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Cooperative enhancement of insulinotropic action of GLP-1 by acetylcholine uncovers paradoxical inhibitory effect of beta cell muscarinic receptor activation on adenylate cyclase activity

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Abstract

The cooperative effect of glucagon-like peptide 1 (GLP-1) and acetylcholine (ACh) was evaluated in a beta cell line model (BRIN BD11). GLP-1 (20 nM) and ACh (100 μM) increased insulin secretion by 24–47%, whereas in combination there was a further 89% enhancement of insulin release. Overnight culture with 100 ng/mL pertussis toxin (PTX) or 10 nM PMA significantly reduced the combined insulinotropic action ($P < 0.05$ and $P < 0.001$, respectively) and the sole stimulatory effects of GLP-1 (PTX treatment; $P < 0.01$) or ACh (PMA treatment; $P < 0.05$). Under control conditions, ACh (50 nM–1 mM) concentration-dependently inhibited by up to 40% ($P < 0.001$) the 10-fold ($P < 0.001$) elevation of cyclic 3′,5′-adenosine monophosphate (cAMP) induced by 20 nM GLP-1. The paradoxical inhibitory action of ACh was abolished by PTX pre-treatment, suggesting involvement of G_i and/or G_o G protein alpha subunit. Effects of selective muscarinic receptor antagonists on the concentration-dependent insulinotropic actions of ACh (50 nM–1 mM) on 20 nM GLP-1 induced insulin secretion revealed inhibition by ρ-FHHSiD (M3 antagonist, $P < 0.05$), stimulation with pirenzepine (M1 antagonist, $P < 0.001$) and no significant effects of either methoctramine (M2 antagonist) or MT-3 (M4 antagonist). Antagonism of M2, M3 and M4 muscarinic receptor effects with methoctramine (3–100 nM), ρ-FHHSiD (3–30 nM) or MT-3 (10–300 nM) did not significantly affect the inhibitory action of ACh on GLP-1 stimulated cAMP production. In contrast, M1 receptor antagonism with pirenzepine (3–300 nM) resulted in a concentration-dependent decrease in the inhibitory action of ACh on GLP-1 stimulated cAMP production ($P < 0.001$). These data indicate an important functional cooperation between the cholinergic neurotransmitter ACh and the incretin hormone GLP-1 on insulin secretion mediated through the M3 muscarinic receptor subtype. However, the insulinotropic action of ACh was associated with a paradoxical inhibitory effect on GLP-1 stimulated cAMP production, achieved through a novel PTX- and pirenzepine-sensitive M1 muscarinic receptor activated pathway. An imbalance between these pathways may contribute to dysfunctional insulin secretion.

Keywords: Muscarinic receptor; Insulin; Beta cell; cAMP; Acetylcholine; GLP-1

1. Introduction

Even though insulin secretion is mainly regulated by changes in circulating concentrations of glucose and other metabolic fuels, stimuli such as neurotransmitters and gastrointestinal hormones make an important contribution to the overall regulation of pancreatic beta cell function. A clear example of non-metabolic regulation comes from the early enhancement of insulin secretion during feeding

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Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; cAMP, cyclic 3′,5′-adenosine monophosphate; GLP-1, glucagon-like peptide 1; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IBMX, isobutylmethylxanthine; MT-3, muscarinic toxin-3; PMA, phorbol-12-myristate-13-acetate; PTX, pertussis toxin; ρ-FHHSiD, hexahydro-sila-difenidol ρ-fluoro hydrochloride.

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behaviour, which precedes any increase in blood glucose. This "cephalic phase" insulin response is largely mediated by cholinergic stimulation and the release of ACh from nerve terminals innervating the pancreatic islets [1–9].

Gut hormones play an important role in the regulation of endocrine pancreatic function, following meal ingestion. Postprandial release of incretin hormones from endocrine cells in the small intestine stimulates insulin secretion through the enteroinsular axis. For example, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like-peptide-1 (7–36) amide (GLP-1) are potent physiological insulin-releasing incretin hormones [1,3,10–12].

A recent study in humans indicated coordinated and sequential actions of cholinergic neurotransmitters and incretin hormones on meal-induced glucose-stimulated insulin secretion [8]. Furthermore, it is well established in normal subjects that the insulin response to oral glucose is much greater than that to a similar intravenous glucose load [13,14]. This difference has been ascribed to an increase of insulin secretion and a decrease of insulin clearance. In addition, it seems that at least in rodents, cholinergic stimulation has a priming effect on glucose, GIP and GLP-1 induced insulin secretion [15,16].

The cholinergic effects of ACh on insulin secretion are mediated through muscarinic receptors, located on the beta cell plasma membrane [4,17–20]. The clonal pancreatic beta cell line BRIN BD11 expresses at least four (M1–M4) of the five known muscarinic receptors, sharing a similar profile to isolated pancreatic rat beta cells and other beta cell lines [21–24]. The odd numbered muscarinic receptors, M1 and M3, are preferentially coupled to phosphoinositide hydrolysis and the generation of inositol 1,4,5 triphosphate and diacylglycerol [25–28]. A recent study indicates that an atypical protein kinase C (PKC), subtype zeta, is involved [29]. Studies in pancreatic beta cells indicate that the M3 muscarinic receptor is the major subtype coupled to stimulation of insulin secretion [20–24]. Interestingly, recent observations have indicated a novel role of ACh to inhibit insulin secretion through the even numbered M2 and/or M4 receptors [24]. In other cell types, those receptors are thought to be preferentially coupled to inhibition of adenylate cyclase [30,31].

Recently, GLP-1 has been the focus of great research interest as a possible pharmacological agent for the treatment of type 2 diabetes [32–36]. Thus, unlike established sulphonylurea drugs, GLP-1 stimulation of insulin secretion is strictly glucose dependent, thereby reducing the risk of hypoglycaemia. GLP-1 receptor activation stimulates adenylate cyclase and elevates both cAMP and intracellular Ca^{2+}, with both distal and proximal effects on the insulin secretory machinery [37]. In addition, GLP-1 is involved in key steps of insulin biosynthesis as well as in restoring glucose sensitivity of diabetic beta cells [35,38–42]. The present study was undertaken to determine the interactions between ACh and GLP-1 in the stimulation of insulin secretion. The effects of both physiological agents on cAMP production were evaluated together with the diversity of the signal pathways triggered by the various muscarinic receptor subtypes.

2. Methods

2.1. Materials

Reagents of analytical grade and deionised water (Purite) were used. RPMI 1640 tissue culture medium, foetal calf serum, penicillin and streptomycin were purchased from Gibco Life Technologies. $Na^{125}I$ (IMS30, 100 mCi/mL) for iodination of insulin and [2-^{3}H]adenine (TRK311, 1 mCi/mL) were from Amersham Pharmacia Biotech. Rat insulin standard was obtained from Novo Industri. Human glucagon-like peptide 1 (7–36) amide (GLP-1) was purchased from the American Peptide Company. Methoctramine tetrahydrochloride, hexahydro-sila-difenidol ρ-fluoro hydrochloride (ρ-FHHSiD), pirenzepine dihydrochloride, muscarinic toxin-3 (MT-3), ACh, PTX, phorbol-12-myristate-13-acetate (PMA) and all other reagents were obtained from Sigma Chemical Co. Ltd.

2.2. Culture of insulin-secreting cells

BRIN BD11 cells (passage 18–27) were cultured in RPMI 1640 tissue culture medium containing 10% (v/v) foetal calf serum, 1% (v/v) antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin) and 11.1 mM glucose. The production and characterisation of BRIN BD11 cells are described elsewhere [43]. Cells were maintained in sterile tissue culture flasks (Corning) at 37° in an atmosphere of 5% CO_2 and 95% air using an LEEC incubator (Laboratory Technical Engineering).

2.3. Acute tests for insulin secretion

Insulin release was determined using monolayers of BRIN BD11 cells. The cells were harvested with the aid of trypsin/EDTA (Gibco Life Technologies), seeded into 24-well plates (Nunc) at a density of 2×10^5 cells per well and allowed to attach during overnight culture. Acute studies of insulin release were preceded by 40-min pre-incubation at 37° in 1.0 mL Krebs–Ringer bicarbonate buffer (115 mM NaCl, 4.7 mM KCl, 1.28 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM $NaHCO_3$ and 0.1% (w/v) BSA, pH 7.4) supplemented with 1.1 mM of glucose. Test incubations were performed at 37° using the same buffer supplemented with 5.6 mM of glucose, 20 μM of the cholinesterase inhibitor neostigmine, 100 μM of isobutylmethylxanthine (IBMX), different concentrations of ACh and the muscarinic antagonists as indicated in the respective figures. Similar data could be obtained using carbamylcholine rather than the naturally occuring neurotransmitter ACh. After 20-min incubation, the buffer was

removed from each well and aliquots were stored at −20° for subsequent measurement of insulin by radioimmunoassay [44].

2.4. Measurement of cAMP

The cAMP was determined in extracts of BRIN BD11 cells labelled with [2-^{3}H]adenine. The cells were seeded into 24-well plates (Nunc) at a density of 3×10^5 cells per well with 25 μCi/mL of [2-^{3}H]adenine and incubated in RPMI media for 24 hr at 37°, 5% CO_2. Following incubation, the cells were washed with ice-cold HEPES-balanced saline buffer (130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.9 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 20 mM HEPES, 0.25 mM phenol red, 5.6 mM glucose, pH 7.4). Test incubations were then performed at 37°, for 20 min, using 200 μL of the same buffer, supplemented with 20 μM of neostigmine, 1 mM of IBMX, in absence (basal) and presence of 20 nM of GLP-1. Different concentrations of ACh and the muscarinic antagonists were also tested. The incubations were terminated by removal of test solution and addition of 300 μL per well of ice-cold stop solution (306 mM trichloroacetic acid, 104 mM sodium dodecyl sulphate, 5 mM 5′-adenosine triphosphate, 210 μM cAMP). Addition of the stop solution also disrupted the cells. Samples were stored at −20° for subsequent separation by liquid chromatography achieved using sequential elution through cation-exchange (Dowex 50WX8), affinity (aluminium oxide) and anion-exchange (Dowex 1X8) columns [45–47]. In brief, samples were loaded into 2 mL bed volume Dowex 50WX8 resin, packed in Poly-Prep chromatography columns (BioRad Laboratories), washed with 4 mL of H_2O and eluted into 1 mL bed volume aluminium oxide columns, with 20 mL of H_2O. Samples were then transferred to 1 mL bed volume Dowex 1X8 resin with 10 mL 600 mM imidazole. The remaining adenine was washed from the Dowex 1X8 columns with 20 mL of 3.16 mM HCl. Formed cAMP was eluted with 3 mL of 1 M HCl and measured by liquid scintillation counting. Results were expressed as a percentage of the stimulatory effect of GLP-1 alone (20 nM) or as a percentage of the total inhibitory effect of ACh on GLP-1 induced cAMP generation.

2.5. Statistical methods

Groups of data are presented as means ± SEM. One-way ANOVA with Bonferroni post-test and Student's *t*-test were performed using GraphPad Prism version 3.02 for Windows (GraphPad Software). Concentration–response curves were fitted with a sigmoidal concentration–response equation (Response = Min + (Max − Min)/$(1 + 10^{((\log EC_{50} - \text{Concentration}) \times \text{Hill slope})})$), using the same software package. Response was calculated as stated in the respective figures. Differences were considered to be significant if $P < 0.05$.

3. Results

3.1. Effects of ACh and GLP-1 on insulin secretion

In preliminary experiments, we found that the addition of neostigmine and IBMX during the acute test enhanced acetylcholine-induced insulin response by inhibiting respectively acetylcholinesterase and phosphodiesterase activities (data not shown). ACh (100 μM) and GLP-1 (20 nM) stimulated insulin secretion in control experiments (Fig. 1a and b, left panel). In addition, when both secretagogues were added during the acute test, a combined effect could be observed. ACh alone increased insulin response by 24% whereas GLP-1 increased secretion by 47% ($P < 0.05$ and $P < 0.001$, respectively). The combined insulinotropic effect of both secretagogues was 89% greater than the control ($P < 0.001$, Fig. 1a and b, left panel).

Overnight culture (18 hr) of BRIN BD11 cells with PTX (100 ng/mL) modestly decreased both the stimulatory effect of GLP-1 ($P < 0.01$) and the combined effect of

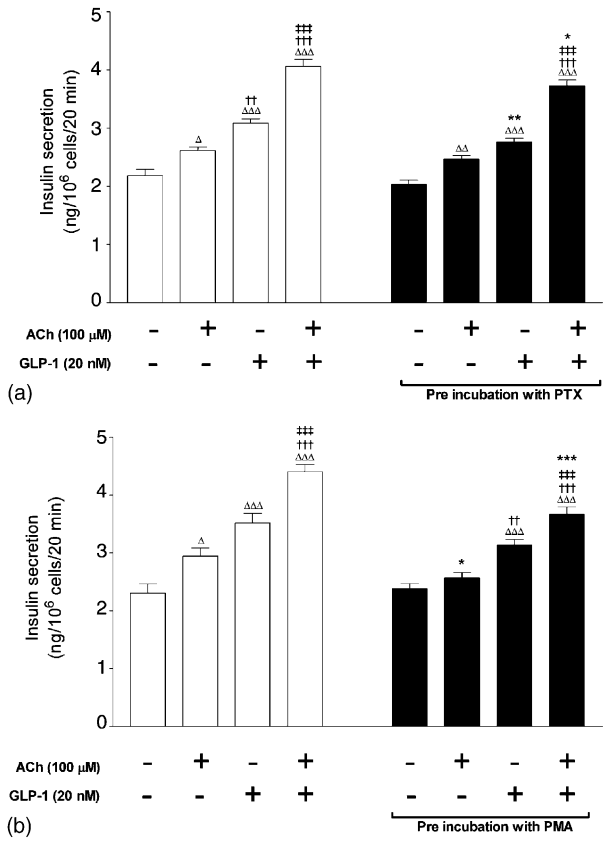


Fig. 1. Effects of ACh and GLP-1 on insulin secretion from BRIN BD11 cells at 5.6 mM glucose. Results are mean (SEM) of 12 independent observations. (a) Cells were cultured overnight under normal conditions (white bars) or in the presence of 100 ng/mL of PTX (black bars). (b) Cells were cultured overnight under normal conditions (white bars) or in the presence of 10 nM of PMA (black bars). (△) $P < 0.05$, (△△) $P < 0.01$, (△△△) $P < 0.001$ compared with control. (††) $P < 0.01$, (†††) $P < 0.001$ compared with ACh alone. (‡‡‡) $P < 0.01$ compared with GLP-1 alone. (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ compared with respective group without pre-incubation.

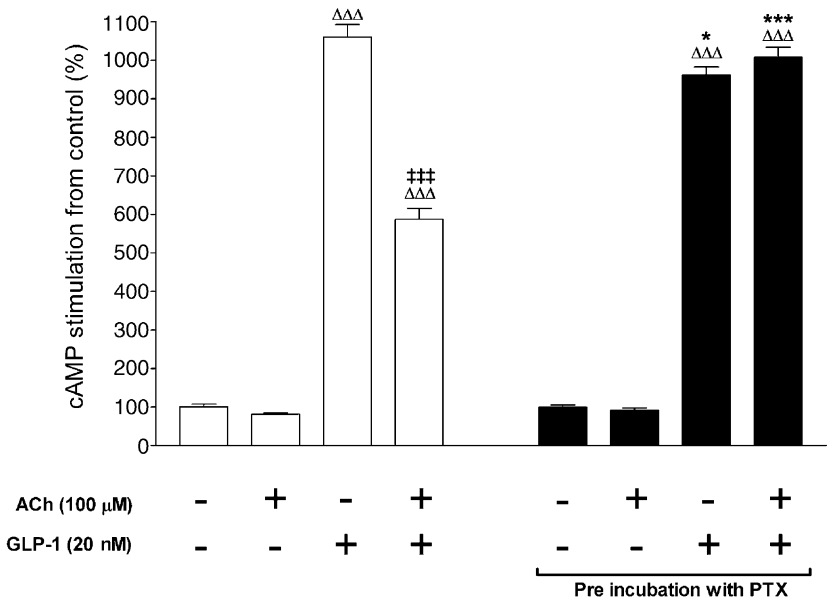


Fig. 2. Effects of ACh and GLP-1 on cAMP production by BRIN BD11 cells at 5.6 mM glucose. Results are mean (SEM) of six independent observations. Cells were cultured overnight under normal conditions (white bars) or in the presence of 100 ng/mL of PTX (black bars). (△△△) $P < 0.001$ compared with control and ACh alone. (‡‡‡) $P < 0.001$ compared with GLP-1 alone. (∗) $P < 0.05$, (∗∗∗) $P < 0.001$ compared with respective group without pre-incubation.

ACh and GLP-1 ($P < 0.05$). There was no significant reduction in the stimulatory effect of ACh (Fig. 1a, right panel). Conversely, 18-hr incubation culture of BRIN BD11 cells with 10 nM of PMA significantly reduced the stimulatory effect of ACh ($P < 0.05$) as well as the combined effect of ACh and GLP-1 ($P < 0.001$), while the stimulatory effect of GLP-1 alone was unchanged (Fig. 1b, right panel).

3.2. Effect of ACh on GLP-1 induced cAMP production

The stimulatory effect of GLP-1 on cAMP production is clearly observed in Fig. 2. ACh alone had no effect on cAMP production (Fig. 2, right and left panels). However, under normal incubation, ACh inhibited the stimulatory effect of GLP-1 by 49% (Fig. 2, left panel). Overnight pre-incubation of BRIN BD11 cells with 100 ng/mL of PTX completely abolished the inhibitory effect of ACh on GLP-1 stimulated cAMP production (Fig. 2, right panel). Atropine (100 nM) also completely abolished the inhibitory effect of ACh on GLP-1 stimulated cAMP production, indicating that the effect is mediated by muscarinic receptors (data not shown). Overnight pre-incubation with PTX significantly reduced the action of GLP-1 (Fig. 2, right panel).

3.3. GLP-1 stimulated insulin secretion and muscarinic receptor subtypes

The combined insulinotropic action of ACh and GLP-1 was further investigated through a series of experiments evaluating the effect of different concentrations of ACh on GLP-1 stimulated insulin secretion. Muscarinic receptor antagonists [25–28] were used to identify the involvement of different muscarinic receptor subtypes in the overall insulinotropic effect (Fig. 3). Basal insulin release under these conditions was approximately 3.07 (±0.29) ng/10^6 cells/20 min. Addition of ACh to 20 nM

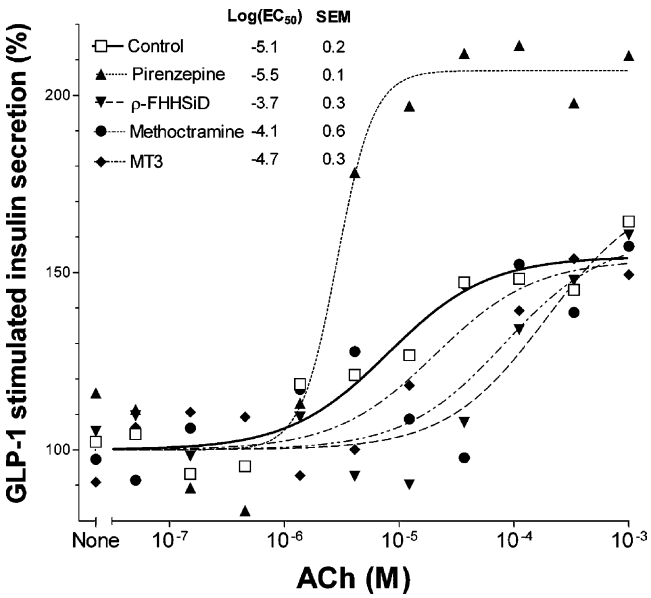
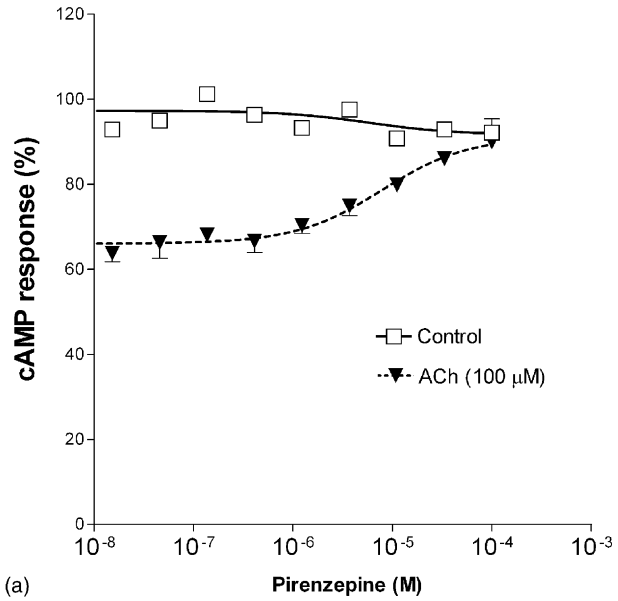


Fig. 3. Effects of different muscarinic antagonists on GLP-1 induced insulin secretion from BRIN BD11 cells. Cells were incubated with 5.6 mM of glucose and 20 nM of GLP-1, at different ACh concentrations without muscarinic antagonists (control, white squares), and with pirenzepine (100 nM, black triangles), ρ-FHHSiD (300 nM, black inverted triangles), methoctramine (300 nM, black circles) or MT-3 (100 nM, black diamonds). Results are mean of 12 independent observations. Standard error bars are omitted for clarity. Dose–response curves were fitted as described in the text. Inset shows the resulting log(EC_{50}) with respective SEM. Basal insulin release from BRIN BD11 cells under GLP-1 stimulation was approximately 3.07 (±0.29) ng/10^6 cells/20 min.

GLP-1 resulted in a concentration-dependent stepwise increase in insulin secretion. In the presence of pirenzepine, a preferentially selective M1 antagonist, the insulinotropic effect of ACh with GLP-1 was modest at lower concentrations of ACh but shifted abruptly to a more potent insulinotropic action at concentrations above 1 μM (Fig. 3). The overall concentration–response curve for pirenzepine had a Hill coefficient of 3.3 (±1.4), which was significantly different from 1 ($P < 0.05$, Fig. 3). The preferential M3 antagonist, ρ-FHHSiD, inhibited the combined insulinotropic action of ACh and GLP-1 ($P < 0.01$, Fig. 3). ρ-FHHSiD, like pirenzepine, also tended to decrease the combined insulinotropic effect of ACh and GLP-1 at lower concentrations of the neurotransmitter. Methoctramine, a preferential M2 antagonist and the M4 muscarinic antagonist MT-3 had no significant effect on the combined insulinotropic action of ACh and GLP-1 (Fig. 3, $P > 0.05$). None of the antagonists tested affected insulin secretion at



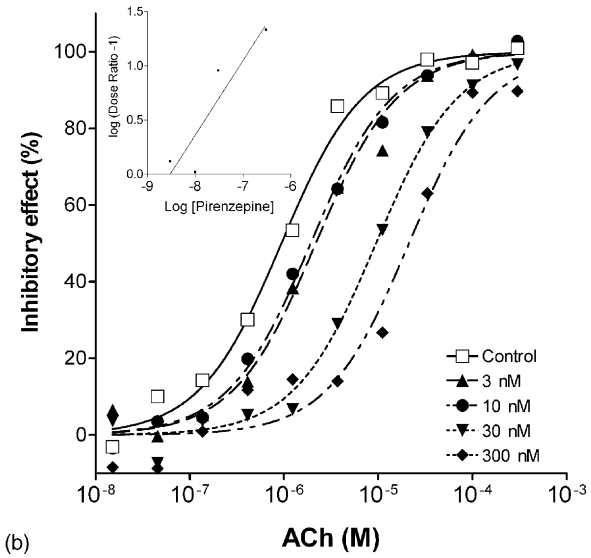
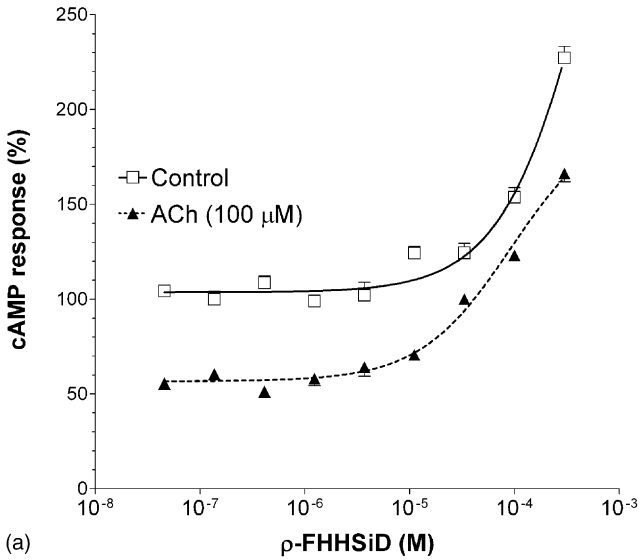


Fig. 4. Effects of ACh and the M1 antagonist pirenzepine on GLP-1 induced cAMP production by BRIN BD11 cells at 5.6 mM glucose. (a) Cells were incubated with GLP-1 (20 nM) and different concentrations of pirenzepine with (black triangles) or without (white squares) 100 μM of ACh. (b) Cells were incubated with GLP-1 (20 nM) and different concentrations of ACh without pirenzepine (control, white squares) or with increasing concentrations of the antagonist (black symbols). Results are expressed as percentage of the maximal inhibitory effect. Inset is the resulting Schild plot from the calculated dose ratios. Results are mean of four independent observations. Standard error bars are omitted for clarity.



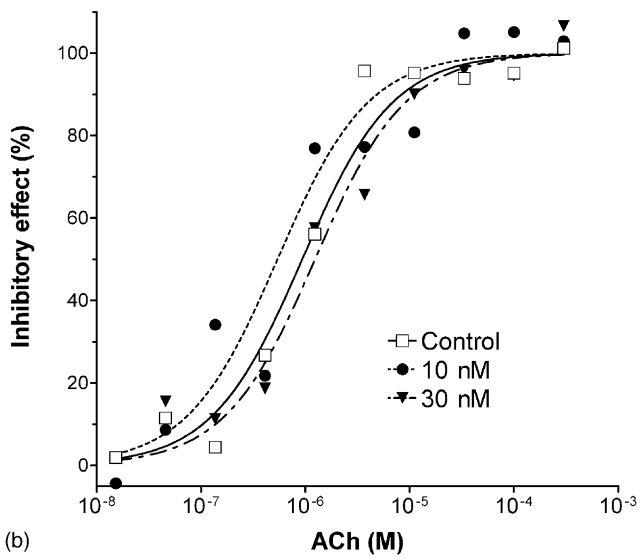


Fig. 5. Effects of ACh and the M3 antagonist ρ-FHHSiD on GLP-1 induced cAMP production by BRIN BD11 cells at 5.6 mM glucose. (a) Cells were incubated with GLP-1 (20 nM) and different concentrations of ρ-FHHSiD with (black triangles) or without (white squares) 100 μM of ACh. (b) Cells were incubated with GLP-1 (20 nM) and different concentrations of ACh without ρ-FHHSiD (control, white squares) or with increasing concentrations of the antagonist (black symbols). Results are expressed as percentage of the maximal inhibitory effect. Results are mean of four independent observations. Standard error bars are omitted for clarity.

5.6 mM glucose in the absence of ACh or GLP-1, indicating freedom from non-specific effects (data not shown).

3.4. GLP-1 stimulated cAMP production and muscarinic receptor subtypes

ACh consistently antagonised the stimulatory effect of GLP-1 on cAMP production, in a concentration-dependent manner (Figs. 4–7). Analyses of the concentration–response curves indicated an overall EC_{50} of 1.1 ± 0.2 μM and a slope not divergent from 1 ($P > 0.05$).

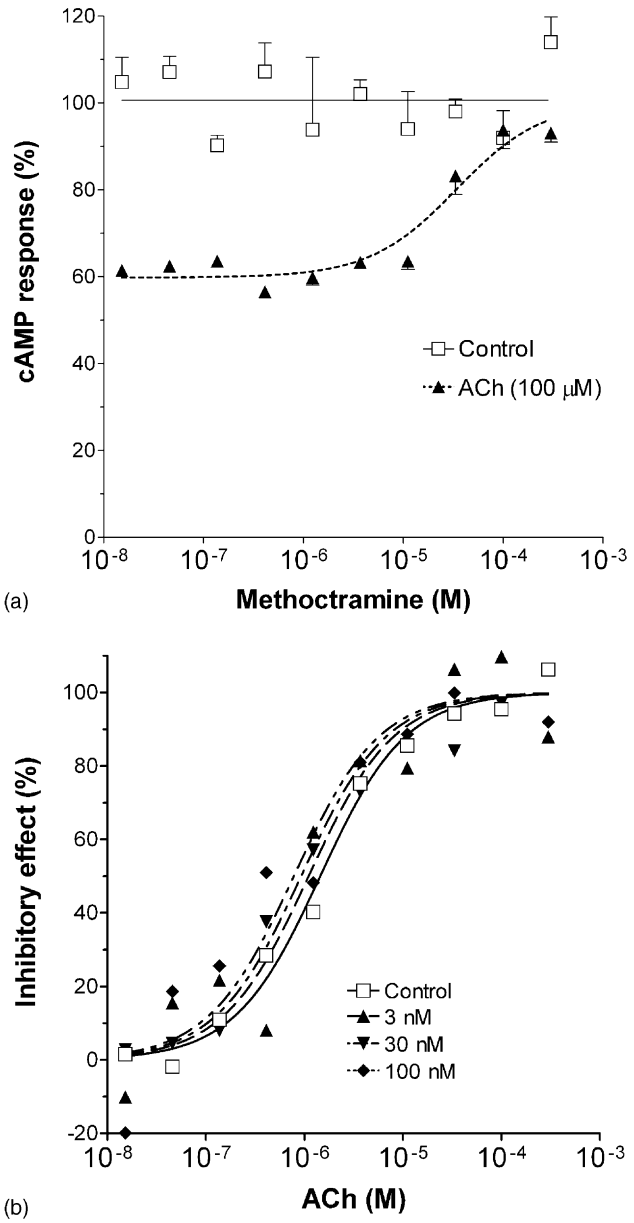


Fig. 6. Effect of ACh and the M2 antagonist methoctramine on GLP-1 induced cAMP production by BRIN BD11 cells at 5.6 mM glucose. (a) Cells were incubated with GLP-1 (20 nM) and different concentrations of methoctramine with (black triangles) or without (white squares) 100 μM of ACh. (b) Cells were incubated with GLP-1 (20 nM) and different concentrations of ACh without methoctramine (control, white squares) or with increasing concentrations of the antagonist (black symbols). Results are expressed as percentage of the maximal inhibitory effect. Results are mean of four independent observations. Standard error bars are omitted for clarity.

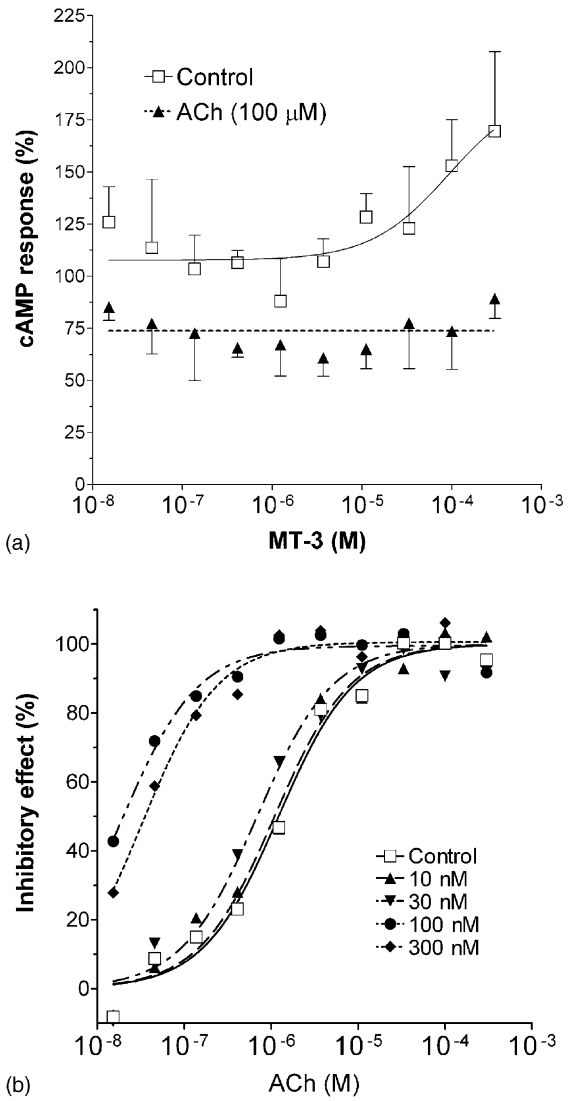


Fig. 7. Effect of ACh and the M4 antagonist MT-3 on GLP-1 induced cAMP production by BRIN BD11 cells at 5.6 mM glucose. (a) Cells were incubated with GLP-1 (20 nM) and different concentrations of MT-3 with (black triangles) or without (white squares) 100 μM of ACh. (b) Cells were incubated with GLP-1 (20 nM) and different concentrations of ACh without MT-3 (control, white squares) or with increasing concentrations of the antagonist (black symbols). Results are expressed as percentage of the maximal inhibitory effect. Results are mean of four independent observations. Standard error bars are omitted for clarity.

Pirenzepine, a preferential M1 muscarinic antagonist, counteracted the inhibitory effect of ACh on GLP-1 stimulated cAMP production ($P < 0.001$, Fig. 4a and b). The antagonic action was concentration dependent with a calculated K_i of 3 nM (Fig. 4b, inset graph). Pirenzepine alone had no effect on GLP-1 stimulated cAMP production (Fig. 4a).

Conversely, ρ-FHHSiD, a preferential antagonist of M3 muscarinic subtype receptors, stimulated cAMP production even in absence of ACh ($P < 0.001$, Fig. 5a). Low concentrations of ρ-FHHSiD failed to significantly alter

the inhibitory action of ACh on GLP-1 stimulated cAMP production (Fig. 5b).

Methoctramine, a preferential antagonist of the M2 muscarinic receptor subtype, also failed to modify the inhibitory effect of ACh on GLP-1 stimulated cAMP production (Fig. 6a and b). Methoctramine alone had no effect on GLP-1 stimulated cAMP production (Fig. 6a).

The preferential antagonist of M4 muscarinic subtype receptors, MT-3 stimulated cAMP production even in absence of ACh ($P < 0.001$, Fig. 7a). MT-3 failed to alter the inhibitory action of ACh on GLP-1 stimulated cAMP production at concentrations up to 30 nM (Fig. 7b). However, at concentrations of 100 and 300 nM, MT-3 markedly increased the inhibitory effect of ACh on GLP-1 stimulated cAMP production ($P < 0.001$, Fig. 7b).

4. Discussion

The present study was undertaken to explore interactions between ACh and GLP-1 in the stimulation of insulin secretion, as well as to examine the second messenger pathways involved. The neurotransmitter ACh alone increased insulin secretion by 24% at 5.6 mM glucose, which is in agreement with a previous study [24]. The incretin hormone GLP-1 also increased insulin secretion by 47%. Our results clearly indicate a cooperative insulinotropic effect of ACh and GLP-1 on the beta cell, corresponding to an 89% increase of insulin secretion when both agents were present. Such a synergistic action has been previously shown in perifused rat islets [15,48,49].

ACh released from nerve terminals upon cholinergic stimulation is thought to be one of the key elements responsible for the pre-absorptive insulin response initiated by meal ingestion [4,8]. The insulinotropic hormones GLP-1 and GIP are secreted as well, although these incretin peptides are not thought to participate to any great extent in the early pre-absorptive cephalic phase insulin response [8]. However, under physiological conditions when blood glucose rises following feeding the islet beta cells are subjected to simultaneous stimulation by both ACh and GLP-1.

Our results confirm a recent study [50] and indicate the involvement of PTX-sensitive G alpha subunits in GLP-1 stimulated insulin secretion; conversely, those PTX-sensitive G protein subtypes do not seem to be involved in the insulinotropic action of ACh. However, the cooperative effect of ACh and GLP-1 was reduced under PTX pretreatment. The insulinotropic effect of ACh, as previously shown, was dependent on PKC [20,51–55], and pre-treatment with PMA greatly reduced both the individual effect of ACh and the cooperative response to ACh and GLP-1. The effect of GLP-1 alone was not affected by prior exposure to PMA, indicating that the PKC pathway plays little role in the insulinotropic action of this incretin hormone.

In a series of experiments, we explored the participation of different muscarinic receptor subtypes in the cooperative actions of GLP-1 and ACh, using selective muscarinic receptor antagonists. Our results indicate that the stimulatory effect of ACh on insulin secretion in the presence of GLP-1 was concentration dependent, attaining near maximal values at 100 μM. Pirenzepine, a selective M1 muscarinic receptor antagonist increased the Hill coefficient of the insulin concentration–response curve; corresponding to a further significant concentration-dependent enhancement of the stimulatory effect of ACh on GLP-1 induced insulin release. This suggests that the M1 receptor counteracts the cooperative interactions between ACh and GLP-1 and that other muscarinic receptor subtypes normally mediate the overall stimulatory effects on insulin secretion. Antagonism of M2 and M4 with methoctramine and MT-3, respectively, did not significantly affect cooperative ACh and GLP-1 stimulated insulin secretion. However, blockade of the M3 receptor with ρ-FHHSiD inhibited the combined secretory response, suggesting that the M3 receptor is the main muscarinic subtype involved in the cooperative actions of ACh and GLP-1. This is in agreement with previous studies, showing the involvement of the M3 receptor on the insulinotropic action of ACh [21–24,56].

As previously suggested, the adenylate cyclase-cAMP system is an important second messenger involved in GLP-1 induced insulin secretion [37,47,57]. Our results indicate that ACh alone does not affect cAMP production, adding to conflicting reports in the literature that ACh may stimulate cAMP production indirectly, via Ca^{2+} and PKC [58–60]. More importantly, we have shown that ACh greatly reduces GLP-1 stimulated cAMP production. This inhibitory effect of ACh is mediated through a PTX-sensitive pathway, suggesting the involvement of a G_i and/or G_o G protein alpha subunit. The fact that combination of ACh with GLP-1 greatly potentiates insulin release despite lower cAMP production indicates an important synergy at other sites in the secretory pathway, possibly arising from activation at or downstream to phospholipase C [25,26,29,51,61,62]. Partial involvement of PKC is supported by the inhibitory effect of prior exposure to PMA on the cooperative actions of ACh and GLP-1.

Experiments on the inhibitory effects of ACh on GLP-1 induced cAMP production using different antagonists of muscarinic subtypes surprisingly indicated the involvement of M1. This is supported by direct observation that M1 receptor blockage with pirenzepine counteracted ACh-induced inhibition of GLP-1 stimulated cAMP production. Further, M4 antagonism with MT-3 at concentrations of 100 nM or above increased the inhibitory action of ACh on cAMP. However, since MT-3 toxin has an intrinsic inhibitory action on cAMP, albeit at much higher concentrations, the involvement of the M4 receptor in cAMP production remains to be established. The M2 and M3 receptor antagonists (methoctramine and ρ-FHHSiD, respectively) had no significant effect on cAMP production.

The involvement of M1 receptors in mediating inhibitory effects of ACh on GLP-1 stimulated cAMP production contrasts with observations in other cellular systems. Thus the muscarinic receptor subtypes usually involved with $G_{i/o}$ G protein alpha subunits are M2 and M4, whereas the M1 and M3 receptor subtypes increase cAMP production via G protein beta/gamma subunits [63–65]. To our knowledge, this is the first study demonstrating an inhibitory effect of M1 on cAMP production in beta cells. Interestingly, interactions between M1 receptors and G_i G protein alpha subunits have been shown in isolated prostate cells [66]. It could be argued that the inhibitory effect of M1 receptors on cAMP could be through stimulation of intracellular Ca^{2+} [67–69], but our results clearly ruled out this possibility since a PTX-sensitive pathway is involved.

Summarising, our results indicate an important functional cooperation between the cholinergic stimulatory effect of ACh and the incretin GLP-1 on insulin secretion, mostly mediated by M3 muscarinic receptors. We have demonstrated also that ACh is capable of inducing a paradoxical inhibitory effect on GLP-1 stimulated cAMP production and insulin secretion, mediated through a novel PTX- and pirenzepine-sensitive M1 receptor activated pathway. The mechanisms underlying the cooperative effects of ACh and GLP-1 are likely to involve interactions between multiple signal transduction pathways. Since neural and hormonal components of the enteroinsular axis are key to the regulation of insulin secretion and metabolic response to feeding, ACh- and GLP-1-mediated pathways are of great importance for the control of the beta cell function. The intricate details of the underlying signalling systems remain to be established but the present study clearly demonstrates the importance of muscarinic receptor subtypes not only in the physiological control of beta cells, but also as a possible cause of dysfunctional insulin secretion.

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References

[1] Morgan LM. Insulin secretion and the entero-insular axis. In: Flatt PR, editor. Nutrient regulation of insulin secretion. London: Portland Press; 1992, p. 1–22.
[2] Holst JJ. Role of classical and peptidergic neurotransmitters in insulin secretion. In: Flatt PR, editor. Nutrient regulation of insulin secretion. London: Portland Press; 1992, p. 23–37.
[3] Berggren PO, Rorsman P, Efendic S, Ostenson CG, Flatt PR, Nilsson T, Arkhammar P, Juntti-Berggren L. Mechanisms of action of entero-insular hormones, islet peptides and neural input on the insulin secretory process. In: Flatt PR, editor. Nutrient regulation of insulin secretion. London: Portland Press; 1992, p. 289–318.
[4] Ahren B. Autonomic regulation of islet hormone secretion—implications for health and disease. Diabetologia 2000;43:393–410.
[5] Berthoud HR, Jeanrenaud B. Sham feeding-induced cephalic phase insulin release in the rat. Am J Physiol Endocrinol Metab 1982;242: E280–5.
[6] Sjostrom L, Garellick G, Krotkiewski M, Luyckx A. Peripheral insulin in response to the sight and smell of food. Metabolism 1980;29:901–9.
[7] Strubbe JH, Steffens AB. Neural control of insulin secretion. Horm Metab Res 1993;25:507–12.
[8] Ahren B, Holst JJ. The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia. Diabetes 2001;50: 1030–8.
[9] Teff K. Nutritional implications of the cephalic-phase reflexes: endocrine responses. Appetite 2000;34:206–13.
[10] Schmidt WE, Siegel EG, Creutzfeldt W. Glucagon-like peptide-1 but not glucagon-like peptide-2 stimulates insulin release from isolated rat pancreatic islets. Diabetologia 1985;28:704–7.
[11] Creutzfeldt W, Ebert R. New developments in the incretin concept. Diabetologia 1985;28:565–73.
[12] Fehmann HC, Goke R, Goke B. Cell and molecular biology of the incretin hormones glucagon-like peptide-I and glucose-dependent insulin releasing polypeptide. Endocr Rev 1995;16:390–410.
[13] Shapiro ET, Tillil H, Miller MA, Frank BH, Galloway JA, Rubenstein AH, Polonsky KS. Insulin secretion and clearance. Comparison after oral and intravenous glucose. Diabetes 1987;36:1365–71.
[14] Tillil H, Shapiro ET, Miller MA, Karrison T, Frank BH, Galloway JA, Rubenstein AH, Polonsky KS. Dose-dependent effects of oral and intravenous glucose on insulin secretion and clearance in normal humans. Am J Physiol Endocrinol Metab 1988;254:E349–57.
[15] Fehmann HC, Goke R, Goke B, Arnold R. Carbachol priming increases glucose- and glucagon-like peptide-1 (7–36) amide-, but not arginine-induced insulin secretion from the isolated perfused rat pancreas. Z Gastroenterol 1990;28:348–52.
[16] Zawalich WS, Zawalich KC, Rasmussen H. Cholinergic agonists prime the beta-cell to glucose stimulation. Endocrinology 1989; 125:2400–6.
[17] Sharp R, Culbert S, Cook J, Jennings A, Burr IM. Cholinergic modification of glucose-induced biphasic insulin release in vitro. J Clin Invest 1974;53:710–6.
[18] Gilon P, Henquin JC. Mechanisms and physiological significance of the cholinergic control of pancreatic beta-cell function. Endocr Rev 2001;22:565–604.
[19] Berthoud HR, Trimble ER, Siegel EG, Bereiter DA, Jeanrenaud B. Cephalic-phase insulin secretion in normal and pancreatic islet-transplanted rats. Am J Physiol Endocrinol Metab 1980;238: E336–40.
[20] Mathias PC, Best L, Malaisse WJ. Stimulation by glucose and carbamylcholine of phospholipase C in pancreatic islets. Cell Biochem Funct 1985;3:173–7.
[21] Verspohl EJ, Tacke R, Mutschler E, Lambrecht G. Muscarinic receptor subtypes in rat pancreatic islets: binding and functional studies. Eur J Pharmacol 1990;178:303–11.
[22] Boschero AC, Szpak-Glasman M, Carneiro EM, Bordin S, Paul I, Rojas E, Atwater I. Oxotremorine-m potentiation of glucose-induced insulin release from rat islets involves M3 muscarinic receptors. Am J Physiol Endocrinol Metab 1995;268:E336–42.
[23] Iismaa TP, Kerr EA, Wilson JR, Carpenter L, Sims N, Biden TJ. Quantitative and functional characterization of muscarinic receptor subtypes in insulin-secreting cell lines and rat pancreatic islets. Diabetes 2000;49:392–8.

[24] Miguel JC, Abdel-Wahab YH, Mathias PC, Flatt PR. Muscarinic receptor subtypes mediate stimulatory and paradoxical inhibitory effects on an insulin-secreting beta cell line. Biochim Biophys Acta 2002;1569:45–50.
[25] Bonner TI. The molecular basis of muscarinic receptor diversity. Trends Neurosci 1989;12:148–51.
[26] Buckley NJ, Bonner TI, Buckley CM, Brann MR. Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. Mol Pharmacol 1989;35:469–76.
[27] Bonner TI, Buckley NJ, Young AC, Brann MR. Identification of a family of muscarinic acetylcholine receptor genes. Science 1987;237:527–32.
[28] Bonner TI, Young AC, Brann MR, Buckley NJ. Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. Neuron 1988;1:403–10.
[29] Tang SH, Sharp GW. Atypical protein kinase C isozyme zeta mediates carbachol-stimulated insulin secretion in RINm5F cells. Diabetes 1998;47:905–12.
[30] Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 1998;50:279–90.
[31] Wess J. Molecular basis of receptor/G-protein-coupling selectivity. Pharmacol Ther 1998;80:231–64.
[32] Vilsboll T, Krarup T, Deacon CF, Madsbad S, Holst JJ. Reduced postprandial concentrations of intact biologically active glucagon-like peptide 1 in type 2 diabetic patients. Diabetes 2001;50:609–13.
[33] Vilsboll T, Krarup T, Madsbad S, Holst JJ. No reactive hypoglycaemia in Type 2 diabetic patients after subcutaneous administration of GLP-1 and intravenous glucose. Diabet Med 2001;18:144–9.
[34] Larsen J, Hylleberg B, Ng K, Damsbo P. Glucagon-like peptide-1 infusion must be maintained for 24 hr/day to obtain acceptable glycemia in type 2 diabetic patients who are poorly controlled on sulphonylurea treatment. Diabetes Care 2001;24:1416–21.
[35] Holst JJ. Gut hormones as pharmaceuticals. From enteroglucagon to GLP-1 and GLP-2. Regul Pept 2000;93:45–51.
[36] Larsen PJ, Fledelius C, Knudsen LB, Tang-Christensen M. Systemic administration of the long-acting GLP-1 derivative NN2211 induces lasting and reversible weight loss in both normal and obese rats. Diabetes 2001;50:2530–9.
[37] Gromada J, Bokvist K, Ding WG, Holst JJ, Nielsen JH, Rorsman P. Glucagon-like peptide 1 (7–36) amide stimulates exocytosis in human pancreatic beta-cells by both proximal and distal regulatory steps in stimulus-secretion coupling. Diabetes 1998;47:57–65.
[38] Holst JJ, Orskov C, Nielsen OV, Schwartz TW. Truncated glucagon-like peptide I, an insulin-releasing hormone from the distal gut. FEBS Lett 1987;211:169–74.
[39] Fehmann HC, Habener JF. Insulinotropic hormone glucagon-like peptide-I (7–37) stimulation of proinsulin gene expression and proinsulin biosynthesis in insulinoma beta TC-1 cells. Endocrinology 1992;130:159–66.
[40] Gromada J, Holst JJ, Rorsman P. Cellular regulation of islet hormone secretion by the incretin hormone glucagon-like peptide 1. Pflugers Arch 1998;435:583–94.
[41] Buteau J, Roduit R, Susini S, Prentki M. Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in beta (INS-1)-cells. Diabetologia 1999;42:856–64.
[42] Holz GG, Kuhtreiber WM, Habener JF. Pancreatic beta-cells are rendered glucose-competent by the insulinotropic hormone glucagon-like peptide-1 (7–37). Nature 1993;361:362–5.
[43] McClenaghan NH, Barnett CR, Ah-Sing E, Abdel-Wahab YH, O'Harte FP, Yoon TW, Swanston-Flatt SK, Flatt PR. Characterization of a novel glucose-responsive insulin-secreting cell line, BRIN-BD11, produced by electrofusion. Diabetes 1996;45:1132–40.
[44] Flatt PR, Bailey CJ. Abnormal plasma glucose and insulin responses in heterozygous lean (ob/+) mice. Diabetologia 1981;20:573–7.
[45] Farndale RW, Allan LM, Martin BR. Adenylate cyclase and cAMP. In: Milligan G, editor. Signal transduction: a practical approach. Oxford: IRL Press; 1995, p. 75–103.
[46] Salomon Y, Londos C, Rodbell M. A highly sensitive adenylate cyclase assay. Anal Biochem 1974;58:541–8.
[47] Widmann C, Burki E, Dolci W, Thorens B. Signal transduction by the cloned glucagon-like peptide-1 receptor: comparison with signaling by the endogenous receptors of beta cell lines. Mol Pharmacol 1994;45:1029–35.
[48] Zawalich WS, Zawalich KC, Rasmussen H. Interactions between cholinergic agonists and enteric factors in the regulation of insulin secretion from isolated perifused rat islets. Acta Endocrinol (Copenhagen) 1989;120:702–7.
[49] Zawalich WS, Zawalich KC, Rasmussen H. Influence of glucagon-like peptide-1 on beta cell responsiveness. Regul Pept 1993;44:277–83.
[50] Hallbrink M, Holmqvist T, Olsson M, Ostenson CG, Efendic S, Langel U. Different domains in the third intracellular loop of the GLP-1 receptor are responsible for Galpha(s) and Galpha(i)/Galpha(o) activation. Biochim Biophys Acta 2001;1546:79–86.
[51] Zawalich WS, Zawalich KC. Regulation of insulin secretion by phospholipase C. Am J Physiol Endocrinol Metab 1996;271:E409–16.
[52] Zawalich WS, Zawalich KC, Kelley GG. Regulation of insulin release by phospholipase C activation in mouse islets: differential effects of glucose and neurohumoral stimulation. Endocrinology 1995;136:4903–9.
[53] Tian YM, Urquidi V, Ashcroft SJ. Protein kinase C in beta-cells: expression of multiple isoforms and involvement in cholinergic stimulation of insulin secretion. Mol Cell Endocrinol 1996;119:185–93.
[54] Wollheim CB, Regazzi R. Protein kinase C in insulin releasing cells. Putative role in stimulus secretion coupling. FEBS Lett 1990;268:376–80.
[55] Gao ZY, Gilon P, Henquin JC. The role of protein kinase-C in signal transduction through vasopressin and acetylcholine receptors in pancreatic B-cells from normal mouse. Endocrinology 1994;135:191–9.
[56] Tang SH, Sharp GW. Identification of muscarinic receptor subtypes in RINm5F cells by means of polymerase chain reaction, subcloning, and DNA sequencing. Diabetes 1997;46:1419–23.
[57] Holz GG, Leech CA, Habener JF. Activation of a cAMP-regulated Ca^{2+}-signaling pathway in pancreatic beta-cells by the insulinotropic hormone glucagon-like peptide-1. J Biol Chem 1995;270:17749–57.
[58] Tian Y, Laychock SG. Protein kinase C and calcium regulation of adenylyl cyclase in isolated rat pancreatic islets. Diabetes 2001;50:2505–13.
[59] Guenifi A, Simonsson E, Karlsson S, Ahren B, Abdel-Halim SM. Carbachol restores insulin release in diabetic GK rat islets by mechanisms largely involving hydrolysis of diacylglycerol and direct interaction with the exocytotic machinery. Pancreas 2001;22:164–71.
[60] Liu YJ, Grapengiesser E, Gylfe E, Hellman B. Crosstalk between the cAMP and inositol trisphosphate-signalling pathways in pancreatic beta-cells. Arch Biochem Biophys 1996;334:295–302.
[61] Gromada J, Dissing S, Rorsman P. Desensitization of glucagon-like peptide 1 receptors in insulin-secreting beta TC3 cells: role of PKA-independent mechanisms. Br J Pharmacol 1996;118:769–75.
[62] Tang SH, Yaney GC, Sharp GW. Unusual carbachol responses in RINm5F cells: evidence for a distal site of action in stimulus-secretion coupling. Mol Pharmacol 1995;47:863–70.
[63] Olianas MC, Adem A, Karlsson E, Onali P. Identification of rat brain muscarinic M4 receptors coupled to cyclic AMP using the selective antagonist muscarinic toxin 3. Eur J Pharmacol 1998;357:235–42.
[64] Murthy KS, Makhlouf GM. Differential coupling of muscarinic m2 and m3 receptors to adenylyl cyclases V/VI in smooth muscle. Concurrent M2-mediated inhibition via Galphai3 and m3-mediated stimulation via Gbetagammaq. J Biol Chem 1997;272:21317–24.

[65] Peralta EG, Ashkenazi A, Winslow JW, Ramachandran J, Capon DJ. Differential regulation of PI hydrolysis and adenylyl cyclase by muscarinic receptor subtypes. Nature 1988;334:434–7.
[66] Luthin GR, Wang P, Zhou H, Dhanasekaran D, Ruggieri MR. Role of m1 receptor-G protein coupling in cell proliferation in the prostate. Life Sci 1997;60:963–8.
[67] Cooper DM, Mons N, Karpen JW. Adenylyl cyclases and the interaction between calcium and cAMP signalling. Nature 1995;374:421–4.
[68] Boyajian CL, Garritsen A, Cooper DM. Bradykinin stimulates Ca^{2+} mobilization in NCB-20 cells leading to direct inhibition of adenylylcyclase. A novel mechanism for inhibition of cAMP production. J Biol Chem 1991;266:4995–5003.
[69] DeBernardi MA, Seki T, Brooker G. Inhibition of cAMP accumulation by intracellular calcium mobilization in C6-2B cells stably transfected with substance K receptor cDNA. Proc Natl Acad Sci USA 1991;88: 9257–61.