British Journal of Pharmacology (2000) 129, 257–264
www.nature.com/bjp

Mechanisms of AVP-induced glucagon release in clonal α-cells In-R1-G9: involvement of Ca^{2+}-dependent and -independent pathways

[1]Sirintorn Yibchok-anun, [1]Henrique Cheng, [2,3]Ter-Hsin Chen & *,[1]Walter H. Hsu

[1]Department of Biomedical Sciences, Iowa State University, Ames, IA 50011-1250, U.S.A.; [2]Pig Research Institute of Taiwan, Chunan, Miaoli, Taiwan 35099, Republic of China and [3]Department of Occupational Safety and Health, China Medical College, Taichung, Taiwan 40421, Republic of China

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

2 AVP increased $[Ca^{2+}]_i$ and glucagon release in a concentration-dependent manner. After the administration of AVP, glucagon was released within 30 s, quickly reached the maximum within 2 min, and maintained a steady-state concentration for at least 15 min.

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

4 Pretreatment with thapsigargin, a microsomal Ca^{2+} ATPase inhibitor, abolished both the Ca^{2+} peak and sustained plateau.

5 AVP increased intracellular concentration of IP_3.

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

7 Pretreatment with nimodipine, an L-type Ca^{2+} channel blocker failed to alter AVP-induced glucagon release or increase in $[Ca^{2+}]_i$.

8 The results suggest that AVP causes glucagon release through both Ca^{2+}-dependent and -independent pathways. For the Ca^{2+}-dependent pathway, the G_q protein activates phospholipase C, which catalyzes the formation of IP_3. IP_3 induces Ca^{2+} release from the endoplasmic reticulum, which, in turn, triggers Ca^{2+} influx. Both Ca^{2+} release and Ca^{2+} influx may contribute to AVP-induced glucagon release.

British Journal of Pharmacology (2000) **129,** 257–264

Keywords: U-73122; intracellular Ca^{2+}; phospholipase C

Abbreviations: AVP, arginine vasopressin; BAPTA-AM, 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester; CL-4-84, 4-OH-phenacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2; ER, endoplasmic reticulum; PIP_2, phosphatidylinositol 4, 5-bisphosphate; PLA_2, phospholipase A_2, PLC-β, phospholipase C-β; PLD, phospholipase D; U73122, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole,2,5-dione; U-73343, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidine-dione; VDCC, voltage-dependent Ca^{2+} channel; WK-3-6, desGly^{9}d$(CH_2)_5$[Tyr(Et)^2]AVP; zLYCK, carbobenzyloxy-leucine-tyrosine-chloromethylketone

Introduction

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

AVP induces glucagon release from clonal α-cells (Yibchok-anun & Hsu, 1998) and rat pancreas (Yibchok-anun *et al*., 1999) through V_{1B} receptors in a concentration-dependent manner. AVP at the concentrations existed in the plasma (3–30 pM) increases glucagon release from perfused rat pancreas, which suggests that AVP may physiologically regulate glucagon release (Yibchok-anun *et al*., 1999). However, the mechanisms underlying AVP-induced glucagon release remain unknown. Typically, AVP activates V1 receptors, which couple to G_q, and thus activates phospholipase C-β (PLC-β), which in turn hydrolyzes phosphatidylinositol 4, 5-bisphosphate (PIP_2) to DAG and IP_3 (Thibonnier, 1992). DAG activates PKC, whereas IP_3 promotes Ca^{2+} release from endoplasmic reticulum (ER), leading to an increase in $[Ca^{2+}]_i$. The increase in $[Ca^{2+}]_i$ induces Ca^{2+} influx through voltage-dependent (VDCC) and -independent Ca^{2+} channels (VICC) (Chen *et al*., 1994; Li *et al*., 1992; Thorn & Petersen, 1991).

In-R1-G9 cells are clonal glucagon-secreting cells derived from the hamster pancreatic islet (Takaki *et al*., 1986). The synthesis and secretion of glucagon by In-R1-G9 cells share the basic characteristics of α-cells of the endocrine pancreas

*Author for correspondence; E-mail: whsu@iastate.edu

(Rorsman *et al*., 1991); for instance, glucagon secretion from these cells is stimulated by forskolin, arginine, and theophylline and is inhibited by somatostatin (Fehmann *et al*., 1995). We therefore used this cell line as a model for the α-cell of the pancreatic islet to study the mechanisms underlying AVP-induced glucagon release.

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

Methods

Cell culture

The hamster glucagonoma In-R1-G9 cells were maintained in RPMI 1640 with 10% foetal bovine serum and aerated with 5% CO_2-95% air at 37°C. All experiments were performed using cells from passages 23–30.

Glucagon release

In-R1-G9 cells were plated into Corning 24-well plates at approximately 10^5 cells $well^{-1}$ 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): NaCl 136, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 5, HEPES 10, glucose 1.67 and 0.1% BSA, pH 7.4. For determination of the dose response to AVP, cells were incubated at 37°C with AVP for 15 min after preincubation with KRB for 15 min. For Ca^{2+}-free experiments, cells were washed with Ca^{2+}-free KRB containing 10 μM EGTA for 3 times, then incubated at 37°C with AVP in Ca^{2+}-free KRB containing 10 μM EGTA for 15 min after preincubation with Ca^{2+}-free KRB containing 10 μM EGTA for 15 min. For time course study of glucagon release in response to AVP, cells were treated with AVP (100 nM) in 0.5 ml KRB after preincubation with KRB for 15 min, the samples were collected at 0.5, 1, 2, 5, and 15 min after the administration of AVP. At the end of the experiments, cells were disrupted using Sonifier Cell Disruptor (Heat Systems Co., Melville, NY, U.S.A.), then glucagon content of the cells was measured using radioimmunoassay (RIA). The following drugs were used in the study: U-73122 or U-73343, an inactive analogue of U-73122, was given 100 s; nimodipine was given 5 min; carbobenzyloxy-leucine-tyrosine-chloromethylketone (zLYCK), an inhibitor of PLD (Kusaka, *et al*., 1996), was given 1 h prior to the administration of AVP. 1,2-bis-(o-Aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid acetoxymethyl ester (BAPTA-AM), an intracelluar Ca^{2+} chelator, was given 30 min in Ca^{2+}-free KRB containing 10 μM EGTA before the administration of AVP. The cells were then treated with AVP in Ca^{2+}-free KRB containing 10 μM EGTA and BAPTA-AM. The concentration of glucagon in the media was measured by RIA, following the procedures provided by Linco Research Inc.

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

20×10^6 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 ($300 \times g$, 2 min), then resuspended at a concentration of 2×10^6 cells/ml with KRB containing (in mM): NaCl 136, KCl 4.8, $CaCl_2$ 1.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 10, glucose 1.67 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, U.S.A.). The Ca^{2+}-free environment was created by centrifugation ($300 \times g$, 30 s) and the cells were resuspended in the Ca^{2+}-free KRB. When needed, cells were pretreated with U-73122, U-73343, 4-OH-phenacetyl-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2 (CL-4-84), an antagonist with potent V_{1B} blocking activity (Thibonnier *et al*., 1997), desGly^{9}d$(CH_2)_5$[-Tyr(Et)^2]AVP (WK-3-6), a V_{1A}/V_2 antagonist (Jard *et al*., 1986), nimodipine for 100 s before the AVP application. The cells were pretreated with thapsigargin (TG) for 30 min before $[Ca^{2+}]_i$ measurement. The $[Ca^{2+}]_i$ was calibrated as previously described (Hsu *et al*., 1991).

Measurement of IP_3

Intracellular IP_3 was measured using a competitive radioreceptor-binding assay kit purchased from Dupont Co., Boston, MA, U.S.A. 2×10^6 cells in 1 ml of KRB were placed in polypropylene tubes and equilibrated in a shaking water bath at 37°C for 15 min. Incubation with AVP was terminated by adding ice-cold 20% (w v^{-1}) trichloroacetic acid in 15 s. The concentration of IP_3 was determined by following the instructions provided by the manufacturer. Briefly, after removal of trichloroacetic acid from the extracts, 100 μl of each sample or standard was added to minitubes, then 400 μl of the working receptor or [^{3}H]-IP_3 tracer solution was added to all the tubes, then incubated for 1 h at 4°C. After incubation, the tubes were centrifuged at 4°C for 10 min at $1000 \times g$. The supernatant was decanted and the membrane pellets were solubilized in 50 μl of 0.15 M NaOH. The tubes were subjected to scintillation counting. This assay is ~100 times more specific for IP_3 than other IP isoforms (Dupont Co., Boston, MA, U.S.A.).

Cyclic AMP measurement

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

Drugs

All reagents were from Sigma Chemical (St. Louis, MO, U.S.A.), except the fura-2AM was from Molecular Probes (Eugene, OR, U.S.A.), U-73122, U-73343, BAPTA-AM were from Biomol Research Laboratory (Plymouth Meeting, PA, U.S.A.), and ^{125}I-glucagon was from Linco Research Inc. (St. Charles, MO, U.S.A.).

Data and statistics

All values were presented as mean ± s.e. Results from glucagon release experiment were analysed using the SAS PROC MIXED procedure and a randomized block design. There were two factors, treatment and block. Individual mean

comparisons were performed using the F test. The significance level was set at $P<0.05$.

Results

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

AVP (1–1000 nM) increased glucagon release (Figure 1A) and $[Ca^{2+}]_i$ (Figure 1B) in a concentration-dependent manner. Because AVP at 100 nM caused submaximal increases in glucagon release and $[Ca^{2+}]_i$, we chose this concentration to study the mechanisms underlying AVP-induced glucagon release and increases in $[Ca^{2+}]_i$. AVP (100 nM) significantly increased glucagon release to ~3 times of the basal control level in Ca^{2+}-containing medium. The concentration of glucagon release in the basal control group was 144 ± 17 pg 10^5 cells^{-1} 15 min^{-1} ($n=6$ cultures with triplicates; cell passages 23–26). The basal glucagon release was not significantly lower in Ca^{2+}-free medium (127 ± 33 pg 10^5 cells^{-1}, $n=6$ cultures with triplicates; cells passages 23–26) than in Ca^{2+}-containing medium. In Ca^{2+}-free medium, AVP (100 nM) increased glucagon release to 2.1 times of the basal control level (control = 127 ± 33 pg 10^5 cells^{-1}; AVP = 268 ± 51 pg 10^5 cells^{-1}, $n=6$ cultures with triplicates; passage 23–26). In addition, although $[Ca^{2+}]_i$ was deprived by preincubating the cells in Ca^{2+}-free medium containing 50 μM BAPTA-AM for 30 min, AVP still increased glucagon release to 1.6 times of the basal control level. BAPTA-AM alone did not significantly change glucagon release (Figure 2).

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

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

The data in Figure 4 show that AVP increased glucagon release within 30 s of the administration (control = 100 ± 19 pg 10^5 cells^{-1}; AVP = 325 ± 40 pg 10^5 cells^{-1}, $n=4$ cultures with quadruplicates; passages 23–26), which reached the maximum within 2 min (control = 110 ± 9 pg 10^5 cells^{-1}; AVP = 572 ± 52 pg 10^5 cells^{-1}; passages 23–26), and maintained a steady-state at 15 min (control = 96 ± 10 pg 10^5 cells^{-1}; AVP = 476 ± 50 pg 10^5 cells^{-1}; passages 23–26).

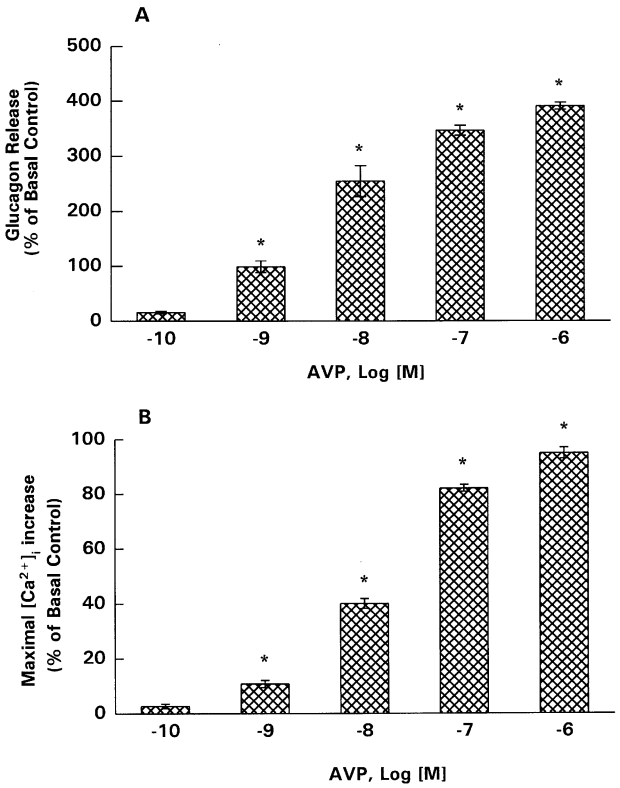

Figure 1 Effects of AVP on glucagon release (A) and $[Ca^{2+}]_i$ increase (B) in In-R1-G9 cells. In (A) static incubation was performed for 15 min to determine glucagon release. The concentration of glucagon release in the basal control group was 235 ± 17 pg 10^5 cells^{-1}; cell passages 25–29. In (B), the basal $[Ca^{2+}]_i$ was 97 ± 4 nM. Values are mean$\pm$s.e.mean ($n=4$). Values are mean$\pm$s.e.mean ($n=3$ cultures with quadruplicates). *$P<0.05$, compared with the control group.

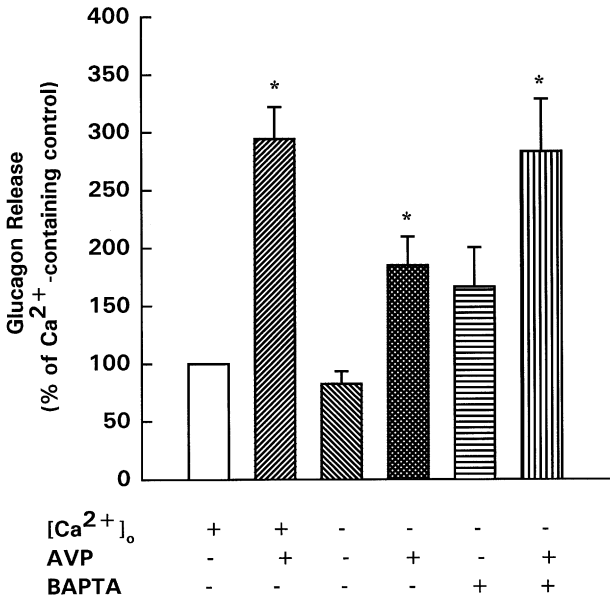

Figure 2 Effects of AVP (100 nM) on glucagon release in Ca^{2+}-containing, Ca^{2+}-free media and 50 μM BAPTA-AM in Ca^{2+}-free media. BAPTA-AM in Ca^{2+}-free media was given 30 min before the administration of AVP. Static incubation was performed for 15 min to determine the glucagon release. The concentration of glucagon release in the Ca^{2+}-containing control was 144 ± 17 pg 10^5 cells^{-1}. Values are mean$\pm$s.e.mean ($n=6$ cultures with quadruplicates). *$P<0.05$, compared with the basal control group at the $[Ca^{2+}]_i$ of 2.5 mM. The results of this experiment were obtained from the cells of passage 23–26.

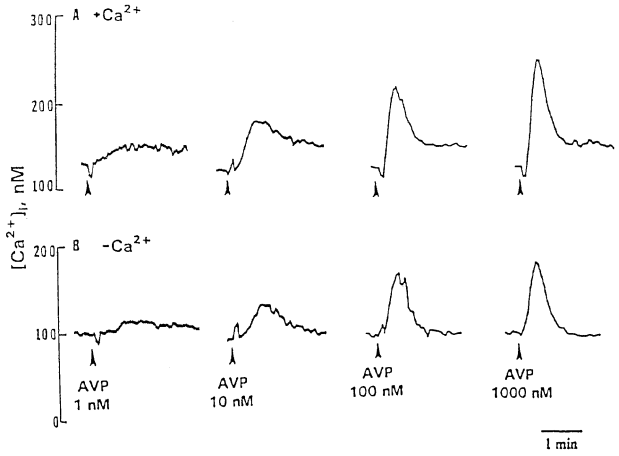

Figure 3 Effects of AVP on $[Ca^{2+}]_i$ in Ca^{2+}-containing (A) and Ca^{2+}-free (B) media. Data shown are representative of four experiments.

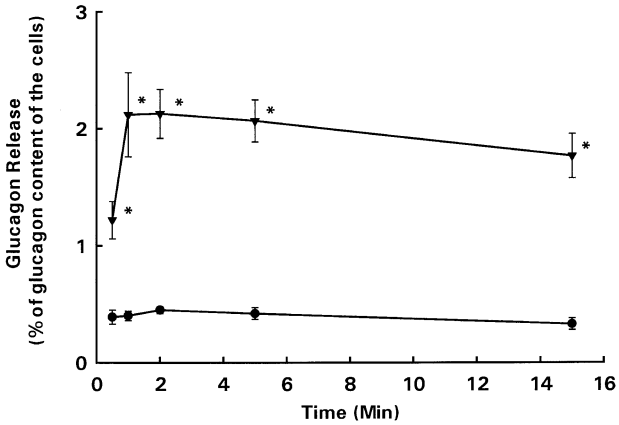

Figure 4 Time course of glucagon release from In-R1-G9 cells in response to AVP (100 nM). Values are mean ± s.e.mean ($n=4$ cultures with quadruplicates). Mean glucagon content of the cells; basal = 25 ± 1.6 ng 10^5 cells^{-1}, AVP = 27 ± 2.8 ng 10^5 cells^{-1}. ● Basal control; ▼ AVP 100 nM. *$P<0.05$, compared with the basal control group. The results of this experiment were obtained from the cells of passage 23–26.

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

AVP increases glucagon release from In-R1-G9 cells (Yibchok-anun & Hsu, 1998) and perfused rat pancreas (Yibchok-anun *et al*., 1999) by activating V_{1B} receptors. To study if AVP-induced $[Ca^{2+}]_i$ increase is also mediated by V_{1B} receptors, we pretreated the cells with different AVP receptor antagonists for 100 s before the application of AVP (100 nM). CL-4-84 (0.1–100 nM), a V_{1A}/V_{1B} receptor antagonist (Thibonnier *et al*., 1997), inhibited AVP-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner (Figure 5). CL-4-84 at the highest concentration studied (100 nM) abolished the AVP-induced rise in $[Ca^{2+}]_i$. IC_{50} of CL-4-84 was 2 ± 0.5 nM. WK-3-6 (1 μM), a potent V_{1A}/V_2 receptor antagonist (Jard *et al*., 1986), failed to block AVP-induced $[Ca^{2+}]_i$ increase (data not shown). Neither CL-4-84 nor WK-3-6 alone significantly changed basal $[Ca^{2+}]_i$ (data not shown).

Effects of U-73122, U-73343, and zLYCK *on AVP-induced glucagon release and $[Ca^{2+}]_i$ increase*

To determine whether AVP-induced increases in glucagon release and $[Ca^{2+}]_i$ are mediated through PLC pathway, U-

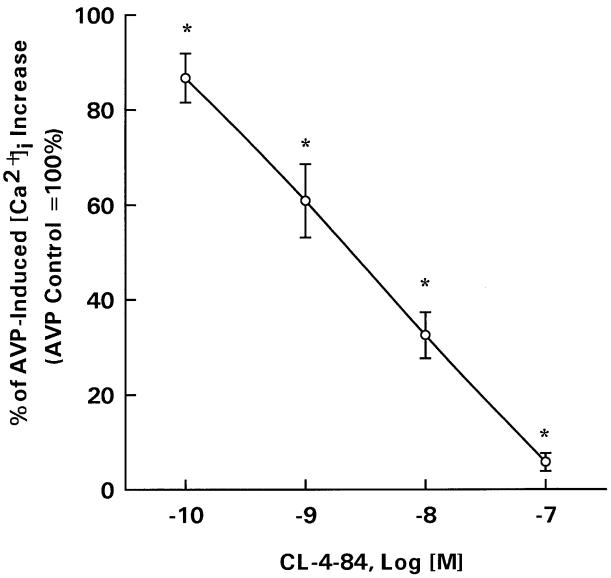

Figure 5 Effects of CL-4-84 on AVP (100 nM)-induced maximal increase in $[Ca^{2+}]_i$. CL-4-84 was given 100 s before the administration of AVP. Values are mean ± s.e.mean ($n=4$). *$P<0.05$, compared with the AVP control group, which had a maximal $[Ca^{2+}]_i$ increase of 188 ± 11 nM.

73122 were used to antagonize the effect of AVP. U-73122 (2, 4 and 8 μM) inhibited AVP-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner (Figure 6A). At highest concentration studied (8 μM), U-73122 abolished AVP-induced the rise in $[Ca^{2+}]_i$, but only reduced AVP-induced glucagon release by 39% (Figure 6B). The lower concentrations of U-73122 (2 and 4 μM) partially inhibited AVP-induced $[Ca^{2+}]_i$ increase, but did not reduce AVP-induced glucagon release. U-73122 alone did not change $[Ca^{2+}]_i$ until 8 μM was applied, which increased $[Ca^{2+}]_i$ by ~15%, and then gradually returned to the basal level within 100 s. To determine if U-73122 was specific to AVP-induced $[Ca^{2+}]_i$ increase, ionomycin (300 nM), a Ca^{2+} ionophore, was used for this purpose. Ionomycin elicited a biphasic rise in $[Ca^{2+}]_i$ with a pattern similar to that induced by AVP (100 nM). U-73122 (8 μM) failed to inhibit ionomycin-induced rise in $[Ca^{2+}]_i$ (ionomycin = 244 ± 41 nM; U73122 + ionomycin = 300 ± 34 nM, $n=4$; $P>0.05$) (Figure 7A). In addition, U-73343 (8 μM), an inactive analogue of U-73122, failed to inhibit the AVP-induced $[Ca^{2+}]_i$ increase (AVP = 266 ± 52 nM; U-73343 + AVP = 278 ± 59 nM, $n=6$; $P>0.05$) and glucagon release (basal = 105 ± 24 pg 10^5 cells^{-1}; AVP = 497 ± 38 pg 10^5 cells^{-1}; U-73343 + AVP = 479 ± 47 pg 10^5 cells^{-1}, $n=3$ cultures with quadruplicates obtained from the cells of passage 25–27; $P>0.05$).

To determine whether the phospholipase D (PLD) pathway is involved in AVP-induced glucagon release, zLYCK, a PLD inhibitor, was used to antagonize the effect of AVP. Pretreatment of the cells with 10 μM zLYCK for 1 h reduced AVP-induced glucagon release by 60% (basal = 184 ± 38 pg 10^5 cells^{-1}; AVP = 1175 ± 263 pg 10^5 cells^{-1}; zLYCK = 214 ± 44 pg 10^5 cells^{-1}; zLYCK + AVP = 551 ± 244 pg 10^5 cells^{-1}, $n=3$ cultures with quadruplicates; passages 24–27; $P<0.05$).

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

We next hypothesized that AVP increases $[Ca^{2+}]_i$ by inducing Ca^{2+} release from ER, which in turn increases

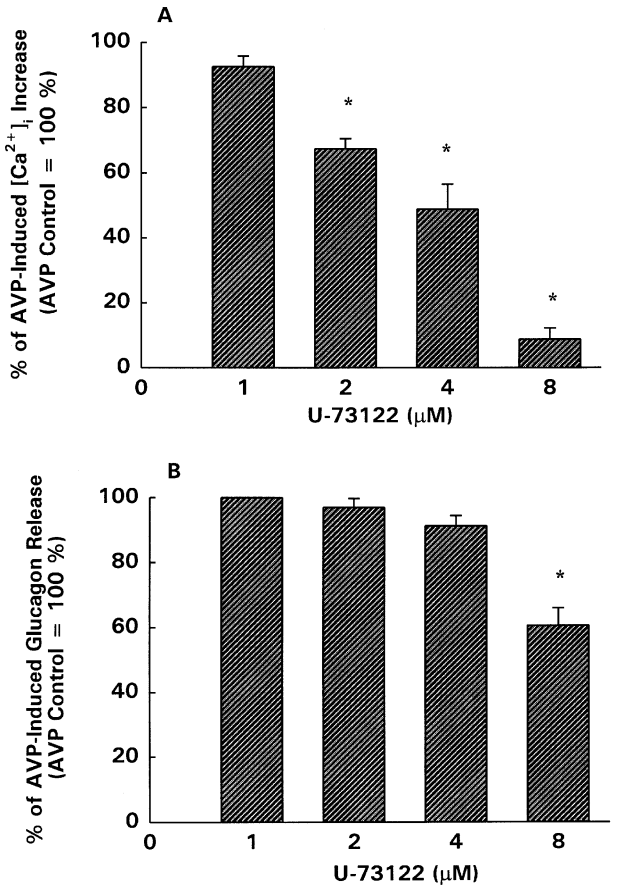

Figure 6 Effects of U-73122 on AVP-induced maximal $[Ca^{2+}]_i$ increase (A) and glucagon release (B) in In-R1-G9 cells. U-73122 was given 100 s before AVP (100 nM). Values are mean ± s.e.mean ($n=4$ for $[Ca^{2+}]_i$ experiments and $n=3$ cultures with quadruplicates for secretion). *$P<0.05$, compared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

Ca^{2+} influx. We depleted intracellular Ca^{2+} stores of In-R1-G9 cells by pretreating the cells with 1 μM TG, a microsomal Ca^{2+}-ATPase inhibitor (Thastrup *et al.*, 1990), for 30 min, which abolished both AVP-induced Ca^{2+} release and influx (Figure 7B). The $[Ca^{2+}]_i$ after TG treatment was 129 ± 19 nM ($n=4$). To investigate if the AVP-induced Ca^{2+} influx was attributable to the opening of VDCCs, we pretreated In-R1-G9 cells with 1 μM nimodipine, an L-type VDCC blocker, for 100 s. Nimodipine failed to inhibit AVP-induced $[Ca^{2+}]_i$ increase (Figure 7C) and glucagon release (basal = 262 ± 53 pg 10^5 cells^{-1}; AVP = 594 ± 153 pg 10^5 cells^{-1}; nimodipine = 261 ± 61 pg 10^5 cells^{-1}; nimodipine + AVP = 667 ± 230 pg 10^5 cells^{-1}, $n=3$ cultures with quadruplicates; passages 28 – 30).

Effects of AVP on intracellular IP_3 and cyclic AMP concentrations

To confirm whether AVP induces glucagon release and $[Ca^{2+}]_i$ increase through the PLC-IP_3 system, but not the adenylyl cyclase-cyclic AMP system, we measured the AVP-induced changes in the intracellular concentration of IP_3 and cyclic-AMP. AVP significantly increased intracellular IP_3 concentration by 147% (control = 8 ± 2 pmol 10^6 cells^{-1}; AVP (100 nM) = 20 ± 2 pmol 10^6 cells^{-1}, $n=5$; $P<0.05$). AVP failed to change intracellular cyclic-AMP concentrations (control = 3.6 ± 0.2 pmol well^{-1}; AVP (1 μM) = 3.8 ± 0.2 pmol well^{-1}, $n=4$; $P>0.05$).

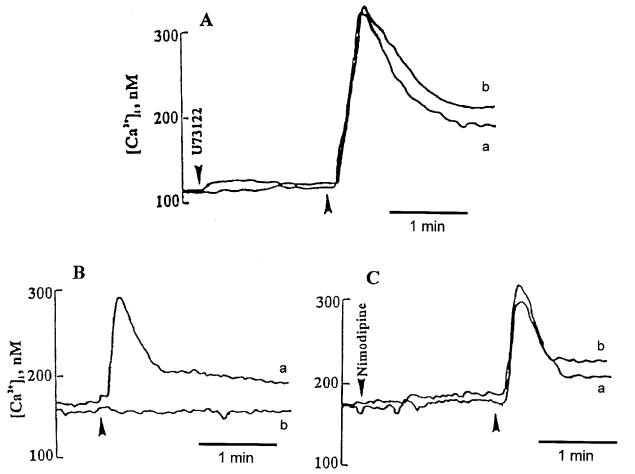

Figure 7 Effects of AVP and ionomycin on $[Ca^{2+}]_i$ increase in In-R1-G9 cells. (A) Effects of U-73122 on ionomycin-induced $[Ca^{2+}]_i$ increase. Curve a shows data of ionomycin (300 nM) alone as a control; curve b shows the effect of U-73122 (8 μM) pretreatment for 100 s before ionomycin administration. (B) Effect of TG on AVP-induced $[Ca^{2+}]_i$ increase. Curve a shows data of AVP (100 nM) alone as a control; curve b shows the effect of TG pretreatment (1 μM) for 30 min before AVP administration. (C) Nimodipine did not affect AVP-induced $[Ca^{2+}]_i$ increase. Curve a shows data of AVP (100 nM) alone as a control; curve b shows the effect of nimodipine pretreatment (1 μM) for 100 s before AVP administration. Arrow indicates AVP or ionomycin administration. Data shown are representative of four experiments.

Discussion

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

The V_{1B} receptor has seven transmembrane-binding domains, and is coupled to a PTX-insensitive G-protein, probably G_q (Thibonnier *et al.*, 1993). The activation of PLC *via* G_q is responsible for the hydrolysis of PIP_2 into DAG and IP_3, which causes Ca^{2+} release from the ER. In this study, since AVP increased IP_3 formation and it had no effect on cyclic AMP production, the activation of PLC-β-IP_3 system should be one of the signal transduction pathways through which AVP induces glucagon release. In addition, we found that 8 μM U-73122, the specific PLC inhibitor, abolished AVP-induced $[Ca^{2+}]_i$ increase, but only reduced AVP-induced glucagon release by 39%. U-73343, an inactive analogue of U-73122 failed to inhibit AVP-induced glucagon release and [Ca2 +]$_i$ increase. These findings confirm that AVP activates PLC-β to increase IP_3 production, leading to the elevations of $[Ca^{2+}]_i$ and glucagon release.

An increase of $[Ca^{2+}]_i$ frequently triggers exocytosis (Wollheim & Pozzan, 1984; Li *et al.*, 1992; Tse *et al.*, 1993; Gromada *et al.*, 1997). Ca^{2+} oscillation is the pacemaker of pulsatile glucagon release in α-cells (Bode *et al.*, 1994). Thus, Ca^{2+} is thought to be a major signal for

glucagon release in In-R1-G9 cells. AVP induced $[Ca^{2+}]_i$ increase in In-R1-G9 cells in a biphasic pattern; a peak followed by a sustained plateau. The Ca^{2+} increase induced by AVP usually reached the maximum within 30 s, which was consistent with the results of the time course study of glucagon release, in which glucagon was released within 30 s of the AVP administration. These results suggest that Ca^{2+} is a signal for AVP to trigger glucagon release. In Ca^{2+}-free experiments, AVP only evoked a Ca^{2+} peak without a sustained phase. The Ca^{2+} peak evoked by AVP in the absence of extracellular Ca^{2+} was lower than in the presence of extracellular Ca^{2+}. These results suggest that the Ca^{2+} peak evoked by AVP is partly due to the release from the intracellular stores and partly due to the influx, whereas the sustained plateau is attributed to an increase in Ca^{2+} influx. When the intracellular Ca^{2+} stores were depleted by TG, a microsomal Ca^{2+}-ATPase inhibitor, the AVP-induced $[Ca^{2+}]_i$ increase including the sustained phase was totally abolished. This finding suggests that the AVP-induced Ca^{2+} influx depends on AVP-induced Ca^{2+} release. The involvement of Ca^{2+}-channels was further studied. Nimodipine did not alter AVP-induced glucagon release or $[Ca^{2+}]_i$ increase. Thus, the AVP-induced Ca^{2+} influx was not mediated through L-type VDCCs. These results are consistent with those of Bode *et al*. (1994) in which TG and U-73122 inhibited the spontaneous Ca^{2+} oscillation in single In-R1-G9 cells, but the L-type VDCC antagonists verapamil and nifedipine did not. In contrast to α-cells, AVP induces Ca^{2+} influx in clonal β-cells RINm5F partly by the opening of L-types VDCCs (Chen *et al*., 1994; Li *et al*., 1992).

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

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

In a smooth muscle cell line A_7r_5, V_1 receptors are coupled to several signaling pathways including PLC, PLA_2 and PLD (Thibonnier *et al*., 1991). AVP stimulates secretion of endothelin-1 and prostanoids from human brain endothelial cells by a receptor-mediated activation of PLC and PLA_2 (Spatz *et al*., 1994). However, $cPLA_2$ seems to be present at very low levels in rat islets, and clonal β-cells, RINm5F and HIT-T15 (Loweth *et al*., 1995). These observations indicate that PLA_2 may not be involved in AVP-induced insulin and glucagon release in rodents.

AVP activates phospolipase D (PLD) in rat Leydig (Vinggaard & Hansen, 1991) and glomerular mesangial cells (Kusaka *et al*., 1996). Activation of PLD leads to phosphatidic acid (PA) formation, thereby stimulating insulin release (Metz & Dunlop, 1990). In the present study, we found that zLYCK, an inhibitor of PLD, at the concentration used in the previous study (Kusaka *et al*., 1996) inhibited AVP-induced glucagon release by 60%, which suggested that PLD pathway may play a role in the PLC-independent mechanism of AVP-induced glucagon release. Activation of PLC requires Ca^{2+} (Chaudry & Rubin, 1990; Gardner, 1989), thus PLC-independent pathway may be one of the Ca^{2+}-independent pathways. The G protein-coupled PLD pathway in rat myocardium is both Ca^{2+}-dependent and -independent (Lindmar & Loffelholz, 1998). However, whether PLD plays a role in the PLC-independent and/or Ca^{2+}-independent pathway of AVP-induced glucagon release remains to be determined.

Another possibility is that AVP acts independently of Ca^{2+} at a distal site to trigger exocytosis. This would be similar to carbachol-induced insulin release in RINm5F cells, in which carbachol may stimulate insulin release by acting at a distal site beyond the point of increased $[Ca^{2+}]_i$ (Tang *et al*., 1995). Although U-73122 (8 μM) abolished AVP-induced $[Ca^{2+}]_i$ increase, the small increases in $[Ca^{2+}]_i$ occurred at the level close to the secretory granules might not have been detected by our method. These small increases in $[Ca2+]_i$ might have triggered glucagon release. Further work is needed to identify the other pathways, particularly the Ca^{2+}-independent ones, which are coupled to AVP-induced glucagon release.

In the present study, there was an inconsistency of glucagon release between experiments, particularly when compared the data of the experiments where the cells of both low and high passages had been used. Our experience with In-R1-G9 cells is that the lower passage cells tend to yield lower levels of basal glucagon release than the higher passage cells. For instance, when the cells of passages 23–26 were used (Figure 2), the basal control group had 114 ± 17 pg 10^5 cells^{-1}; when the cells of passages 28–30 were used (nimodipine experiments), the basal control group had 262 ± 53 pg 10^5 cells^{-1}. As a result of the high basal glucagon release in the high passage cells, AVP tended to cause a smaller increase in glucagon release than the lower passage cells.

Taken together, U-73122 inhibited AVP-induced $[Ca^{2+}]_i$ increase in clonal α-cells In-R1-G9 in a concentration-dependent manner, but only partially antagonized AVP-induced glucagon release. In this cell line, AVP may induce glucagon release through multiple signalling pathways that are

both Ca^{2+}-dependent and -independent. For the Ca^{2+}-dependent pathway, G_q activates PLC, which promotes the formation of IP_3 and DAG. IP_3 stimulates Ca^{2+} release from the ER, which in turn triggers Ca^{2+} influx *via* non-L-type Ca^{2+} channels. Both Ca^{2+} release and Ca^{2+} influx may contribute to AVP-induced glucagon release. The Ca^{2+}-independent pathway for AVP-induced glucagon release is not well-understood and needs further investigations.

This work has been supported by NSC 88-2313-B-059-014. In addition, we thank The Royal Thai Government for partial support of this study; Dr Bruce Chertow of Marshall University School of Medicine, Huntington, WV for providing In-R1-G9 cells; Dr David L. Hopper of Iowa State University, Ames, IA for assistance in data analysis; Dr Joseph Dunbar of Wayne State University, Detroit, MI, U.S.A. for providing antiserum against glucagon; Dr Maurice Manning of the Medical College of Ohio, Toledo, OH, U.S.A. for providing CL-4-84 and WK-3-6 and Eli Lilly Laboratories, Indianapolis, IN, U.S.A. for donating glucagon.

References

BLEASDALE, J.E. & FISHER, S.K. (1993). Use of U-73122 as an inhibitor of phospholipase C-dependent processes. *Neuroprotocols,* **3,** 125 – 133.

BLEASDALE, J.E., THAKUR, N.R., GREMBAN, R.S., BUNDY, G.L., FITZPATRICK, F.A., SMITH R.J. & BUNTING, S. (1990). Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J. Pharmacol. Exp. Ther.,* **225,** 756 – 768.

BODE, H.P., YULE, D.I., FEHMANN, H.C., GOKE, B. & WILLIAMS, J.A. (1994). Spontaneous calcium oscillations in clonal endocrine pancreatic glucagon-secreting cells. *Biochem. Biophys. Res. Commun.,* **205,** 435 – 440.

CHAUDHRY, A. & RUBIN, R.P. (1990). Mediators of Ca^{2+}-dependent secretion. Mediators of Ca^{2+}-dependent secretion. *Environ. Health Perspect.,* **84,** 35 – 39.

CHEN, T.H., LEE, B. & HSU, W.H. (1994). Arginine vasopressin-stimulated insulin secretion and elevation of intracellular Ca^{++} concentration in rat insulinoma cells: Influences of a phospholipase C inhibitor 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73122) and a phospholipase A_2 inhibitor N-(p-Amylcinnamoyl)anthranilic acid. *J. Pharmacol. Exp. Ther.,* **270,** 900 – 904.

DIBAS, A.I., REZAZADEH, S.M., VASSAN, R., MIA, A.J. & YORIO, T. (1997). Mechanism of vasopressin-induced increase in intracellular Ca^{2+} in LLC-PK1 porcine kidney cells. *Am. J. Physiol.,* **272,** C810 – C817.

DUNNING, B.E., MOLTZ, J.H. & FAWCETT, C.P. (1984). Modulation of insulin and glucagon secretion from the perfused rat pancreases by the neurohypophysial hormones and by desamino-D- arginine vasopressin (DDAVP). *Peptides,* **5,** 871 – 875.

FEHMANN, H.C., STROWSKI, M. & GOKE, B. (1995). Functional characterization of somatostatin receptors expressed on hamster glucagonoma cells. *Am. J. Physiol.,* **268,** E40 – E47.

FOX, A.W., FRIEDMAN, P.A. & ABEL, P.W. (1987). Vasopressin receptor mediated contraction and [^{3}H]inositol metabolism in rat tail artery. *Eur. J. Pharmacol.,* **135,** 1 – 10.

GARDNER, P. (1989). Calcium and T lymphocyte activation. *Cell,* **59,** 15 – 20.

GROMADA, J., BOKVIST, K., DING, W.G., BARG, S., BUSCHARD, K., RENSTROM, E. & RORSMAN, P. (1997). Adrenaline stimulates glucagon secretion in pancreatic A-cells by increasing the Ca^{2+} current and the number of granules close to the L-type Ca^{2+} channels. *J. Gen. Physiol.,* **110,** 217 – 228.

HAWES, B.E., WATERS, S.B., JANOVIK, J.A., BLEASDALE, J.A. & CONN, P.A. (1992). Gonadotropin-releasing hormone-stimulated intracellular Ca^{2+} fluctuations and luteinizing hormone release can be uncoupled from inositol phosphate production. *Endocrinology* **130,** 3475 – 3483.

HSU, W.H., XIANG, H., RAJAN, A.S., KUNZE, D.L. & BOYD, A.E. III. (1991). Somatostatin inhibits insulin secretion by a G-protein-mediated decrease in Ca^{2+} entry through voltage-dependent Ca^{2+} channels in the beta cell. *J. Biol. Chem.,* **266,** 837 – 843.

JARD, S., GAILARD, R.C., GUILLON, G., MARIE, J., SCHOENENBER, G.P., MULLER, A.F., MANNING, M. & SAWYER, W.H. (1986). Vasopressin antagonists allow demonstration of a novel type of vasopressin receptor in the rat adenohypophysis. *Mol. Pharmacol.,* **30,** 171 – 177.

KIMURA, M. & OGIHARA, M. (1997). Proliferation of adult rat hepatocytes by hepatocyte growth factor is potentiated by phenylephrine and metaproterenol. *J. Pharmacol. Exp. Ther.,* **282,** 1146 – 1154.

KIRK, C.J., RODRIGUES, L.M. & HEMS, D.A. (1979). The influence of vasopressin and related peptides on glycogen phosphorylase activity and phosphatidylinositol metabolism in hepatocytes. *Biochem. J.,* **178,** 493 – 496.

KUSAKA, I., ISHIKAWA, S., HIGASHIYAMA, M., SAITO, T., NAGASAKA, S. & SAITO, T. (1996). The activation of phospholipase D partcipates in the mitogenic action of arginine vasopressin in cultured rat glomerular mesangial cells. *Endocrinology,* **137,** 5421 – 5428.

LI, G., PRALONG, W.F., PITTET, D., MAYR, G.W., SCHLEGE, W. & WOLLHEIM, C.B. (1992). Inositol tetrakisphosphate isomers and elevation of cytosolic Ca^{2+} in vasopressin-stimulated insulin-secreting RINm5F cells. *J. Biol. Chem.,* **267,** 4349 – 4356.

LINDMAR, R. & LOFFELHOLZ, K. (1998). Phospholipase D in rat myocardium: formation of lipid messengers and synergistic activation by G-protein and protein kinase C. *Biochem. Pharmacol.,* **56,** 799 – 805.

LOWETH, A.C., SCARPELLO, J.H. & MORGAN, N.G. (1995). Phospholipase A_2 expression in human and rodent insulin-secreting cells. *Mol. Cell Endocrinol.,* **112,** 177 – 183.

MCNICHOL, A.M., MURRY, J.E. & MCMEEKIN, W. (1990). Vasopressin stimulation of cell proliferation in the rat pituitary gland in vitro. *J. Endocrinol.,* **126,** 255 – 259.

METZ, S.A. & DUNLOP, M. (1990). Stimulation of insulin release by phospholipase D. *Biochem. J.* **270,** 427 – 435.

RICHARDS, J.S., JONASSEN, J.A., ROLFERS, A.I., JERSEY, K. & REICHERT, JR. L.E. (1979). Adenosine 3′, 5′-monophosphate, luteinizing hormone receptor, and progesterone during granulosa cell differentiation: Effect of estradiol and follicle-stimulating hormone. *Endocrinology,* **104,** 765 – 773.

RORSMAN, P., ASHCROFT, F.A. & BERGGREN, P.O. (1991). Regulation of glucagon release from pancreatic A-cells. *Biochem. Pharmacol.,* **41,** 1783 – 1790.

RUSSELL, J.T., BROWNSTEIN, M.J. & GAINER, H. (1990). Biosynthesis of vasopressin, oxytocin and neurophysin: Isolation and characterization of two common precursors (propressophysin and prooxyphysin). *Endocrinology,* **107,** 1880 – 1891.

SMALLRIDGE, R.C., KIANG, J.G., GIST, I.D., FEIN, H.G. & GALLOWAY, R.J. (1992). U-73122, an aminosteroid phospholipase C antagonist, noncompetitively inhibits thyrotropin-releasing hormone effects in GH_3 rat pituitary cells. *Endocrinology,* **131,** 1883 – 1888.

SPATZ, M., STANIMIROVIC, D., BACIC, F., UEMATSU, S. & MCCARRON, R.M. (1994). Vasoconstrictive peptides induce endothelin-1 and prostanoids in human cerebromicrovascular endothelium. *Am. J. Physiol.,* **266,** C654 – C660.

SPERTI, G. & COLUCCI, W.S. (1991). Calcium influx modulates DNA synthesis and proliferation in A_7r_5 vascular smooth muscle cells. *Eur. J. Pharmacol.,* **206,** 279 – 284.

SWOPE, S.L. & SCHONBRUNN, A. (1988). The biphasic stimulation of insulin secretion by bombesin involves both cytosolic free calcium and protein kinase C. *Biochem. J.,* **253,** 193 – 202.

TAKAKI, R., ONO, J., NAKAMURA, M., YOKOGAWA, Y., KUMAE, S., HIRAOKA, T., YAMAGUCHI, K., HAMAGUCHI, K., UCHIDA, S. (1986). Isolation of glucagon-secreting cell lines by cloning insulinoma cells. *In Vitro Cell Dev. Biol.,* **22,** 120 – 126.

TANG, S.H., YANEY, G.C. & SHARP, G.W. (1995). Unusual carbachol responses in RINm5F cells: Evidence for a 'distal' site of action in stimulus-secretion coupling. *Mol. Pharmacol.,* **47,** 863 – 870.

THASTRUP, O., CULLEN, B.K., DRBAK, HANLEY, M.R. & DAWSON, A.P. (1990). Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} store by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. U.S.A.,* **87,** 2466 – 2470.

THIBONNIER, M. (1992). Signal transduction of V_1-vascular vasopressin receptors. *Regul. Pept.,* **38,** 1 – 11.

THIBONNIER, M., BAYER, A.L. & LENG, Z. (1993). Cytoplasmic and nuclear signaling pathways of V_1-vascular vasopressin receptors. *Regul. Pept.,* **45,** 79 – 84.

THIBONNIER, M., BAYER, A.L., SIMONSON, M.S. & KESTER, M. (1991). Multiple signaling pathways of V_1-vascular vasopressin receptors of A_7r_5 cells. *Endocrinology,* **129,** 2845–2856.

THIBONNIER, M., PRESTON, J.A., DULIN, N., WILKINS, P.L., BERTI-MATTERA, L.N. & MATTERA, R. (1997). The human V_3 pituitary vasopressin receptor: ligand binding profile and density-dependent signaling pathways. *Endocrinology,* **138,** 4109–4122.

THOMPSON, A.K., MOSTAFAPOUR, S.P., DELINGER, L.C., BLEASDALE, J.E. & FISHER, S.K. (1991). The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. *J. Biol. Chem.,* **266,** 23586–23862.

THORN, P. & PETERSEN, O.H. (1991). Activation of voltage-sensitive Ca^{2+} currents by vasopressin in an insulin-secreting cell line. *J. Membr. Biol.,* **124,** 63–71.

TSE, A., TSE, F.W., ALMERS, W. & HILLE, B. (1993). Rhythmic exocytosis stimulated by GnRH-induced calcium oscillations in rat gonadotropes. *Science,* **260,** 82–84.

VINGGAARD, A.M. & HANSEN, H.S. (1991). Phosbol ester and vasopressin activate phospholipase D in leydig cells. *Mol. Cell Endocrinol.,* **79,** 157–165.

WOLLHEIM, C.B. & POZZAN, T. (1984). Correlation between cytosolic free Ca^{2+} and insulin release in an insulin-secretion cell-line. *J. Biol. Chem.,* **259,** 2262–2267.

WU, H., JAMES-KRACKE, M.R. & HALENDA, S.P. (1992). Direct relationship between intracellular calcium mobilization and phospholipase D activation in prostaglandin E-stimulated human erythroleukemia cells. *Biochemistry,* **31,** 3370–3377.

YANG, C., LEE, B., CHEN, T.H. & HSU, W.H. (1997). Mechanisms of bradykinin-induced insulin secretion in clonal beta cell line RINm5F. *J. Pharmacol. Exp. Ther.,* **282,** 1247–1252.

YIBCHOK-ANUN, S., CHENG, H., HEINE, P.A. & HSU, W.H. (1999). Characterization of receptors mediating AVP- and OT-induced glucagon release from the rat pancreas. *Am. J. Physiol.,* **277,** E56–E62.

YIBCHOK-ANUN, S. & HSU, W.H. (1998). Effect of arginine vasopressin and oxytocin on glucagon release from clonal α-cell line In-R1-G9: involvement of V_{1b} receptors. *Life Sci.,* **63,** 1821–1829.

YOSHIDA, S. & PLANT, S. (1992). Mechanism of release of Ca^{2+} from intracellular stores in response to ionomycin in oocytes of the frog *Xenopus laevis*. *J. Physiol. (Lond.),* **458,** 307–318.

YULE, D.I. & WILLIAMS, J.A. (1992). U-73122 inhibits Ca^{2+} oscillations in response to cholecystokinin and carbachol but not to JVM-180 in rat pancreatic acinar cells. *J. Biol. Chem.,* **267,** 13830–13835.

(Received April 27, 1999
Revised October 14, 1999
Accepted October 15, 1999)