Effects of galnon, a non-peptide galanin-receptor agonist, on insulin release from rat pancreatic islets

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Abstract

Galanin is a neurotransmitter peptide that suppresses insulin secretion. The present study aimed at investigating how a non-peptide galanin receptor agonist, galnon, affects insulin secretion from isolated pancreatic islets of healthy Wistar and diabetic Goto–Kakizaki (GK) rats. Galnon stimulated insulin release potently in isolated Wistar rat islets; 100 μM of the compound increased the release 8.5 times ($p < 0.001$) at 3.3 mM and 3.7 times ($p < 0.001$) at 16.7 mM glucose. Also in islet perifusions, galnon augmented several-fold both acute and late phases of insulin response to glucose. Furthermore, galnon stimulated insulin release in GK rat islets. These effects were not inhibited by the presence of galanin or the galanin receptor antagonist M35. The stimulatory effects of galnon were partly inhibited by the PKA and PKC inhibitors, H-89 and calphostin C, respectively, at 16.7 but not 3.3 mM glucose. In both Wistar and GK rat islets, insulin release was stimulated by depolarization of 30 mM KCl, and 100 μM galnon further enhanced insulin release 1.5–2 times ($p < 0.05$). Cytosolic calcium levels, determined by fura-2, were increased in parallel with insulin release, and the L-type Ca^{2+}-channel blocker nimodipine suppressed insulin response to glucose and galnon. In conclusion, galnon stimulates insulin release in islets of healthy rats and diabetic GK rats. The mechanism of this stimulatory effect does not involve galanin receptors. Galnon-induced insulin release is not glucose-dependent and appears to involve opening of L-type Ca^{2+}-channels, but the main effect of galnon seems to be exerted at a step distal to these channels, i.e., at B-cell exocytosis.
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Galanin is a widely distributed peptide neurotransmitter that activates G-protein-coupled receptors to regulate a variety of physiologic processes, including feeding, insulin release, lactation, spinal reflex, gut contractility, growth, learning, memory, and depression [1–4]. Most galanin receptor ligands are peptides, which are vulnerable to enzymatic degradation and unable to cross the blood–brain barrier. In 2002, the first nonpeptide galanin receptor agonist, galnon, was discovered by application of a combinatorial library approach to the galanin pharmacophores [5]. Galnon was found to have the ability to displace [125I]galanin with micromolar affinity at Bowes cellular and rat hippocampal membranes. When injected into the hippocampus area in rats, galnon reversed the proconvulsant effects of the galanin receptor antagonist M35 [5]. Galnon was also shown to prolong heat withdrawal latency in nerve-injured rats [6]. This anti-hyperalgesic effect of galnon was prevented by intrathecal M35 [6].

Insulin release processes are controlled by a large number of factors, of which the most important one is

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glucose. This process involves metabolism of glucose, leading to an increase in ATP/ADP ratio, causing closure of K_{ATP}-channels, depolarizing the membranes, opening of L-type Ca^{2+}-channels, an increase in the cytosolic free calcium concentration ($[Ca^{2+}]_i$), and eventually, exocytosis of insulin [7]. Sulfonylurea drugs stimulate insulin release by closing the K_{ATP}-channels. In contrast, diazoxide opens the K_{ATP}-channels and inhibits insulin release [7]. Galanin suppresses insulin release from pancreas in animals [8] and human [9], and this effect of galanin is mediated by a G_i-protein [10].

The Goto–Kakizaki (GK) rat is an animal model of type 2 diabetes, developed by repeated inbreeding of glucose tolerant Wistar rats with high-normal plasma glucose levels in oral glucose tolerance test as selection index [11]. In GK rats, there are significantly higher fasting plasma glucose levels, altered glucose tolerance, and a very poor insulin secretory response to glucose in vivo and in vitro compared with Wistar controls [12,13]. These changes may be due to a defect late in stimulus-secretion coupling for glucose that impairs insulin secretion [14] and deficient oxidative metabolism of glucose in islet mitochondria [13].

The present study aimed to investigate the effect of galnon on insulin secretion and the mechanisms behind galnon's stimulation of insulin secretion using isolated islets from both normal Wistar and diabetic GK rats.

Materials and methods

Materials

Drugs and chemicals. Galnon (Fmoc-cycleo-hexylalanine-Lys-amidomethylcoumarin) was synthesized as described [5] and galanin was synthesized as described in [15]. Galnon was dissolved in DMSO first, and then diluted 100 times or more to get the concentrations that were used in the experiments. Identical concentrations of DMSO were also present in control media and did not affect insulin release or $[Ca^{2+}]_i$ responses. The antagonist of galanin receptor, M35, was obtained from Bachem AG Company (Bubendorf, Switzerland). Diazoxide, pertussis toxin, calphostin C, H-89, mastoparan, and other chemicals were bought from Sigma–Aldrich (Stockholm, Sweden). Fura-2 acetoxymethyl ester was purchased from Molecular Probes Europe (Leiden, The Netherlands).

Animals. Male Wistar rats (B & K Universal, Sollentuna, Stockholm, Sweden), 2–3 months old, weighing 295 ± 25 g ($n = 26$) and age-matched male Goto–Kakizaki (GK) rats, weighing 258 ± 21 g ($n = 4$), from our own breeding were used. The blood glucose concentrations in Wistar and GK rats were 4.9 ± 0.3 and 7.9 ± 0.5 mM ($p < 0.001$), respectively, prior to islet isolation starting at noon. The animals were kept in a room with 22 °C and were allowed free access of food during five days before isolation of islets. Data on glucose tolerance and other characteristics of GK rats of the Stockholm colony have been published previously [11].

Methods

Islet isolation. Rats were killed by decapitation, being unconscious after inhalation of carbon dioxide. The study was approved by the Ethics Committee of Animal Research at the Karolinska Institutet.

The islets were isolated by injecting collagenase A in Hanks' solution (9 mg/10 ml for Wistar rats and 24 mg/10 ml for GK rats) into pancreas through the pancreatic duct. Then the gland was removed, incubated for 24 min at 37 °C, washed with Hanks' solution, and islets were picked up after separation on Histopaque gradient (Sigma Diagnostics). Isolated islets were cultured overnight free floating in Petri dishes in RPMI 1640 medium (Flow Laboratory) with 11 mM glucose, 2 mM glutamine, 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin at 37 °C, atmosphere 95% O_2, 5% CO_2.

Batch-incubation experiments. After culture, islets were washed and pre-incubated for 30–45 min at 37 °C in 5 ml Krebs–Ringers bicarbonate (KRB) buffer containing 118.4 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$ (equilibrated with 5% CO_2 and 95% O_2), 10 mM Hepes, and 0.2% bovine albumin with 3.3 mM glucose, pH 7.4.

After preincubation, batches of three islets were transferred to tubes containing 300 μl KRB with glucose concentrations and substances as indicated in the following. Islets were incubated for 1 h at 37 °C in a shaking water-bath. Four tubes were run for each experimental condition. Incubations were stopped by cooling the tubes on ice. The islets were picked up and aliquots of the media were stored at −20 °C until insulin were measured by radioimmunoassay with our own anti-porcine insulin antiserum and rat insulin standard [16].

In the first series of batch incubations, the Wistar rat islets were incubated in KRB buffer with different concentrations of galnon (1, 10, and 100 μM) in both 3.3 and 16.7 mM glucose to study the dose–response of galnon on insulin secretion and choose the most suitable concentration of galnon for the following experiments.

In the second series of batch incubations, to elucidate the role of galanin receptor in galnon-induced insulin secretion, Wistar rat islets were incubated with medium containing 100 μM galnon without or with the galanin receptor antagonist M35 at 10, 100 nM, and 1 μM concentrations. These experiments were performed at both basal 3.3 and 16.7 mM glucose. In addition, the impact of galanin (10 and 100 nM) on galnon-induced insulin release was studied in separate incubations.

In the third series of batch incubations, Wistar rat islets were incubated in KRB buffer supplemented with 0.25 mM diazoxide to see if galnon exerted its effect on sites before or distal to K_{ATP}-channels. Furthermore, both Wistar and GK rat islets were exposed to diazoxide (0.25 mM) to keep the K_{ATP}-channels open and 30 mM KCl to depolarize the B-cell membrane. Then galnon was added in the buffer to see if galnon had any further effect. This experiment was performed in both 3.3 and 16.7 mM glucose conditions.

In the fourth series of batch incubations, galnon's effect on insulin response was studied in Wistar rat islets cultured overnight with medium as described above and adding 100 ng/ml pertussis toxin (PTX), which inhibits pertussis toxin-sensitive G-proteins. Batches of islets were also incubated with 1 nM somatostatin in 16.7 mM glucose and with or without PTX, to check if the inhibitory effect of somatostatin on glucose-induced insulin secretion was blocked by PTX used in these experiments or not. The interaction between galnon and mastoparan, a peptide known to stimulate the exocytotic machinery of B-cells [17,18], on insulin release from Wistar rat islets was also assessed.

In the fifth series of batch incubations, Wistar rat islets were incubated in the presence of 5 μM nimodipine to block L-type of Ca^{2+} channels, and see in that condition if galnon still stimulated insulin secretion.

In sixth series of incubations, dependency of galnon-induced insulin release on protein kinase A (PKA) and protein kinase C (PKC) by incubations in the presence of PKA inhibitor H-89 and PKC-inhibitor calphostin C.

Perifusion experiment. After overnight culture, 50 Wistar rat islets were pre-incubated for 30–45 min in KRB buffer supplemented with 3.3 mM glucose. Then, those islets were put into a perifusion chamber,

layered between two layers of bio-gel (Bio-Rad). The volume of the chamber was 0.5 ml. The islets were perifused at a flow rate of 200 μl/min, at 37 °C, with KRB and 3.3 mM glucose for 20 min prior to the start of collecting samples. To see the dynamic response of insulin secretion, the islets were first perifused for 10 min with 3.3 mM glucose, then for 20 min with 16.7 mM glucose plus 100 μM galnon, followed by 30 min with 3.3 mM glucose, only. Samples were collected every 2 min and stored at −20 °C until insulin was analyzed by radioimmunoassay [16].

Measurement of cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Dispersed Wistar rat islet cells plated on glass coverslips were incubated for 35 min at 37 °C in RPMI 1640 medium supplemented with 0.1% bovine serum albumin and 1 μM fura-2 acetoxymethyl ester. Cells were then incubated for an additional 10 min in KRB buffer and 3.3 mM glucose. Coverslips were mounted as the exchangeable bottom of an open perifusion chamber on the stage of an inverted epifluorescence microscope (Olympus CK 40). The superfusion chamber was designed to allow rapid exchange of fluids. The chamber was thermostatically controlled to maintain a temperature of 37 °C in the perifusate inside. The microscope was connected to a fluorescence system (M-39/2000 RatioMaster, PhotoMed) for dual wavelength excitation fluorometry. The excitation wavelengths generated by a monochromator (DeltaRam, PhotoMed) were directed to the cell by a dichroic mirror. The emitted light selected by a 510 nm filter was monitored by a photomultiplier. The excitation wavelengths were alternated at a frequency of 1 Hz, and the duration of data collection at each wavelength was 0.33 s. The emission at the excitation wavelength of 340 nm (F_{340}) and that of 380 nm (F_{380}) were used to calculate the fluorescence ratio ($R_{340/380}$). Single cells isolated optically by means of a diaphragm were studied by using a 40× 1.3 NA oil immersion objective (40× UV APO).

Displacement of galanin by galnon. A binding assay was used to quantify ability of galnon to displace [^{125}I]porcine-galanin in membrane preparations from Rinm5F cells. The assay is described in detail in a previous study [5].

Cell viability test. Assessment of cell viability was performed by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [19] of Rinm5F cells as well as single rat islet cells, prepared as previously described [20], after exposure to galnon (100 μM) or control buffer during 60 min. Results were expressed as a percentage viable cells as compared to control, untreated cells.

Statistical analysis. Data were analyzed using Sigma Plot. All data are expressed as means ± SEM and comparisons of data have been done by unpaired Student's *t* test or ANOVA, as appropriate.

Results

Effects of galnon on insulin secretion from Wistar rat islets

Insulin release from batch-incubated pancreatic Wistar rat islets was stimulated 2.4-fold by 16.7 mM glucose in comparison with 3.3 mM glucose ($p < 0.001$, Table 1). At both glucose concentrations, 1 μM galnon had no effect on insulin secretion. At 10 μM, galnon stimulated insulin secretion 2.3-fold ($8.4 ± 1.8$ μU/islet/h vs. $19.9 ± 3.3$ μU/islet/h, $p < 0.001$) in 3.3 mM glucose but only tended to enhance glucose-stimulated (16.7 mM glucose) insulin secretion ($p = 0.07$). At 100 μM, however, galnon had very marked effects on both basal and glucose-stimulated insulin secretion, enhancing insulin release up to 8.4- and 3.7-fold at 3.3 and

Table 1
Effects of galnon on basal and glucose-stimulated insulin release from batch-incubated Wistar rat islets

Addition to the medium	3.3 mM glucose	16.7 mM glucose
None	$8.4 ± 1.8$	$19.9 ± 2.5$
1 μM galnon	$9.7 ± 2.2$	$28.4 ± 6.3$
10 μM galnon	$19.9 ± 3.3^{**}$	$31.4 ± 6.2$
100 μM galnon	$71.4 ± 7.1^{***}$	$74.0 ± 8.1^{***}$

Results of insulin release (μU/islet/h) are means ± SEM of five batch incubations, each representing the mean of quadruplicate incubations, at each condition. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to control group (without galnon).

16.7 mM glucose, respectively, compared to control groups ($p < 0.001$, Table 1).

Effect of galnon on kinetics of insulin secretion

To study the effect of galnon on the kinetics of insulin release and to elucidate whether the insulinotropic effect of galnon is reversible or not, the perifusion experiments were done. Galnon increased strongly the insulin response to 16.7 mM glucose, the stimulation mainly affecting the late phase of glucose stimulation (Fig. 1). When galnon was withdrawn and the glucose concentration of the medium was switched back to 3.3 mM, insulin release decreased gradually to the same levels as seen in the control group (Fig. 1).

Effects of galanin receptor antagonist M35 and galanin on galnon-induced insulin secretion

The addition of the galanin receptor antagonist M35 at concentrations of 10, 100 nM, and 1 μM did not affect insulin secretion or block the effect of galnon on insulin
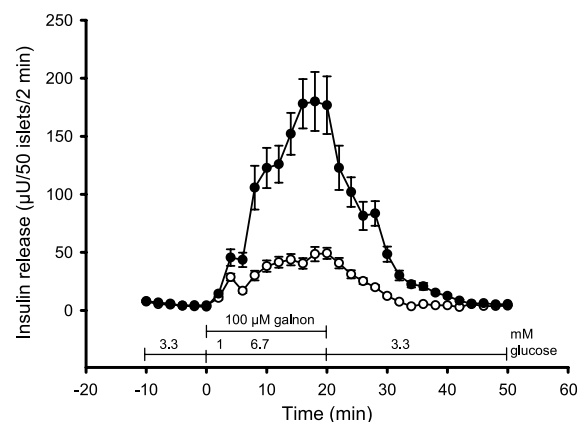

Fig. 1. Effect of galnon on insulin release from perifused Wistar rat islets. Batches of 50 islets were perifused at a flow rate of 200 μl/min with KRB and 3.3 mM glucose. At min 0, galnon (100 μM) was added with 16.7 mM glucose (●–●), and at min 20 galnon was omitted, and the glucose concentration again decreased to 3.3 mM during 30 min. Control perifusions (○–○) were performed with vehicle (1% DMSO) dissolved in KRB buffer. The data are shown as means ± SEM of four experiments.

Table 2
Interaction between galanin and galnon on insulin release from isolated Wistar rat islets

Addition to the medium	3.3 mM glucose	16.7 mM glucose
None	7.5 ± 0.8	34.0 ± 2.0
Galanin (10 nM)	10.2 ± 0.8	18.2 ± 2.2[a]
Galanin (100 nM)	13.3 ± 1.1	18.4 ± 1.1[a]
Galanin (10 nM) + M35 (1 μM)	11.8 ± 2.0	30.6 ± 3.5
Galnon (100 μM)	62.2 ± 7.8	91.1 ± 4.3
Galnon (100 μM) + galanin (10 nM)	55.0 ± 5.0	95.8 ± 10.3
Galnon (100 μM) + galanin 100 nM	76.9 ± 9.3	102.3 ± 11.2

Results of insulin release (μU/islet/h) are means ± SEM of 5–6 batch incubations at each condition.

[a] $p < 0.001$ vs. no addition.

secretion from Wistar rat pancreatic islets (data not shown). Similarly, galanin (10 and 100 nM) did not affect galnon-induced insulin release, although these concentrations of the peptide inhibited glucose-stimulated insulin release by almost 50% ($p < 0.001$), an effect that was blocked by the receptor antagonist, M35 (Table 2).

Effect of 0.25 mM diazoxide on galnon-induced insulin secretion

In 3.3 mM glucose, 0.25 mM diazoxide that kept K_{ATP}-channels open did not affect basal or galnon-in-

Table 3
Effect of 100 μM galnon in combination with 0.25 mM diazoxide

Addition to the medium	3.3 mM glucose	16.7 mM glucose
None	9.4 ± 1.9	41.2 ± 6.6
Diazoxide (0.25 mM)	9.8 ± 1.6	12.6 ± 2.1[a]
Galnon (100 μM)	77.3 ± 13.3[a]	88.4 ± 6.4[a]
Galnon (100 μM) + diazoxide (0.25 mM)	66.4 ± 5.1[a]	54.2 ± 3.0[b]

Results of insulin release (μU/islet/h) are means ± SEM of three batch incubations, each representing the mean of quadruplicate incubations, at each condition.

[a] $p < 0.001$ vs. control group (without galnon).
[b] $p < 0.001$ vs. galnon alone.

duced insulin secretion (Table 3). In 16.7 mM glucose, 0.25 mM diazoxide blocked glucose-induced insulin secretion to a level similar to that seen in 3.3 mM glucose ($p < 0.001$). In contrast to our finding at 3.3 mM glucose, addition of 0.25 mM diazoxide suppressed partly the combination effect of galnon and high glucose concentration (16.7 mM) on insulin secretion from 88.4 ± 6.4 to 54.2 ± 3.0 μU/islet/h ($p < 0.001$, Table 3).

The effects of galnon in islets depolarized by KCl

When islets were depolarized by 30 mM K^+ and in the presence of 0.25 mM diazoxide (to keep the potassium channels open), insulin release at 3.3 mM glucose increased 3.7- and 2.2-fold in Wistar rat and GK rat islets, respectively (Table 4). At similar conditions, but at 16.7 mM glucose, insulin release increased 2.3- and 4.2-fold in Wistar rat and GK rat islets, respectively. In this context, it is of interest that GK rat islets did not respond to 16.7 mM glucose under normal conditions, but increased insulin response 2.4-fold at depolarizing conditions (Table 4).

In the experiments with pancreatic islets of normal Wistar rat, the effects of galnon on insulin secretion were 1.6-fold ($p < 0.001$) and 1.5-fold ($p < 0.05$) stronger in comparison with the effects of depolarizing condition (30 mM KCl and 0.25 mM diazoxide) in 3.3 and 16.7 mM glucose, respectively (Table 4). The insulin release stimulating effect of galnon was similar both in normal islets and in depolarized islets (by 30 mM KCl), and in the presence of 0.25 mM diazoxide (Table 4). Galnon also exerted a marked effect on insulin release from diabetic GK rat islets. The galnon-induced insulin secretion in normal Wistar rat islets and GK rat islets was similar.

Effect of galnon on insulin release from Wistar rat islets pre-incubated with PTX

Pre-treating islets with 0.1 μg/ml PTX did not affect basal or glucose-induced insulin secretion by itself.

Table 4
Effect of 100 μM galnon on depolarized islets from Wistar and GK rats

Addition to the medium	Wistar rat islets		GK rat islets	
	3.3 mM glucose	16.7 mM glucose	3.3 mM glucose	16.7 mM glucose
Control	10.8 ± 3.3	31.8 ± 5.8	11.7 ± 3.6	14.8 ± 2.8
30 mM KCl + 0.25 mM diazoxide	39.6 ± 7.2[a]	72.6 ± 16.1[a]	26.2 ± 5.0[b]	61.7 ± 10.2[a]
100 μM galnon	65.5 ± 9.9[a]	110.7 ± 17.8[a]	72.1 ± 5.9[a]	103.0 ± 15.6[a]
100 μM galnon + 30 mM KCl + 0.25 mM diazoxide	71.4 ± 6.5[a,c]	98.8 ± 17[a,e]	74.4 ± 8.9[a,c]	124.0 ± 16.6[a,d]

Results of insulin release (μU/islet/h) are means ± SEM of three batch incubations, each representing the mean of quadruplicate incubations, at each condition.

[a] $p < 0.001$ vs. control.
[b] $p < 0.05$ vs. control.
[c] $p < 0.001$ vs. KCl + diazoxide.
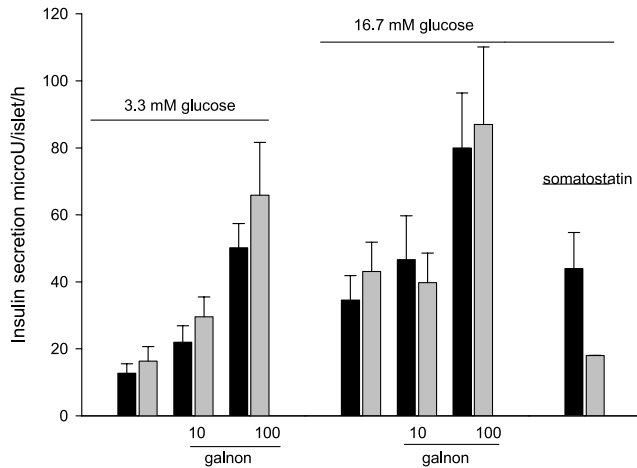[d] $p < 0.01$ vs. KCl + diazoxide.
[e] $p < 0.05$ vs. KCl + diazoxide.

Fig. 2. Effect of 10 and 100 μM galnon on Wistar rat pre-treated with 100 ng/ml PTX islets (■ black bars) or control islets (without pre-treated with PTX, ▨ grey bars). Results of insulin release (μU/islet/h) are means ± SEM of three batch incubation experiments, each representing the mean of triplicate incubations.

Somatostatin (1 nM) decreased glucose-induced insulin secretion from 56.2 ± 8.7 to 18.0 ± 0.9 μU/islet/h, $p < 0.01$ (Fig. 2). When Wistar rat islets were pre-treated with PTX, the inhibitory effect of somatostatin on glu-cose-induced insulin secretion was abolished ($p < 0.05$) (Fig. 2). Galnon stimulated insulin release similarly in is-lets pre-treated with PTX and in islets without pre-treat-ment with PTX (Fig. 2).

Interaction between galnon and mastoparan on insulin release

At 3.3 mM glucose, insulin release was 8.1 ± 1.0 μU/islet/h ($n = 6$), and the insulin responses to 10 μM mas-toparan 36.8 ± 4.3 ($p < 0.01$ vs. control), to 100 μM galnon 63.5 ± 7.1 ($p < 0.001$ vs. control), and to mastop-aran together with galnon 92.7 ± 8.5 μU/islet/h ($n = 6$; $p < 0.05$ vs. each substance alone). At 16.7 mM glucose, insulin release was stimulated to 39.8 ± 5.0 μU/islet/h ($p < 0.001$ vs. 3.3 mM glucose), and insulin responses to the same concentrations of mastoparan and galnon were 67.6 ± 8.2 ($p < 0.05$) and 85.9 ± 7.0 μU/islet/h ($p < 0.01$), respectively, and the combined effect 143.5 ± 18.4 μU/is-let/h ($p < 0.05$ vs. each substance alone).

Effect of galnon on the ($[Ca^{2+}]_i$) in B-cells

We measured intracellular free Ca^{2+} ($[Ca^{2+}]_i$) from single dispersed islet cells loaded with fura-2. The cell was first perifused with 3.3 mM glucose (Fig. 3A). Addi-tion of 100 μM galnon in the presence of 3.3 mM glu-cose caused a gradual increase of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was reduced on wash out of galnon but it did not reach the baseline. In other experiments, the cell was first stim-ulated with 16.7 mM glucose (Fig 3B). This caused a
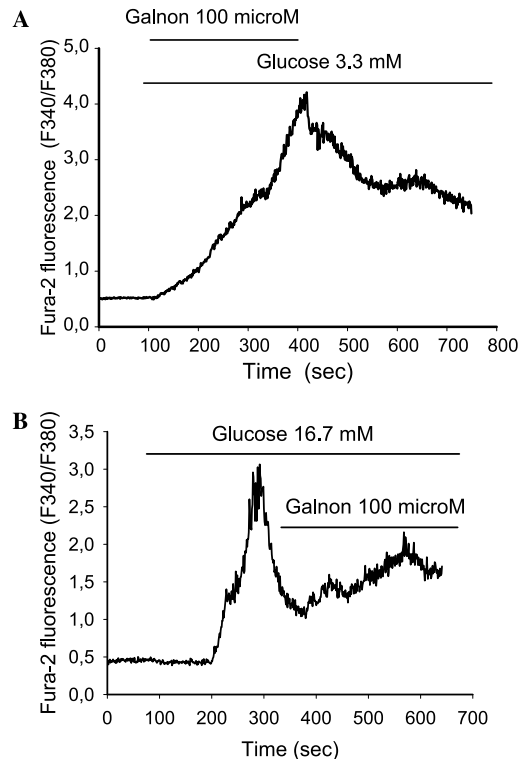

Fig. 3. (A) Effect of galnon on $[Ca^{2+}]_i$ in the presence of 3.3 mM glucose. $[Ca^{2+}]_i$ was measured in single dispersed islet cells loaded with fura-2. The cell was first perifused with 3.3 mM glucose. Galnon was added during the time indicated by the horizontal bar. The trace is representative of four experiments. (B) Effect of galnon on $[Ca^{2+}]_i$ in the presence of 16.7 mM glucose. $[Ca^{2+}]_i$ was measured using fura-2 technique as described in the methods section. The cell was first stimulated by 16.7 mM glucose which caused an increase of $[Ca^{2+}]_i$. Addition of galnon (100 μM) in the continued presence of 16.7 mM glucose caused a renewed increase of $[Ca^{2+}]_i$. The trace is represen-tative of four experiments.

transient increase of $[Ca^{2+}]_i$. Addition of 100 μM galnon in the continued presence of 16.7 mM glucose induced renewed increase of $[Ca^{2+}]_i$.

Effect of galnon in combination with nimodipine

Glucose-induced insulin secretion from Wistar rat is-lets was inhibited by 5 μM nimodipine from 30.6 ± 2.9 to 6.5 ± 2.3 μU/islet/h ($p < 0.001$), i.e., to a level similar to that seen at 3.3 mM glucose (Table 5). At 3.3 and 16.7 mM glucose, galnon exerted markedly stimulating effects on insulin secretion ($p < 0.001$), but only at 16.7 mM glucose, the addition of 5 μM nimodipine re-duced that effect of galnon from 91.2 ± 6 to 52.5 ± 11.4 μU/islet/h ($p < 0.01$).

Effects of PKA and PKC inhibitors on galnon-induced insulin release

At 3.3 mM glucose, the PKA inhibitor H-89 and the PKC inhibitor calphostin C did not affect insulin release

Table 5
Effects of L-type calcium channel blocker, nimodipine, on galnon-induced insulin secretion of Wistar rat islets

Additions to the medium	3.3 mM glucose	16.7 mM glucose
None	4.5 ± 1.0	30.6 ± 2.9
Galnon (100 μM)	66.8 ± 14.8[a]	91.2 ± 6.0[a]
Nimodipine (5 μM)	6.9 ± 2.3	6.5 ± 2.3[a]
Galnon (100 μM) + nimodipine (5 μM)	73.0 ± 12.3	52.5 ± 11.4[b]

Results of insulin release (μU/islet/h) are means ± SEM of three batch incubation experiments, each representing the mean of triplicate incubations at each condition.

[a] $p < 0.001$ vs. no addition.
[b] $p < 0.01$ vs. galnon alone.

either in the absence or in the presence of galnon (Table 6). At 16.7 mM glucose both H-89 and calphostin C reduced insulin secretion, each by approximately 50%, whereas galnon-induced insulin secretion was decreased by 38% and 51%, respectively.

Galanin receptor binding assay

Previous work showed that galnon binds to galanin receptors and displaces [125I]galanin in membranes from rat ventral hippocampus with a K_D value of 4.8 μM [5], from rat spinal cord membranes with a K_D value of 6.0 μM [6], and from rat hypothalamus membranes with a K_D of 6.2 μM [21]. Present displacement studies from Rinm5F cellular membranes displayed a similar affinity, with a K_D of 8.5 μM (data not shown).

Cell viability test

The MTT cell viability test demonstrated that 60 min incubation with 100 μM galnon did not significantly affect the percentage of viable pancreatic islet cells (97 ± 3% of control incubations) or Rinm5F cells (96 ± 4% of control).

Table 6
Effects of protein kinase A and protein kinase C inhibition on galnon-induced insulin release

Additions to the medium	3.3 mM glucose	16.7 mM glucose
None	7.2 ± 0.5	48.1 ± 4.9
H-89 (10 μM)	10.8 ± 2.6	23.2 ± 2.9[a]
Calphostin C (1.5 μM)	8.1 ± 0.6	27.1 ± 3.6[a]
Galnon (100 μM)	57.0 ± 3.0	101.4 ± 10.6
Galnon (100 μM) + H-89 (10 μM)	65.4 ± 9.2	63.1 ± 8.3[b]
Galnon (100 μM) + calphostin C (1.5 μM)	68.1 ± 6.7	48.7 ± 6.5[c]

Results of insulin release (μU/islet/h) are means ± SEM of nine batch incubations at each condition. H-89, inhibitor of PKA, and Calphostin C, inhibitor of PKC.

[a] $p < 0.01$ vs. no addition.
[b] $p < 0.05$ vs. galnon at 16.7 mM glucose.
[c] $p < 0.01$ vs. galnon at 16.7 mM glucose.

Discussion

We have demonstrated in isolated rat pancreatic islets that galnon, a non-peptide galanin receptor agonist, is a potent stimulator of insulin release both at 3.3 and 16.7 mM glucose. In addition, galnon markedly enhanced glucose-stimulated insulin secretion in perifused islets. The release gradually returned to basal levels when galnon was abolished from the perfusion medium, suggesting that the compound has got a specific, reversible effect on the insulin-secreting B-cells. This view is further supported by our results showing the effects of galnon on cytosolic calcium concentrations. Furthermore, exposure of single rat pancreatic islet cells and Rinm5F cells for 60 min to 100 μM galnon did not show significant evidence of cell damage as judged by cell viability assessment.

The effect of galnon was also studied in islets of diabetic GK rat, a spontaneous model of type 2 diabetes mellitus that is characterized by a markedly impaired insulin response to glucose [11,13]. In the GK rat islets, there are not only abnormalities in islet glucose metabolism and the function of the K_{ATP}-channels and L-type Ca^{2+} channels but important defect(s) reside late in signal transduction, i.e., in the exocytotic machinery [11]. Interestingly, galnon enhanced strongly insulin secretion from GK pancreatic rat islets both at 3.3 and 16.7 mM glucose similar to its effect in Wistar rat islets.

At a first glance it may appear surprising that galnon could stimulate insulin release, since galanin has been shown to inhibit B-cell secretion [22]. However, it has been demonstrated previously that a cell-penetrating, galanin-related peptide, galparan, similar to galnon enhances insulin release markedly at both low and high glucose levels [23]. The effect of galparan seemed to be mediated through the exocytotic machinery and not linked to the galanin receptors of B-cells [23]. In a recent study, galnon had shown a moderate affinity to galanin receptors [5]. To elucidate the role of galanin receptors in the insulin stimulating effect of galnon, we used M35, a chimeric galanin-based peptide, which has been shown to act as a high-affinity, selective antagonist at galanin receptors on insulin-producing cells [24]. In our experiments, 1 μM M35 itself had no effect on basal and glucose stimulated insulin secretion (in agreement with the results of another study) [24] and did not decrease the insulin stimulating effects of galnon. With 1 μM M35, when almost all of galanin receptors are expected to be occupied, galnon still stimulated insulin secretion, suggesting that this effect of galnon was linked to a site unrelated to galanin receptors. This notion was further supported by the observation that galanin suppressed glucose- but not galnon-induced insulin release.

In the insulin release process, the important role of the K_{ATP}-channels has been proven. Hypoglycemic agents, such as tolbutamide, close K_{ATP}-channels and

increase insulin secretion [25]. In contrast, the openers of K_{ATP}-channels, such as diazoxide, decrease insulin secretion [25]. We studied the role of K_{ATP}-channels for the insulin stimulating effect of galnon. The presence of 0.25 mM diazoxide, to keep the K_{ATP}-channels open, did not alter the effect of galnon on insulin secretion in 3.3 mM glucose, suggesting that the effect of galnon was distal to K_{ATP}-channels. This was further supported by the result from experiment in depolarized pancreatic islets. An addition of 100 μM galnon caused stronger effect on insulin release in comparison with the effect by 30 mM K^+ in Wistar rat islets both in low and high glucose concentrations. Interestingly, in the same conditions, GK rat islets had also a similar response in insulin release, suggesting the intact insulin release mechanisms related to the galnon effect in the B-cell of diabetic GK rat.

In this study, we investigated the role of pertussis toxin (PTX)-sensitive G-proteins in insulinotropic effect of galnon. The majority of known hormones, neurotransmitters, and other regulatory molecules that alter cellular processes do so by signal transduction pathways that involve GPT-binding proteins (G proteins). There are several subtypes of G-proteins in which G_i (which inactivates adenylyl cyclase), G_t (or tranducin, which regulates a cGMP-specific phosphodiesterase), G_k (which opens ligand-gated K^+ channels), G_{pla} (which activates phospholipase A_2), G_{plc} (which activates phospholipase C), and G_o (function uncertain) are sensitive to PTX [26]. In the pancreatic islet, G proteins act as important modulators of its function. G_s and G_i positively and negatively, respectively, modulate adenylyl cyclase activity [26]. Pre-treating islets with PTX reversed the inhibitory insulin release effects of epinephrine, galanin, somatostatin, and prostaglandin E_2, indicating that these inhibitors of glucose-induced insulin secretion are at least partially dependent on the activity of PTX-sensitive G-protein [26]. Also G-proteins involved in exocytotic mechanisms, i.e., G_e-proteins, have been proposed to be inhibited by PTX [18]. In our experiments, pre-treating islet with 100 ng/ml PTX did not affect galnon-induced insulin secretion from Wistar rat islets. This suggested that the effect of galnon was not mediated via PTX-sensitive G-proteins. Interestingly, galnon and mastoparan appeared to exert additive stimulatory effects on insulin release, suggesting that these two compounds act on the exocytotic machinery by different mechanisms. It is likely that mastoparan, in contrast to galnon, acts via a PTX-sensitive G-protein [17,18]. In addition, the results with inhibitors of PKA and PKC supported a partial role of these kinases in galnon-stimulated insulin secretion, specifically by modulation of exocytosis [27].

To further study at which site galnon exerted its effect on insulin release, we measured directly the concentration of $[Ca^{2+}]_i$ in a single dispersed B-cell during perifus-

ing with 100 μM galnon. The addition of 100 μM galnon in the continued presence of high glucose concentration caused renewed increase of $[Ca^{2+}]_i$. Because of heterogeneity of responses in $[Ca^{2+}]_i$ to high glucose of single dispersed B-cell, it was difficult to confirm that galnon increases $[Ca^{2+}]_i$. Hence, we repeated these experiments in the presence of 3.3 mM glucose only and saw a clear effect of galnon on increasing $[Ca^{2+}]_i$. We could conclude that galnon-induced Ca^{2+} increase had, at least partly, a role in galnon-induced insulin secretion. However, the effect of galnon on $[Ca^{2+}]_i$ was not stronger than the effect of 16.7 mM glucose, in contrast to the effect of galnon on insulin release which was much more stronger that that of 16.7 mM glucose, suggesting that increase of $[Ca^{2+}]_i$ contributed partly on galnon-induced insulin release. This was further supported because addition of 5 μM nimodipine, a L-type Ca^{2+}-channel blocker [28,29], could not reduce or abolish galnon-induced insulin secretion in 3.3 and 16.7 mM glucose, respectively. Thus, the main effect of galnon seemed to be independent of L-type Ca^{2+}-channels.

In conclusion, galnon is a potent stimulator of insulin release in both normal Wistar and diabetic GK pancreatic rat islets. In this context, it should be pointed out that this effect is not glucose-dependent, and thus confers the compound as such a limited role as a therapeutic tool in type 2 diabetes. The mechanism of this stimulatory action does not seem to involve galanin receptors. Galnon-induced insulin release appears to involve modulation through the PKA and PKC systems, and opening of L-type Ca^{2+}-channels, but the main effect of galnon is likely to be exerted at a step distal to these channels, i.e., at the exocytotic machinery of the B-cells.

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