Vasopressin potentiates corticotropin-releasing hormone-induced insulin release from mouse pancreatic β-cells

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Abstract

Arginine vasopressin (AVP) and corticotropin-releasing hormone (CRH) have both been implicated in modulating insulin secretion from pancreatic β-cells. In the present study, we investigated the insulin-secreting activities of AVP and CRH in wild-type and AVP VIb receptor knockout mice. Both neuropeptides stimulated insulin secretion from isolated mouse pancreatic islets. The response of islets to CRH was increased fourfold by concomitant incubation with a subthreshold dose of AVP that alone did not stimulate insulin secretion. Activation of the endogenously expressed M3 receptor by the cholinergic agonist carbachol also potentiated CRH-induced insulin secretion, indicating that the phenomenon may be pathway specific (i.e. Ca^{2+}-phospholipase C) rather than agonist specific. The protein kinase C (PKC) inhibitors Ro-31-8425 and bisindolylmaleimide I attenuated the potentiating effect of AVP on CRH-stimulated insulin secretion and blocked AVP-stimulated insulin secretion. A possible interaction between the PKC and protein kinase A pathways was also investigated. The phorbol ester phorbol myristate acetate (PMA) stimulated insulin secretion, while the addition of both PMA and CRH enhanced insulin secretion over that measured with either PMA or CRH alone. Additionally, no AVP potentiation of CRH-stimulated insulin secretion was observed upon incubation in Ca^{2+}-free Krebs–Ringer buffer. Taken together, the present study suggests a possible synergism between AVP and CRH to release insulin from pancreatic β-cells that relies at least in part on activation of the PKC signaling pathway and is dependent on extracellular Ca^{2+}. This is the first example of a possible interplay between the AVP and CRH systems outside of the hypothalamic–pituitary–adrenal axis.

Journal of Endocrinology (2008) **197,** 231–239

Introduction

The main function of the pancreatic β-cell is to secrete insulin to maintain glucose homeostasis. Insulin secretion is a complex process that is primarily regulated by the levels of circulating glucose and is fine-tuned by additional factors such as other nutrients (e.g., amino acids) and growth factors, as well as by intra-islet autocrine and paracrine interactions. The stimulatory effects of glucose on insulin secretion are mediated by changes in intracellular Ca^{2+} levels and are modulated by signals generated by neurotransmitter and hormone binding to G protein-coupled receptors (GPCR) present on islet β-cells (Lang 1999, Henquin 2000, Ahren *et al.* 2006). Several GPCR agonists including norepinephrine, dopamine, galanin, and apelin inhibit insulin secretion following binding to pertussis toxin-sensitive $G_{i/o}$-coupled receptors (Lang 1999, Rubi *et al.* 2005, Sorhede Winzell *et al.* 2005, Ahren *et al.* 2006). On the other hand, neurotransmitters and hormones like acetylcholine, angiotensin II, and cholecystokinin act on their receptors via coupling to the G protein $G_{q/11}$ to potentiate glucose-induced insulin secretion by promoting phosphoinositide hydrolysis with a consequent increase in inositol 1,4,5-trisphosphate ($InsP_3$) production, elevation of intracellular Ca^{2+}, and activation of protein kinase C (PKC; Lang 1999, Ahren *et al.* 2006, Ramracheya *et al.* 2006). Other potentiators, such as the incretin hormones, glucagon-like peptide 1 and glucose-dependent insulinotropic polypeptide, and pituitary adenylate cyclase-activating protein and glucagon, stimulate adenylate cyclase and increase cAMP levels via an interaction with the stimulatory G protein G_s and activate cAMP-dependent protein kinase A (PKA; Lang 1999). There is considerable evidence that cAMP/PKA directly or indirectly regulates components of the Ca^{2+}-signaling system including $InsP_3$ generation, $InsP_3$ receptors, plasma membrane Ca^{2+}-ATPase, and L-type Ca^{2+} channels (Henquin *et al.* 1987, Lang 1999, Henquin 2000, Bruce *et al.* 2003). A feature of stimulus-secretion coupling in β-cells is this intracellular cross talk, where compounds that activate PKC and PKA act synergistically to enhance insulin secretion (Henquin *et al.* 1987, Henquin 2000).

The hypothalamic neuropeptide corticotropin-releasing hormone (CRH) plays a central role in the mammalian response to stress and exerts a wide range of roles in the brain (e.g., mediating anxiety behavior) and peripheral tissues (e.g., female reproductive tract; Bale & Vale 2004, Kalantaridou *et al.* 2004).

CRH and the related urocortin (Ucn) peptides, Ucn, Ucn II and Ucn III, elicit their effects by binding to two GPCRs, CRH-type 1 and 2 receptors (CRHR1 and CRHR2), which primarily stimulate the adenylate cyclase/cAMP second messenger system (Hillhouse & Grammatopoulos 2006). CRHR1 and CRHR2 are widely distributed and have recently been demonstrated in rodent pancreas islets by immunocyto-chemistry (Kanno *et al.* 1999). In addition, Ucn III is expressed in mouse β-cells suggesting that the CRH system may be involved in the local regulation of pancreatic endocrine secretions (Li *et al.* 2003). Indeed, accumulating evidence suggests that the CRH family of peptides stimulate Ca^{2+} influx into isolated islets and enhance insulin release in the presence of glucose (Kanno *et al.* 1999, Li *et al.* 2003, Kageyama *et al.* 2006).

CRH is a major regulator of the hypothalamic–pituitary–adrenal (HPA) axis under basal and stressful conditions (Bale & Vale 2004). It rapidly stimulates adrenocorticotropin (ACTH) secretion by binding to the CRHR1 on pituitary corticotropes. This action is potentiated by the neurohypo-physeal hormone arginine vasopressin (AVP) that has modest effects on ACTH release acting by itself (Gillies *et al.* 1982, Vale *et al.* 1983). The CRH-potentiating role of AVP in the anterior pituitary is mediated through the $G_{q/11}$-coupled AVP V1b receptor (AVPR1b) and is PKC dependent (Abou-Samra *et al.* 1986, Lolait *et al.* 1995). The AVPR1b is also expressed in extra-pituitary sites, principally the brain, adrenal, and pancreas (Lolait *et al.* 1995, Saito *et al.* 1995). Extensive studies have shown that AVP causes a PKC-dependent rise in intracellular Ca^{2+} and potentiates glucose-induced insulin release from insulin-secreting cell lines and isolated β-islets (see Gao *et al* 1990, 1994, Lee *et al.* 1995, Schaeffer *et al.* 2004). Moreover, in one study immuno-reactive AVP (and the related nonapeptide oxytocin) was found in the rat and human pancreas, suggesting that like CRH, AVP may play a local regulatory role in pancreatic function (Amico *et al.* 1988). More recently pharmacological and gene knockout studies have unequivocally demonstrated that AVP-stimulated insulin release from β-islets is mediated by the AVPR1b (Folny *et al.* 2003, Oshikawa *et al.* 2004).

In the present study, we hypothesized that AVP and CRH act synergistically to release insulin from pancreatic β-cells. We show that AVP potentiates CRH-induced insulin secretion from isolated mouse islets at a dose at which AVP alone has no effect on insulin secretion, suggesting a synergism between these two peptides that is involved in paracrine or autocrine stimulation of insulin secretion.

Materials and Methods

Animals

Adult male (12–16 weeks) mice (a mix of the C57BL/6J and 129X1/SvJ strains) were group housed (three to four per cage) under controlled light and temperature (21 ± 2 °C) with food and water available *ad libitum* and maintained on a 14 h light:10 h darkness cycle (lights on at 0500 h). Mice deficient for the AVPR1b were generated from crosses using mice heterozygous for the AVPR1b mutation (Wersinger *et al.* 2002), identified by PCR analysis of DNA isolated from tail clips. Studies were performed between 0900 and 1200 h. All procedures were conducted in accordance with the Animal Scientific Procedures Act (1986) United King-dom and the appropriate University of Bristol Ethical Review Process.

Pancreatic islet isolation and insulin assay

Pancreatic islets were isolated from mice by collagenase (NB8 Serva GmbH/AMS Biotechnology Abingdon, Oxfordshire, UK) digestion and subsequent centrifugation in a Histopaque (1083-1, 1077-1, 1119-1 Sigma) gradient. Briefly, mice were killed by cervical dislocation and medium (RPMI 1640 (Gibco), 1% Pen/Strep, 1·1% glucose (RPMI−); 2·5 ml) was injected into the pancreas. The swollen pancreas was surgically removed, cut into small pieces in RPMI− medium containing collagenase (0·5 mg/ml), incubated at 37 °C for 17 min, and digestion terminated by adding 10 ml ice-cold RPMI medium containing 10% fetal bovine serum (RPMI+). Digested pancreata were then filtered through a sieve, washed twice in RPMI− medium, and resuspended in a 5 ml layer of Histopaque 1119-1 and overlaid with successive 5 ml layers of Histopaque 1083-1, Histopaque 1077-1, and RPMI− medium. The sample was centrifuged at 863 *g* for 20 min at 4 °C. Islets were collected from the interface and resuspended in RPMI+ medium and cultured overnight at 37 °C at 95% O_2/5% CO_2.

Following culture, islets were washed twice with Krebs–Ringer buffer (KRB; 2·5 mM $CaCl_2$, 5·4 mM KCl, 0·8 mM $MgSO_4$, 116 mM NaCl, 20 mM $NaHCO_3$, 0·9 mM NaH_2PO_4) containing 10 mM HEPES, 1% BSA, and 2·8 mM glucose, and preincubated in the same buffer for 60 min at 95% O_2/5% CO_2. Islets were handpicked under a microscope and batches of five were then transferred to borosilicate tubes and incubated, in triplicate, at 37 °C with testing agents diluted in KRB containing 10 mM HEPES, 1% BSA, and 10 mM glucose for a further 60 min. For incubations with antagonists, islets were pretreated with antagonist for 15 min followed by incubation with testing agents for a further 60 min. The incubation was terminated by brief centrifugation and the supernatants collected and stored at −20 °C until assay for insulin by ELISA (Diagenics Ltd, Milton Keynes, UK). ELISA was performed according to the manufacturer's instructions and analyzed using a microplate reader (Microplate 5.1, Bio-Rad Laboratories). Experiments on the effects of extracellular Ca^{2+} were performed in Ca^{2+}-free KRB supplemented with 1 mmol/l EGTA. The PKC inhibitors Ro-31-8425 (2-[8-(aminomethyl)-6,7,8,9-tetrahydropyr-ido[1,2-a]indol-3-yl]-3-(1-methyl-1H-indol-3-yl)maleimide, HCl) and bisindolylmaleimide I (2-[1-(3-dimethyl-aminopropyl)-1H-indol-3-yl]-3-(1H-indol-3-yl)-maleimide), phorbol-12-myristate-13-acetate (PMA), and 4α-phorbol

(4α,9α,12β,13α,20-pentahydroxytigilia-1,6-dien-3-one) were obtained from Merck Chemicals Ltd. Carbachol and astressin$_2$-B (trifluoroacetate salt) were obtained from Sigma–Aldrich.

Statistical analysis

All values were expressed as means ± S.E.M. and presented as the mean percentage change from control, assigned an arbitrary value of 100. Results were analyzed using one-way ANOVA followed by Newman–Keuls multiple comparison *post hoc* test using GraphPad Prism (version 4.0b) software (San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Stimulation of insulin secretion from pancreatic islets by AVP and CRH

Mouse pancreatic islets were isolated and the absolute levels of insulin secreted in the presence of 2·8 mM glucose, 10 mM glucose, and 56 mM KCl were determined to be 0·586 ± 0·003, 1·1 ± 0·1, and 8·18 ± 2·43 ng/ml per h per islet respectively. As AVP stimulation of glucose-induced insulin secretion is effective only at glucose levels above 7 mM (Gao *et al.* 1990), islets were incubated in 10 mM glucose for experimental procedures. Both AVP and CRH caused a significant dose-dependent increase in insulin secretion from wild-type mouse pancreatic islets in the presence of 10 mM glucose. AVP stimulated insulin secretion in the dose range from 0·25 to 100 nM (Fig. 1A) while CRH, at a relatively high dose (50 nM), also stimulated insulin secretion (Fig. 1B). Recent studies indicate an important role for Ucn III, acting through CRHR2, in insulin secretion from rat islets (Li *et al.* 2003, 2006). However, in our system, Ucn III failed to stimulate insulin secretion at any dose tested (Fig. 1B). To determine whether endogenous Ucn III contributes to the effect mediated by CRH, mouse islets were incubated with 100 nM CRH and the CRHR2-selective antagonist astressin$_2$–B (Ast$_2$–B). Pretreatment with 1 μM Ast$_2$-B had no effect on the stimulatory effect of CRH on insulin release (Fig. 2).

Potentiation of CRH-induced insulin secretion from pancreatic islets by AVP

To investigate an interaction between AVP and CRH in insulin secretion, isolated mouse islets were incubated with 100 nM CRH, a dose at which this peptide was shown to stimulate insulin release, either alone or in combination with a range of AVP doses (0·05–100 nM). Concomitant incubation of CRH with doses of AVP shown to stimulate insulin secretion when given alone (0·25–100 nM) did not further increase insulin secretion above that seen with either AVP or CRH alone (Fig. 3). However, incubation of CRH with subthreshold doses of AVP (0·05 and 0·1 nM), doses at which AVP alone did not stimulate insulin secretion, induced a significant enhancement of insulin secretion when compared with CRH or AVP
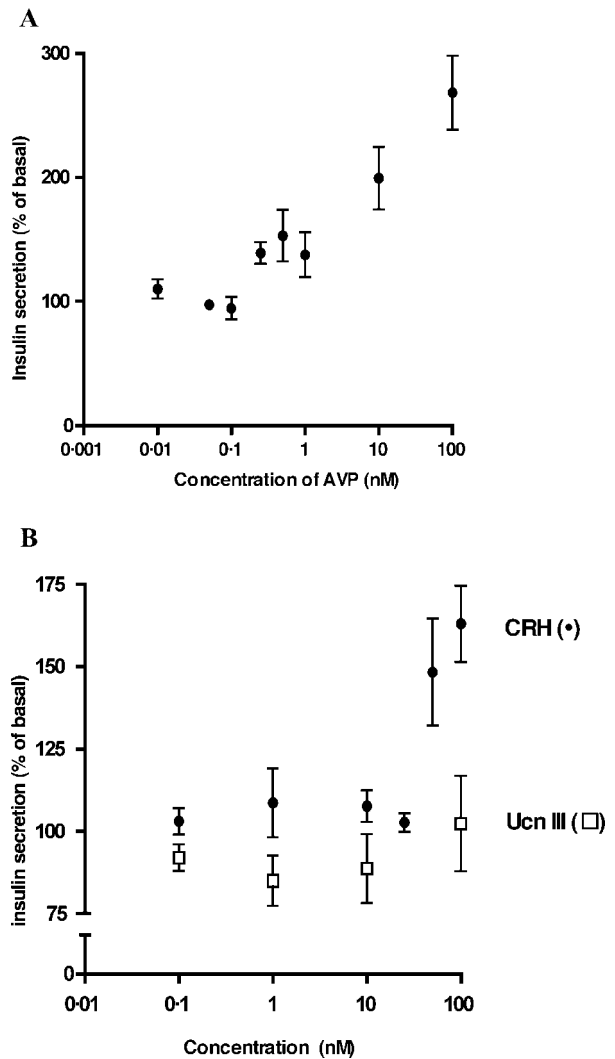
Figure 1 Dose–response curves of (A) AVP and (B) CRH and Ucn III on insulin secretion from isolated mouse pancreatic islets. (A) AVP and (B) CRH (●) and Ucn III (□) at the indicated doses were incubated with isolated pancreatic islets (batches of five) for 60 min in the presence of 10 mM glucose. Insulin secreted is represented as the percentage of insulin secreted in the presence of 10 mM glucose whose value was set as 100%. The level of insulin secreted was determined by ELISA. Values represent mean ± S.E.M. of at least three different experiments in triplicate.

treatment alone (Figs 3 and 4A). Concomitant incubation of 0·05–0·1 nM AVP with 100 nM CRH increased CRH-induced insulin secretion approximately threefold.

Effects of muscarinic receptor activation on CRH-induced insulin secretion

AVP causes a PKC-dependent rise in intracellular Ca^{2+} and potentiates glucose-induced insulin release from insulin-secreting cell lines and isolated β-islets (Gao *et al.* 1990,
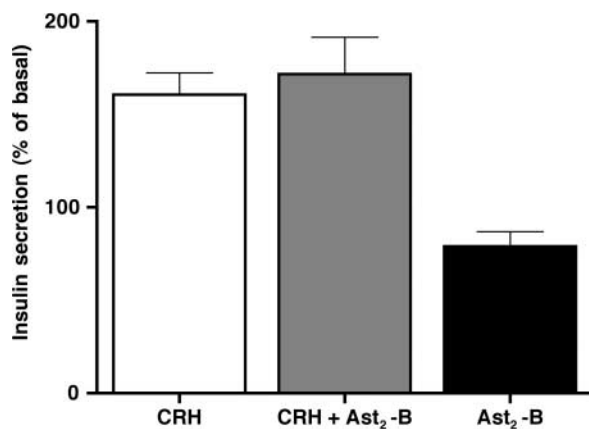
Figure 2 Effects of Ast$_2$-B on CRH-induced insulin secretion from isolated mouse pancreatic islets. Agonists at the indicated doses were incubated with isolated islets (batches of five) for 60 min in the presence of 10 mM glucose. CRH (100 nM) stimulated insulin secretion in the presence of 10 mM glucose. Pretreatment with 1 μM Ast$_2$-B had no effect on CRH-induced insulin secretion. Paired *t*-tests showed no significant difference between CRH and CRH+ Ast$_2$-B levels of insulin secretion and between Ast$_2$-B and basal levels of insulin secretion. Insulin secreted is represented as the percentage of insulin secreted in the presence of 10 mM glucose whose value was set as 100%. Values represent mean±S.E.M. of at least three different experiments in triplicate.

1994, Lee *et al.* 1995, Schaeffer *et al.* 2004). To determine whether the potentiation of CRH-induced insulin secretion by AVP is specific for AVP alone or is also characteristic of other PKC activators, the interaction between CRH and the cholinergic agonist carbachol (CCh) in insulin secretion was investigated. CCh primarily activates the M3 muscarinic receptor to enhance insulin secretion from β-cells (Zawalich *et al.* 2004). CCh, at doses of 1 nM and above, stimulated insulin secretion (data not shown) from *in vitro* mouse islets. Incubation of subthreshold doses of CCh (0·1 nM) with CRH (100 nM) increased CRH-induced insulin secretion over twofold (Fig. 4B), indicating that the potentiation of CRH-induced insulin secretion may be the result of activation of the Ca^{2+}-phospholipase C-signaling pathway.

Effect of PKC inhibition on AVP potentiation of CRH-stimulated insulin secretion

To investigate whether the AVP potentiation of CRH-stimulated insulin secretion may be mediated by PKC activation, isolated mouse islets were incubated in the presence of PKC inhibitors Ro-31-8425 and bisindolylmaleimide I (Bis). Ro-31-8425 is a potent and selective PKC inhibitor, which exhibits slight selectivity for PKCα, PKCβ, and PKC$_\gamma$ over the Ca^{2+}-independent PKC isoenzyme PKC$_\varepsilon$, whereas Bis shows high selectivity for PKCα-, βI-, βII-, γ-, δ-, and ε-isozymes. As shown in Fig. 5A, incubation with either Ro-31-8425 or Bis attenuated the potentiating effect of AVP on CRH-induced insulin secretion to a level of secretion seen with 100 nM CRH alone, while AVP (100 nM)-induced insulin secretion was
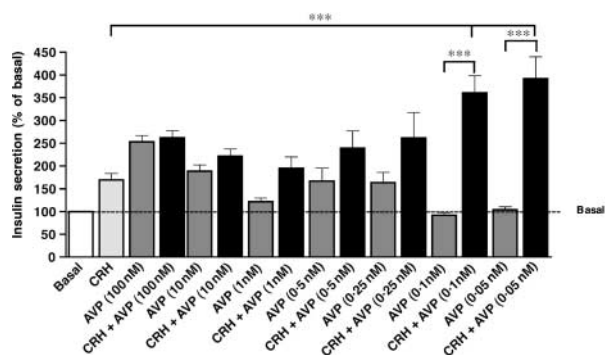

Figure 3 Effects of AVP and CRH interaction on insulin secretion from isolated mouse pancreatic islets. Insulin secretion after incubation of isolated islets (batches of five) for 60 min in the presence of 10 mM glucose with the indicated doses of AVP and/or CRH. Concomitant incubation of 100 nM CRH with doses of AVP shown to stimulate insulin secretion alone (0·25–100 nM) showed no significant increase in insulin secretion above that seen with either peptide alone. Incubation of 100 nM CRH with either 0·05 nM or 0·1 nM AVP, doses at which AVP does not stimulate insulin secretion, induced a significant enhancement of insulin secretion when compared with CRH treatment alone. Insulin secreted is represented as the percentage of insulin secreted in the presence of 10 mM glucose whose value was set as 100%. Values represent mean±S.E.M. of at least three different experiments in triplicate. ***$P<0·001$ indicates significant differences from islets treated with 100 nM CRH, 0·05 nM AVP, or 0·1 nM AVP alone.

reduced to basal levels. Incubation with the PKC inhibitors had no effect on insulin secretion induced by 100 nM CRH (Fig. 5A) probably owing to cellular signaling that is largely independent of PKC activation.

Stimulation of insulin secretion from pancreatic islets by PMA

To investigate the interaction between the PKC and PKA signaling pathways in the AVP potentiation of CRH-stimulated insulin secretion, isolated mouse islets were incubated with 100 nM CRH, a dose at which this peptide was shown to stimulate insulin release, either alone or in combination with a range of PMA doses (0·01–1 nM). PMA alone stimulated insulin secretion while concomitant incubation with CRH potentiated insulin secretion over that measured after the addition of either PMA or CRH given alone; however, significance was only achieved with the addition of lower doses of PMA with CRH (Fig. 5B). No insulin secretion or potentiation of CRH-induced insulin secretion was seen after incubation with the inactive phorbol compound, 4-α-phorbol (0·01 and 1 nM).

Effect of extracellular Ca^{2+} on the stimulation of insulin secretion from pancreatic islets

The dependence of the observed interplay between AVP and CRH on extracellular Ca^{2+} was examined. Isolated mouse islets were incubated either in KRB or in Ca^{2+}-free KRB, with either 0·1 nM AVP, a dose at which this peptide does not stimulate insulin release, 100 nM CRH, or both agonists added
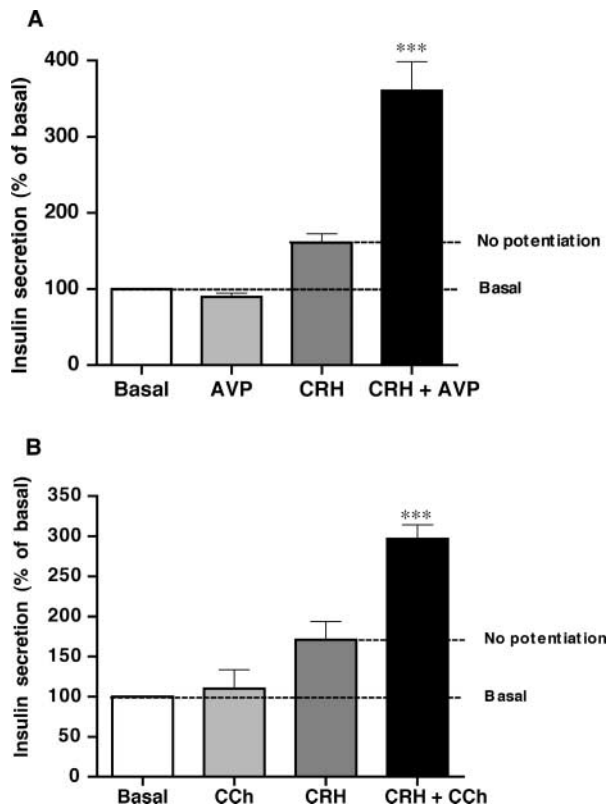
Figure 4 Effects of AVP and carbachol (CCh) on CRH-induced insulin secretion from isolated mouse pancreatic islets. (A) AVP (0·1 nM) and CRH (100 nM) and (B) CCh (0·1 nM) and CRH (100 nM) were incubated, either alone or together, with isolated islets for 60 min. Incubation with a subthreshold dose of (A) AVP alone and (B) CCh alone failed to stimulate insulin secretion. Concomitant incubation of peptides induced a significant potentiation of (A) CRH-induced insulin secretion by AVP and (B) CRH-induced insulin secretion by CCh. Insulin secreted is represented as the percentage of insulin secreted in the presence of 10 mM glucose whose value was set as 100%. Values represent mean±S.E.M. of at least three different experiments in triplicate. ***$P<0.001$ indicates significant differences from islets treated with single peptide.

together. Concomitant incubation of AVP (0·1 nM) with CRH (100 nM) in KRB increased CRH-induced insulin secretion approximately threefold (Fig. 6), whereas no potentiation was observed upon incubation in Ca^{2+}-free KRB, suggesting that the potentiation effect of AVP was absolutely dependent on Ca^{2+} influx from the extracellular space.

Stimulation of insulin secretion from pancreatic islets isolated from AVPR1b KO mice

Pancreatic islets isolated from AVPR1b knockout (KO) mice were used to examine the effect of deleting the AVPR1b on AVP potentiation of CRH-induced insulin secretion. In AVPR1b KO mice, AVP-stimulated insulin secretion was completely absent (Fig. 7A) and AVP failed to potentiate CRH-induced insulin secretion (Fig. 7B).
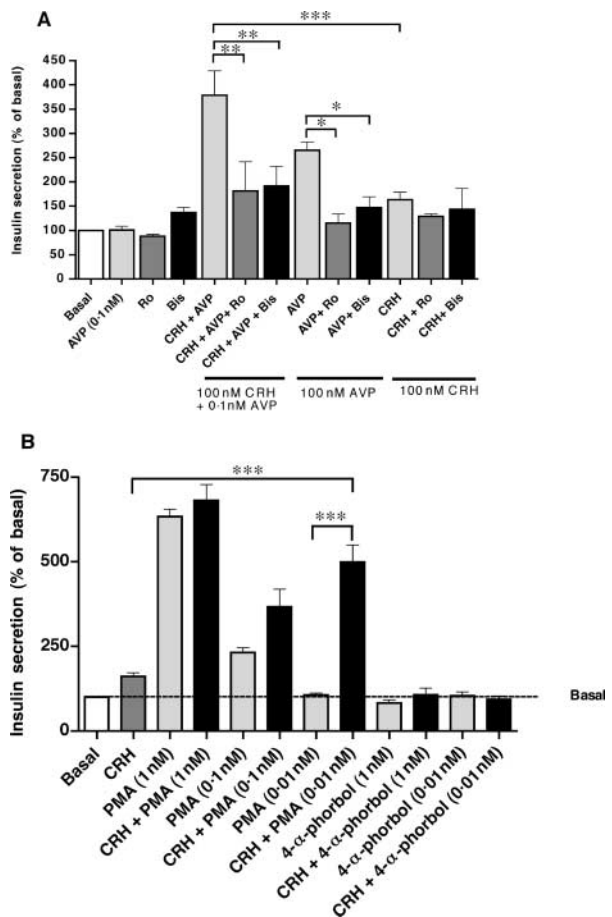

Figure 5 Effects of (A) PKC inhibitors Ro-31-8425 and bisindolylmaleimide I and (B) PMA on AVP- and CRH-induced insulin secretion from isolated mouse pancreatic islets. (A) Incubation with AVP (100 nM) or CRH (100 nM) alone stimulated insulin secretion, whereas incubation with AVP (0·1 nM) did not stimulate insulin secretion. Concomitant incubation of isolated islets with AVP (0·1 nM) and CRH (100 nM) induced a significant enhancement of insulin secretion. Pretreatment with 200 nM Ro-31-8425 (Ro) or 2 μM bisindolylmaleimide I (Bis) for 15 min before stimulation with testing agents for 60 min attenuated the effect of 0·1 nM AVP on CRH-induced insulin secretion and abolished the effect of 100 nM AVP on AVP-induced insulin secretion. Pretreatment with either inhibitor had no effect on 100 nM CRH-induced insulin secretion. ***$P<0.001$ indicates significant differences of concomitant incubation of 100 nM CRH and 0·1 nM AVP from islets treated with 100 nM CRH alone; **$P<0.01$ indicates concomitant incubation of 100 nM CRH and 0·1 nM AVP from CRH/AVP/Ro-31-8425 and from CRH/AVP/Bis; and *$P<0.05$ indicates incubation of 100 nM AVP from AVP/Ro-31-8425 and from AVP/Bis. (B) PMA alone stimulated insulin secretion while concomitant incubation with CRH potentiated insulin secretion over that measured after the addition of either PMA or CRH given alone. No insulin secretion or potentiation of CRH-induced insulin secretion was seen after incubation with the inactive phorbol compound, 4-α-phorbol. Insulin secreted is represented as the percentage of insulin secreted in the presence of 10 mM glucose whose value was set as 100%. Values represent mean±S.E.M. of at least three different experiments in triplicate. ***$P<0.001$ indicates significant differences of concomitant incubation of 100 nM CRH and 0·01 nM PMA from islets treated with 100 nM CRH or 0·01 nM PMA alone.
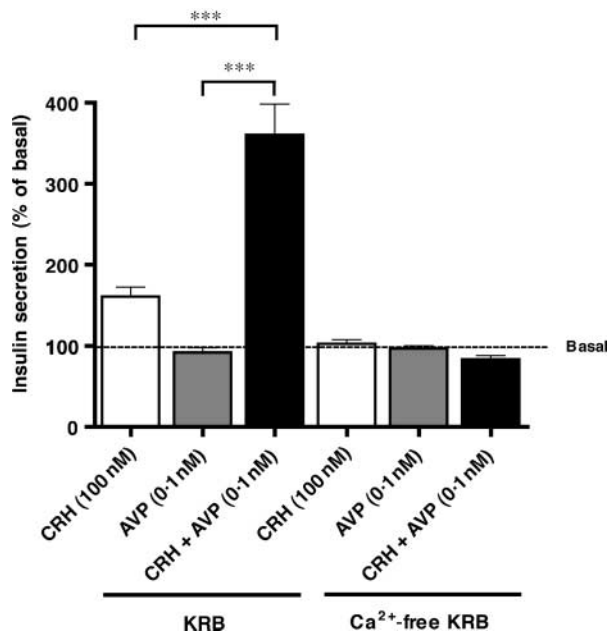
Figure 6 Effect of extracellular Ca^{2+} on the stimulation of insulin secretion from isolated mouse pancreatic islets. Isolated mouse pancreatic islets were incubated for 60 min either in KRB or in Ca^{2+}-free KRB, with either 0·1 nM AVP, 100 nM CRH, or both agonists added together. In KRB, CRH stimulated insulin secretion and concomitant incubation with AVP increased the CRH-induced insulin secretion approximately threefold. In Ca^{2+}-free KRB, no stimulation of insulin secretion was observed upon incubation with AVP, CRH, or both agonists added together. ***$P < 0.001$ indicates significant differences from islets treated with 100 nM CRH or 0·1 nM AVP alone.

Discussion

The secretion of insulin is under complex control and intra-islet feedback mechanisms exist to ensure proper levels of insulin under given glycemic conditions. AVP and CRH have both been implicated in modulating insulin secretion from the endocrine pancreas and the clonal β-cell line, RINm5F (Gao *et al.* 1990, Kanno *et al.* 1999, Lee *et al.* 1995).

While AVP has been shown to work as a positive modulator of glucose-stimulated insulin release by regulating plasma membrane depolarization and the CD38–cyclic ADP ribose signal system (Okamoto & Takasawa 2002), the role of AVP in the regulation of insulin release from the endocrine pancreas and the AVP receptor subtypes responsible for this modulation have only recently been determined. Using a combined pharmacological and knockout approach, Oshikawa and coworkers have clearly demonstrated that the AVP-stimulated insulin release from the pancreatic β-cells is mediated via the AVPR1b (Oshikawa *et al.* 2004). In the rat pancreas, CRH, acting through CRH receptors present on isolated β-cells, appears to have stimulatory effects on insulin secretion (Kanno *et al.* 1999), while the related peptide Ucn III has also been reported to regulate insulin secretion, most likely via CRHR2, in rat pancreatic β-cells (Li *et al.* 2003). In this study, we show for the first time,
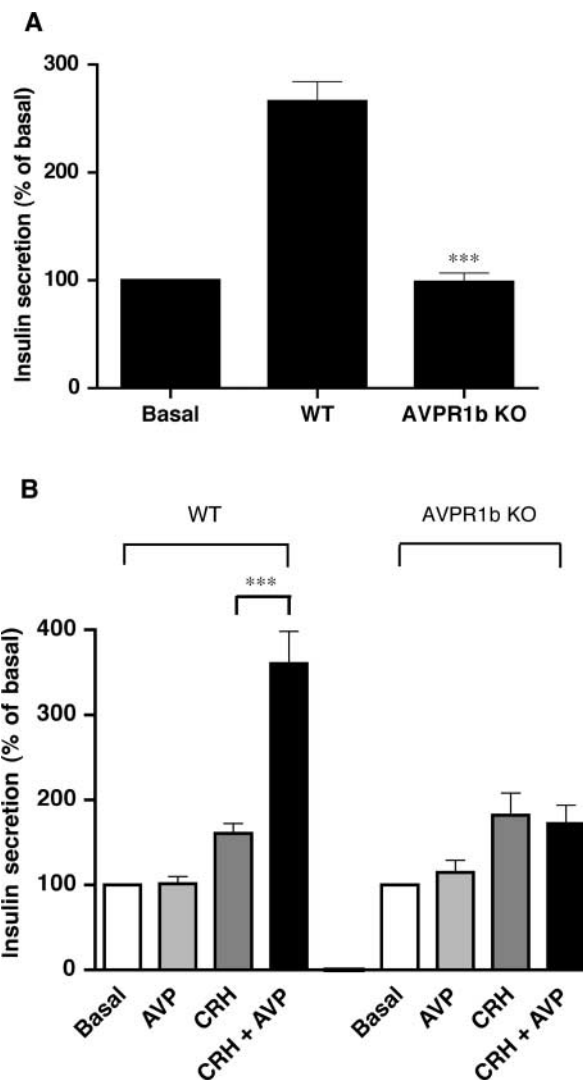
Figure 7 AVP-induced insulin secretion in mouse pancreatic islets isolated from wild-type and AVPR1b KO mice. (A) AVP (100 nM) induced insulin secretion in pancreatic islets isolated from wild-type (WT) mice. In islets isolated from AVPR1b KO mice, AVP-stimulated insulin secretion was completely abolished. (B) Concomitant incubation of 0·1 nM AVP and 100 nM CRH for 60 min with pancreatic islets isolated from AVPR1b KO mice failed to produce the potentiation of CRH-induced insulin secretion seen in the WT mouse. Insulin secreted is represented as the percentage of insulin secreted in the presence of 10 mM glucose whose value was set as 100%. Values represent mean ± S.E.M. of at least three different experiments in triplicate. ***$P < 0.001$ indicates significant differences from wild-type mouse islets and concomitant incubation of 100 nM CRH and 0·1 nM AVP from 100 nM CRH alone in wild-type mouse islets.

to our knowledge, that CRH induces insulin secretion in mouse pancreatic β-cells and confirm that AVP stimulates insulin secretion from pancreatic islets isolated from wild-type control mice, concurring with previous studies (Gao *et al.* 1990, Oshikawa *et al.* 2004). CRH stimulated insulin secretion only at

relatively high concentrations, as has been previously reported in the rat (Li *et al.* 2003), whereas AVP appears to be more effective with significant secretion being seen at 0·25 nM. This low efficacy of CRH in inducing insulin secretion appears to be a limiting effect of the pancreatic CRH system as high concentrations of the peptide are required to induce insulin secretion at both high (10 mM) and low (2·8 mM) glucose levels in the rat (Li *et al.* 2003). These stimulatory effects of CRH appear to be acting through the CRHR1, as preincubation with a selective CRHR2 antagonist, astressin$_2$-B, failed to block the observed induced insulin secretion. CRH has been reported to be less potent than Ucn III, a high-affinity ligand for the CRHR2, in stimulating insulin secretion from isolated rat islets (Li *et al.* 2003). By contrast, we did not observe insulin secretion induced by Ucn III from mouse islets, thus reinforcing the suggestion that CRH signaling in our system is acting through the CRHR1. It has been suggested that Ucn III plays a role in high glucose-induced insulin secretion as blockage of CRHR2 attenuates high (16·8 mM) but not low (10 mM) glucose-induced insulin secretion from isolated rat islets *in vitro* (Li *et al.* 2006). It is possible also that the glucose concentration (10 mM) used in our study was not optimal for Ucn III-stimulated insulin secretion, and that additional glucose dose–response and CRH antagonist studies would clarify the respective roles of CRH and Ucn III in insulin secretion from isolated mouse islets.

It is well known that AVP, through the AVPR1b, stimulates ACTH secretion from anterior pituitary corticotropes and potentiates the release of ACTH induced by CRH. CRH is widely recognized as the primary factor maintaining basal HPA activity and mediating the HPA axis response to acute stress. However, studies have revealed that AVP and CRH are also synthesized at low levels (compared with brain) in many other tissues including the pancreas (Hashimoto *et al.* 1984, Sasaki *et al.* 1987). For example, there is evidence that the rat adrenal medulla contains a CRH-ACTH system, duplicating that existing at the hypothalamic–pituitary level, which may be involved in paracrine stimulation of glucocorticoid secretion (Mazzochi *et al.* 1997). We speculated that AVP, acting through the AVPR1b receptor, might modulate CRH-induced insulin secretion in the endocrine pancreas. The present study demonstrates that AVP at high concentrations stimulated insulin release from isolated mouse pancreatic islets, whereas at low concentrations, which by themselves did not stimulate insulin secretion, AVP potentiated CRH-induced insulin secretion approximately threefold. This suggests a possible synergism between AVP and CRH and indicates that AVP may be acting on islet β-cells to increase their responsiveness to CRH. The potentiating effect was only seen with subthreshold concentrations of AVP. If doses of AVP greater than the stimulus threshold were used, then the process of insulin secretion became insensitive to potentiation. It should be emphasized that the synergism seen between AVP and CRH on ACTH secretion from pituitary corticotropes also occurs with subthreshold concentrations of AVP (Yates *et al.* 1971, Rivier & Vale 1983). Reduced availability of receptors (e.g. caused by receptor internalization) or changes in intracellular transduction

processes may contribute to this desensitization. Our study on pancreatic islets is the first example of a possible interplay between the AVP and CRH systems outside of the HPA axis.

We also demonstrate that the pancreatic AVP response is specific for the AVPR1b, as AVP-induced and AVP potentiation of CRH-induced insulin secretion was completely absent in mice lacking functional AVPR1b receptors. Assuming that both AVP and CRH are acting directly on β-cells, and not acting through some unknown intermediate, our results imply that AVP1Rb and CRHR1/CRHR2 are co-localized in a percentage of β-cells.

The specificity of the observed AVP/CRH potentiation was investigated. In the pituitary, CRH-stimulated ACTH secretion and cAMP accumulation are potentiated by PKC activators in general (e.g. phorbol myristate acetate, PMA) and by activation of specific PKC-linked GPCRs such as AVPR1b and the angiotensin II receptor (Abou-Samra *et al.* 1986). In the pancreas, similar to AVP, agents such as acetylcholine have also been shown to cause a PKC-dependent rise in intracellular Ca^{2+} and to stimulate insulin secretion (Zawalich *et al.* 2004). We investigated whether endogenously expressed receptors (e.g. M3 and/or M1 muscarinic receptors) that activate PKC also potentiate CRH-induced insulin secretion and found that incubation of islets with CRH and a subthreshold dose of carbachol, a cholinergic agonist, resulted in a greater than twofold increase in CRH-induced insulin secretion. As the potentiating effect of AVP can be imitated by carbachol, this indicates a phenomenon that may be specific to the Ca^{2+}-phospholipase C-signaling pathway, such as activation of PKC or InsP$_3$ production, or enhanced Ca^{2+}-influx, rather than specific for a certain agonist.

To determine whether this effect was mediated, in part or in full, by the PKC pathway, isolated pancreatic islets were preincubated in the presence or absence of either of two PKC inhibitors, Ro-31-842 and Bis, prior to repeating the potentiation studies. The 11 known PKC isozymes can be divided into three major classes: the conventional (cPKC) that are activated by both Ca^{2+} and diacylglycerol (DAG); the novel (nPKC) that require only DAG; and the atypical (aPKC) that bind neither Ca^{2+} nor DAG. The PKC inhibitors Ro-31-842 and Bis show selectivity for cPKC and nPKC isozymes. Preincubation with either PKC inhibitor attenuated the potentiating effect of AVP on CRH-induced insulin secretion to a non-potentiating level seen with CRH alone, while the insulin-secreting effects of AVP alone were virtually abolished, i.e., incubation with the PKC inhibitors removed the potentiating effects seen with subthreshold doses of AVP. These data suggest that, although low doses of AVP do not stimulate insulin secretion, AVP potentiation of CRH-stimulated insulin secretion appears to rely on PKC activation. This study suggests that the pancreatic AVP/CRH potentiation is occurring intracellularly where the peptides may activate signaling pathways and downstream signaling molecules such as cPKC and nPKC. We cannot discount at this time however that effects occurring at the cell surface level, where either peptide

might influence each other's release or receptor expression, may also have a role to play.

Additionally, the possible interaction between PKC-dependent (AVP) and PKA-dependent (CRH) signaling was examined. Phorbol esters, such as PMA, can substitute for DAG as high-affinity ligands for cPKC and nPKC isoforms. Incubation of the isolated pancreatic islets with PMA stimulated insulin secretion, while the addition of both lower doses of PMA and CRH enhanced insulin secretion over that measured after the addition of either PMA or CRH given alone, indicating that both the PKA and PKC systems are involved in the observed AVP potentiation of CRH-stimulated insulin secretion in mouse pancreatic islets. The specificity of the phorbol ester effect was demonstrated by the lack of insulin secretion or potentiation of CRH-induced insulin secretion after incubation with the inactive phorbol compound, 4-α-phorbol.

Experiments on the effects of extracellular Ca^{2+} on AVP potentiation of CRH-induced secretion showed that potentiation did not occur in the absence of extracellular Ca^{2+}, suggesting that the interaction between AVP and CRH was also critically dependent on Ca^{2+} influx from the extracellular space. As a rise in cytosolic calcium is a critical trigger for insulin release, measurements of cytosolic calcium done at the level of both the whole islet and a single β-cell could provide evidence as to whether the observed potentiating effect on insulin secretion is dependent on the dynamics and levels of intracellular Ca^{2+} within the β-cell.

The physiological relevance of AVP and CRH-induced insulin secretion is unclear and assigning significance to any particular role is speculative. However, increased AVP and CRH levels are well-known correlates of stressful situations, and it is possible that during some physiological and pathophysiological situations (e.g. stress, pregnancy, adrenal deficiency, Cushing's syndrome, severe hemorrhage) elevated circulating plasma AVP and CRH may contribute to increasing insulin secretion to cope with immediate metabolic demands. In addition, the presence of both AVP and CRH and their respective receptors in the pancreatic β-islets supports a role for these peptides in the local regulation of insulin secretion and the interplay between them suggests a new intra-islet regulatory mechanism that adds an additional level of complexity to modulation of insulin secretion. Whether or not AVP potentiates CRH effects in other aspects of pancreatic endocrine function, e.g. glucagon secretion and/or β-cell growth and differentiation, remains to be determined.

In conclusion, in this study we have shown an interplay between the AVP and CRH systems within the mouse pancreas. AVP potentiated CRH-induced insulin secretion from the isolated mouse pancreatic islets at concentrations where, by itself, it failed to stimulate secretion, and this potentiation was specific for the AVPR1b receptor subtype. This interplay appeared to be mediated at least in part by the PKC signaling pathway and was critically dependent on extracellular Ca^{2+}. This observation supports the existence in the pancreas of an *in vivo* synergism between these two peptides, which is involved in paracrine or autocrine stimulation of insulin secretion.

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