ~11- and ~35-fold of the baseline level, respectively (Fig 3A and B). In the presence of 1.4 mmol/L, AVP (30 pmol/L) increased glucagon release with a peak and a sustained increase of ~5- and ~7-fold of the baseline level, respectively, whereas AVP (300 pmol/L) caused a peak and a sustained phase of ~5- and ~21-fold of the baseline level, respectively (Fig 3A and B). By calculating the AUCs for AVP (30 and 300 pmol/L) in 0 or 1.4 mmol/L glucose groups and comparing them with those of the 0 or 1.4 mmol/L glucose groups, both 30 and 300 pmol/L AVP significantly had higher glucagon release than 0 or 1.4 mmol/L glucose alone.

The higher concentration of glucose (20 mmol/L) significantly inhibited glucagon release, under this conditions, AVP (30 pmol/L) failed to change glucagon release (Fig 3D). However, AVP (300 pmol/L) at 20 mmol/L glucose induced a significant glucagon release for 5 minutes when compared to the glucose alone group (Fig 3D). A summary of AVP (30 and 300 pmol/L)-induced glucagon release in various glucose concentrations is shown in Fig 4B, which demonstrates that the glucagonotropic effect of AVP is glucose-dependent.

Effects of AVP on Somatostatin Release

Neither the various concentrations of glucose (0, 1.4, 5.5, or 20 mmol/L) alone, nor AVP (30 or 300 pmol/L) in glucose (0, 1.4, 5.5, or 20 mmol/L) changed somatostatin release from the perfused pancreas (data not shown).

DISCUSSION

In the present study, AVP evoked both the release of insulin and glucagon from the perfused rat pancreas in a glucose concentration-dependent manner. AVP (30 and 300 pmol/L) failed to induce insulin release at glucose concentrations of 0 and 1.4 mmol/L. However, in the presence of basal glucose level (5.5 mmol/L), AVP (300 pmol/L) increased insulin release, and it induced a greater increase in insulin release at 20 mmol/L glucose. These findings suggested that the higher the glucose concentration, the greater the enhancement of AVP-induced insulin release. In mouse islets, AVP (100 nmol/L) in the presence of 0, 3, or 7 mmol/L glucose failed to induce insulin release, while at higher glucose concentrations (10-30 mmol/L), AVP (1-100 nmol/L) enhanced glucose-induced insulin release.[20] These authors concluded that AVP is not an initiator of insulin release but only enhanced glucose-induced insulin release. In contrast, our study demonstrated that lower concentration of AVP (30 pmol/L) not only enhanced glucose-induced insulin release, but also initiated insulin release at the basal level of glucose. The discrepancy between these findings may be attributed to the use of two different preparations. In our study we used the perfused pancreas, which requires a much less invasive procedure than the use of isolated islets, in which the pancreas remains intact. In addition, Gao et al.[20] preincubated the mouse islets for 60 minutes to permit the recovery from the isolation procedure. This period of incubation may not be sufficient, because a minimum incubation of 24-48 hours is essential for receptors recovery.[26] This might also explain why in the mouse islets high concentrations of AVP were used to demonstrate AVP's effects and why AVP failed to initiate insulin release.

We also investigated the effects of different glucose concentrations on insulin and glucagon release from the perfused rat pancreas. The rate of insulin release in the presence of 0 and 1.4 mmol/L glucose was ~70-80% and ~60-70% less, respectively, than the baseline level. The basal glucose concentration (5.5 mmol/L), maintained a constant level of insulin through out the perfusion period, whereas a higher glucose concentration (20 mmol/L) induced a biphasic release pattern; a transient peak followed by a sustained phase with a greater magnitude. Similar findings have been reported in the perfused rat pancreata[27,28] and human islets[29,30]. On the other hand, the rates of glucagon release in the presence of 0 and 1.4 mmol/L glucose was ~3- and ~2-fold more, respectively, than the baseline level. During hypoglycemic stress, glucagon release is increased and insulin release is decreased. Increased glucagon release is the main counterregulatory factor in the recovery of hypoglycemia[31,32]. However, the mechanism underlying hypoglycemia-induced glucagon release is uncertain. A number of studies suggest that the increase in glucagon release is due to the activation of the autonomic nervous system[33-36]. In contrast, our results suggested that a low glucose level directly increases glucagon release, since these experiments were performed in isolated perfused pancreas where there was no input from the autonomic nervous system. This finding is consistent with results observed in the isolated islets[36] and perfused pancreata.[37] The basal glucose concentration maintained a constant level of glucagon through out the perfusion period, whereas a higher concentration of glucose (20 mmol/L) inhibited glucagon release, which was ~60-70 % less than the baseline level.

AVP (30 and 300 pmol/L) increased glucagon release in the presence of 0, 1.4, or 5.5 mmol/L glucose, whereas only 300 pmol/L AVP increased glucagon release at 20 mmol/L glucose. Thus, this increase was in a glucose concentration-dependent manner; the lower the

glucose concentration, the greater the enhancement of AVP-induced glucagon release. In experiments using mouse islets, as expected, AVP induced a large increase in glucagon release at 0 glucose and less glucagon release in the presence of glucose concentrations of 3-7 mmol/L.[20] Surprisingly, in mouse islets at higher glucose concentrations (15-20 mmol/L), AVP increased glucagon release from the mouse islets to the same extent as at 0 glucose.[20] We failed to observe such changes in the perfused rat pancreas. AVP (3-30 pmol/L) increased glucagon but not insulin release from the perfused rat pancreas (our unpublished data). These findings suggested that α-cells are more sensitive to AVP than β-cells in hormone release. Our findings are consistent with those obtained from the perfused rat pancreas[10] and isolated rat islets[18] and different from those obtained from the mouse islets.[20] In addition, these findings indicated that AVP may physiologically increase glucagon release, since AVP increased glucagon release at physiological concentrations of AVP plasma level (≤30 pmol/L)[39], while it failed to increase insulin release at these concentrations.

AVP increased insulin and glucagon release but did not affect somatostatin release. This precludes that somatostatin may influence AVP- or glucose-induced insulin and glucagon release. The findings that AVP induced a large increase in glucagon release at low glucose concentrations without changing insulin release supports the notion that AVP has a direct effect on α-cells. In addition, the higher glucose concentration (20 mmol/L) inhibited glucagon release, which was ~60-70% less than the baseline level. Under these conditions, AVP (30 pmol/L) failed to change glucagon release, whereas administration of AVP (300 pmol/L) only induced a transient glucagon release. On the other hand, AVP (300 pmol/L) induced a large increase in insulin release, which supports the notion that AVP has a direct effect on β-cells.

In conclusion, AVP may increase insulin and glucagon release by a direct action on β- and α-cells, respectively. These increases are glucose-dependent; the higher the glucose concentration, the greater the enhancement of AVP-induced insulin release. In contrast, the lower the glucose concentration, the greater the enhancement of AVP induced glucagon release. AVP not only can enhance glucose-induced insulin release, but also can initiate insulin release. α-cells are much more sensitive to AVP than β-cells in hormone release. Furthermore, our results confirmed the previous findings that hypoglycemia directly increases glucagon and decreases insulin release

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Fig 1. Effect of glucose (0, 1.4, 5.5, and 20 mmol/L) on insulin (A) and glucagon (B) release from the perfused rat pancreas. In this and all other figures, basal glucose (5.5 mmol/L) was administered during the equilibration period of 20 minutes preceded time 0 followed by another 12 minutes of the baseline period. Various concentrations of glucose were given for 30 minutes (glucose treatment), followed by 8 minutes of the washout period (wash). By calculating the areas under the curves for glucose (0, 1.4, and 20 mmol/L) and comparing them with those of the control group (5.5 mmol/L glucose), both 0 and 1.4 mmol/L glucose significantly ($P < .05$) decreased insulin release, whereas 20 mmol/L glucose significantly increased insulin release. In contrast, both 0 and 1.4 mmol/L glucose significantly increased glucagon release, whereas 20 mmol/L glucose significantly decreased glucagon release. Values are the mean ± SE (n=4). □, Basal control (5.5 mmol/L glucose); ○, 0 glucose; ▽, 1.4 mmol/L glucose; ◇, 20 mmol/L glucose. Range of baseline insulin and glucagon concentrations of effluents were 1045-2507 and 50-68 pg/ml, respectively.

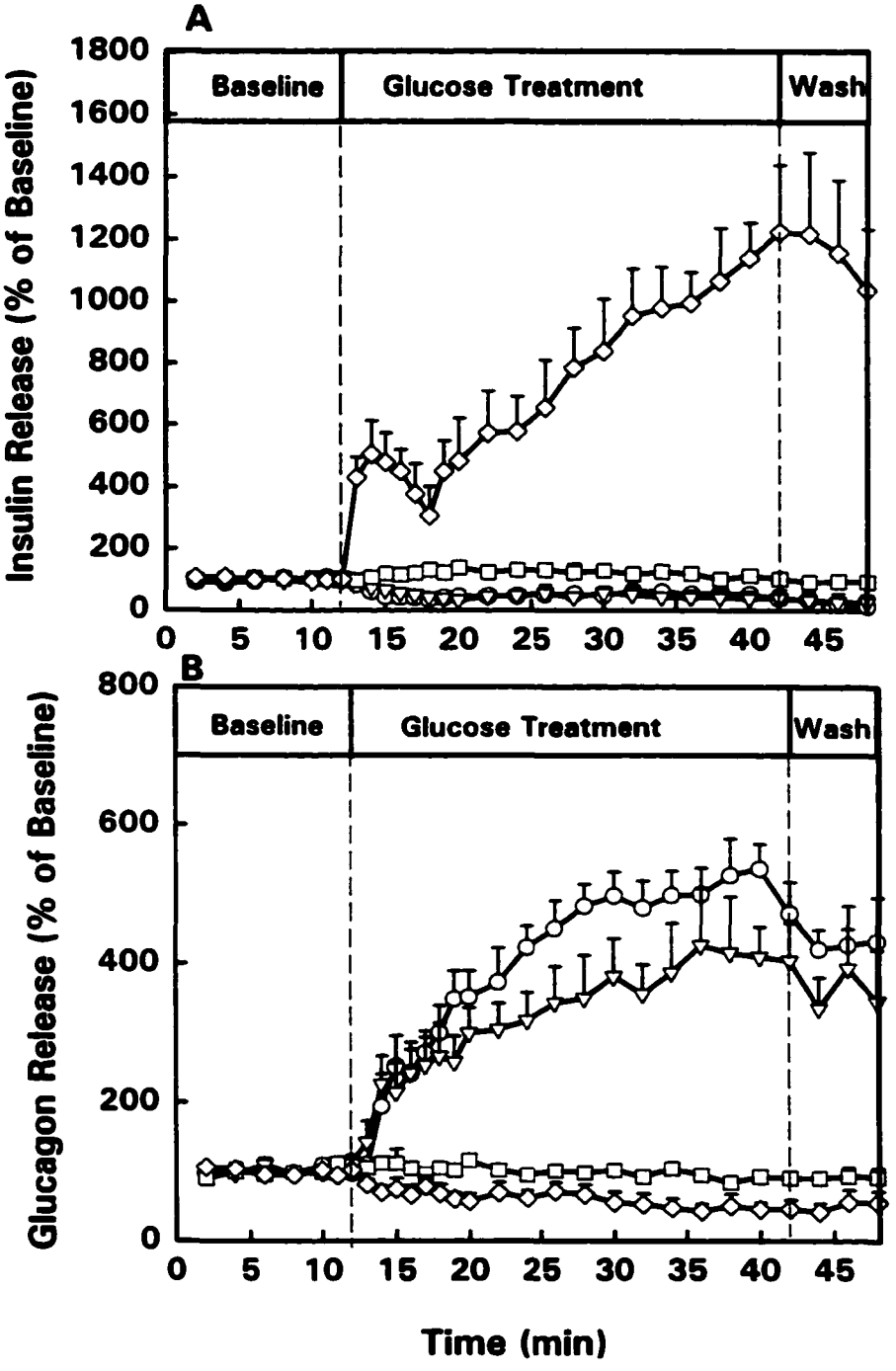

A
Baseline
Glucose Treatment
Wash
Insulin Release (% of Baseline)
1800
1600
1400
1200
1000
800
600
400
200
0
0 5 10 15 20 25 30 35 40 45
B
Baseline
Glucose Treatment
Wash
Glucagon Release (% of Baseline)
800
600
400
200
0
0 5 10 15 20 25 30 35 40 45
Time (min)

Fig 2. Effect of AVP (30 and 300 pmol/L) on insulin release from the perfused rat pancreas. The treatment protocol was the same as in Fig 1. AVP was given for 30 minutes. Glucose concentrations were 0 (A), 1.4 (B), 5.5 (C), and 20mmol/L (D), respectively. By calculating the areas under the curves for AVP and comparing them with those of the control group, 30 pmol/L AVP did not significantly changed insulin release in all tested glucose concentrations, whereas 300 pmol/L AVP significantly ($P <$ .05) increased insulin release in 5.5 and 20 mmol/L glucose groups. Values are the mean ± SE (n=4). O, Control (glucose without AVP); ▽, AVP (30 pmol/L); □, AVP (300 pmol/L). Range of baseline insulin concentrations of effluents was 1045-3137 pg/ml. The F ratios are as follows: AVP concentration, $P <$ .0001; glucose concentration, $P <$ .0001; AVP concentration x glucose concentration, $P <$ .0001.

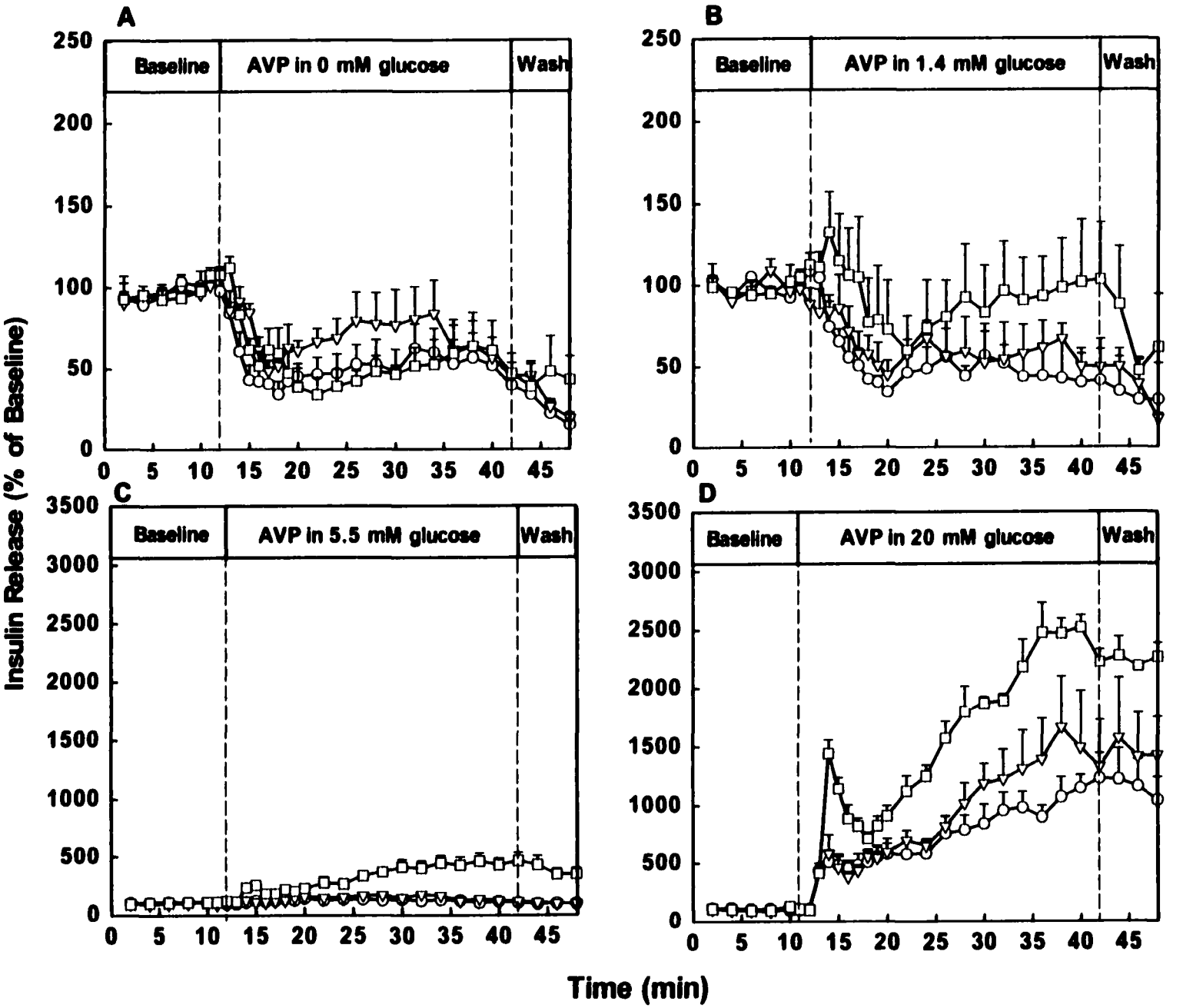

A
Baseline
AVP in 0 mM glucose
Wash
B
Baseline
AVP in 1.4 mM glucose
Wash
C
Baseline
AVP in 5.5 mM glucose
Wash
D
Baseline
AVP in 20 mM glucose
Wash
Insulin Release (% of Baseline)
Time (min)

Fig 3. Effect of AVP (30 and 300 pmol/L) on glucagon release from the perfused rat pancreas. The treatment protocol was the same as in Fig 1. AVP was given for 30 minutes. Glucose concentrations were 0 (A), 1.4 (B), 5.5 (C), and 20mmol/L (D), respectively. By calculating the areas under the curves for AVP and comparing them with those of the control group, both 30 and 300 pmol/L AVP significantly ($P < .05$) increased glucagon release in 0, 1.4, and 5.5 mmol/L glucose groups, whereas only 300 pmol/L AVP significantly ($P < .05$) increased glucagon release at 20 mmol/L glucose group. Values are the mean ± SE (n=4). ○, Control (glucose without AVP); ▽, AVP (30 pmol/L); □, AVP (300 pmol/L). Range of baseline glucagon concentrations of effluents was 50-75 pg/ml. The F ratios are as follows: AVP concentration, $P < .0001$; glucose concentration, $P < .0001$; AVP concentration x glucose concentration, $P < .0001$.

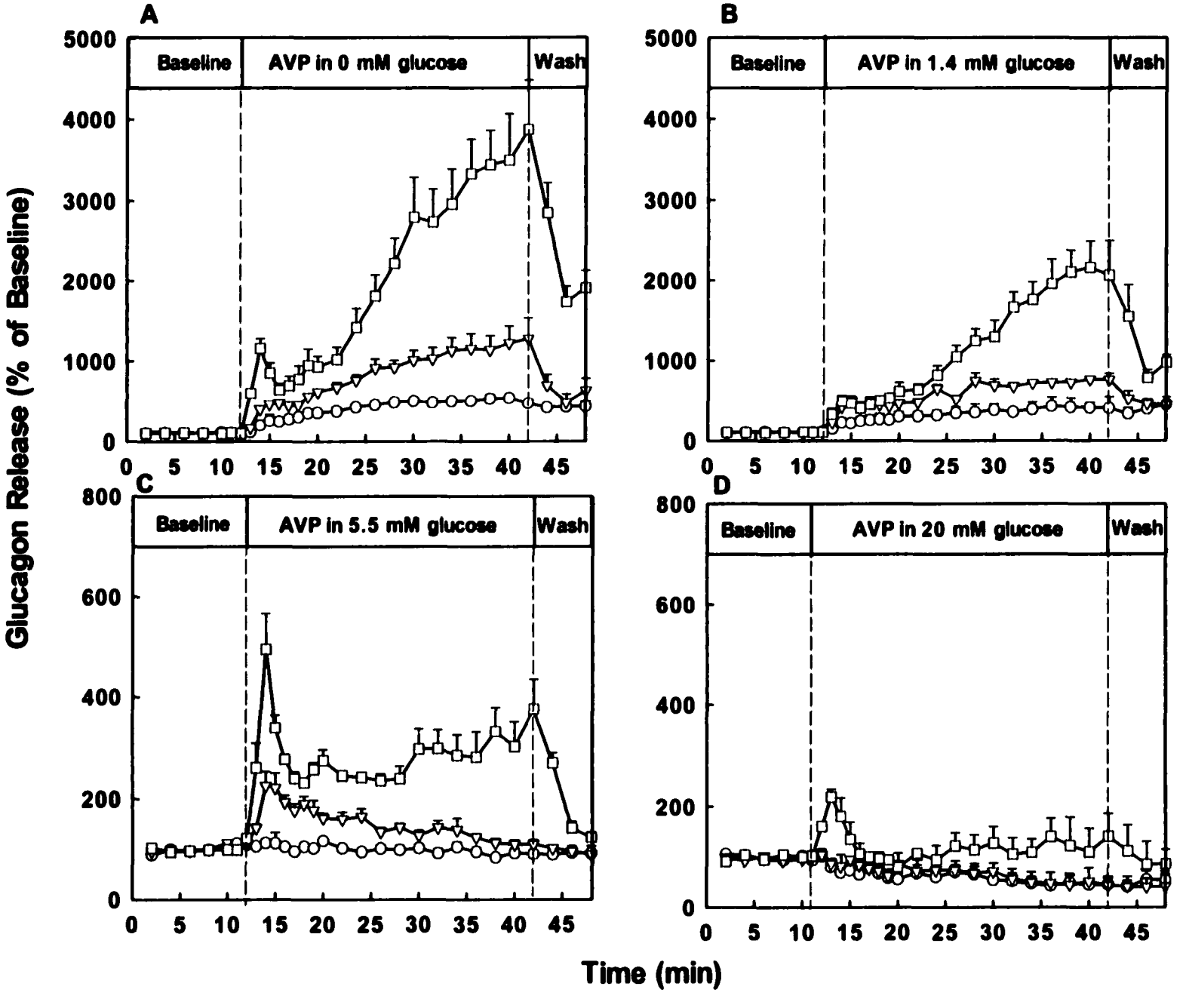
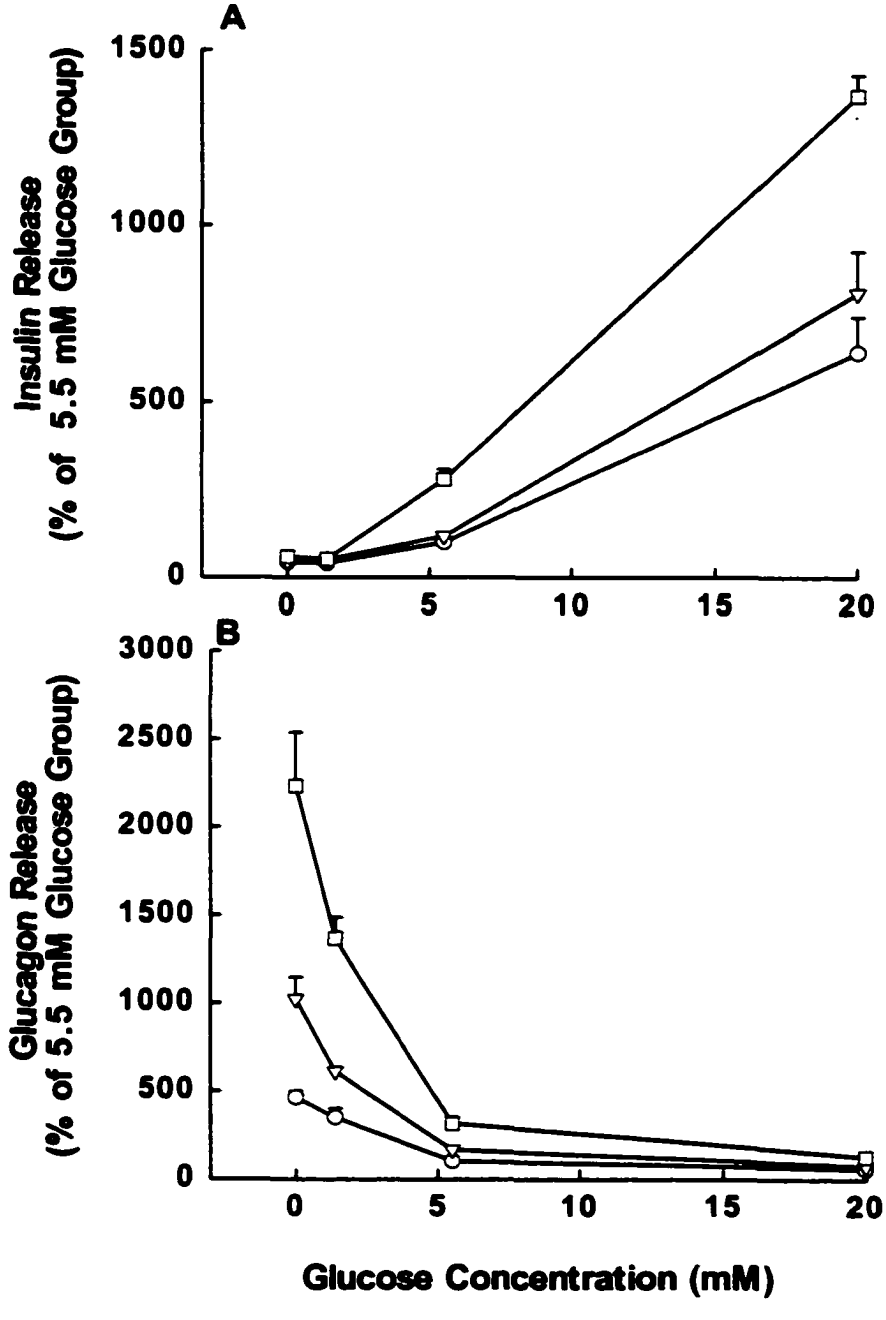

A
Baseline
AVP in 0 mM glucose
Wash
B
Baseline
AVP in 1.4 mM glucose
Wash
C
Baseline
AVP in 5.5 mM glucose
Wash
D
Baseline
AVP in 20 mM glucose
Wash
Glucagon Release (% of Baseline)
Time (min)

Fig 4. Schematic representation of data plotted showing the glucose dependency. Values are the mean ± SE (n=4) obtained by calculating the areas under the curves of the 30 minutes insulin (A) or glucagon (B) release and expressed as a percentage of basal glucose (5.5 mmol/L) control group. ○, Control (glucose without AVP); ▽, AVP (30 pmol/L); □, AVP (300 pmol/L).


A
Insulin Release
(% of 5.5 mM Glucose Group)
1500
1000
500
0
0
5
10
15
20
B
Glucagon Release
(% of 5.5 mM Glucose Group)
3000
2500
2000
1500
1000
500
0
0
5
10
15
20
Glucose Concentration (mM)

CHAPTER III. CYLIC AMP-DEPENDENT PROTEIN KINASE ENHANCES ARGININE VASOPRESSIN-INDUCED GLUCAGON RELEASE BY INCREASING THE READILY RELEASABLE POOL: INVOLVEMENT OF SYNAPSIN I

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Running title: cAMP/PKA enhances AVP-induced glucagon release

ABSTRACT

The purpose of this study was to investigate the intracellular mechanism of cAMP/PKA-dependent enhancement of arginine vasopressin (AVP)-induced glucagon release. Increasing intracellular cAMP levels, by using forskolin or by preventing cAMP degradation with the phosphodiestrease inhibitor IBMX, enhanced AVP-induced glucagon release from the perfused rat pancreas and the clonal α-cells InR1G9. Pre-treatment with SQ-22536, an adenylyl cyclase inhibitor, abolished both the effect of forskolin on glucagon release and the enhancement effect of forskolin on AVP-induced glucagon release. In addition, SQ-22536 abolished forskolin-induced cAMP production. Furthermore, the inactive analog of forskolin neither increased glucagon release nor enhanced the effect of AVP-induced glucagon release. cAMP produced its effects through the stimulation of PKA, as a selective PKA inhibitor H-89 reduced the effect of forskolin on AVP induced-glucagon release and abolished the effect of forskolin on glucagon release. The effect of forskolin and IBMX on glucagon release was Ca^{2+}-independent since both chemicals induced glucagon release to the same extent in the presence or absence of Ca^{2+}. However, forskolin and IBMX enhanced AVP-induced glucagon release in Ca^{2+}-containing medium but not in Ca^{2+}-free medium. CaMK II plays a partial role in AVP-induced glucagon release, since the selective inhibitor, KN93, partially blocked AVP-induced glucagon release and forskolin/AVP-induced glucagon release.

The fact that glucagon release was prolonged with forskolin treatment in the perfused rat pancreas and from InR1G9 cells led us to hypothesize that PKA promotes the mobilization of secretory granules from the reserve pool (RP) to the readily releasable pool (RRP). To test this hypothesis, InR1G9 cells were loaded with styryl dye FM1-43. The

combination of AVP and forskolin induced more increase in fluorescence intensity (~ 4.8 fold of the control group) than AVP or forskolin alone (~ 2.1 and ~ 1.5 fold of the control group, respectively), which reflects an increase in the number of secretory granule membranes fused with the plasma membrane and in the size of the RRP. Secretory granules in the RP are thought to be reversibly connected to the actin-based cytoskeleton by synapsin I. Synapsin I has been proposed to play a major structural role in the assembly and maintenance of the RP secretory granules as well as a dynamic role in controlling secretory granules transitions from RP to RRP. Pretreatment with antisynapsin I antibody abolished the effect of forskolin/AVP-induced glucagon release but not glucagon release in the control, AVP, or forskolin groups. In addition, FM1-43 loading experiments showed that synapsin I is involved in recruitment of secretory granules from RP to RRP. Our finding suggested that 1) cAMP/PKA does not increase $[Ca^{2+}]_i$ nor does it enhance AVP-induced $[Ca^{2+}]_i$ increase, 2) cAMP/PKA enhancement of AVP-induced glucagon release is Ca^{2+}-dependent, 3) CaMK II plays a partial role in the enhancement of AVP-induced glucagon release, 4) cAMP, acting through PKA, increases the number of secretory granules in RRP by mobilization of granules from the RP, an action mediated by Synapsin I.

KEY WORDS

Arginine vasopressin; Glucagon; Exocytosis; cAMP/PKA, FM1-43, readily releasable pool, Synapsin I.

INTRODUCTION

Glucagon plays a key role in maintaining glucose homeostasis. It is secreted from the pancreatic α-cells in response to low blood glucose levels and stimulates hepatic glucose output by increasing glycogenolysis and gluconeogenesis, while at the same time inhibiting glycolysis (Burcelin et al., 1996). Understanding the regulation of glucagon release may contribute to a better control of type 2 diabetes because excessive glucagon release in diabetic patients further aggravates the hyperglycemia caused by low insulin release (Unger and Oric, 1995).

Arginine vasopressin (AVP), a neurohypophysial nonapeptide hormone, stimulates the release of a number of hormones including catecholamine (Grazzini and Lodboerer, 1996), insulin (Dunning et al., 1984; Chen et al., 1994) and glucagon (Yibchok-anun and Hsu, 1998; Yibchok-anun et al., 1999). AVP induces glucagon release through Ca^{+2} – dependent and –independent pathways (Yibchok-anun, 2000). In the case of the Ca^{+2} – dependent pathway, V_{1b} receptor-coupled G_q protein activates phospholipase C, which in turn catalyzes the hydrolysis of phospatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol and inositol 1,4,5-triphosphate (IP_3). Diacylglycerol activates PKC and IP_3 induces Ca^{+2} release from the endoplasmic reticulum, which leads to Ca^{+2} influx. Both Ca^{+2} release and Ca^{+2} influx contribute to AVP-induced glucagon release (Yibchok-anun et al., 2000).

Changes in the intracellular cyclic AMP (cAMP) concentration play an important role in the adjustment of cellular processes initiated by hormones, neurotransmitters and nutrients (Sutherland, 1972). In pancreatic β-cells, cAMP has been found to potentiate Ca^{2+} - dependent exocytosis (Wollheim et al., 1987; Gillis and Misler, 1993). The ability of cAMP

to stimulate insulin release can be prevented by Rp-cAMPS, a specific inhibitor of PKA (Wollheim et al., 1987). Experiments on permeabilized islets or β-cells suggest that the principal action of cAMP is exerted downstream of $[Ca^{2+}]_i$ elevation(Ammala et al., 1993; Renstrom et al., 1997). Ammala et al. (1993) used capacitance measurements to quantify exocytosis and showed that this effect accounted for up to 80% of the exocytosis. cAMP – induced stimulation of exocytosis involves both increasing the release probability of secretory granules already in the readily releasable pool (RRP) and by accelerating the refilling of this pool (Renstrom et al., 1997). The ability of cAMP to stimulate exocytosis is not exclusive to the pancreatic β-cell, since it also enhances glucagon release from pancreatic α-cells (Gromada et al., 1997). Forskolin evokes PKA-dependent potentiation of exocytosis by enhancing Ca^{2+} influx through L-type channels, which accounts for less than 30% of total stimulatory effect (Gromada et al., 1997). The majority of the stimulatory effect of forskolin (70%) resulted from acceleration of granule mobilization and thus increasing the size of the RRP (Gromada et al., 1997).

The transition of secretory granules between the reserve pool (RP) and RRP are subject to extensive regulation, much of which is mediated by Ca^{2+} (Pyle et al., 2000). The secretory granule cycle is regulated by increasing the number of vesicles in RRP through mobilization of vesicles from RP (regulation of the releasable pools), or by increasing the number of docked vesicles that are ready for fusion (release probability of docked vesicles) (Turner et al., 1999). The filling of RRP, measured as recovery from depletion, is enhanced by elevating $[Ca^{2+}]_i$ (Von Ruden and Neher, 1993). Protein Kinase C (PKC) (Gillis et al., 1996; Stevens et al., 1998; Smith et al., 1998; Sudhof, 2000) and PKA (Renstrom et al.,

1997; Gromada et al., 1997) are also involved in the regulation of the size of RPP in other cell systems by mobilizing the secretory vesicles from RP to RRP.

The RRP is thought to correspond to the number of synaptic vesicles morphologically docked to the presynaptic membrane that can be released (Rosenmund and Stevens, 1996; Dobrunz and Stevens, 1997). Synaptic vesicles in the RP are organized in clusters in which the vesicles are reversibly linked to the actin-based cytoskeleton by a family of abundant synaptic vesicle-associated phosphoproteins, the synapsins (Benfenati et al., 1993; Ceccaldi et al., 1995; Pieribone et al., 1995; Kuromi and Kidokoro, 1998). Synapsin I has been proposed to play a major structural role in the assembly and maintenance of the RP of synaptic vesicles as well as a dynamic role in controlling synaptic vesicle transitions from RP to RRP (Greengard et al., 1993; Brodin et al., 1997; Benfenati et al., 1999). Synapsin I links synaptic vesicles to the cytoskeleton, thus regulating the availability of synaptic vesicles for RRP (Benfenati et al., 1993, Ceccaldi et al., 1995). Synapsin I is phosphorylated at the N-terminus by CaMK II and PKA (Sudhof, 1995). Phosphorylation of synapsin I releases the connected vesicles and allow mobilization of these vesicles from RP to RRP (Greengard et al., 1993; Brodin et al., 1997; Benfenati et al., 1999; Hosaka et al., 1999). Neither the function of synapsin I phosphorylation nor the significance of phosphorylation of PKA is clear in pancreatic α-cells.

In a preliminary study, we found that elevation of cAMP via forskolin, enhanced AVP-induced glucagon release from the perfused rat pancreas and InR1G9 cells. Thereafter, we investigated the intracellular molecular mechanism of cAMP/PKA-dependent enhancement of AVP-induced glucagon release, particularly at the level of exocytosis. Although the effects of cAMP on secretion have been widely studied, in this study, we

present the first work demonstrating the effects of cAMP/PKA on single cell exocytotic episodes and the involvement of synapsin I on glucagon-secreting cells (InR1G9). Our results suggested that cAMP, acting through PKA, increases the number of secretory granules in the RRP by mobilization of granules from the RP, an action mediated by Synapsin I.

MATERIALS AND METHODS

Pancreatic perfusion

Male Sprague-Dawley rats (300-400 g) were randomly used. The rats were anesthetized with pentobarbital sodium (60 mg/kg, intrapertoneally), and were maintained at 37°C on a hot plate during the experiment. Pancreatic perfusion was performed as previously described (Yang and Hsu, 1995). Briefly, after cannulation of the celiac artery, the rat pancreas was immediately perfused with Krebs-Ringer bicarbonate (KRB) solution supplemented with 20 mmol/L HEPES, 5.5 mmol/L glucose, 1% dextran, and 0.2% bovine serum albumin as a basal medium. The KRB was maintained at pH 7.4 and continuously aerated with 95% O_2 - 5% CO_2. The rats were euthanatized by the induction of pneumothorax immediately following the cannulation of the portal vein and the beginning of the flow.

The first 20 min of perfusion was considered as an equilibration period. Subsequently, the effluent fluid was collected every minute from the cannula in the portal vein. The flow rate was set at 1 ml/min. After a baseline period of 12 min, the perfusate containing AVP (30 pmol/L), forskolin (10 μM), or forskolin + AVP was administered for 30

min. This was followed by a washout period during which the basal medium was administered for 10 min. Control experiments were performed following the same protocol except that KRB was used in place of the drugs. In all experiments, arginine (1 mmol/L) was administered at the end of the experiment for 8 minutes to confirm the normal secretory capacity under experimental condition. The effluent fluids were kept at 4°C and assayed within 6 h for glucagon using radioimmunoassay as previously described (Yibchock-anun and Hsu, 1998).

Cell culture

The hamster glucagonoma InR1G9 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.

Perifusion of InR1G9 cells

Glucagon release was measured under perifusion condition as previously described (Kikuchi et al., 1974; Daniel et al., 1999). Briefly, InR1G9 cells were grown in custom-made coverslips, which were placed in a perifusion chamber. Each coverslip was exposed to one of the following treatments: control, which was treated with Krebs-Ringer bicarbonate (KRB) alone; AVP (100 nM); forskolin (10 μM); and the combination of AVP and forskolin. At the end of the experiment, the cells were treated with KCl 40 mM to stimulate glucagon release, in order to confirm that the cells retained the normal secretory capacity under the experimental conditions.

Static incubation

InR1G9 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 AVP, forskolin (or IBMX), forskolin (or IBMX) + AVP effects on glucagon release, the cells were incubated at 37°C for 15 min after preincubation with KRB for 15 min. For Ca^{2+}-free experiments, cells were incubated at 37°C with AVP or other agonists in Ca^{2+}-free KRB containing 30 μM EGTA for 15 min after preincubation with Ca^{2+}-containing KRB for 15 min. The following drugs were used in the study: SQ-22536, an adyenylyl cyclase inhibitor; KN93, a selective CaM kinase II inhibitor, were given 30 minutes prior to the administration of AVP or other agonists. 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), an intracellular Ca^{2+} chelator, was given 30 min in Ca^{2+}-containing KRB before the administration of different agonists. H-89, a selective inhibitor for PKA, was given 24 h before the experiment. The cells were then treated with AVP or other agonist. The concentration of glucagon in the media was measured by radioimmunoassay as previously described (Yibchock-anun and Hsu, 1998).

Intracellular delivery of antisynapsin I antibody

Intracellular delivery of antisynapsin I antibody was performed using a recently developed lipid-mediated delivery system (BioPORTER, Gene Therapy System, San Diego, CA). This protein delivery system is fast and displays no significant toxicity and makes protein delivery into cultured cells as convenient, effective, and reliable as DNA transfection

(Zelphati et al., 2001). It is composed of a new trifluoroacetylated lipopolyamine (TFA-DODAPL) and dioleoyl phosphatidylethanolamine (DOPE). The cationic formulation successfully delivered antibodies, dextran sulfates, phycobiliproteins, albumin, and enzymes (β-galactosidase and proteases) into the cytoplasm of numerous adherent and suspension cells (Zelphati et al., 2001). Undiluted antisyapsin I antibody (Calbiochem-Novabiochem Co., San Diego, CA) was delivered into InR1G9 cells following the manufacture's protocol. Briefly, In-R1-G9 cells were plated into Corning 48-well plates at 10^5 cells/well (or on cover slips at 10^4 cells/cover slip) and were grown for 2 days. Antisynapsin I antibody (1.5 μl) (or normal rabbit plasma as control) was mixed with 1.5 μl of the protein delivery reagent and 150 μl of serum free RPMI medium and added to each well. The cells were incubated at 37°C for 4 h. The culture medium was then removed and replaced by KRB following the same procedures as described in static incubation for the 48-well plates and the single cell exocytosis for the cover slips.

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 $[Ca^{2+}]_i$ was calibrated as previously described (Hsu *et. al.*, 1991).

cAMP measurement

Intracellular cAMP concentrations were measured in InR1G9 cells after 15 min of static incubation with AVP, forskolin (or IBMX), or the combination of AVP and forskolin (or IBMX). SQ-22536, an adyenylyl cyclase inhibitor, was given 30 min before the administration of different agonists. After the experiment, cells were scraped from the plate in 0.01 N HCl and incubated in the water bath at 75 °C for 20 min to inactivate phosphodiesterase (PDE). After centrifugation, the supernatant was removed and neutralized by 0.01 NaOH and diluted in the assay buffer as needed. The cAMP concentrations were determined using RIA as previously described (Richards et al., 1979; Chen and Hsu, 1994).

Single cell exocytosis

InR1G9 cells were grown in custom-made circular cover slips. The cells were loaded with styryl dye FM1-43 (2 μM) and exposed to one of the 4 treatments for 4 min: control, treated with KRB alone; 100 nM AVP; 10 μM forskolin, and the combination of AVP and forskolin. Antisynapsin antibody I (1:100) was given 4 h before the experiment as described above in the intracellular delivery of antisynapsin I antibody with TFA-DODAPL: DOPE. Then the cells were exposed to the same 4 treatment groups above. At the end of the experiment, cells were rinsed 8 times with KRB to remove the dye from the external plasma membrane and fixed with 4% paraformaldehyde.

Image Analysis

Fluorescence imaging and quantitative analysis of fluorescence images were performed as previously described (Uemura and Greenlee, 2001). Briefly, digital images of

the cells were captured using a Leica TCS NT laser confocal microscope under a 60X objective. The NIH image software was used to analyze relative fluorescence intensity of FM1-43.

Test Agents

AVP, cAMP, forskolin, 1,9 dideoxyforskolin and IBMX were purchased from Sigma Chemical Co. (St. Lous, MO). FM1-43 was purchased from molecular Probes (Eugene, OR), BAPTA-AM, KN-93 and H-89 were purchased from Biomol Research Laboratories (Plymouth Meeting, PA). SQ-22536 was purchased from Squipp (Princeton, NJ). Glucagon standard was donated by Eli Lilly laboratories (Indianapolis, IN). Glucagon antibody was donated by Dr. Joseph Dunbar of Wayne State University (Detroit, MI). ^{125}I-glucagon was purchased from Linco Research (St. Charles, MO). The antiserum against cAMP was a gift of the National Institute of Diabetes and Digestive and Kidney Disease, National Hormone and Pituitary Program, and the University of Maryland, School of Medicine.

Data Expression and Statistical Analysis

The effluent concentrations of glucagon in rat pancreatic perfusion and the perifused InR1G9 cells were expressed as a percentage of the baseline level (mean of last 5 baseline values) in mean ± SE. The area under the curve for the treatment period of 30 min was calculated using the Transforms and Regressions (Sigma Plot 5.0; SPSS Inc., Chicago, IL). Data were analyzed by analysis of variance using the SAS Proc General Linear Means procedure. Individual mean comparisons were calculated using the *F* test. The significance level was set at $P < .05$.

RESULTS

Enhancement by forskolin of AVP-induced glucagon release from the perfused rat pancreas. We determined the possible involvement of the interaction between cAMP/PKA and PLC pathways in rat pancreatic perfusion. The effects of forskolin, an adenylyl cyclase activator, on AVP-induced glucagon release were investigated. Forskolin (10 μM) enhanced the effect of AVP (30 pM) on glucagon release (a peak of ~2.4 fold and ~4.4 fold of the baseline level for AVP and the combination of AVP and forskolin, respectively) (Fig. 1). This enhancement was more prominent in the sustained phase than that in the peak phase (~1.5-2.0 fold and ~5-6 fold of the baseline level for AVP and the combination of AVP and forskolin, respectively) (Fig. 1). Forskolin alone caused a small increase in glucaogon release (~ 1.6 fold of the baseline level) (Fig. 1).

Enhancement by forskolin of AVP-induced glucagon release from perifused InR1G9 cells. The synthesis and release of glucagon by InR1G9 cells share the basic characteristics of α-cells of the endocrine pancreas (Rorsman et al., 1991). Therefore, we used InR1G9 cells as a model for the α-cells of the pancreas to study the mechanisms underlying this enhancement. Glucagon release was measured under perifusion condition (Kikuchi et al., 1974; Daniel et al., 1999). Forskolin (10 μM) enhanced the effect of AVP (100 nM) on glucagon release (a peak of ~3 fold and ~7 fold of the baseline level for AVP and the combination of AVP and forskolin, respectively) (Fig. 2). This enhancement was still present in the sustained phase (~1.5 fold and ~2-3 fold of the baseline level for AVP and the combination of AVP and

forskolin, respectively) (Fig. 2). Forskolin alone caused a small increase in glucagon release (~ 1.7 fold of the baseline level) (Fig. 2). In all experiments, administration of 40 mM KCl increased glucagon release at the end of the experiment (data not shown).

Enhancement by forskolin and IBMX of AVP-induced glucagon release in static secretion. Forskolin (10 μM) and IBMX (100 μM) respectively enhanced AVP (100 nM)-induced glucagon release from InR1G9 cells in static secretion experiments (Fig. 3). Forskolin (0.1-10 μM) enhanced the effect of AVP on glucagon release in a concentration-dependent manner (Fig. 4).

The above data suggest that an increase in intracellular cAMP level may be involved in the enhancement of AVP-induced glucagon release. In order to investigate this possibility, we examined the effect of SQ-22536, an adenylyl cyclase inhibitor, on forskolin-induced enhancement. Pre-incubation with SQ-22536 (100 μM for 30 min), abolished both the effect of forskolin-induced glucagon release and the enhancement effect of forskolin on AVP-induced glucagon release (Fig. 5). Furthermore, the inactive analog of forskolin, 1,9 dideoxyforskolin, neither increased glucagon release nor enhanced the effect of AVP on glucagon release (Fig. 6).

To determine whether PKA plays a role in this enhancement, we studied the effect of H-89, a selective inhibitor of PKA (Dodge and Sanborn, 1998). Preincubation of H-89 (1 μM for 24 h) reduced the effect of forskolin (1 μM)-induced enhancement (Fig. 7). In addition, H-89 abolished the effect of forskolin-induced glucagon release, while it failed to inhibit AVP-induced glucagon release (Fig. 7).

Effect of SQ-22536 on forskolin- and IBMX-induced increase in cAMP. The addition of forskolin or IBMX would be expected to promote an increase in cAMP level and consequently activate PKA. Therefore, we next measured the cAMP levels in forskolin, IBMX and AVP treated groups. Forskolin (10 μM) caused ~7.7 fold of the baseline level (Fig. 8). Neither AVP (100 nM) alone nor the combination of AVP and forskolin caused a further increase in cAMP production when compared with the control and forskolin groups, respectively (Fig. 8). IBMX caused ~2.6 fold of the baseline level (Fig. 9). As expected, the cAMP level in the combination of AVP and IBMX group was not different from IBMX alone (Fig. 9). Therefore, the effects of forskolin and IBMX on AVP-induced glucagon release cannot be explained by further increase in cAMP level (Figs. 8 and 9).

Similar to the results obtained in the glucagon release experiments, pretreatment with SQ-22536 elicited a remarkable reduction on forskolin-induced cAMP production (Fig. 10). Furthermore, the inactive analog of forskolin did not change the cAMP levels when compared with the control group (Fig. 8). Therefore, the increase in cAMP production is essential for the enhancement of AVP-induced glucagon release.

Enhancement by cAMP/PKA on AVP-induced glucagon release at a site distal to the elevation of $[Ca^{2+}]_i$. Forskolin (10 μM) did not enhance AVP (100 nM)-induced $[Ca^{2+}]_i$ in cell suspension experiments (Fig. 11). Forskolin alone did not increase $[Ca^{2+}]_i$ (data not shown). These results suggest that cAMP/PKA enhances AVP-induced glucagon release in InR1G9 cells at a site distal to the elevation of $[Ca^{2+}]_i$.

Ca^{2+}-dependency of the interaction between cAMP/PKA and AVP. Forskolin (10 μM) and IBMX (100 μM) enhanced AVP (100 nM)-induced glucagon release in Ca^{2+}-containing medium but not in Ca^{2+}-free medium (Figs. 12 and 13). The basal rate of glucagon release in Ca^{2+}-free medium was ~46% less than that in Ca^{2+}-contianing medium. In Ca^{2+}-free medium, AVP (100 nM) still increased glucagon release ~2.2-fold of the basal control level (Figs. 12 and 13). Forskolin and IBMX induced glucagon release in both Ca^{2+}-containing and Ca^{2+}-free medium (Figs. 12 and 13). The effect of forskolin and IBMX on glucagon release was Ca^{2+}-independent since both chemicals induced glucagon release to the same extent in the presence or absence of Ca^{2+}.

Furthermore, preincubating the cells with 25 μM BAPTA-AM, an intracellular Ca^{2+} chelator, in Ca^{2+}-containing medium for 30 min reduced the effect of forskolin on AVP-induced glucagon release (Fig. 14). This concentration of BAPTA-AM abolished AVP-induced $[Ca^{2+}]_i$ increase (data not shown). AVP still increased glucagon release ~1.6-fold of the basal control level (Fig. 14). Forskolin increased glucagon release to the same extent in the presence or absence of BAPTA-AM (Fig. 14). BAPTA-AM alone did not significantly change glucagon release (Fig. 14).

Role of Ca^{2+}/ calmodulin-dependent protein kinase II (CaMK II). A Ca^{2+}-dependent pathway largely mediates AVP-induced glucagon release (Yibchok-anun et al., 2000). Ca^{2+}-dependent regulatory mechanisms are often mediated by activation of calmodulin (CaM), which, in turn, activates a range of cellular proteins, such as CaMK II (Colbran et al., 1989; Means et al., 1991). Several lines of evidence suggest that CaM and/or CaMK II could be

involved in the regulation of PLC-linked Ca^{2+} signals (Hosey et al., 1986; Hill et al., 1988; McCarron et al., 1992; Zhang et al., 1993). To investigate the role of CaMK II activity in PLC-linked Ca^{2+} signals in InR1G9 cells and its role in the enhancement of AVP-induced glucagon release, we studied the effects of KN-93, a selective CaMK II inhibitor. KN93 (1 μM) partially blocked AVP-induced glucagon release and forskolin/AVP-induced glucagon release (Fig. 15). In contrast, KN-93 failed to inhibit forskolin-induced glucagon release (Fig. 15).

Involvement of RRP. Our results from the perfused rat pancreas and InR1G9 cells suggested that cAMP/PKA may increase the size of RRP, leading to enhancement of AVP-induced glucagon release. Therefore, we used FM1-43 as a fluorescent probe to study the effect of cAMP/PKA on RRP. AVP- and forskolin-induced exocytosis and increased the fluorescent intensity by ~2.1-fold and ~1.45-fold of the control group, respectively (Fig. 16). Forskolin enhanced AVP-induced exocytosis and increased the fluorescent intensity by ~4.8-fold of the control group (Fig. 16), which reflects an increase in the number of secretory granule membranes fused with the plasma membrane and an increase in the size of RRP.

Involvement of Synapsin I. In neurons, synapsin I plays a key role in controlling the mobilization of the secretory vesicles from RP to RRP (Greengard et al., 1993; Benfenati et al., 1999). We next investigated the involvement of synapsin I in cAMP/PKA enhanced AVP-induced glucagon release and its role in granule mobilization in InR1G9 cells. Pretreatment with antisynapsin I antibody in the presence of forskolin (10 μM) and AVP (100 nM) for 4 h abolished the enhancement effect of forskolin on AVP-induced glucagon

release (Fig 17). Antisynapsin I antibody did not change glucagon release in the control, AVP or forskolin groups (Fig. 17).

We further tested the involvement of synapsin I on the size of RRP. InR1G9 cells were pretreated with antisynapsin I antibody or normal rabbit plasma for 4 h in the presence of forskolin (10 μM) and AVP (100 nM). Similar to the results obtained from glucagon release experiments, antisynapsin I antibody abolished the effect of forskolin on AVP-induced exocytosis (Fig. 18). AVP- and forskolin-increased fluorescent intensity by ~175% and ~197% of the control group, respectively (Fig. 18). Antisynapsin I antibody did not change the exocytotic rate in AVP or forskolin groups when compared with AVP or forskolin groups that did not receive antisynapsin I antibody (Fig. 18). These results suggest that synapsin I is involved in the release of secretory vesicles from RP to refill RRP.

DISCUSSION

In the present study we investigated the contribution of the cAMP/PKA system to the regulation of glucagon release and the kinetics of an episode of exocytotic release. For this, the interaction between AVP and cAMP/PKA was studied using rat pancreatic perfusion and perifused clonal α-cells InR1G9 cells. The results suggested that AVP-induced glucagon release is enhanced by cAMP/PKA system. Data from static secretion, $[Ca^{2+}]_i$ measurements, and exocytosis from individual InR1G9 cells were used to explore the mechanism of this enhancement.

The cAMP signal transduction pathway is clearly involved in the enhancement of AVP-induced glucagon release. We first observed that increasing intracellular cAMP

formation by using forskolin or preventing cAMP degradation with the phosphodiestrease inhibitor IBMX, enhanced AVP-induced glucagon release. SQ-22536, an adenylyl cyclase inhibitor, abolished the stimulatory effect of forskolin on glucagon release and the enhancement effect of forskolin on AVP-induced glucagon release. In addition, SQ-22536 abolished forskolin-induced cAMP production, which provides evidence that adenylyl cyclase activation is crucial to this enhancement. Furthermore, the inactive analog of forskolin neither increased glucagon release nor enhanced the effect of AVP on glucagon release. The signaling pathway used by cAMP to produce its effects seems to be through the stimulation of PKA, as H-89, a selective PKA inhibitor, reduced the effect of forskolin/AVP-induced glucagon release and abolished the effect of forskolin induced glucagon release.

The enhancement effect of cAMP/PKA on AVP-induced glucagon release is Ca^{2+}-dependent because forskolin and IBMX enhanced AVP-induced glucagon release in Ca^{2+}-containing medium but not in Ca^{2+}-free medium. Furthermore, preincubating the cells with BAPTA-AM, an intracellular Ca^{2+} chelator, in Ca^{2+}-containing medium reduced the effect of forskolin/AVP-induced glucagon release. This was expected since most of the exocytotic fusion is Ca^{2+}-dependent and the granule fusion, per se, occurs independently of protein kinase involvement and is thought to be mediated by the direct action of Ca^{2+} influx through Ca^{2+}/phospholipid binding protein synaptotagmin (Lang et al., 1997; Gerber and Sudhof, 2002). High $[Ca^{2+}]_i$ plays a critical role in secretagogue-induced glucagon secretion (Pipeleer et al., 1985; Hii and Howell, 1986). Stimulation of pancreatic α-cells with secretagogues such as AVP results in a rise in $[Ca^{2+}]_i$ due to Ca^{2+} release from endoplasmic reticulum and Ca^{2+} influx from the extracellular space (Yibchok-anun et al., 2000). Many effects of Ca^{2+} are mediated through Ca^{2+}-binding proteins such as calmodulin (CaM). The

effects of Ca^{2+}/CaM may be mediated by CaMK II (Hanson and Schulman 1992; Miyamoto et al., 1997; Easom, 1999). The CaMK II may play a partial role in glucagon release since the selective inhibitor, KN93, partially blocked AVP-induced glucagon release and forskolin/AVP-induced glucagon release. How Ca^{2+} increase regulates the complex series of steps that results in neurotransmitter/hormonal release remains largely unknown, but SNARE complex proteins and synaptotagmin play important roles in regulation of this process (for review see Sudhof, 1995; Hilfiker et al., 1999; Gerber and Sudhof, 2002).

Previous studies showed that Ca^{2+} mobilization and IP_3 accumulation induced by ethanol and vasopressin in rat hepatocytes were potentiated by cpt-cAMP (Higashi et al., 1996). Agents that increase cAMP levels have no significant effects on basal inositol phosphate synthesis but enhance the effect of AVP-induced IP3 accumulation (Pittner and Fain, 1989a,b). IP_3 induces Ca^{2+} release from the endoplasmic reticulum, therefore any increase in IP_3 formation will be detected by an increase in $[Ca^{2+}]_i$. In addition, 30% of the total stimulatory effect of cAMP enhanced glucagon release from pancreatic α-cells was by enhancement of Ca^{2+} influx through L-type channels (Gromada et al., 1997). We determined if in our system cAMP also mediated its effect through further increase in $[Ca^{2+}]_i$. In contrast to the above studies, our findings suggested that the enhancement effect of cAMP/PKA on AVP-induced glucagon release is not mediated through the modulation of $[Ca^{2+}]_i$ and it occurs at a site distal to the elevation of $[Ca^{2+}]_i$.

How can activation of PKA (e.g., in response to forskolin) enhances exocytosis? PKA must do more than simply change the Ca^{2+} affinity in the fusion machinery (Ammala et al., 1993). The most straightforward explanation for these results is that phosphorylation by PKA increases the number of granules available for Ca^{2+}-regulated fusion. The fact that

glucagon release was prolonged with forskolin treatment in the perfused rat pancreas and from InR1G9 cells suggests that PKA promotes the mobilization of secretory granules from RP to RRP. Styryl dyes, which were originally developed as membrane potential sensors, have become useful tools in the study of exocytosis, endocytosis and synaptic vesicle recycling (Betz et al, 1996; Cochilla et al, 1999). This is due to their ability to reversibly stain membranes, their inability to penetrate membranes, and their fluorescence (Betz et al, 1996; Cochilla et al, 1999). FM1-43 is one of the most extensively used styryl dyes to study secretory activity (Cochilla et al, 1999). It is non-fluorescent in aqueous solution, but becomes fluorescent and its quantum yield increases by ~ 350 times when incorporated into the plasma membrane (Betz et al, 1992). Therefore, the fluorescence intensity is proportional to the amount of membrane exposed to FM1-43. Upon stimulation, exocytosis causes the vesicular membrane to fuse with the plasma membrane, and the dye binds to the exocytosing membrane resulting in an increase of fluorescence intensity. Therefore, the fluorescence intensity can be used as an index for the size of RRP (Cochilla et al., 1999; Cousin and Robinson, 1999; Kuromi and Kidokoro, 1998; 2000, Sudhof, 2000). The advantage of this imaging method as an indicator of exocytosis, over traditional methods which measure glucagon release, is that it enables measurement of exocytosis from individual cells with high temporal resolution. Using this method we demonstrated that the combination of AVP and forskolin induced more increase in fluorescent intensity (Fig. 16), which reflects an increase in the number of secretory granule membranes fused with the plasma membrane and therefore an increase in the size of RRP.

One possible mechanism for the recruitment of the secretory granules from RP is the release of granules from the actin-based cytoskeleton induced by phosphorylation of

synapsin I (Greengard et al., 1993; Brodin et al., 1997; Benfenati et al., 1999; Hosaka et al., 1999). In pancreatic β- and α-cells, the actin network beneath the plasma membrane may prevent the interaction between secretory granules and plasma membranes (Orci, 1982). By analogy with neuronal cells, synapsin I in α-cells cells may function as a linker between glucagon secretory granules and the cytoskeletal network. Synapsin I is found in glucagon-expressing α-cells including InR1G9 cells (Matsumoto et al., 1999) and is phosphorylated at its N-terminus by CaMK II and PKA (Sudhof, 1995). Until now, neither the function of synapsin I phosphorylation in α-cells nor the significance of phosphorylation by PKA is clear in pancreatic α-cells. The present study showed that synapsin I is likely involved in forskolin/AVP-induced glucagon release as well as in the mobilization of secretory granules from RP to refill RRP. Pretreatment with antisynapsin I antibody abolished the effect of forskolin/AVP-induced glucagon release but not glucagon release in the control, AVP, or forskolin groups. These findings were confirmed by the exocytosis experiments using FM1-43 loaded cells.

In this study, we present the first work demonstrating the effects of cAMP/PKA on single exocytotic events and the involvement of synapsin I on glucagon-secreting cells (InR1G9). Our findings suggested that cAMP, acting through PKA, increases the number of secretory granules in RRP by mobilization of granules from RP, an action mediated by Synapsin I (Fig. 19). In order for exocytosis to continue during extensive stimulation, RRP must be supplied with secretory granules from RP. Agents that increase intracellular cAMP concentrations cause sustained exocytosis (Eddlestone et al., 1985; Hill et al., 1987; Gills and Misler, 1993) and have been used in combination with secretagogues to augment exocytosis (Wiedenkeller and Sharp, 1983; Henquin 1985; Wang et al., 1993) This effect may be due to

an increase in the size of RRP as indicated in this study and others (Renstrom et al., 1997; Gromada et al., 1997). Because of the similarities between glucagon granules and other neuroendocrine cells such as pancreatic β-cells, chromaffin cells, pituitary somatotrophs, pituitary melanotrophs and lactotrophs, it is probable that our results that elevated intracellular cAMP concentrations can modulate the size of RRP can be extrapolated to these endocrine cells.

Diabetic patients have elevated levels of plasma AVP (Grimaldi et al., 1988; Tinder et al., 1994) and the α-cells from diabetic subject may exhibit an exaggerated response to AVP (our unpublished data). Therefore, our present findings that elevation of cAMP concentrations enhances AVP-induced glucagon may partially account for the clinical observations that hypersecretion of glucagon aggravates the hyperglycemia associated with type 2 diabetes (Unger and Orci 1995; Dinnen et al., 1995).

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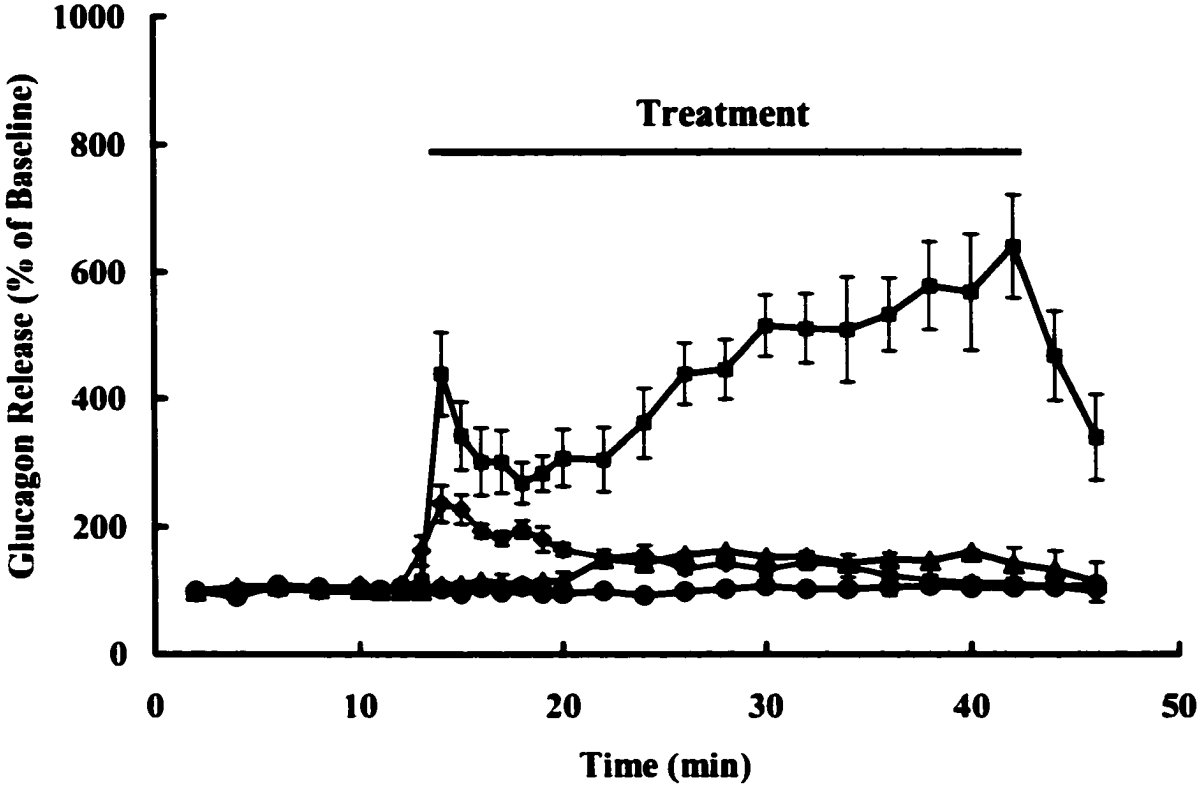


Fig 1. Effects of forskolin on AVP-induced glucagon release from the perfused rat pancreas. In these experiments, a 20-min equilibration period preceded time 0. After a baseline period of 12 min, AVP (30 pM), forskolin (10 μM), or the combination of forskolin and AVP were administered for 30 min (heavy line), followed by a 6 min washout period. Values are the mean ± SE (n = 4). Basal control (●); forskolin (▲); AVP (◆); and the combination of forskolin and AVP (■). Range of baseline glucagon concentrations of effluents was 32-70 pg/ml.

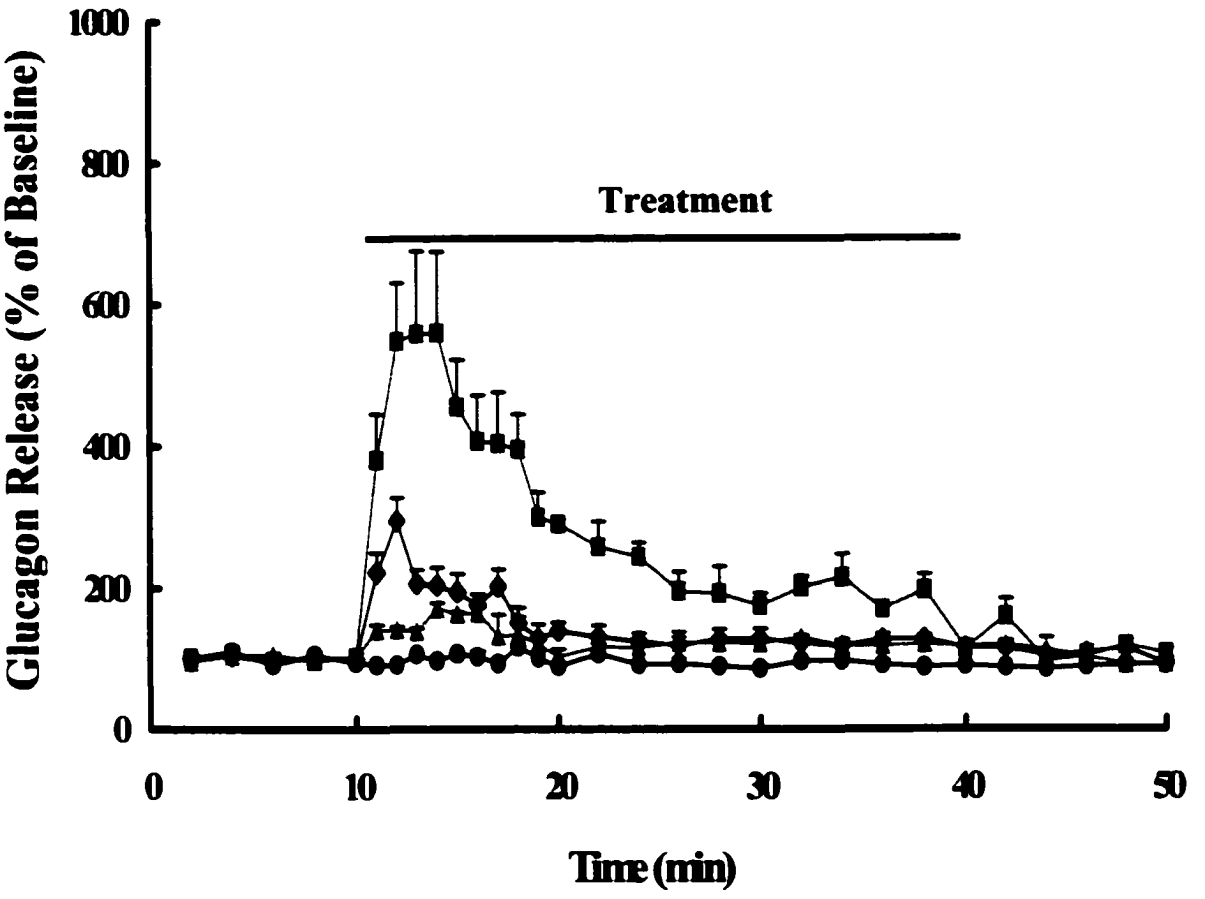


Fig 2. Effects of forskolin on AVP-induced glucagon release in the perifusion of InR1G9 cells. In these experiments, a 15-min equilibration period preceded time 0. After a baseline period of 10 min, AVP (100 nM), forskolin (10 μM), or the combination of forskolin and AVP were administered for 30 min (heavy line), followed by a 10 min washout period. Values are the mean ± SE (n = 3). Basal control (●); forskolin (▲); AVP (◆); and the combination of forskolin and AVP (■). Range of baseline glucagon concentrations of effluents was 25-72 pg/ml.

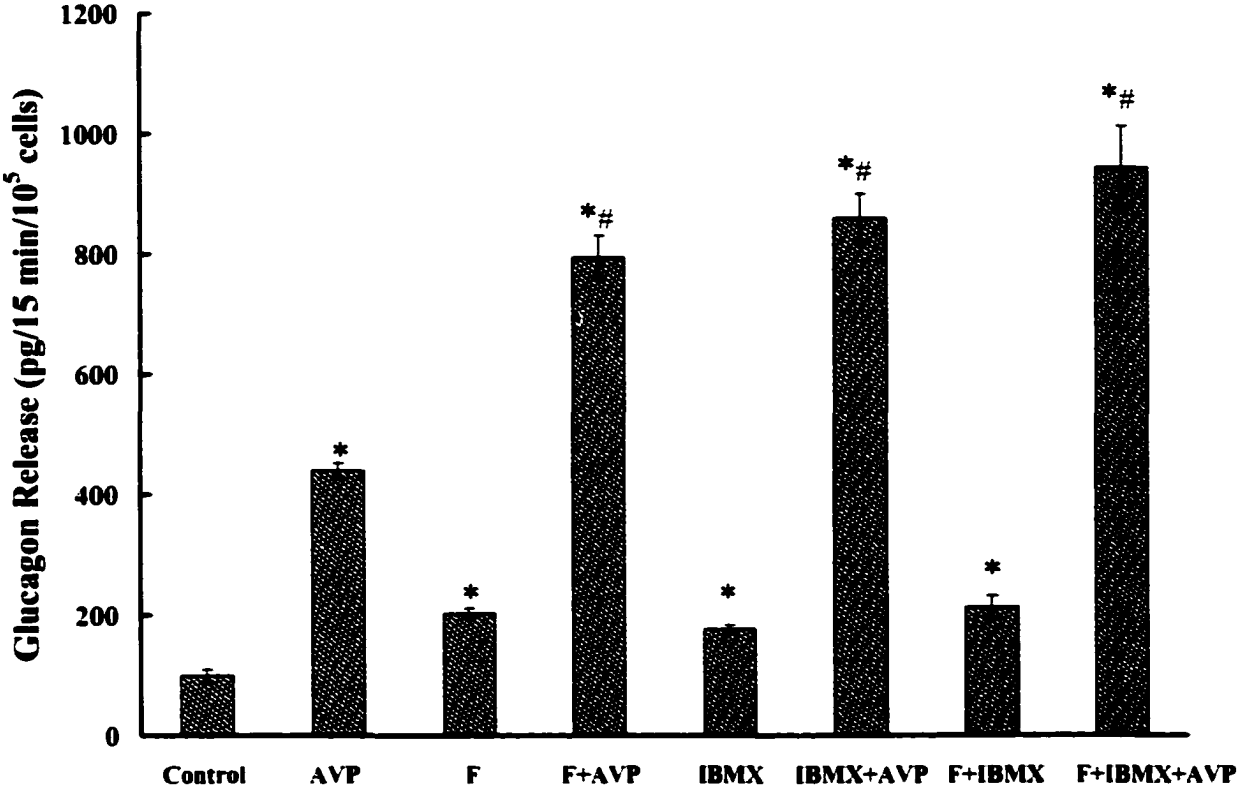


Fig 3. Effects of forskolin (F) (10 μM) and IBMX (100 μM) on AVP (100 nM)-induced glucagon release in InR1G9 cells. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n = 4 cultures with triplicates). *P < .05 vs. Control; # P< .05 vs. AVP alone.

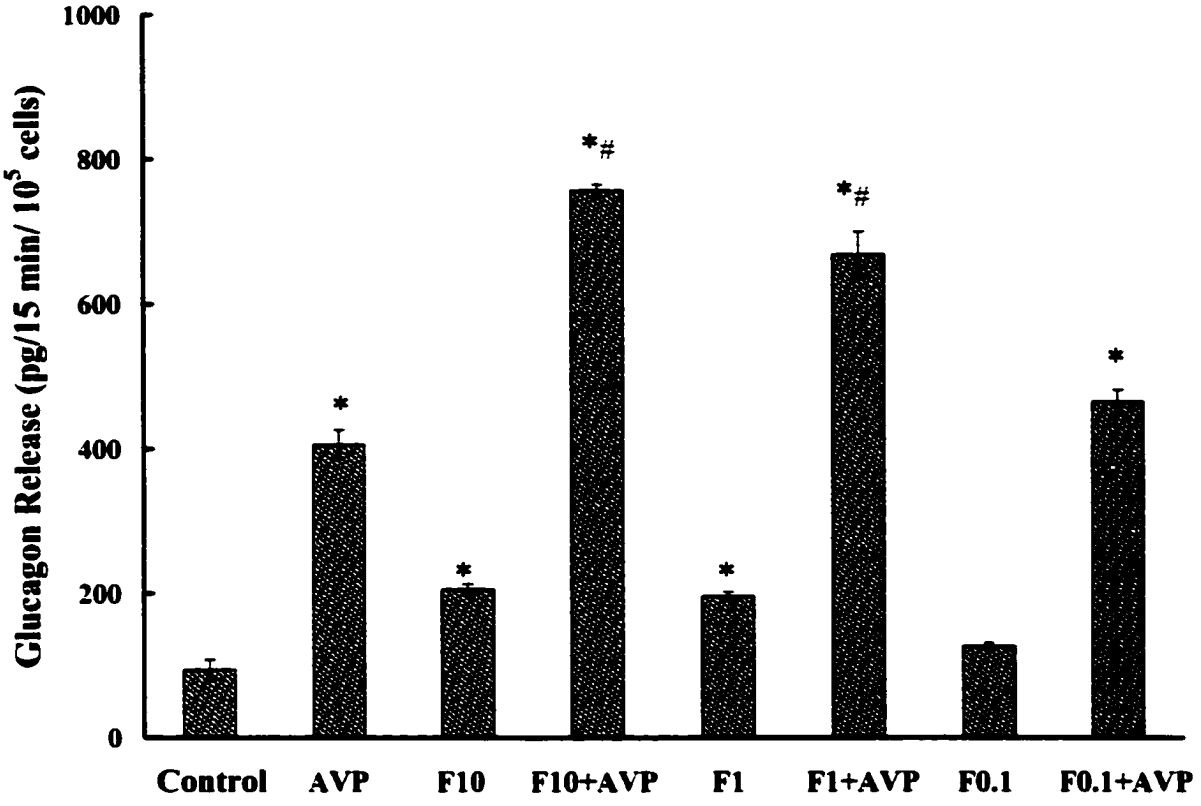


Fig 4. Effects of forskolin (F) (F10= 10 μM, F1= 1 μM, or F0.1= 0.1 μM) on AVP (100 nM)-induced glucagon release in InR1G9 cells. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n = 4 cultures with triplicates). *P < .05 vs. Control; # P< .05 vs. AVP alone.

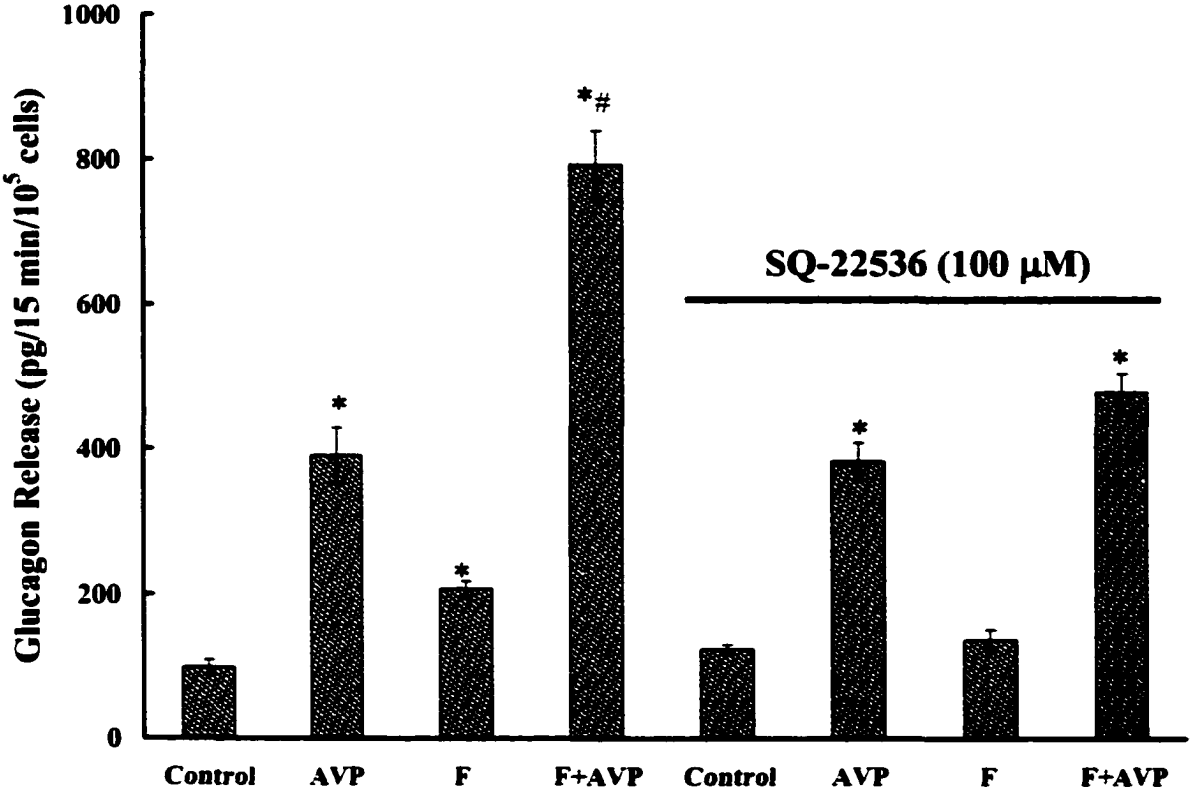


Fig 5. Effects of SQ-22536 (SQ) (100 μM) on forskolin (F)- (10 μM), AVP- (100nM) and the combination of forskolin and AVP-induced glucagons release in InR1G9 cells. SQ was given 30 min before the administration of AVP. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n = 4 cultures with triplicates). *P < .05 vs. Control; # P< .05 vs. AVP alone.

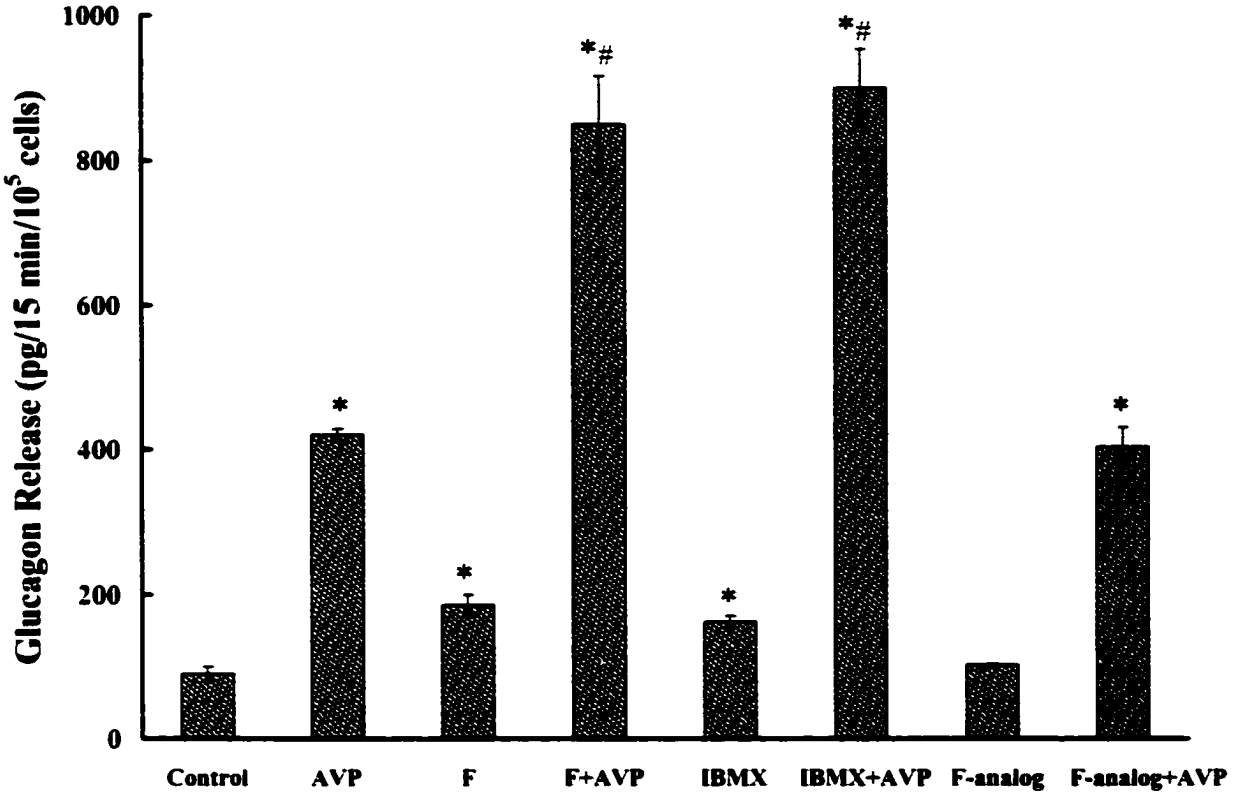


Fig 6. Effects of forskolin (F) (10 μM) and the inactive analog of forskolin, 1,9 dideoxyforskolin (F-analog) (10 μM) on AVP (100 nM)-induced glucagon release in InR1G9 cells. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n = 4 cultures with triplicates). *P < .05 vs. Control; # P< .05 vs. AVP alone.

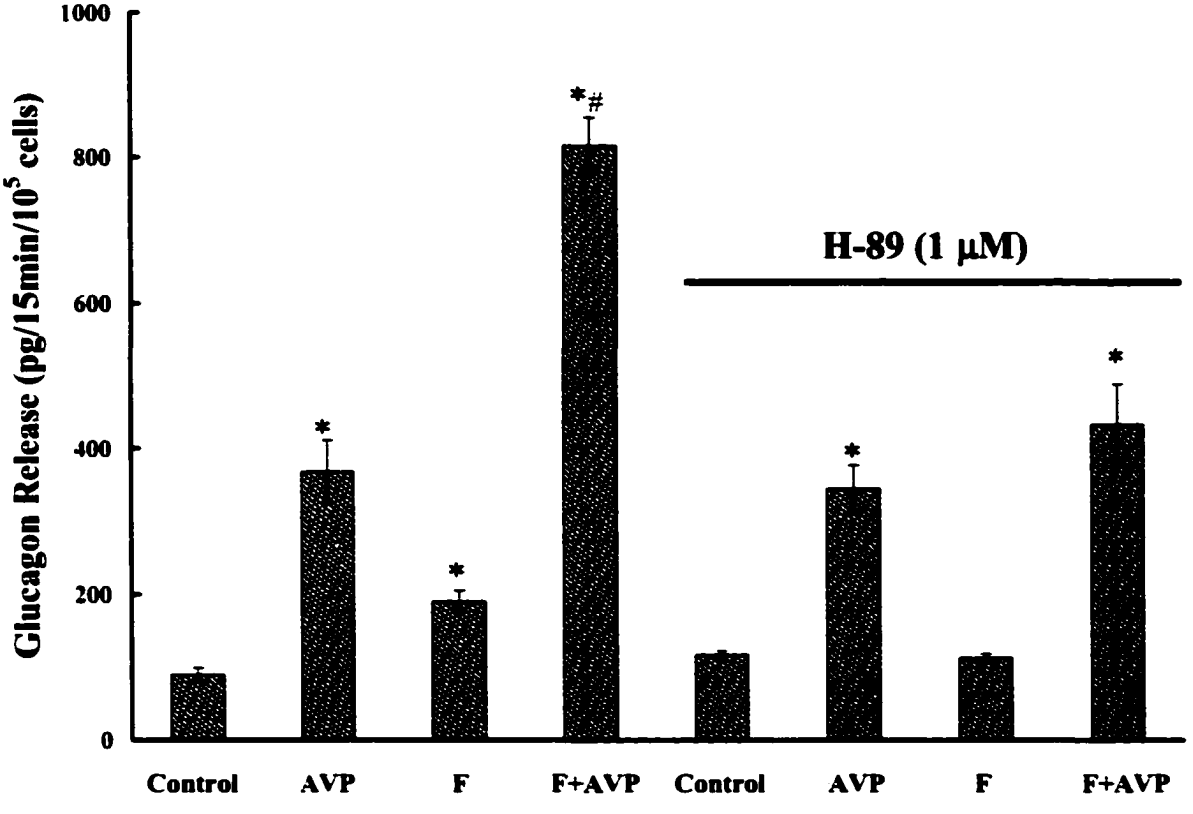


Fig 7. Effects of H-89 (1 μM), a selective inhibitor of PKA, on forskolin (F) (1 μM)/ AVP (100 nM) induced glucagon release in InR1G9 cells. H-89 was pretreated for 24 h. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n = 4 cultures with triplicates). *P < .05 vs. Control; # P< .05 vs. AVP alone.

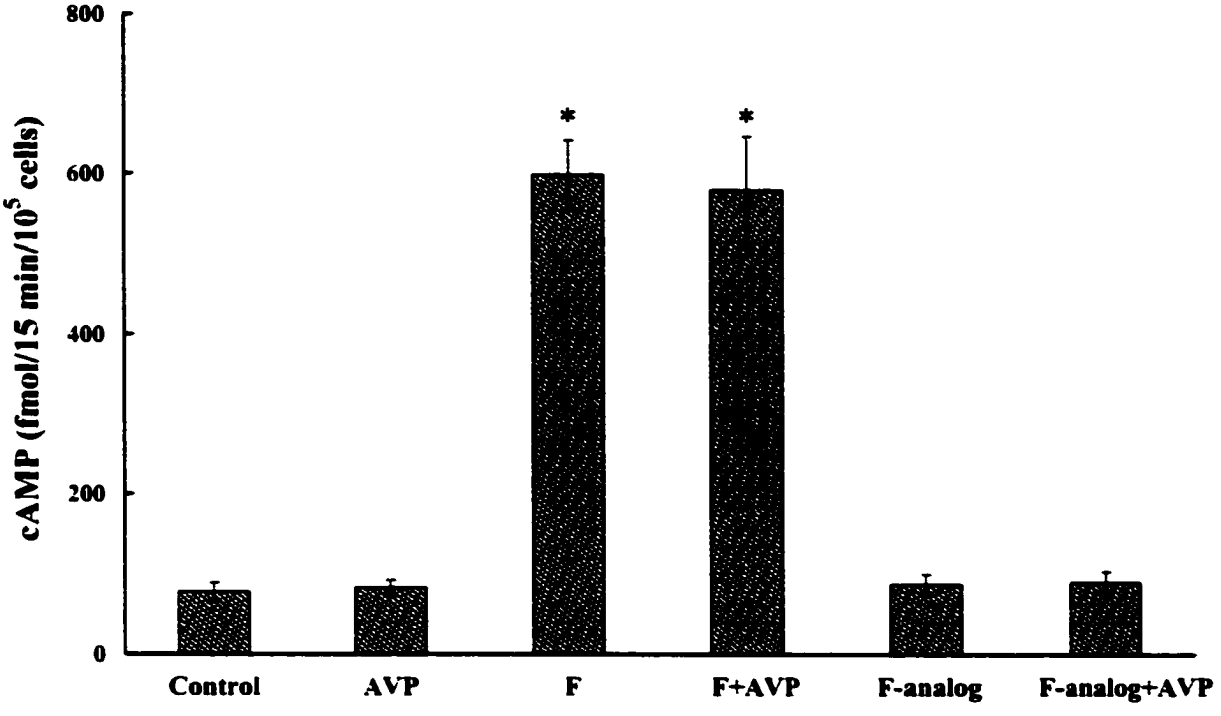


Fig 8. Effects of forskolin (F) (10 μM) and the inactive analog of forskolin, 1,9 dideoxyforskolin (F-analog) (10 μM) and AVP (100nM) on cAMP production in InR1G9 cells. Static incubation was performed for 15 min to determine cAMP levels. Values are mean ± SE (n = 3 cultures with quadruplicates). *P < .05 vs. control.

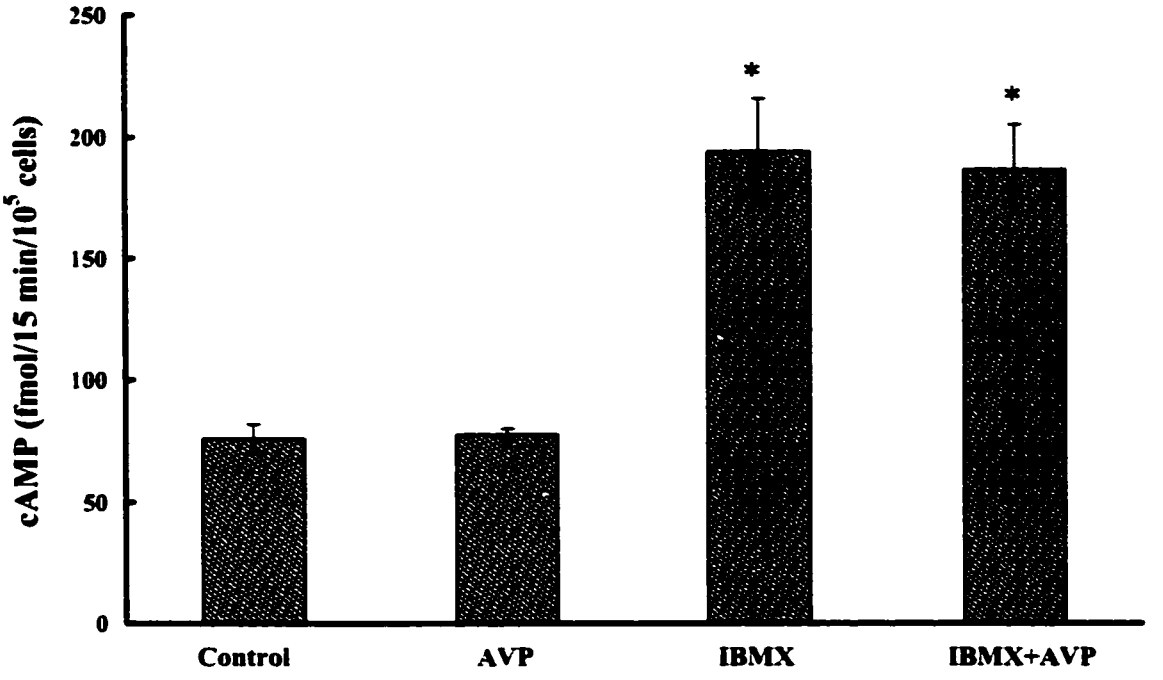


Fig 9. Effects of IBMX (100 μM) on cAMP production in InR1G9 cells. Static incubation was performed for 15 min to determine cAMP levels. Values are mean ± SE (n = 3 cultures with quadruplicates). *P < .05 vs. control.

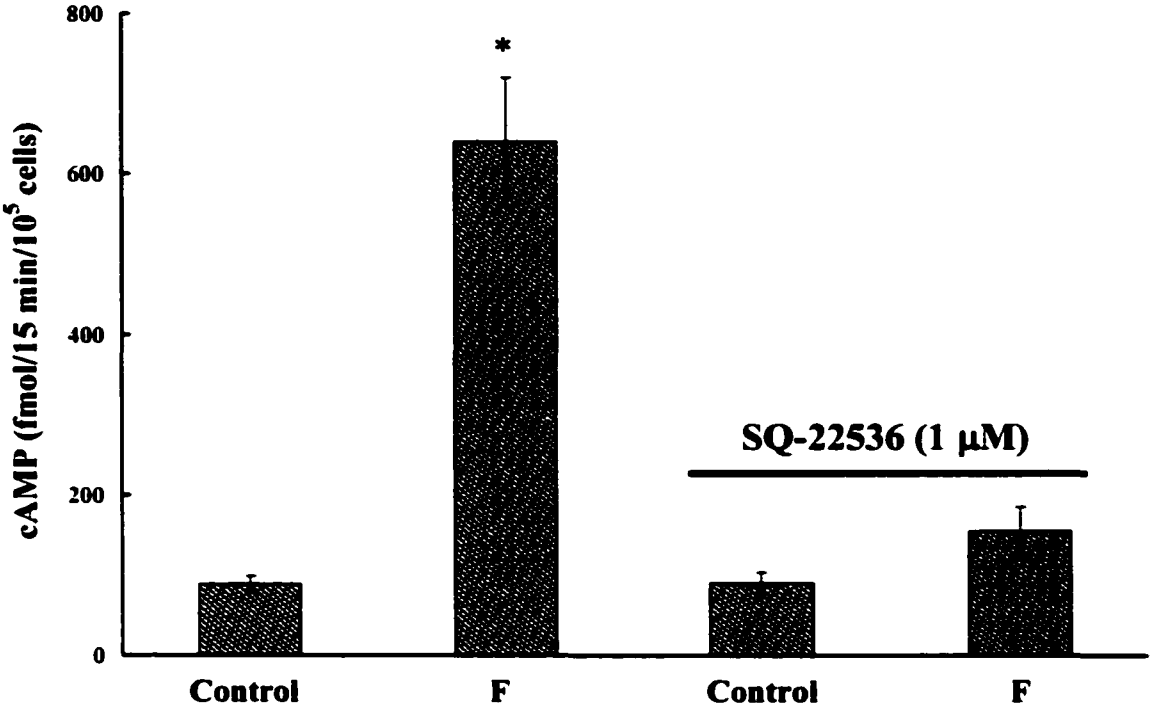


Fig 10. Effects of SQ-22536 (SQ) (100 μM) on forskolin (10 μM)-induced cAMP production in InR1G9 cells. SQ was given 30 min before the administration of forskolin. Static incubation was performed for 15 min to determine cAMP levels. Values are mean ± SE (n = 3 cultures with quadruplicates). *P < .05 vs. Control.

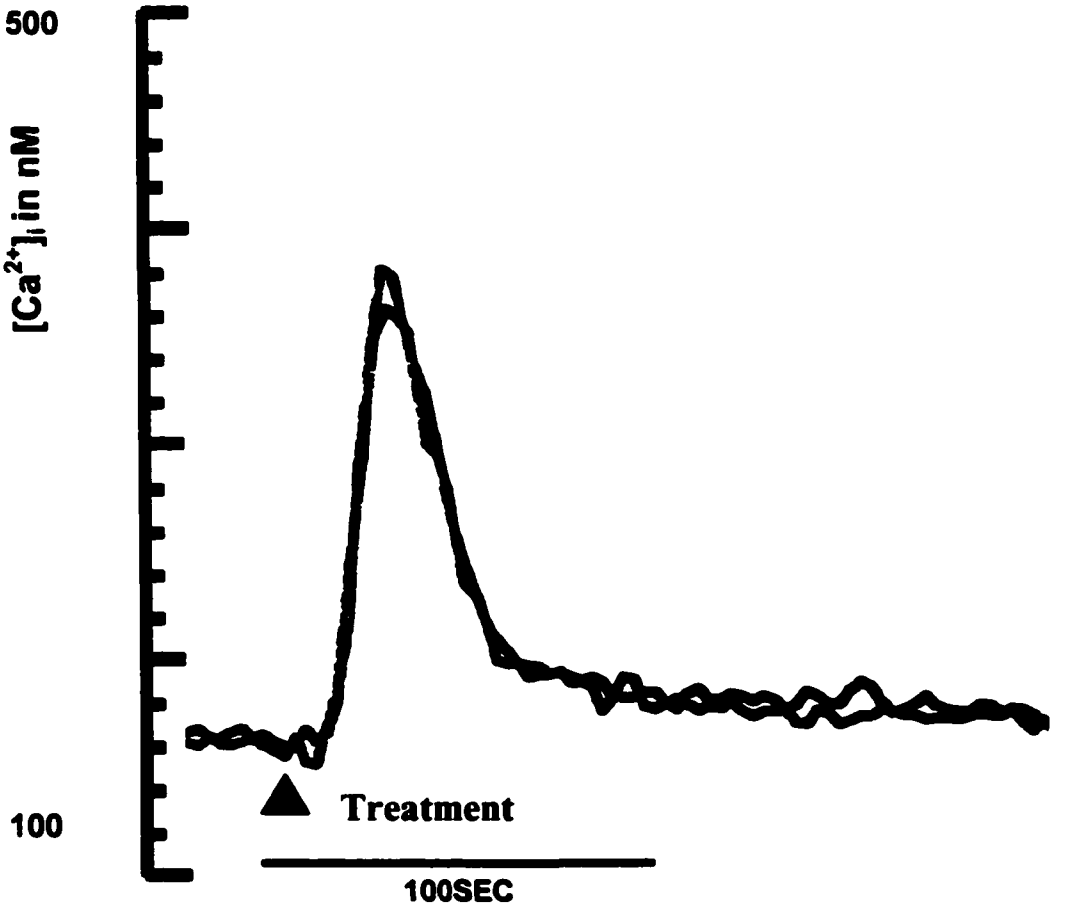


Fig 11. Effects of forskolin (10 μM) on AVP (100 nM)-induced increase in $[Ca^{2+}]_i$. Forskolin failed to further increase AVP-induced $[Ca^{2+}]_i$. Traces are representative of 3 experiments.

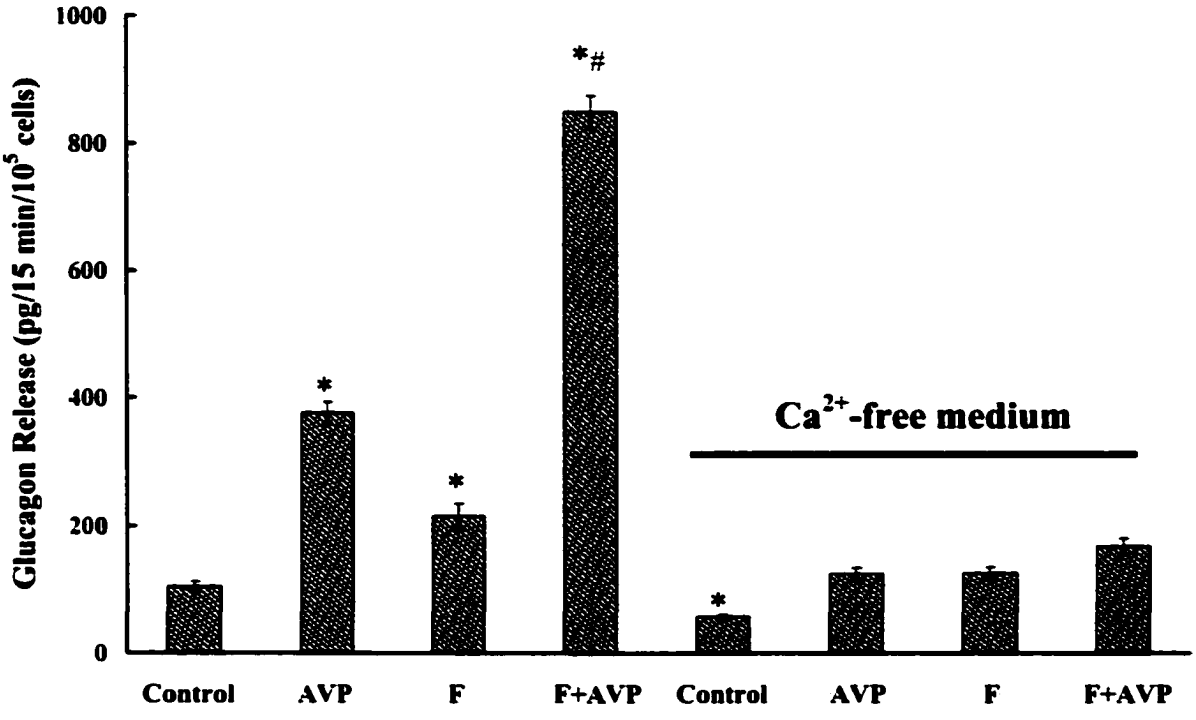


Fig 12. Effects of forskolin (F) (10 μM)/AVP (100 nM)-induced glucagon release in Ca^{2+}-containing and Ca^{2+}-free KRB. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n=4 cultures with triplicates). *P < .05 vs. Control in Ca^{2+}-containing media; # P< .05 vs. AVP alone.

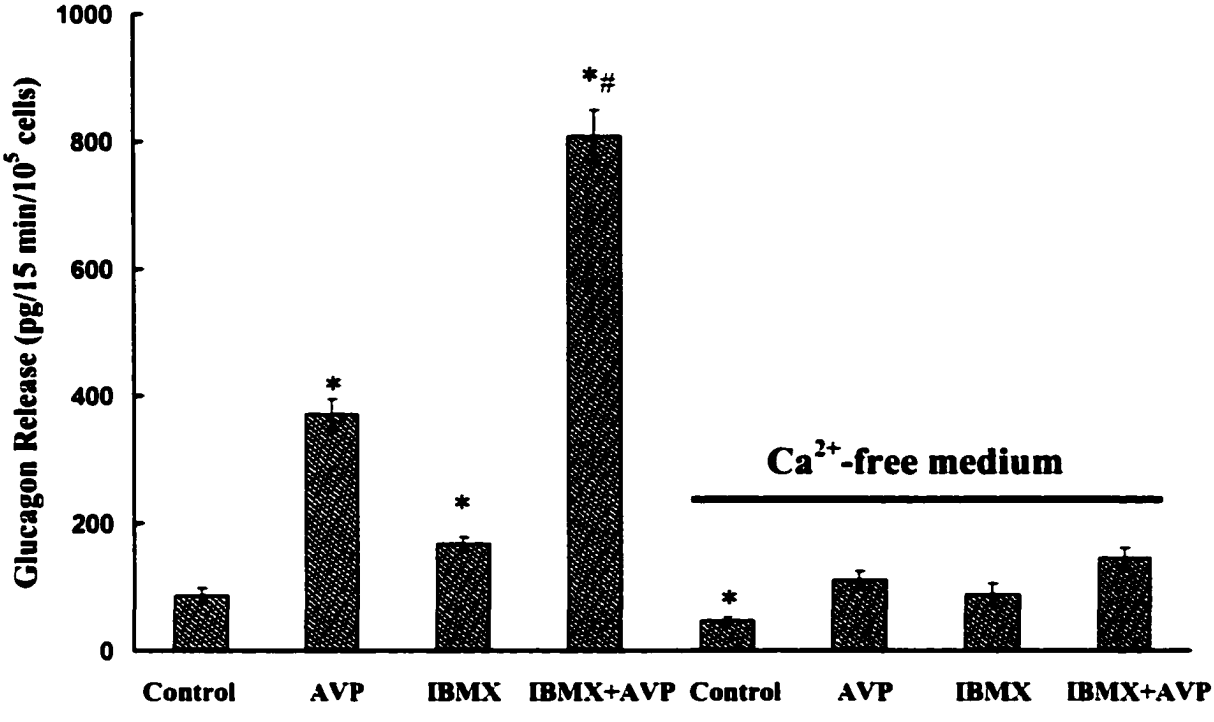


Fig 13. Effects of IBMX (100 μM)/AVP (100 nM)-induced glucagon release in Ca^{2+}-containing and Ca^{2+}-free KRB. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n=4 cultures with triplicates). *P < .05 vs. Control in Ca^{2+}-containing media; # P< .05 vs. AVP alone.

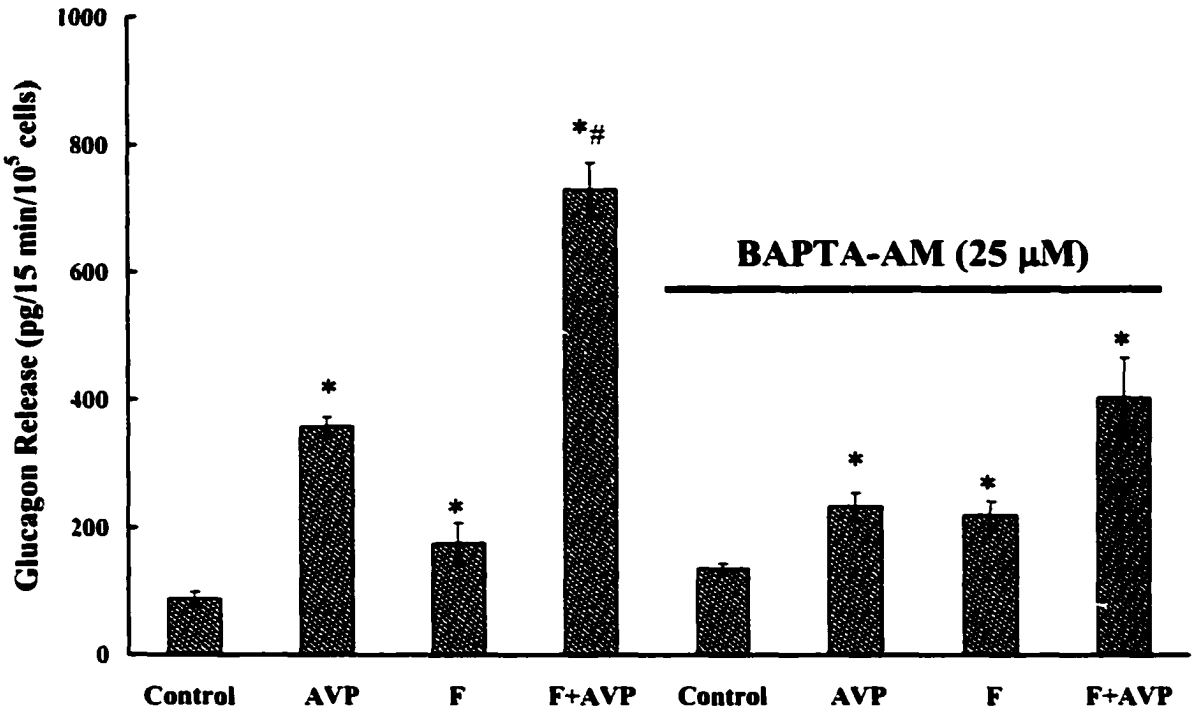


Fig 14. Effects of forskolin (F) (10 μM)/AVP (100 nM)-induced glucagon release in Ca^{2+}-containing media and under deprived Ca^{2+}-condition (25 μM BAPTA-AM in Ca^{2+}-containing media). BAPTA-AM in Ca^{2+}-containing media was given 30 min before the administration of AVP. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n=4 cultures with triplicates). *P < .05 vs. control without BAPTA-AM; # P< .05 vs. AVP alone.

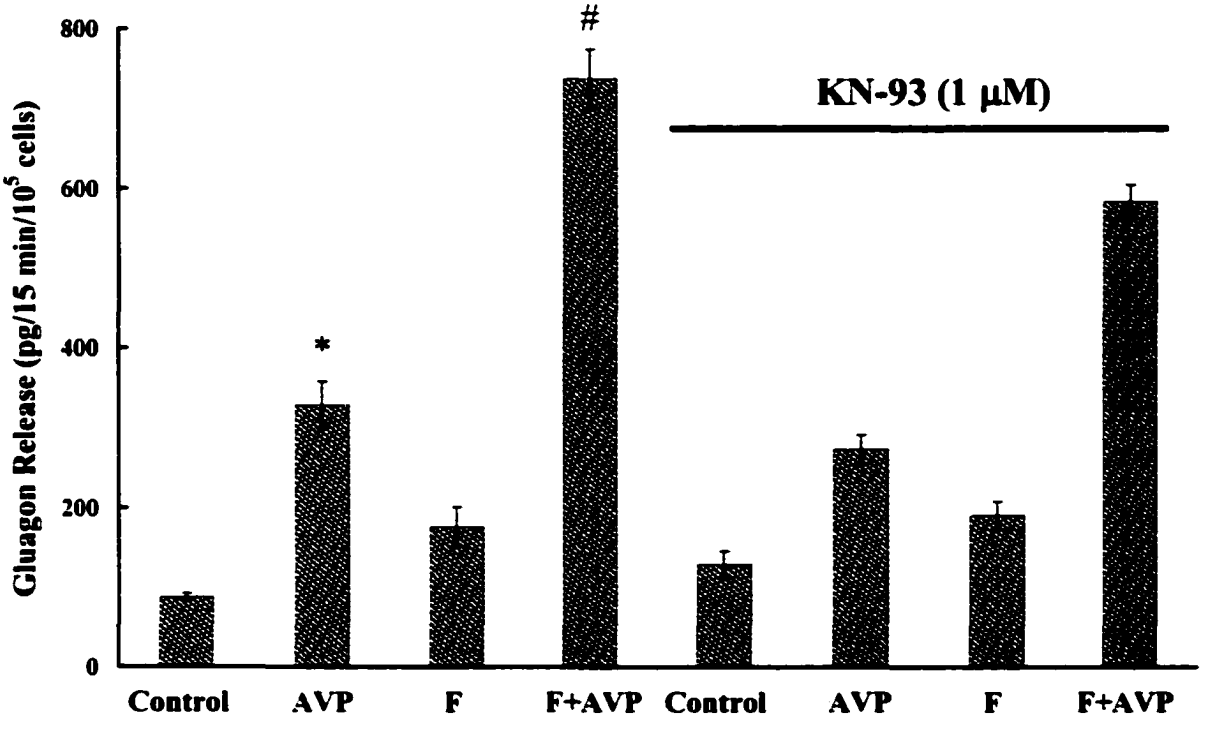
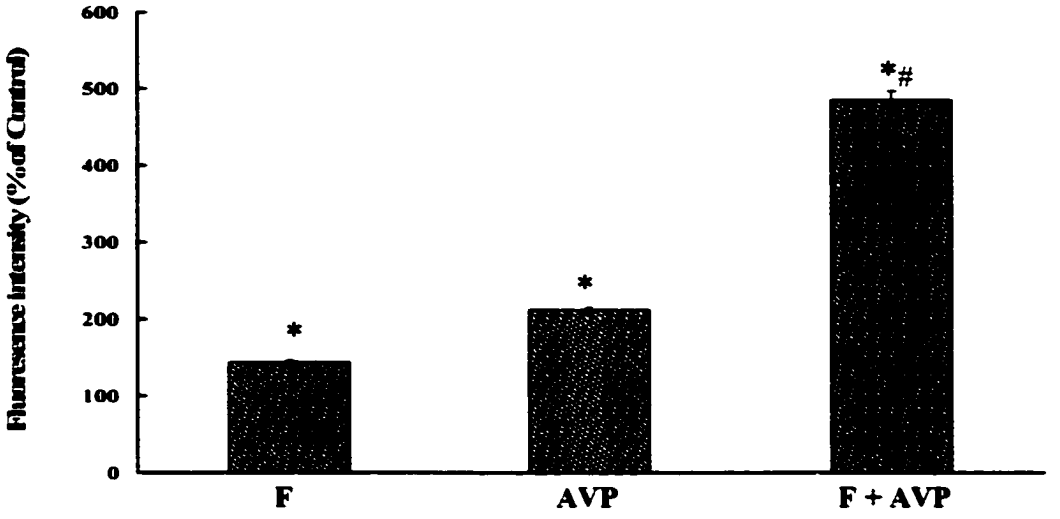


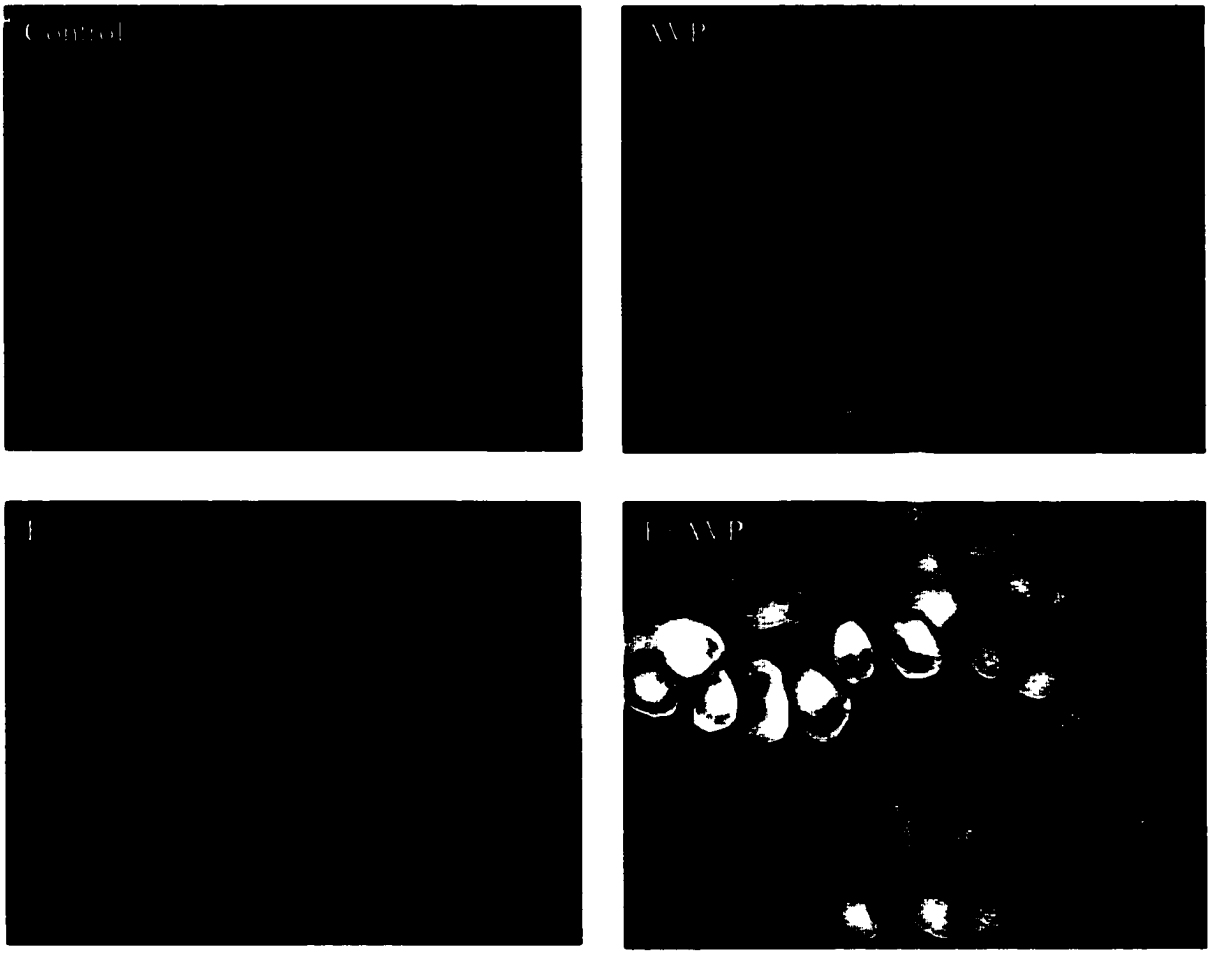
Fig 15. Effects of KN-93 (1 μM), a selective CaM kinase II inhibitor, on forskolin (F) (10 μM)/AVP (100 nM)-induced glucagon release. KN-93 was given 30 min before the administration of AVP. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n=4 cultures with triplicates). *P < .05 AVP vs. AVP in KN-93; # P< .05 F+AVP vs. F+AVP in KN-93 alone.

Fig 16. Effects of forskolin and AVP on FM1-43 fluorescence intensities in InR1G9 cells. A) Forskolin (F) (10 μM) enhanced AVP (100 nM)- induced fluorescence intensities. FM1-43 loading, fluorescent imaging and quantitative analysis of fluorescence intensities are described in the Materials and Methods. Values are mean ± SE (n= at least 200 individual cells for each treatment group). *P < .05 vs. control; # P< .05 vs. AVP alone. B) Fluorescence images of representative cells from (A) loaded by FM1-43 in control, forskolin (F), AVP, and the combination of AVP and forskolin (F + AVP).

A)


Fluorescence Intensity (%of Control)
600
500
400
300
200
100
0
*
*
*#
F
AVP
F + AVP

B)


Control
AVP
F
F+AVP

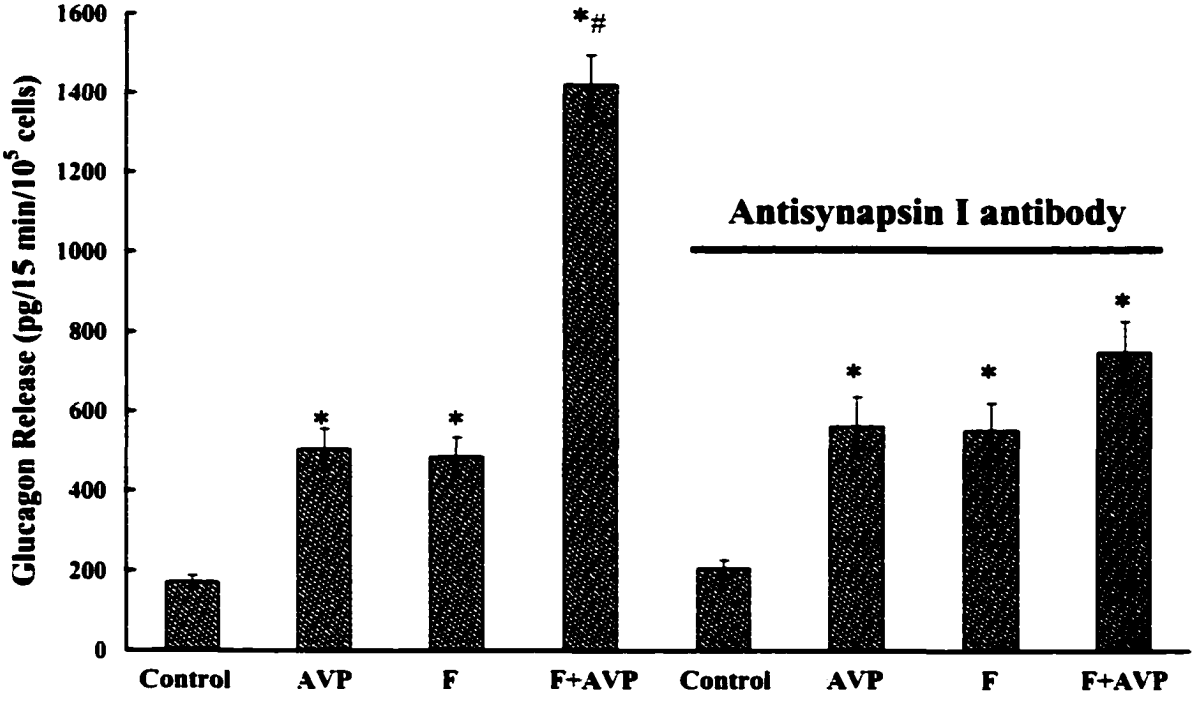
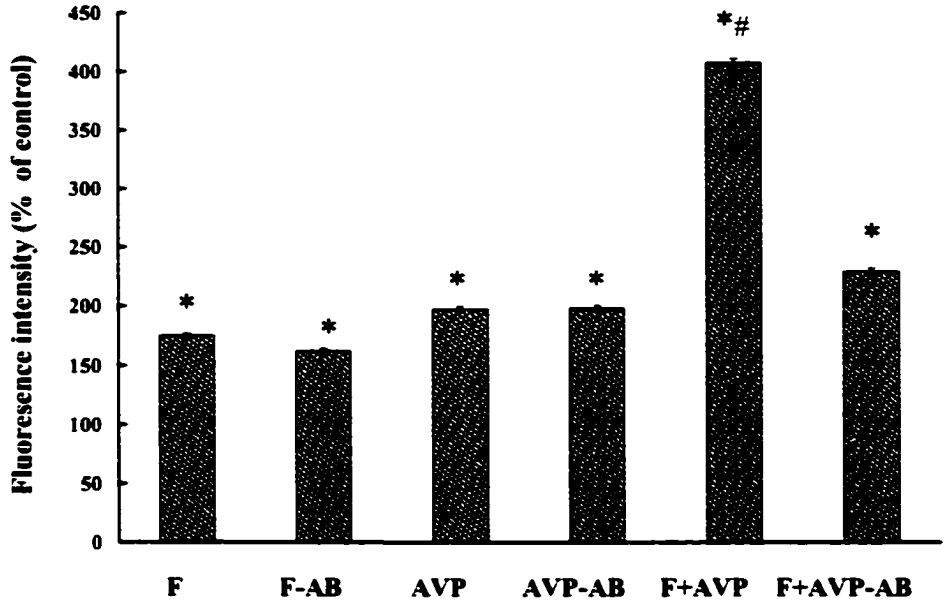


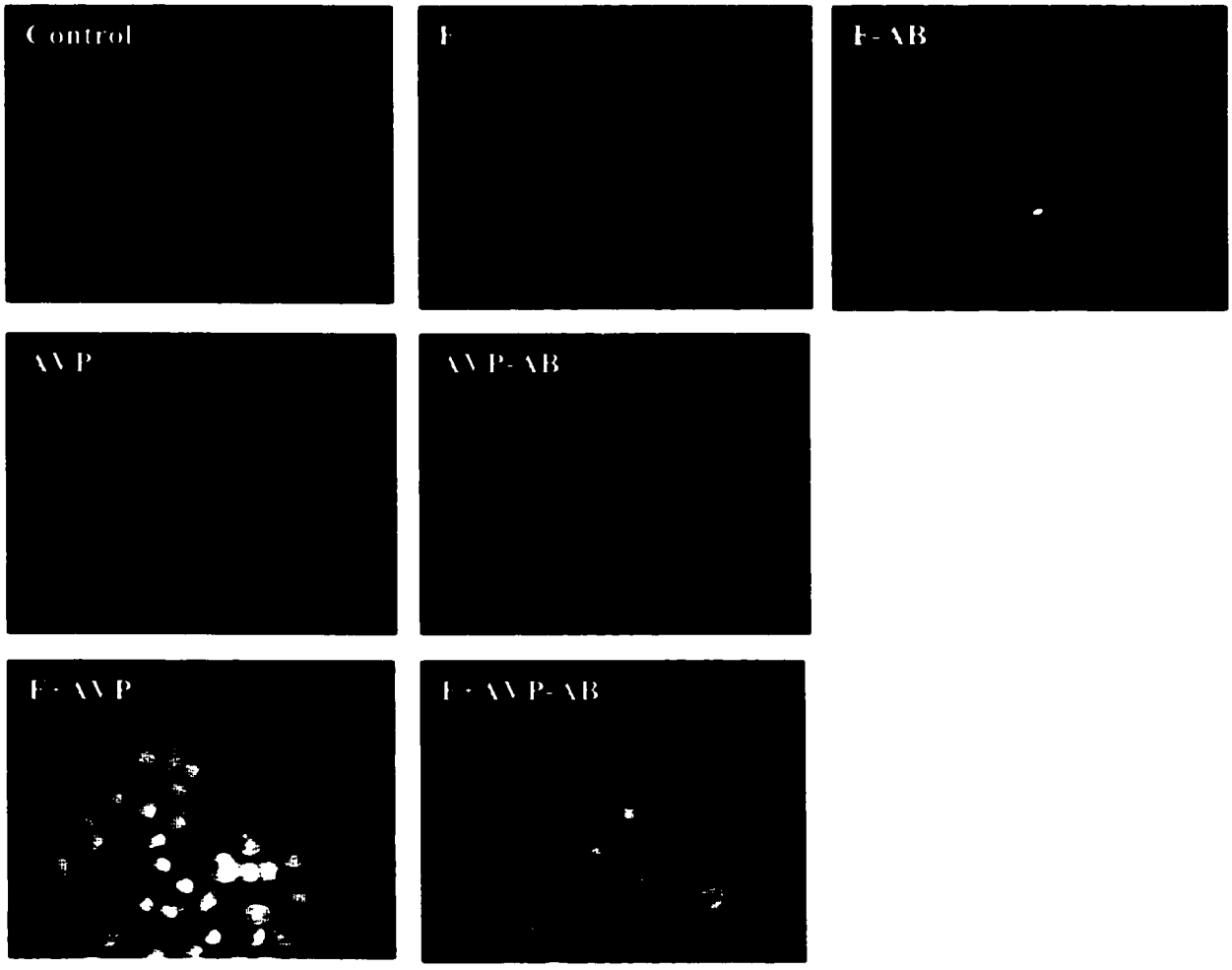
Fig 17. Effects of antisynapsin I antibody on forskolin (F) (10 μM)/AVP (100 nM)-induced glucagon release. Antisynapsin I was given 4 h before the administration of the treatment as described in the Materials and Methods. Static incubation was performed for 15 min to determine glucagon release. Values are mean ± SE (n=4 cultures with triplicates). *P < .05 vs. control without antisyapsin antibody I, # P< .05 vs. AVP alone.

Fig 18. Effect of forskolin and AVP on FM1-43 fluorescence intensities in InR1G9 cells. A) Antisyanpsin I antibody (AB) abolished the effect of forskolin (F) (10 μM) enhancement on AVP (100 nM)- induced fluorescence intensities. FM1-43 loading, fluorescent imaging and quantitative analysis of fluorescence intensities are described in the Materials and Methods. Values are mean ± SE (n= at least 200 individual cells for each treatment group). *P < .05 vs. control; # P< .05 vs. AVP alone. B) Fluorescence images of representative cells from (A) loaded by FM1-43 in control, forskolin (F), AVP, and the combination of forskolin and AVP (F+AVP) and in groups that were pretreated with antisyanpsin I antibody control-AB , forskolin-AB, AVP-AB , and the combination of forskolin-AB and AVP-AB (F+AVP-AB).

A)


Fluoresence intensity (% of control)
450
400
350
300
250
200
150
100
50
0
*
*
*
*
*#
*
F
F-AB
AVP
AVP-AB
F+AVP
F+AVP-AB

B)


Control
F
F-AB
AVP
AVP-AB
F+AVP
F+AVP-AB

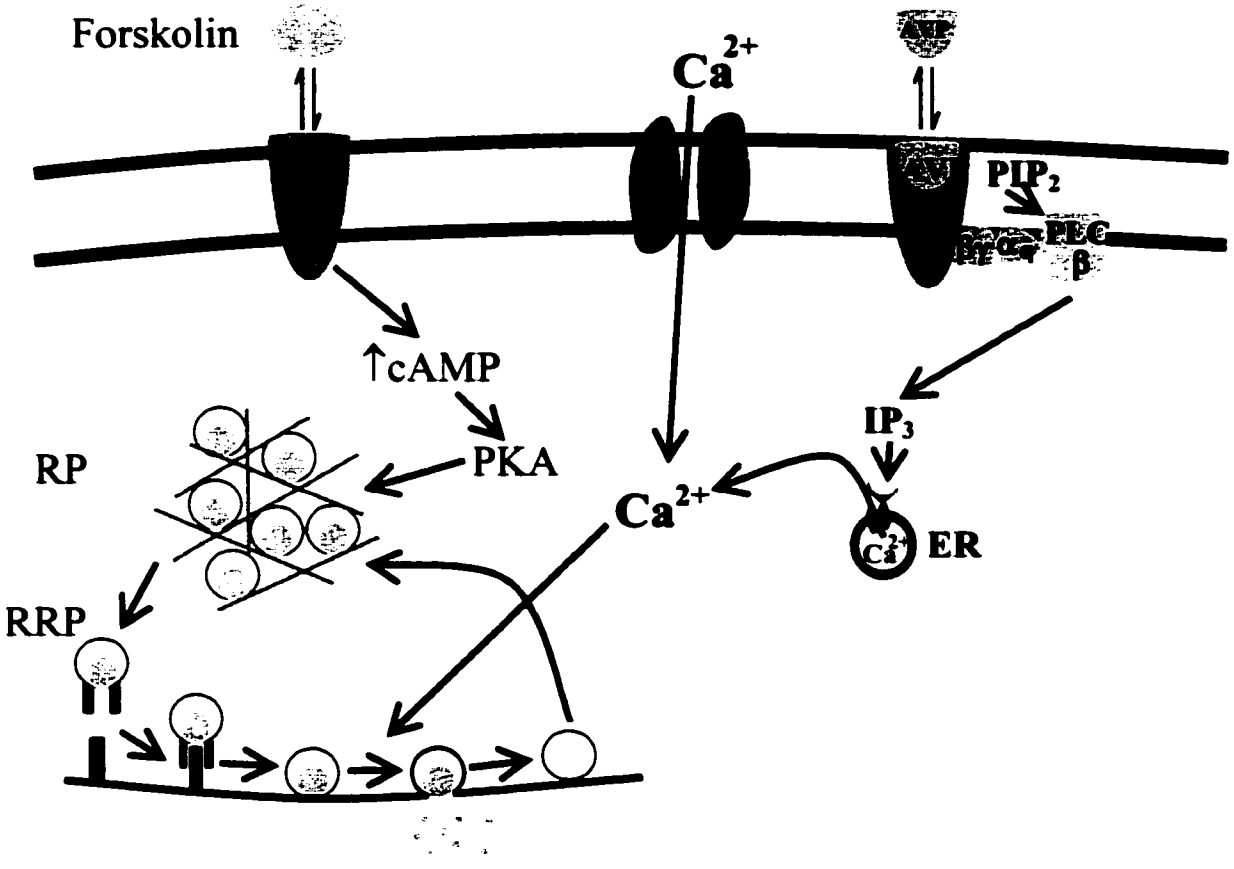


Fig 19. Mechanism of cAMP dependent protein kinase A (PKA) enhanced arginine vasopressin (AVP)-induced glucagon release. PKA may enhance AVP-induced glucagon release by mobilization of secretory granules from the reserve pool (RP) to the readily releasable pool (RRP), an action mediated through synapsin I.

CHAPTER IV. GENERAL CONCLUSIONS

This chapter contains the discussion and the conclusions obtained from the present studies that may be found in the Discussion section of each chapter. In addition, it consists of the possible physiological and clinical implications that are related to our findings. Also, it includes suggestions for the further studies.

Glucose-Dependency of AVP

In the present study, AVP evoked both the release of insulin and glucagon from the perfused rat pancreas in a glucose concentration-dependent manner. Our findings suggest that the higher the glucose concentration, the greater the enhancement of AVP-induced insulin release. In contrast, the lower the glucose, the greater the enhancement of AVP-induced glucagon release. In the pancreatic mouse islets, AVP (100 nmol/L) in the presence of 0, 3, or 7 mmol/L glucose failed to induce insulin release (Gao et al., 1992). These authors concluded that AVP is not an initiator of insulin release but only enhances glucose-induced insulin release. In contrast, our study demonstrated that lower concentration of AVP (300 pmol/L) not only enhanced glucose-induced insulin release, but also initiated insulin release in the basal level of glucose (5.5 mmol/L).

Our findings suggest that AVP has a much greater impact on glucagon than insulin release since AVP (30 pmol/L) increased glucagon but not insulin release. These findings suggest that α-cells are more sensitive to AVP than β-cells in hormone release. These findings are consistent with those obtained from the perfused rat pancreas (Dunning et al., 1984a) and isolated rat islets (Dunning et al., 1984b) and are different from those obtained

from the mouse islets (Gao et al., 1992). In addition, our findings support the notion that AVP has a direct stimulatory effect on glucagon release in α-cells.

During hypoglycemic stress, glucagon release increases and insulin release decreases. Increased glucagon release is the main counterregulatory factor in the recovery of hypoglycemia (Gerich et al, 1979; Cryer, 1981). However, the mechanism underlying hypoglycemia-induced glucagon release is uncertain. A number of studies suggest that the increase in release is due to the activation of autonomic nervous system (Havel and Taborsky, 1989; Havel and Valverde, 1996; Havel et al., 1996). In contrast, our results suggested that the low glucose level directly increases glucagon release, because these experiments were performed in isolated perfused pancreas where there was no input from the autonomic nervous system. These findings are consistent with those found in the isolated islets (Oliver et al., 1976) and perfused pancreata (Weir et al., 1974).

In conclusion, the results from this study suggest that hypoglycemia directly increases glucagon and decreases insulin release. AVP may increase insulin and glucagon release by a direct action on β- and α-cells, respectively. These increases are glucose-dependent; the higher the glucose concentration, the greater the enhancement of AVP induced insulin release. In contrast, the lower the glucose concentration, the greater the enhancement of AVP induced glucagon release. AVP not only may enhance glucose-induced insulin release, but may also initiate insulin release. α-cells are much more sensitive to AVP than β-cells in hormone release. Our findings indicate that AVP may physiologically increase glucagon release, since AVP increased glucagon release at similar plasma

concentrations of AVP (≤ 30 pmol/L) (Thibonnier, 1992), while failed to increase insulin release at these concentrations.

The mechanisms underlying the glucose enhancement of AVP-induced insulin and glucagon releases are not known. Further studies are needed to investigate the mechanisms underlying this enhancement.

cAMP/PKA Enhances AVP-Induced Glucagon Release

In this study, we demonstrated that cAMP/PKA enhance AVP-induced glucagon release from the perfused rat pancreas and InR1G9 cells. Thereafter, we investigated the intracellular molecular mechanism of cAMP/PKA-dependent enhancement of AVP-induced glucagon release, particularly at the level of exocytosis.

The effect of cAMP/PKA on AVP-induced glucagon release is Ca^{2+}-dependent. This is expected since most of the exocytotic fusion is Ca^{2+}-dependent. Granule fusion, per se, occurs independently of protein kinase involvement and is thought to be mediated by the direct action of Ca^{2+} influx through Ca^{2+}/phospholipid binding protein synaptotagmin (Lang et al., 1997; Gerber and Sudhof, 2002). High $[Ca^{2+}]_i$ plays a critical role in secretagogue-induced glucagon secretion (Pipeleers et al., 1985; Hii and Howell, 1986). Stimulation of pancreatic α-cells with secretagogues such as AVP results in a rise in $[Ca^{2+}]_i$ due to Ca^{2+} release from endoplasmic reticulum and Ca^{2+} influx from the extracellular space (Yibchok-anun et al., 2000). Many effects of Ca^{2+} are mediated through Ca^{2+}-binding proteins such as calmodulin (CaM). The effects of Ca^{2+}/CaM may be mediated by CaMK II (Hanson and Schulman 1992; Miyamoto et al., 1997; Easom, 1999). Our findings suggest that CaMK II

may play a partial role in AVP- and forskolin/AVP-induced glucagon release. Although how Ca^{2+} increase regulates the complex series of steps that results in neurotransmitter/ hormonal release remains largely unknown, SNARE complex proteins and synaptotagmin play important roles in regulation of this process (for review see Sudhof, 1995; Hilfiker et al., 1999; Gerber and Sudhof, 2002). Synaptotagmin is an integral Ca^{2+}-binding protein of synaptic vesicle membranes, and binding of Ca^{2+} to synaptotagmin is necessary for fusion of the membranes of secretory granules and the plasma membrane (Gerber and Sudhof, 2002). Gerber and Sudhof (2002) have proposed out a model for the molecular machinery that mediates membrane fusion during exocytosis. However, much remains to be clarified in this cycle including the precise function of certain exocytotic proteins, the validation of the hypothesis that the core complex formation from SNAREs drives fusion, and how Ca^{2+} triggers exocytosis.

The next question is how activation of PKA (e.g., in response to forskolin) enhances AVP-induced glucagon release. PKA must do more than simply changing the Ca^{2+} affinity in the fusion machinary (Ammala et al., 1993) or further enhanceing AVP-induced $[Ca^{2+}]_i$ (as shown in this study). The most straightforward explanation for these results is that phosphorylation by PKA increases the number of granules available for Ca^{2+}-regulated fusion. The fact that glucagon release was prolonged with forskolin treatment in the perfused rat pancreas and from InR1G9 cells led us to hypothesize that PKA promotes the mobilization of secretory granules from RP to RRP. Styryl dyes, which were originally developed as membrane potential sensors, have become useful tools in the study of exocytosis, endocytosis and synaptic vesicle recycling (Betz et al, 1996; Cochilla et al, 1999). The advantage of this imaging method as an indicator of exocytosis, over traditional

methods measuring glucagon release, is that it enables measurement of exocytosis from individual cells with high temporal resolution. Using this method we demonstrated that cAMP/PKA, increases the number of secretory granules in RRP. One limitation of this method is that in order to detect the fluorescent intensity, FM1-43 dye must bind to the exocytosing membrane as secretory granules fuse with plasma membrane during exocytosis. Therefore, an agent that increases the size of RRP can not be detected with this method unless it is used in combination with a secretagogue that induces exocytosis. Thus, the FM1-43 method provides indirect evidence to measure the size of RRP. The use of electron microscopy provides a direct determination for the increase in the size of RRP (Augustin et al., 1999; Neale et al., 1999).

How are the secretory granules released from RP to RRP? One possible mechanism for the recruitment of the secretory granules from RP is the release of granules from the actin-based cytoskeleton induced by phosphorylation of synapsin I (Greengard et al., 1993; Brodin et al., 1997; Benfenati et al., 1999; Hosaka et al., 1999). By analogy with neuronal cells, synapsin I in α-cells cells may function as a linker between glucagon secretory granules and the cytoskeletal network. Synapsin I is found in glucagon-expressing α-cells including InR1G9 cells (Matsumoto et al., 1999) and is phosphorylated at its N-terminus by CaMK II and PKA (Sudhof, 1995). Until this present study, neither the function of synapsin I phosphorylation in α-cells nor the significance of phosphorylation by PKA in pancreatic α-cells was clear. The present study showed that synapsin I is likely involved in forskolin/AVP-induced glucagon release as well as in the mobilization of secretory granules from RP to refill RRP.

Pathophysiological Significance

In this study, we present the first work demonstrating the effects of cAMP/PKA on single exocytotic events and the involvement of synapsin I on glucagon-secreting cells (InR1G9). Our findings suggested that cAMP, acting through PKA, increases the number of secretory granules in RRP by mobilization of granules from RP, an action mediated by synapsin I. In order for exocytosis to continue during extensive stimulation, RRP must be supplied with secretory granules from RP. Agents that increase intracellular cAMP concentrations cause sustained exocytosis (Eddlestone et al., 1985; Hill et al., 1987; Gills and Misler, 1993) and have been used in combination with secretagogues to augment exocytosis (Henquin 1985; Sharp et al., 1993; Wang et al., 1993). This effect is due to an increase in the size of RRP as indicated in this study and others (Renstrom et al., 1997; Gromada et al., 1997). Because of the similarities between glucagon granules and other secretory granules in neuroendocrine cells such as pancreatic β-cells, chromaffin cells, pituitary somatotrophs, pituitary melanotrophs and lactotrophs, it is probable that our results can be extrapolated to these endocrine cells, and suggest that elevated intracellular cAMP concentrations can modulate the size of RRP.

Diabetic patients have elevated levels of plasma AVP (Grimaldi et al., 1988; Tinder et al., 1994) and the α-cells from diabetic subjects may exhibit an exaggerated response to AVP (our unpublished data). Therefore, our present findings that elevation of cAMP concentrations enhances AVP-induced glucagon may partially account for the clinical observations that hypersecretion of glucagon aggravates the hyperglycemia associated with type 2 diabetes (Unger and Orci 1995; Dinnen et al., 1995). AVP may aggravate

hyperglycemia in diabetic patients by increasing glucagon secretion. It is possible that the use of V_{1b} receptor antagonists may decrease glucagon release in these patients. Further work is needed to prove or disprove this hypothesis.

Future studies

Several lines of evidence suggest that phosphorylation of exocytotic proteins play a major role in modulating neurotransmitter/hormonal release. Although, the precise downstream targets of PKA are currently unknown, several intracellular regulatory proteins have been shown to be substrates for PKA in vitro. These include rabphilin-3A (Lonart et al., 1998), synapsin (Sudhof, 1995), syntaxin (Trudeau et al., 1998) and SNAP-25 (Foster et al., 1998). Thus, it is likely that the modulation of glucagon release produced by the activated PKA could occur through a direct phosphorylation of one or more of these exocytotic proteins. In addition, AVP-induced glucagon release is mediated largely by a Ca^{2+}-dependent pathway (Yibchok-anun et al., 2000). It is well known that Ca^{2+} activates CaMK II, which may lead to phosphorylation of a set of exocytotic proteins including rabphilin-3A (Fykse et al., 1995), synaptotagmin (Popoli, 1993), syntaxin (Trudeau et al., 1998) and SNAP-25 (Foster et al., 1998).

Although, both PKA and CaMKII may phosphorylate the mentioned above exocytotic proteins, the information is still largely unknown and controversial. First, most of the studies were performed in the cell-free system using the catalytic subunit of PKA and purified CaMKII to demonstrate the phosphorylation by these kinases. This might not be the case if these studies were performed in cells under physiological conditions. For example, in

intact cells CaMKII but not PKA phosphorylated rabphilin-3A (Fykse, 1998), whereas in cell-free system both PKA and CaMKII phosphorylated rabphilin-3A (Fykse, 1995; Lonart et al., 1998). Second, there are contradictory findings between studies. For example, syntaxin was phosphorylated by PKA in one study (Foster et al., 1998) but not in another (Risinger and Bennett, 1999). SNAP-25 was phosphorylated by PKA in one study (Risinger and Bennett, 1999) but not in another one (Foster et al., 1998). Third, these studies did not compare the magnitude of protein phosphorylation between PKA and CaMKII.

Although, the CaMK II inhibitor, KN93, reduced AVP- and forskolin/AVP-induced glucagon release, the reduction was small. In addition, we recently found that CaMK II antibody failed to reduce the effect of AVP or AVP/forskolin on glucagon release (unpublished data). This suggests that CaMK II may only play a minor role in glucagon release and the increase in Ca^{2+} may directly activate other exocytotic proteins. Investigation of the potential phosphorylation of the above-mentioned proteins by PKA and CaMK II and the magnitude of the phosphorylation could provide better understanding of the efficacy of downstream steps in the exocytotic process.

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