Endothelin-1 (ET-1)–Potentiated Insulin Secretion: Involvement of Protein Kinase C and the ET_A Receptor Subtype

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Endothelin-1 (ET-1), a potent vasoconstrictor peptide of endothelial origin, is capable of influencing hormone secretion from endocrine tissues, eg, pancreatic islet cells. We have shown a direct stimulatory effect of ET-1 on insulin secretion from isolated mouse islets of Langerhans. However, it is unknown as to whether the peptide acts through specific receptors on the islet cells and which mechanisms are involved in this insulinotropic action. We have therefore used the specific ET_A receptor antagonist BQ123, the ET_B receptor agonist BQ3020, and classic α- and β-adrenergic and cholinergic antagonists. ET-1 (100 nmol/L) stimulated insulin secretion from islets incubated at 8.3, 11.1, 16.7, and 25 mmol/L glucose ($P < .05$). At 3.3 mmol/L glucose, no alteration in insulin secretion was found. The cholinergic receptor antagonist atropine (5 μmol/L) or the adrenergic receptor antagonists propranolol (5 μmol/L) or phentolamine (5 μmol/L) did not affect ET-1 (100 nmol/L)-stimulated insulin secretion. BQ123 (10 pmol/L to 10 μmol/L) and BQ3020 (1 nmol/L to 1 μmol/L) had no effect on glucose (16.7 mmol/L)-stimulated insulin secretion, but BQ123 counteracted the stimulatory effect of ET-1 (100 nmol/L) at concentrations of 1 nmol/L to 10 μmol/L ($P < .01$). We also studied the relative role of protein kinase C (PKC) and a Wortmannin-sensitive pathway for ET-1–induced insulin secretion using 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), Calphostin C, and Wortmannin, respectively. At 5.6 mmol/L glucose, ET-1 (100 nmol/L) had no effect per se, whereas in the presence of 1 μmol/L TPA, which acutely stimulates PKC, the peptide did potentiate insulin secretion ($P < .05$). Furthermore, the insulinotropic effect of ET-1 at 16.7 mmol/L glucose was counteracted by the PKC inhibitor Calphostin C ($P < .05$) and by downregulation of PKC by 24 hours of exposure of islets to TPA (0.5 μmol/L, $P < .05$). Wortmannin (1 μmol/L) did not alter ET-1–potentiated insulin secretion. In conclusion, our results suggest that ET-1 acts through specific ET-1 receptors, most likely the ET_A subtype. Furthermore, PKC plays an essential role in the insulinotropic action of ET-1 in mouse islets.

ENDOTHELIN-1 (ET-1) is a peptide that has attracted great interest since its discovery in 1988 by Yanagisawa et al,[1] the potent vasoconstrictor action being the first to be recognized. The peptide, initially found in endothelial cells, is present in many organs including endocrine tissues, eg, the parathyroid and the human pancreas.[2-4] ET-1 interacts with at least 2 receptor subtypes, ET_A and ET_B,[4] of which the ET_A receptor is the most abundant in endocrine tissues.[5,6] The peptide is a putative marker for endothelial damage and, most interestingly, it has been shown that circulating ET-1 levels are increased in subjects with diabetes mellitus and vascular dysfunction.[7-10] There are indications from in vivo studies that ET-1 can modulate blood glucose and insulin levels.[11,12] Recently, we have demonstrated that ET-1 acutely potentiates insulin secretion by a direct action on isolated mouse islets.[13]

Presently, it is not known if ET-1 acts on islet cells via specific receptors and, if so, what receptor subtype, ET_A and/or ET_B, is involved. The possibility exists that the peptide interacts with the adrenergic or cholinergic receptor systems. It is also not known through which intracellular messenger systems the insulinotropic action is produced, and we focus here on the putative role of protein kinase C (PKC) and a wortmannin-sensitive pathway in the ET-1–induced alteration of insulin secretion in mouse islets.

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MATERIALS AND METHODS

Isolation of Islets

Female NMRI (Naval Medical Research Institute–established) mice (Bomholtgård Breeding and Research Center, Ry, Denmark) weighing 20 to 25 g were used. The animals were fed a standard pellet diet (Altromin, Lage, Germany) with tap water ad libitum before the experiments. The dark/light cycle was 12 hours. The animals were anesthetized with pentobarbital (50 mg/kg) intraperitoneally. A midline laparotomy was performed and the common bile duct was ligated at the papilla Vateri, where, after the hepatic duct was cannulated, 3 mL ice-cold Hanks balanced salt solution (HBSS) (Sigma Chemical, St Louis, MO) containing 0.3 mg/mL Collagenase type P (Boehringer Mannheim GmbH, Mannheim, Germany) was injected into the duct system of the pancreas. The inflated pancreas was removed from the animal and placed in a test tube in a water bath at 37°C for 19 minutes. After rinsing with HBSS, the islets were handpicked under a stereomicroscope and immediately transferred for overnight incubation in RPMI 1640 supplemented with 10% (vol/vol) NU-serum, glutamine (2.06 nmol/L), penicillin (100 IE/mL), amphotericin B (2.5 mg/mL), and streptomycin (100 μg/mL) (all GIBCO BRL, Paisley, UK) in an atmosphere of 95% normal air/5% CO_2.

Incubation Studies

The experiments started with a 60-minute preincubation of the islets in HEPES-buffered medium containing either 3.3 or 16.7 mmol/L glucose. The HEPES-buffered medium contained 125 mmol/L NaCl, 5.9 mmol/L KCl, 1.2 mmol/L $MgCl_2$, 1.28 mmol/L $CaCl_2$, 25 mmol/L HEPES, and human serum albumin (Boehringer), pH 7.4. Single islets were incubated in 100 μL HEPES-buffered medium containing D-glucose (16.7 or 3.3 mmol/L). The following substances were supplemented according to the protocols: ET-1, phentolamine, propranolol, atropine, BQ3020 (all Sigma), or BQ123 (Peninsula Laboratories, Belmont, CA). After a 60-minute incubation in a normal atmosphere at 37°C, 50 μL incubation medium was removed for insulin analysis.

In some experiments, islets were exposed long-term to 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) or 4α-phorbol 12,13-didecanoate (PDD). The phorbol esters were added to RPMI 1640 during incubation for 24 hours before the acute incubation experiments. TPA, PDD, Calphostin C, and Wortmannin (all Sigma) were dissolved in dimethylsulfoxide (DMSO) before further dilution in the incubation medium.[14] The final concentration of DMSO was lower than 0.4% in all experiments.

Insulin Assay

Samples of the incubation medium were immediately frozen for insulin analyses. The concentration of insulin was determined by radioimmunoassay with a guinea pig anti-porcine insulin antibody (Novo Nordisk, Bagsvaerd, Denmark) and mono-^{125}I-(Tyr A14)-labeled human insulin (Novo Nordisk) as a tracer and rat insulin (Novo Nordisk) as a standard. Free and bound radioactivity was separated using ethanol. ET-1 (0.1 pmol/L to 1 μmol/L) did not cross-react in the insulin assay. Interassay and intraassay variation was less than 10%.

Statistical Analysis

For statistical comparison, ANOVA and Newman-Keuls test for multiple comparisons were used. Statistical significance was established at a P level less than .05. Results are expressed as the mean ± SEM.

RESULTS

Effect of ET-1 on Glucose-Stimulated Insulin Secretion

The effect of ET-1 on insulin secretion from mouse islets was studied at different glucose concentrations from 3.3 to 25 mmol/L. ET-1 (100 nmol/L) potentiated insulin secretion from isolated mouse islets at 8.3, 11.1, 16.7, and 25 mmol/L glucose, whereas the peptide did not affect glucose output at 3.3 mmol/L glucose (Fig 1).

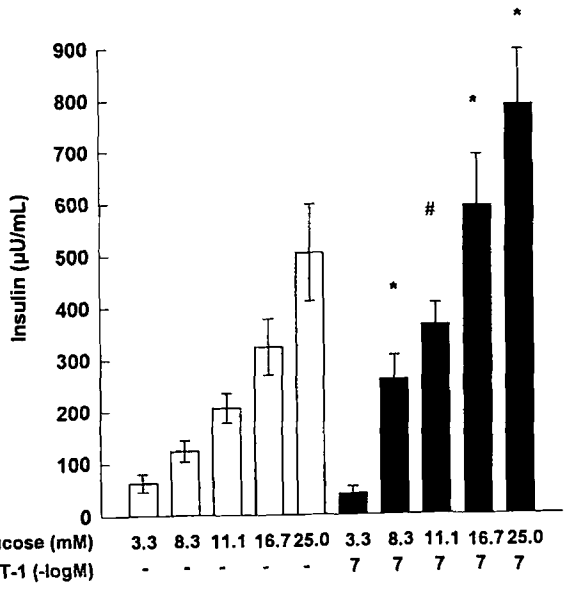

Fig 1. Effects of ET-1 (100 nmol/L) on insulin secretion from mouse islets incubated at a glucose concentration of 3.3-25 mmol/L. Each bar represents the mean ± SEM for 21 incubations of single islets. *$P < .05$ and #$P < .01$ denote probability level of random differences in the presence *v* absence of ET-1.

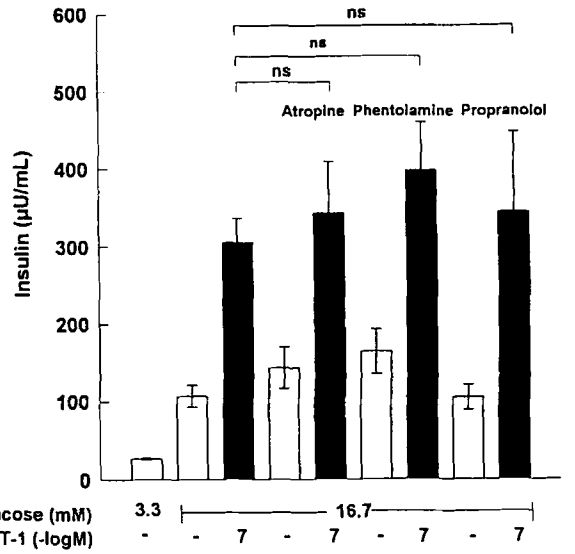

Fig 2. Effects of propranolol (5 μmol/L), phentolamine (5 μmol/L), and atropine (5 μmol/L) on ET-1 (100 nmol/L)-potentiated insulin secretion in the presence of 16.7 mmol/L glucose. Each bar represents the mean ± SEM for 21 incubations of single islets. NS, no statistically significant difference in the presence *v* absence of the respective antagonist.

Effect of Adrenergic and Cholinergic Receptor Blockade

The cholinergic antagonist atropine (5 μmol/L), α-adrenergic antagonist phentolamine (5 μmol/L), and β-adrenergic antagonist propranolol (5 μmol/L) had no effect on glucose (16.7 mmol/L)-stimulated insulin secretion (Fig 2). None of the antagonists altered ET-1 (100 nmol/L)-potentiated insulin secretion at 16.7 mmol/L glucose.

Effect of BQ123 and BQ3020

The ET receptor antagonist BQ123 (10 pmol/L to 10 μmol/L) had no effect per se on glucose (16.7 mmol/L)-stimulated insulin secretion (Table 1). In contrast, BQ123 dose-dependently counteracted the insulinotropic effect of ET-1 (100 nmol/L) in the presence of 16.7 mmol/L glucose at concentrations of 1 nmol/L and higher (Fig 3). We also studied the potential action of an ET_B receptor agonist, BQ3020, compared with the effect of ET-1. BQ3020 (1 nmol/L to 1 μmol/L) had no impact on glucose (16.7 mmol/L)-stimulated insulin secretion (Table 1).

The Role of PKC

Effect of ET-1 on TPA-stimulated insulin secretion. ET-1 (100 nmol/L) potentiated insulin secretion from islets incubated for 60 minutes at 16.7 mmol/L glucose, whereas at 5.6 mmol/L glucose, ET-1 did not influence insulin secretion (Fig 4). At 5.6 mmol/L glucose, the islets responded with an increase in insulin secretion when exposed to 1 μmol/L TPA ($P < .05$), but not to 10 and 100 nmol/L. When ET-1 was added together with 1 μmol/L TPA in the presence of 5.6 mmol/L glucose, the peptide was capable of further exaggerating the insulin response to TPA ($P < .05$).

Table 1. Effects of BQ123 and BQ3020 Alone on Glucose (16.7 mmol/L)-Stimulated Insulin Secretion From Isolated Mouse Islets (mean ± SEM)

	Insulin Secretion (μU/mL)								
		Concentration of Receptor Modifier (-logM)							
Agent	Basal	11	10	9	8	7	6	5	No.*
BQ123	263 ± 32	228 ± 39	234 ± 41	291 ± 44	326 ± 57	295 ± 43	311 ± 55	231 ± 38	21-24
BQ3020	196 ± 24	—	—	154 ± 21	159 ± 21	199 ± 27	203 ± 27	—	32

*Single incubation of islets in 3-4 separate experiments.

Effect of Calphostin C on ET-1–stimulated insulin secretion. When islets were incubated with 100 nmol/L ET-1 in the presence of 16.7 mmol/L glucose, the PKC inhibitor Calphostin C (10 and 100 nmol/L) counteracted the insulinotropic action of ET-1 ($P < .05$). Calphostin C per se did not influence insulin secretion at 16.7 mmol/L glucose (Fig 5).

Effect of 24 hours' treatment of islets with TPA or PDD. The islets were still glucose-responsive after exposure to 0.5 μmol/L TPA for 24 hours, ie, 16.7 mmol/L glucose significantly stimulated insulin secretion compared with 5.6 mmol/L glucose ($P < .05$). However, ET-1 (100 nmol/L) did not potentiate insulin secretion from islets exposed to TPA for 24 hours. In contrast, ET-1 still potentiated insulin secretion from islets incubated for 24 hours with 0.5 μmol/L of the inactive phorbol ester PDD ($P < .05$). As a control for downregulation of PKC, it was shown that acute exposure to TPA (10 μmol/L) at 5.6 mmol/L glucose did not alter insulin secretion from islets treated with TPA for 24 hours, whereas TPA (10 μmol/L) enhanced insulin secretion from PDD-treated islets. As expected, insulin secretion from islets incubated overnight with PDD was increased (Fig 6).

Effect of Wortmannin. Wortmannin (10 nmol/L to 1 μmol/L) did not alter the insulinotropic effect of 100 nmol/L ET-1 in the presence of 16.7 mmol/L glucose. Wortmannin (10 nmol/L to 1 μmol/L) per se did not alter glucose-stimulated insulin secretion (Fig 7).

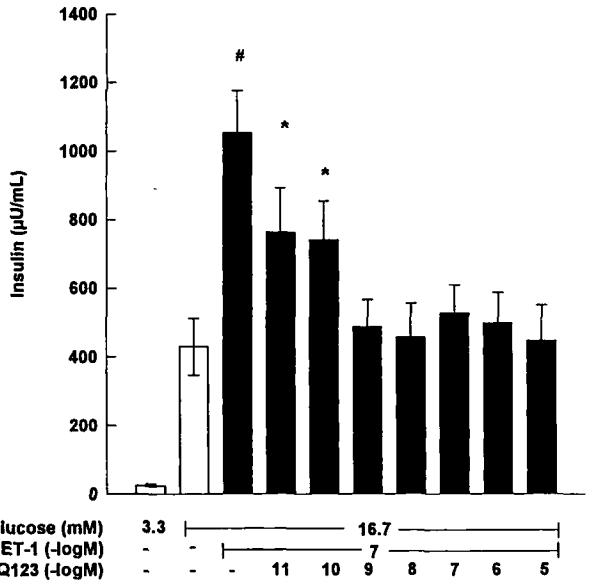

Fig 3. Effects of BQ123 (0.1 pmol/L-10 μmol/L) on ET-1 (100 nmol/L)–stimulated insulin secretion in the presence of 16.7 mmol/L glucose. Each bar represents the mean ± SEM for 21 incubations of single islets. *$P < .05$ and #$P < .01$ denote probability level of random differences *v* control (16.7 mmol/L glucose alone).

DISCUSSION

Binding sites for ET-1 have been demonstrated in the brain and various endocrine tissues, and ET-1 has been shown to modulate hormone secretion from various endocrine glands, eg, the pituitary and adrenal.[15] Paracrine and autocrine actions for locally produced ET-1 have been suggested to be operative in vascular and neuroendocrine tissues.[6,16] In the endocrine pancreas, ET-1 immunoreactivity has been identified in islet cells in normal subjects and patients with chronic pancreatitis,[3] the peptide being colocalized with insulin and glucagon. The presence of ET-1 in pancreatic endocrine cells and endothelium may indicate a role for ET-1 as a local modulator of the endocrine secretion, since we recently showed a direct effect of ET-1 on insulin secretion from isolated islets.[13]

In the present study, we found that the well-characterized ET_A receptor antagonist BQ123[17] dose-dependently counteracted the insulinotropic action of ET-1 in isolated mouse islets. This indicates that the actions of ET-1 on insulin secretion are mediated via ET-1 receptors of the ET_A subtype. This is corroborated by our finding that BQ3020, a specific ET_B receptor agonist,[18] did not alter glucose-stimulated insulin secretion. To further substantiate the hypothesis that the peptide acts via specific receptors, we also showed that ET-1–potentiated insulin secretion was not influenced by antagonists for α- and β-adrenergic or cholinergic receptors applied at concentrations known to block α-adrenergic, β-adrenergic, and cholinergic stimulation in the endocrine pancreas.[19] It can be hypothesized that the ET receptors are present on the β cells, but our study does not erase the possibility that ET-1 interacts with ET receptors on pancreatic α and/or Δ cells. Further studies using purified islet cells and/or clonal islet cell lines are needed to elucidate this possibility. However, there was no apparent ET-1 tonus operating during basal conditions, at least in vitro, since the ET_A receptor blocker per se did not influence the islet insulin release.

To improve our understanding of the mechanisms underlying the insulinotropic effects of ET-1, we studied the putative role of PKC and phospholipase D (PLD). PKC, which is activated by diacylglycerol (DAG), is suggested to be an important intracellular amplifier of the insulin secretory process.[20] We used 3 different approaches to elucidate the role of PKC in ET-1–potentiated insulin secretion. First, we used TPA, a tumor-promoting phorbol ester that substitutes for DAG, to acutely stimulate PKC.[21] We found that ET-1 in combination with TPA is able to potentiate insulin secretion even at low glucose, a condition at which ET-1 per se does not potentiate insulin secretion (Fig 4), corroborating our previous studies.[13] This

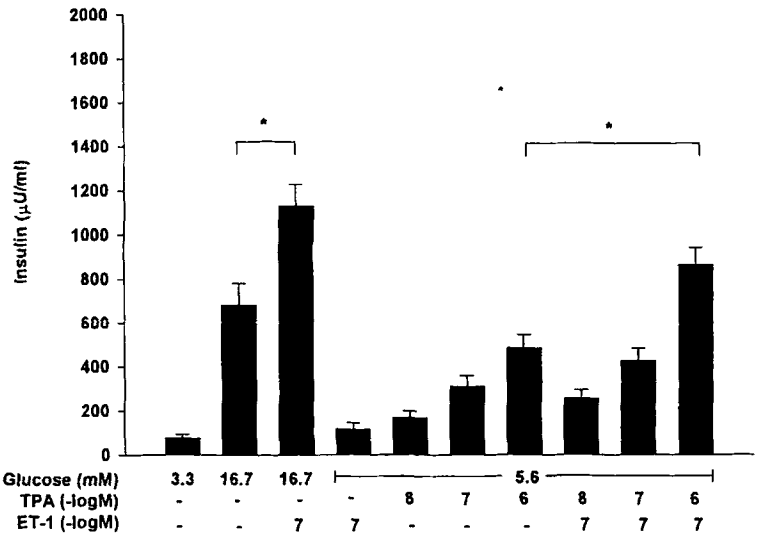

Fig 4. Effects of ET-1 (100 nmol/L) on glucose (16.7 mmol/L)-stimulated and TPA (10 and 100 nmol/L)-stimulated insulin secretion from mouse islets incubated at 5.6 mmol/L glucose. Each column represents the mean ± SEM for 21-24 single islets incubated for 60 minutes. *$P < .05$ indicates probability level of random difference in the presence *v* absence of ET-1.

indicates that ET-1 may further stimulate an already activated PKC, and makes it plausible that ET-1 and TPA synergistically can potentiate insulin secretion through activation of PKC. Second, we used the finding that long-term incubation of islets with TPA downregulates PKC activity.[22,23] We showed that ET-1 cannot potentiate glucose-stimulated insulin secretion from long-term TPA-treated islets, whereas ET-1 retains this capability when islets are incubated with the inactive phorbol ester PDD. Third, we used a selective inhibitor of PKC, Calphostin C,[24] and found that ET-1 was unable to potentiate insulin secretion in the presence of Calphostin C. Thus, the potentiating effect on insulin secretion is abolished by inhibition of islet PKC either by Calphostin C or by long-term exposure of islets to TPA. Taken together, these results suggest that PKC plays a key role in the insulinotropic action of ET-1; however, to be operative, PKC must be activated by either glucose or another activator, eg, TPA. PKC is activated by DAG, which can be formed through at least 2 pathways, ie, via phospholipase C (PLC) or PLD activation.[20,25]

In an attempt to discriminate the relative importance of these pathways for DAG formation and PKC activation, we examined the potential effect of Wortmannin. Wortmannin is a known phosphatidyl-inositol-3 (PI-3) kinase inhibitor when applied in the nanomolar range,[26] whereas higher concentrations inhibit PLD.[27] Importantly, the involvement of a Wortmannin-sensitive pathway in insulin release induced by vasoactive intestinal polypeptide and pituitary adenylate cyclase–activating polypep-

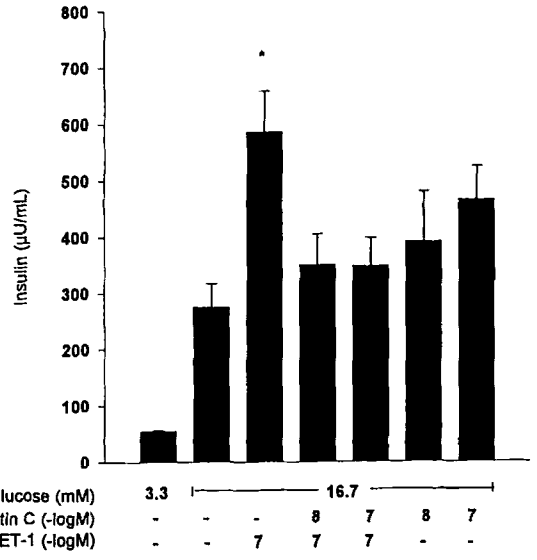

Fig 5. Effects of Calphostin C (10 and 100 nmol/L) on ET-1 (100 nmol/L)-potentiated insulin secretion from mouse islets incubated at 16.7 mmol/L glucose. Each column represents the mean ± SEM for 21-24 single islets incubated for 60 minutes. *$P < .05$ indicates probability level of random difference in the presence *v* absence of ET-1.

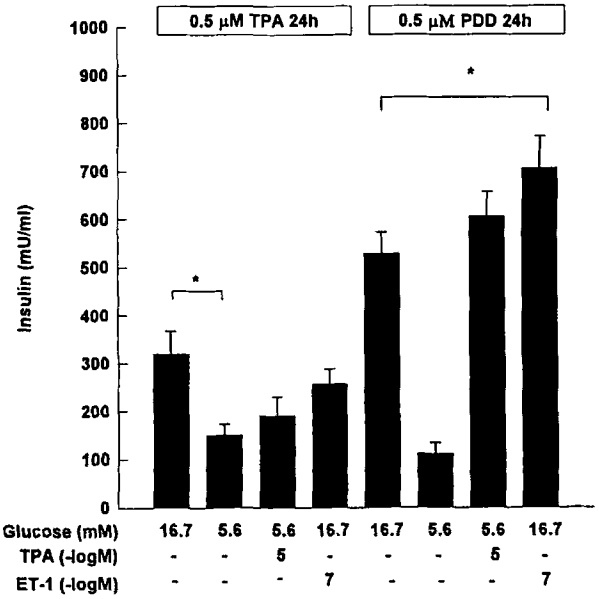

Fig 6. Effects of ET-1 (100 nmol/L) and TPA (10 μmol/L) on insulin secretion from islets incubated overnight with TPA (0.5 μmol/L) or PDD (0.5 μmol/L). Each column represents the mean ± SEM for 14-16 single islets incubated for 60 minutes. *$P < .05$ indicates probability level of random difference in the presence *v* absence of ET-1.

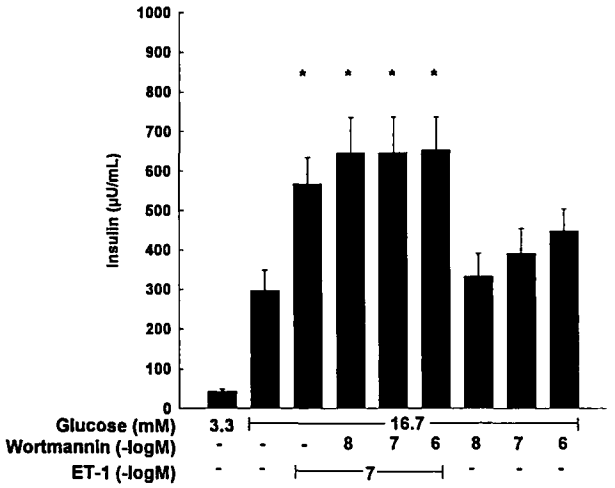

Fig 7. Effects of Wortmannin (10 nmol/L-1 μmol/L) on 100 nmol/L ET-1–potentiated insulin secretion from islets incubated at 16.7 mmol/L glucose. Each column represents the mean ± SEM for 21-24 single islets incubated for 60 minutes. *$P < .05$ indicates probability level of random difference in the presence *v* absence of ET-1.

tide has been found.[27] The lack of an effect of Wortmannin per se on glucose-stimulated insulin secretion corroborates studies in HIT-T15 cells[28] but contrasts with studies using MIN6 cells.[29] When applied at the high concentration of 1 μmol/L, Wortmannin would be expected to inhibit PLD; however, the inhibitor did not alter the insulinotropic effect of ET-1. PLD has been suggested to play a role in insulin secretion involving PKC activation.[25] This study may indicate that this is not an important mechanism in mouse islets, since Wortmannin had no effect on insulin secretion elicited by ET-1–induced PKC activation. As previously mentioned, Wortmannin, in addition to the inhibitory action on PLD, also inhibits PI-3 kinase, although at much lower concentrations. At least this study does not support the proposition that Wortmannin-sensitive pathways play a major role in ET-1–induced insulin secretion. This may indicate that it is unlikely that either PI-3 kinase or PLD are involved in ET-1–induced insulin secretion. The elucidation of this issue will require studies on the specific enzyme expression and activity. However, it is likely that DAG activating PKC is formed through an ET-1–induced activation of PLC, as shown in several other cell systems.[6]

The increased levels of circulating ET-1 found in diabetes mellitus may be secondary to leakage from damaged endothelial cells, thus making circulating ET-1 a potential marker for diabetic angiopathy and hypertension.[9] In this context, it is noteworthy that insulin stimulates the gene expression and secretion of ET-1 in endothelial cells[30] and hyperinsulinemia and acute elevations of glucose increase plasma ET-1 in humans.[31]

Circulating levels of ET-1 are in the picomolar range[4] and low compared with the concentrations used in this in vitro study. However, the action of ET-1 may be paracrine in nature,[6,16] and due to its unipolar mode of secretion,[32] plasma concentrations of ET-1 may only vaguely reflect the local islet concentrations. In other words, under conditions characterized by elevated plasma concentrations of ET-1, eg, diabetes and atherosclerosis, local concentrations of ET-1 may well exceed those found in the periphery. If so, ET-1 may possess the ability to modulate insulin secretion acutely in vivo, as shown by ET-1 infusion in rats.[11,33] Furthermore, the possibility exists that ET-1, which is capable of modulating pancreatic capillary blood flow,[34] might influence islet hormone secretion via modulation of the intraislet portal circulation.

In conclusion, our data indicate that the stimulatory effect of ET-1 on insulin secretion is mediated via ET_A receptors in pancreatic islets. It appears that PKC has an important role as a second messenger for the insulinotropic effect of ET-1 in islets. Further studies are needed to establish the presence of ET-1 receptors in the endocrine pancreas and to explore the physiological and pathophysiological role of ET-1.

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