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Insulin-releasing action of the novel antidiabetic agent BTS 67 582

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1 BTS 67 582 (1,1-dimethyl-2-(2-morpholinophenyl)guanidine fumarate) is a novel antidiabetic agent with a short-acting insulin-releasing effect. This study examined its mode of action in the clonal B-cell line BRIN-BD11.

2 BTS 67 582 increased insulin release from BRIN-BD11 cells in a concentration-dependent manner (10^{-8} to 10^{-4} M) at both non-stimulating (1.1 mM) and stimulating (16.7 mM) concentrations of glucose.

3 BTS 67 582 (10^{-4} M) potentiated the insulin-releasing effect of a depolarizing concentration of K^+ (30 mM), whereas the K^+ channel openers pinacidil (400 μM) and diazoxide (300 μM) inhibited BTS 67 582-induced release.

4 Suppression of Ca^+ channel activity with verapamil (20 μM) reduced the insulin-releasing effect of BTS 67 582 (10^{-4} M).

5 BTS 67 582 (10^{-4} M) potentiated insulin release induced by amino acids (10 mM), and enhanced the combined stimulant effects of glucose plus either the fatty acid palmitate (10 mM), or agents which raise intracellular cyclic AMP concentrations (25 μM forskolin and 1 mM isobutylmethylxanthine), or the cholinoceptor agonist carbachol (100 μM).

6 Inhibition of glucose-stimulated insulin release by adrenaline or noradrenaline (10 μM) was partially reversed by BTS 67 582 (10^{-4} M).

7 These data suggest that the insulin-releasing effect of BTS 67 582 involves regulation of ATP-sensitive K^+ channel activity and Ca^{2+} influx, and that the drug augments the stimulant effects of nutrient insulin secretagogues and agents which enhance adenylate cyclase and phospholipase C. BTS 67 582 may also exert insulin-releasing effects independently of ATP-sensitive K^+ channel activity.

Keywords: BTS 67 582; BRIN-BD11 cells; insulin release; antidiabetic agent

Introduction

BTS 67 582 (1,1-dimethyl-2-(2-morpholinophenyl)guanidine fumarate) is a novel insulin-releasing agent (Figure 1). It lowers blood glucose concentrations in normal and non-insulin-dependent diabetic (NIDD) rodents (Kaul *et al*., 1995a; Jones *et al*., 1997) and in non-diabetic and NIDD patients (Byrom *et al*., 1994; 1997), associated with increased plasma concentrations of insulin.

Insulinotropic drugs presently used to treat NIDD are the sulphonylureas (Gerich, 1989). These drugs act on the pancreatic B-cells to inhibit activity of the adenosine 5′-triphosphate (ATP)-sensitive potassium (K^+-ATP) channels, causing depolarization and increased voltage-dependent Ca^{2+} influx (Nelson *et al*., 1992). Sulphonylureas also augment the insulin-releasing effects of glucose, amino acids and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) (Ostensen *et al*., 1983; Sako *et al*., 1986).

BTS 67 582 is a guanidine derivative which is structurally unrelated to sulphonylureas. BTS 67 582 does not displace binding of the sulphonylurea [^{3}H]-glibenclamide to pancreatic B-cells, and increases insulin release in streptozotocin-treated glucose-primed rats that are unresponsive to glibenclamide (Jones *et al*., 1997). Studies with rat isolated islets indicate that BTS 67 582 exerts a direct and immediate stimulant effect on insulin release (Dickinson *et al*., 1996), although the mechanism of action of BTS 67 582 on the pancreatic B-cell remains to be determined. In the present study the novel glucose-responsive BRIN-BD11 B-cell line (McClenaghan *et al*., 1996a) was used to investigate the mechanism through which BTS 67 582 stimulates insulin secretion.

Methods

BRIN-BD11 cells used in this study were from passages 20–25. This clonal insulin-secreting cell line was derived from the electrofusion of the RINm5F cell line with pancreatic B-cells of New England Deaconess Hospital (NEDH) rats (McClenaghan *et al*., 1996a). The maintenance and characteristics of these cells have been extensively described elsewhere (McClenaghan *et al*., 1996a, b, c). Cells were grown in RPMI-1640 tissue culture medium containing 11.1 mM glucose and 0.3 g l^{-1} L-glutamine, and supplemented with 10% (v/v) foetal calf serum, 100 iu ml^{-1} penicillin and 0.1 g l^{-1} streptomycin at 37°C with 5% CO_2 and 95% air.

For insulin studies, cells were detached from the flasks with 0.025% (w/v) trypsin containing 1 mM EDTA, washed in Hank's balanced saline solution, seeded into 24-multiwell plates at 2.5×10^5 cells per well, and cultured overnight at 37°C. Culture medium was then replaced with 1 ml of a Krebs Ringer Bicarbonate (KRB) buffer supplemented with 0.05% bovine serum albumin and 1.1 mM glucose (McClenaghan *et al*., 1996b). After preincubation for 40 min at 37°C the buffer was replaced with 1 ml of KRB buffer supplemented with glucose at either 1.1 or 16.7 mM, plus test agents as described in the figures and tables. After incubation for 20 min at 37°C, the buffer was removed, stored at −20°C and aliquots assayed

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for insulin (Flatt & Bailey, 1981). Insulin release is expressed as ng per 10^6 cells 20 min^{-1} for means ± s.e.mean of six independent observations. Groups were compared by Student's unpaired t test. Differences were considered significant if $P<0.05$.

Reagents of analytical grade and deionized water were used throughout. RPMI-1640 tissue culture medium, foetal calf serum and antibiotics were from Gibco (Paisley, Strathclyde, U.K.), rat insulin standard was from Novo-Nordisk (Bagsvaerd, Denmark), and [^{125}I]-bovine insulin was from Lifescreen (Watford, U.K.). BTS 67 582 was provided by Knoll Pharmaceuticals Research and Development (Nottingham, Notts). Palmitate (sodium salt) and other chemicals were from Sigma Chemicals and BDH Chemicals (both of Poole, Dorset).

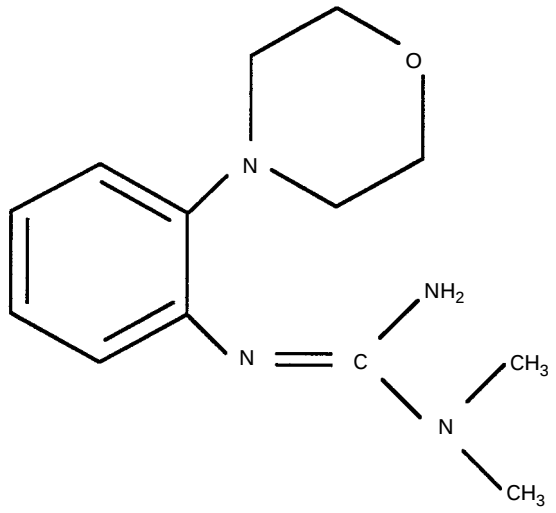

Figure 1 Chemical structure of BTS 67 582 (1, 1-dimethyl-2-(2-morpholinophenyl) guanidine fumarate).

Results

Basal and glucose-stimulated insulin release

BTS 67 582 (10^{-8}–10^{-4} M) evoked a concentration-dependent increase (1.3–2 fold, $P<0.05$ to $P<0.001$) in insulin release in the presence of a non-stimulative (1.1 mM) concentration of glucose (Figure 2a). A similar increase (1.2–1.8 fold, $P<0.05$ to $P<0.001$) in insulin release was observed with equimolar concentrations of the sulphonylurea tolbutamide (Figure 2b). Raising the glucose concentration from 1.1 to 16.7 mM increased insulin release 1.5 fold ($P<0.01$). At 16.7 mM glucose, BTS 67 582 (10^{-4} M) increased insulin release 1.4 fold ($P<0.05$), whereas an equimolar concentration of tolbutamide did not exert a significant effect (Figure 2a and b).

Membrane depolarization, K^+-ATP channel activity and Ca^{2+} influx

Membrane depolarization by incubation with 30 mM KCl (normally 4.7 mM KCl) increased insulin release 2.9 fold ($P<0.001$) at 16.7 mM glucose (Table 1). The combined effect of 16.7 mM glucose and 30 mM KCl was almost doubled ($P<0.001$) in the presence of 10^{-4} M BTS 67 582 (Table 1). Conversely, the insulin releasing effect of glucose (16.7 mM) and BTS 67 582 (10^{-4} M) was inhibited by the potassium channel openers pinacidil (400 μM) and diazoxide (300 μM) (Table 1).

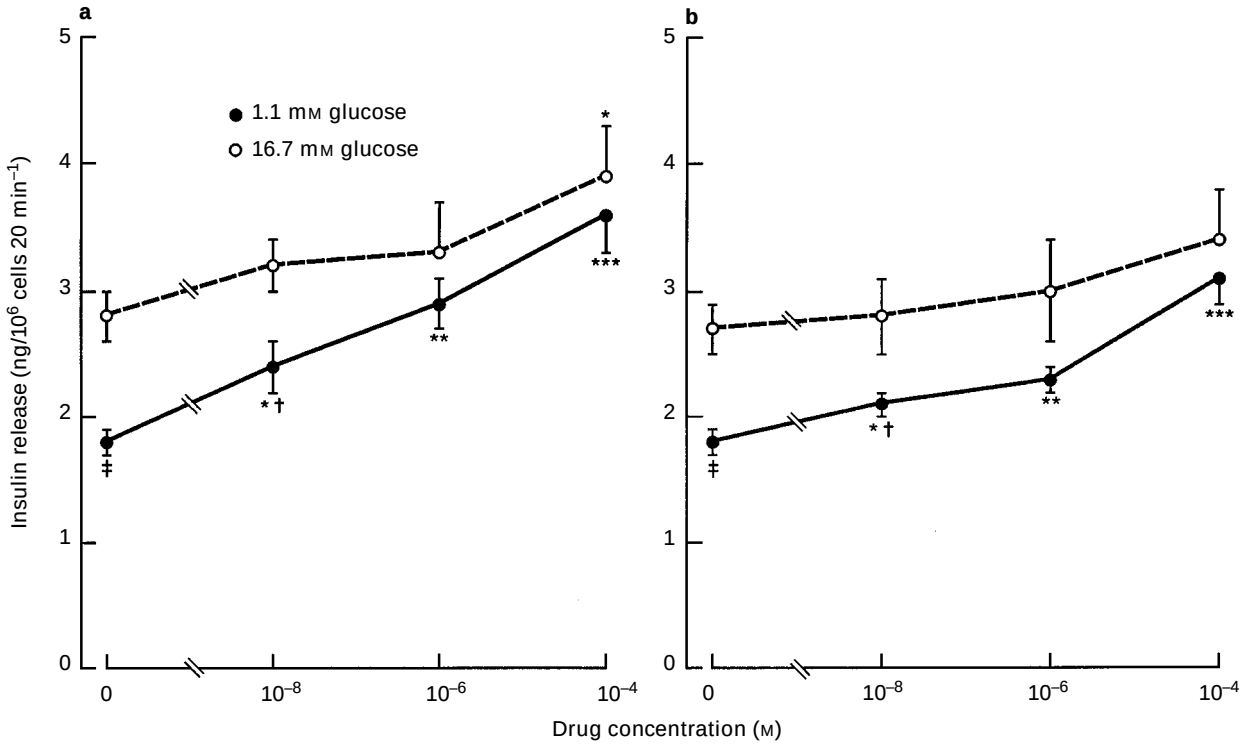

Figure 2 Effect of (a) BTS 67 582 and (b) tolbutamide (10^{-8}, 10^{-6} and 10^{-4} M) on insulin release by BRIN-BD11 cells after 20 min incubation at a glucose concentration of 1.1 mM or 16.7 mM. Values are mean of six incubations; vertical lines show s.e.mean. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus no drug at the same glucose concentration. †$P<0.05$, ‡$P<0.01$ versus 16.7 mM glucose at the same drug concentration. Comparisons between equimolar BTS 67 582 and tolbutamide revealed no significant differences, except at 10^{-6} M in the presence of 1.1 mM glucose where BTS 67 582 released more insulin ($P<0.05$) than tolbutamide.

Omission of extracellular Ca^{2+} abolished the insulin-releasing effects of glucose and BTS 67 582 (10^{-4} M) (Table 1). At 1.1 mM glucose, raising the extracellular Ca^{2+} concentration from 1.28 to 7.68 mM enhanced ($P<0.05$) insulin release (Table 2). At 7.68 mM Ca^{2+}, insulin release was more than doubled ($P<0.01$) by inclusion of 10^{-4} M BTS 67 582, but insulin output was not significantly greater than that observed with BTS 67 582 at 1.28 mM Ca^{2+} (Table 2). While verapamil (20 μM) had no effect on insulin release at a non-stimulative glucose concentration (1.1 mM), the calcium channel blocker effectively inhibited the insulin-releasing effect of BTS 67 582 (Table 2).

Non-glucidic nutrients and other regulators of B-cell function

As shown in Table 3, insulin release was stimulated 1.6–4.8 fold ($P<0.05$ to $P<0.01$) by the amino acids L-alanine, L-leucine and L-arginine, and by the L-leucine metabolite 2-ketoisocaproic acid (KIC). The stimulative effects of each amino acid and KIC were increased 1.4 to 2.1 fold ($P<0.01$ to $P<0.001$) by 10^{-4} M BTS 67 582 (Table 3).

Forskolin (25 μM) or isobutylmethylxanthine (IBMX, 1 mM), which increase intracellular cyclic AMP concentrations, markedly increased glucose-induced insulin release (4.1 and 6.3 fold, respectively; $P<0.001$) (Table 4). BTS 67 582 (10^{-4} M) further enhanced insulin secretion by these agents by 1.5 and 1.6 fold, respectively ($P<0.001$) (Table 4). The cholinoceptor agonist carbachol (100 μM) increased the stimulative effects of 16.7 mM glucose by 1.4 fold ($P<0.01$) (Table 4). Inclusion of BTS 67 582 (10^{-4} M) promoted (1.3 fold, $P<0.01$) the combined effect of 16.7 mM glucose and 100 μM carbachol. Insulin release was stimulated (3.1 fold, $P<0.001$) by addition of the fatty acid palmitate (10 mM) to 16.7 mM glucose, and 10^{-4} M BTS 67 582 effectively promoted this action (1.3 fold, $P<0.05$) (Table 4).

As shown in Table 5, the adrenoceptor agonists adrenaline and noradrenaline (10 μM) inhibited the stimulative effects of 16.7 mM glucose. These inhibitory actions were partially reversed by 10^{-4} M BTS 67 582 (Table 5).

Discussion

BRIN-BD11 cells provide a new and particularly useful clonal cell line to study the mechanisms of insulin secretion. These cells have been thoroughly characterized and display appropriate secretory responsiveness to a wide range of regulators of pancreatic B-cell function (McClenaghan *et al.*, 1996a,b,c). Most importantly BRIN-BD11 cells respond to glucose

Table 1 Effect of BTS 67 582 on insulin release by BRIN-BD11 cells in the presence of 16.7 mM glucose and agents affecting membrane potential, K^+-ATP channel activity and cytoplasmic Ca^{2+}

Test agent (mM)	*BTS 67 582* (M)	*Insulin release* (ng per 10^6 cells 20 min^{-1})
None	–	2.79±0.26
None	10^{-4}	3.85±0.40*
KCl (30)	–	8.06±0.60***
KCl (30)	10^{-4}	15.86±0.70$^{***\Delta\dagger}$
Pinacidil (0.4)	–	1.86±0.14*
Pinacidil (0.4)	10^{-4}	1.47±0.20$^{**\Delta}$
Diazoxide (0.3)	–	1.51±0.08**
Diazoxide (0.3)	10^{-4}	1.46±0.13$^{**\Delta}$
Ca^{2+} omission plus EGTA (0.5)	–	1.10±0.09***
Ca^{2+} omission plus EGTA (0.5)	10^{-4}	1.21±0.08$^{***\Delta\Delta}$

Data are mean±s.e.mean of six observations. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus 16.7 mM glucose alone. $^{\Delta}P<0.05$, $^{\Delta\Delta}P<0.001$ versus BTS 67 582 without test agent. $^{\dagger}P<0.001$ versus effect in the absence of BTS 67 582.

Table 2 Effect of BTS 67 582 on insulin release by BRIN-BD11 cells in the presence of 1.1 mM glucose and elevated Ca^{2+} or Ca^{2+} channel blocker

Test agent (mM)	*BTS 67 582* (M)	*Insulin release* (ng per 10^6 cells 20 min^{-1})
None	–	1.73±0.09
None	10^{-4}	3.63±0.31**
$CaCl_2$ (7.68)	–	2.29±0.16*
$CaCl_2$ (7.68)	10^{-4}	4.60±0.45**†
Verapamil (0.02)	–	2.06±0.13
Verapamil (0.02)	10^{-4}	2.25±0.14$^{*\Delta}$

Data are mean±s.e.mean of six observations. $^*P<0.05$, $^{**}P<0.001$ versus 1.1 mM glucose alone (at 1.28 mM Ca^{2+}). $^{\Delta}P<0.01$ versus BTS 67 582 without test agent. $^{\dagger}P<0.01$ versus $CaCl_2$ in the absence of BTS 67 582.

Table 3 Effect of BTS 67 582 on insulin release by BRIN-BD11 cells in the presence of 1.1 mM glucose and insulinotropic amino acids

Test agent (mM)	*BTS 67 582* (M)	*Insulin release* (ng per 10^6 cells 20 min^{-1})
None	–	1.39±0.08
None	10^{-4}	2.95±0.14**
L-Alanine (10)	–	5.82±0.26**
L-Alanine (10)	10^{-4}	11.58±0.27$^{**\Delta\ddagger}$
L-Leucine (10)	–	2.19±0.19*
L-Leucine (10)	10^{-4}	3.01±0.16**†
KIC (10)	–	2.68±0.16**
KIC (10)	10^{-4}	5.63±0.47$^{**\Delta\ddagger}$
L-Arginine (10)	–	6.67±0.32**
L-Arginine (10)	10^{-4}	12.73±1.05$^{**\Delta\ddagger}$

Data are mean±s.e.mean of six observations. $^*P<0.01$, $^{**}P<0.001$ versus 1.1 mM glucose alone. $^{\Delta}P<0.001$ versus BTS 67 582 without test agent. $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$ versus test agent in the absence of BTS 67 582.

Table 4 Effect of BTS 67 582 on insulin release by BRIN-BD11 cells in the presence of 16.7 mM glucose and modulators of cyclic AMP, carbachol and palmitate

Test agent (mM)	*BTS 67 582* (M)	*Insulin release* (ng per 10^6 cells 20 min^{-1})
None	–	2.90±0.20
None	10^{-4}	3.62±0.15*
Forskolin (0.025)	–	11.98±0.55**
Forskolin (0.025)	10^{-4}	18.06±0.26$^{**\Delta\#}$
IBMX (1)	–	18.38±0.38**
IBMX (1)	10^{-4}	29.34±1.04$^{**\Delta\#}$
Carbachol (0.1)	–	4.05±0.29*
Carbachol (0.1)	10^{-4}	5.25±0.07$^{**\Delta\ddagger}$
Palmitate (10)	–	9.02±0.78**
Palmitate (10)	10^{-4}	11.61±0.36$^{**\Delta\dagger}$

Data are mean±s.e.mean of six observations. $^*P<0.05$, $^{**}P<0.001$ versus 16.7 mM glucose alone. $^{\Delta}P<0.001$ versus BTS 67 582 without test agent. $^{\dagger}P<0.05$, $^{\ddagger}P<0.01$, $^{\#}P<0.001$ versus test agent in the absence of BTS 67 582.

Table 5 Effects of BTS 67 582 on insulin release by BRIN-BD11 cells in the presence of 16.7 mM glucose and sympathomimetic agents

Test agent (mM)	*BTS 67 582* (M)	*Insulin release* (ng per 10^6 cells 20 min^{-1})
None	–	2.90 ± 0.20
None	10^{-4}	3.68 ± 0.19*
Adrenaline (10)	–	1.33 ± 0.28**
Adrenaline (10)	10^{-4}	$2.74 \pm 0.30^{\Delta\dagger}$
Noradrenaline (10)	–	1.45 ± 0.17**
Noradrenaline (10)	10^{-4}	$2.56 \pm 0.14^{\Delta\Delta\ddagger}$

Data are mean ± s.e.mean of six observations. $^*P < 0.05$, $^{**}P < 0.001$ versus 16.7 mM glucose alone. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.001$ versus BTS 67 582 without test agent. $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.001$ versus test agent in the absence of BTS 67 582.

concentrations in the physiological range, probably due to expression of the glucose transporter isoform-2 (GLUT-2) and a high glucokinase : hexokinase ratio (McClenaghan *et al*., 1996a,c).

The present study demonstrates that BTS 67 582 stimulates insulin release from BRIN-BD11 cells in a concentration-dependent manner, enhancing both nutrient-induced insulin release and secretory pathways controlled through adenylate cyclase and phospholipase C. The insulin-releasing effect of BTS 67 582 showed a similar potency to tolbutamide, and both agents were more effective at a non-stimulative (1.1 mM) than a stimulative (16.7 mM) glucose concentration. A recent study with rat perifused islets noted that BTS 67 582 was most effective as an insulin-releasing agent at glucose concentrations in the normal physiological range (5–8 mM) (Dickinson *et al*., 1997). Opening the K^+-ATP channels with either pinacidil or diazoxide inhibited the stimulant effect of BTS 67 582, indicating the critical importance of reduced K^+-ATP channel activity and membrane depolarization to allow the insulin-releasing action of this agent.

The role of membrane depolarization in the effects of BTS 67 582 was further studied by superimposing a depolarizing concentration of K^+ in addition to stimulation with 16.7 mM glucose. Since these conditions are sufficient to depolarize fully the cell membrane, further potentiation of insulin release by BTS 67 582 (Table 1) indicates that this drug may also exert insulin-releasing activity that is independent of K^+-ATP channel activity. Similar K^+-ATP channel-independent actions have recently been demonstrated with tolbutamide, possibly reflecting an intracellular effect mediated by protein kinase C (Flatt *et al*., 1994; Eliasson *et al*., 1996). The role of intracellular Ca^{2+} in the mechanism of action of BTS 67 582 was illustrated by abolition of drug-stimulated insulin release under Ca^{2+}-free conditions. Consistent with this, elevation of extracellular Ca^{2+} augmented the effect of BTS 67 582, whereas blockade of voltage-dependent Ca^{2+}-channels with verapamil abolished the stimulative effects of the drug on insulin release.

In addition to increasing glucose-stimulated insulin release, BTS 67 582 enhanced the stimulative actions of non-glucidic nutrient secretagogues, including amino acids and the fatty acid palmitate. These insulinotropic nutrients have diverse actions on the pancreatic B-cell (Conget *et al*., 1994; Yada, 1994; McClenaghan *et al*., 1997). Whereas L-alanine, L-arginine, L-leucine and 2-ketoisocaproic acid each evoke membrane depolarization and insulin release, only the two latter nutrients act largely through their metabolism and generation of ATP (Yada, 1994; McClenaghan *et al*., 1996b, 1997). Palmitate-induced insulin release has been attributed to stimulation of Ca^{2+} influx and intracellular Ca^{2+} mobilization (Ordway *et al*., 1991; Warnotte *et al*., 1994). The ability of BTS 67 582 to potentiate the actions of these non-glucidic nutrients is a useful attribute and suggests effects of the drug on common elements of the secretory pathway.

BTS 67 582 augmented the stimulative effects of forskolin and IBMX which both increase cyclic AMP and protein kinase A (PKA), and potentiate Ca^{2+}-mediated insulin release (Howell & Montague, 1973; Ullrich & Wollheim, 1984; Hellman *et al*., 1992). The insulin-releasing effect of carbachol, which is mediated via phospholipase C (PLC) (Morgan & Montague, 1992) was also augmented by BTS 67 582, indicating that this agent may interact with PKA and PKC-dependent pathways in its mode of action. Since BTS 67 582 partially reversed the suppression of insulin release by adrenaline and noradrenaline, which act on the pancreatic B-cell via α_2-adrenoceptors (Samols & Wier, 1979; Morgan *et al*., 1994), it is possible that BTS 67 582 might exert some antagonism at this receptor subtype, or affect the G-protein-mediated signalling pathway through which these catecholamines suppress insulin release.

Collectively these data suggest that BTS 67 582 exerts an insulinotropic effect on pancreatic B-cells, acting through K^+-ATP channel inhibition, membrane depolarization and Ca^{2+} influx. The augmentation of nutrient-induced insulin release by BTS 67 582 coupled with amplification of late PKA-mediated and PKC-mediated steps of the exocytotic pathway, suggest that this agent increases insulin release through several different effects on the pancreatic B-cell.

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