The possible mechanisms by which phanoside stimulates insulin secretion from rat islets

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Abstract

We recently showed that phanoside, a gypenoside isolated from the plant *Gynostemma pentaphyllum*, stimulates insulin secretion from rat pancreatic islets. To study the mechanisms by which phanoside stimulates insulin secretion. Isolated pancreatic islets of normal Wistar (W) rats and spontaneously diabetic Goto-Kakizaki (GK) rats were batch incubated or perifused. At both 3·3 and 16·7 mM glucose, phanoside stimulated insulin secretion several fold in both W and diabetic GK rat islets. In perifusion of W islets, phanoside (75 and 150 μM) dose dependently increased insulin secretion that returned to basal levels when phanoside was omitted. When W rat islets were incubated at 3·3 mM glucose with 150 μM phanoside and 0·25 mM diazoxide to keep K-ATP channels open, insulin secretion was similar to that in islets incubated in 150 μM phanoside alone. At 16·7 mM glucose, phanoside-stimulated insulin secretion was reduced in the presence of 0·25 mM diazoxide ($P<0·01$). In W islets depolarized by 50 mM KCl and with diazoxide, phanoside stimulated insulin release twofold at 3·3 mM glucose but did not further increase the release at 16·7 mM glucose. When using nimodipine to block L-type Ca^{2+} channels in B-cells, phanoside-induced insulin secretion was unaffected at 3·3 mM glucose but decreased at 16·7 mM glucose ($P<0·01$). Pretreatment of islets with pertussis toxin to inhibit exocytotic G_e-protein did not affect insulin response to 150 μM phanoside. Phanoside stimulated insulin secretion from W and GK rat islets. This effect seems to be exerted distal to K-ATP channels and L-type Ca^{2+} channels, which is on the exocytotic machinery of the B-cells.

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Introduction

Type 2 diabetes is a common disease that develops on the basis of impaired insulin release and/or insulin resistance (Kuzuya *et al.* 2002). Despite the use of several types of oral anti-diabetic drugs, treatment of type 2 diabetes is still a major problem due to therapy failure (DeFronzo 1999). Such failure is evident in a majority of patients after 10 years treatment with sulfonylurea, a widely used class of drugs that stimulate insulin release by closure of B-cell K-ATP channel (DeFronzo1999, Brown *et al.* 2004).

Glucose-stimulated biphasic insulin secretion involves at least two signaling pathways, the K-ATP channel-dependent and K-ATP channel-independent pathways respectively (Chow *et al.* 1995, Straub & Sharp 2002). In the former, enhanced glucose metabolism increases the cellular ATP/ADP ratio, which closes K-ATP channels, depolarizes the cell and activates the voltage-dependent L-type Ca^{2+} channels. The activation of L-type Ca^{2+} channels increases Ca^{2+} entry (Yang & Gillis 2004) and stimulates insulin release (Hellman *et al.* 1994*a*,*b*). The latter involves second messengers such as cyclic AMP (cAMP) and diacylglycerol (DAG) and exerts its stimulatory effect on exocytosis of insulin (Jones *et al.* 1991, Zawalich & Zawalich 2001, Straub & Sharp 2002, Quynh *et al.* 2005).

To find novel drugs for treatment of type 2 diabetes, we have investigated anti-diabetic effects of extracts of several traditional medicinal herbs in Vietnam. We found that the extract of *Gynostemma pentaphyllum* decreased blood glucose levels in mice and rats due to stimulation of insulin release (Norberg *et al.* 2004). The compound responsible for this effect, phanoside, was further purified, and its structure was characterized (Norberg *et al.* 2004). In the present study, we aimed at elucidating the mechanisms of phanoside-induced insulin secretion.

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Materials and Methods

Animals and chemicals

Normal Wistar (W) rats were purchased from a commercial breeder (B&K Universal, Sollentuna, Stockholm Sweden). Diabetic Goto-Kakizaki (GK) rats, originating from W rats, were bred in our department (Ostenson *et al.* 1993). The animals were kept in room temperature (22 °C) with food and water and allowed to feed *ad libitum* before being killed to get pancreas for isolation of islets. A light–darkness cycle (0600 and 1800 h) was strictly enforced. The rats were fed a chow with 18·5% raw protein, 4·0% fat, and 55·7% carbohydrates, with energy content of 1260 kJ/100 g. The study was approved by the animal research ethics committee of the Karolinska Institute.

Collagenase for isolation of islets was purchased from Roche Diagnostic (Stockholm, Sweden), calphostin-C and H89 were from Calbiochem (Stockholm, Sweden), diazoxide, forskolin, carbachol and other chemicals were from Sigma Aldrich (Stockholm, Sweden). Phanoside was purified from *G. pentaphyllum* following the method described previously (Norberg *et al.* 2004).

Isolation of pancreatic islets

The experiments were performed with islets isolated by collagenase digestion of the pancreas of male Wistar and GK rats (280–320 g; Lacy & Kostianovsky 1967). After isolation, the islets were cultured for 24 h in RPMI 1640 medium (Flow lab Ltd), containing 11 mM glucose, 10% heat inactivated fetal calf serum, 100 IU/ml penicillin, and 0·1 mg/ml streptomycin (Ostenson *et al.* 1993).

Batch incubations

The medium used for islet incubations was Krebs–Ringer bicarbonate (KRB) buffer solution containing 118·4 mM NaCl, 4·7 mM KCl, 1·9 mM $CaCl_2$, 1·2 mM $MgSO_4$, 1·2 mM KH_2PO_4 and 25 mM NaHCO3 (equilibrated with 5% CO_2–95% O_2, pH 7·4) and 0·2% BSA, 10 mM HEPES, and 3·3 or 16·7 mM glucose. Insulin release was assessed in batch incubations of islets following preincubation for 30 min at 3·3 mM glucose. Batches of three islets were incubated for 60 min in KRB with 3·3 or 16·7 mM glucose, and phanoside (150 μM, which was found previously to be a stimulating concentration).

To investigate whether phanoside exerts direct effect on insulin exocytosis, islets were incubated in KRB with 50 mM KCl to depolarize the B-cells, 0·25 mM diazoxide to keep the K-ATP channels open (Sato *et al.* 1999) or just 0·25 mM diazoxide with or without phanoside.

To evaluate the effect of protein kinase A (PKA) and protein kinase C (PKC) on phanoside-induced insulin release, normal W rat islets were incubated with phanoside and the PKA-inhibitor, H89 (10 μM; Filipsson & Ahren 1998) or the PKC inhibitor, calphostin-C (1·5 μM; Thams & Capito 2001) for 60 min in KRB containing 3·3 or 16·7 mM glucose with or without phanoside. The inhibition of PKA and PKC by each appropriate inhibitor was also studied in islets incubated in the presence of forskolin or carbachol respectively.

To evaluate the effect of pertussis toxin on phanoside-induced insulin release, normal W rat islets were pretreated for 24 h at 37 °C in RPMI-1640 culture medium containing 11 mM glucose, 10% heat-inactivated fetal calf serum, 100 ng/ml pertussis toxin, 100 IU/ml penicillin, and 0·1 mg/ml streptomycin.

Perifusion of islets

Perifusion of islets was used to investigate how phanoside affects the kinetics of insulin release. Batches of 30 isolated W rat islets were perifused for 20 min (−20 to min 0) with medium containing 3·3 mM glucose. Perifusion medium was collected in fractions every 2 min to establish the basal insulin secretion rate at 3·3 mM glucose. From min 0 to 20, the glucose concentration was increased to 16·7 mM glucose and then decreased to 3·3 mM. Phanoside (75 or 150 μM) together with 16·7 mM was added from min 0 to 20.

Insulin RIA

After batch incubations or perifusions, aliquots of the medium were analyzed for insulin content by RIA (Herbert *et al.* 1965). The sensitivity of the RIA was 3·9 mU/l, the interassay coefficient of variation was $<$3·8% and the intra-assay coefficient of variation was $<$3·1%.

Cell viability assays

Trypan blue assay After incubation in the absence (control group) or presence of phanoside 150 μM, islet cells prepared as described previously (Pipeleers & Pipeleers-Marichal 1981) were exposed to the membrane-impermeant dye, trypan blue (0·1% w/v) for 15 min at 37 °C. The presence of dye was determined by light microscopy and the numbers of unstained and stained cells in the field were counted to obtain an estimate of the percentage of the cells taking up the dye (Persaud *et al.* 1999).

Measurement of lactate dehydrogenase (LDH) release Batches of 100 pancreatic islets were incubated for 60 min with phanoside (150 μM). LDH release from islets was measured by determining LDH activity (cytotoxicity detection kit-LDH, Roche Applied Science). The amount of color formed in the assay is proportional to the number of lysed islet cells. The LDH activity in the total of dead islet cells (high control) was measured after solubilization of islet cells with 5% (v/v) Triton X-100 (Lash *et al.* 2001). To determine the percentage cytotoxicity, the absorbance at 490 nm was measured in duplicate samples with subtraction of values obtained in control incubation (low control with islets but

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without phanoside), using the following equation:

$$\text{Cytotoxicity (\%)} = (\text{experimental value} - \text{low control value}) \times 100/(\text{high control value} - \text{low control value}).$$

Statistical analysis

The results have been calculated as means ± S.E.M. and comparisons of the data have been done by ANOVA test with Bonferroni correction for multiple testing.

Results

Effects of phanoside on insulin secretion of W and diabetic GK rat islets

Glucose (16·7 mM) stimulated insulin release, relative to the release at 3·3 mM, in W rat islets but not in GK rat islets (Fig. 1). In W rat islets, phanoside (150 μM) stimulated insulin secretion from 4·3 ± 0·9 to 32·0 ± 3·9 μU/islet per h at 3·3 mM glucose and from 15·2 ± 4·0 to 57·7 ± 8·7 μU/islet per h at 16·7 mM glucose ($P<0·001$ for both; Fig. 1). In GK rat islets, at 3·3 mM glucose, phanoside (150 μM) stimulated insulin secretion islets from 10·2 ± 3·6 to 29·5 ± 5·1 μU/islet per h ($P<0·001$; Fig. 1). At 16·7 mM glucose, phanoside (150 μM) also augmented insulin secretion from 12·1 ± 4·8 to 37·3 ± 7·5 μU/islet per h ($P<0·001$).

Kinetics of insulin secretion of isolated islets

Glucose (16·7 mM) induced a biphasic insulin secretion from the perifused islets (Fig. 2). When glucose was decreased to 3·3 mM, insulin release gradually returned to basal levels. Addition of 150 or 75 μM phanoside to 16·7 mM glucose markedly enhanced insulin secretion from the perifused islets when compared with that of islets perifused only with 16·7 mM glucose, and the effect of phanoside was dose-dependent. When phanoside was omitted from the perifusate, the insulin secretion decreased to basal levels (Fig. 2).

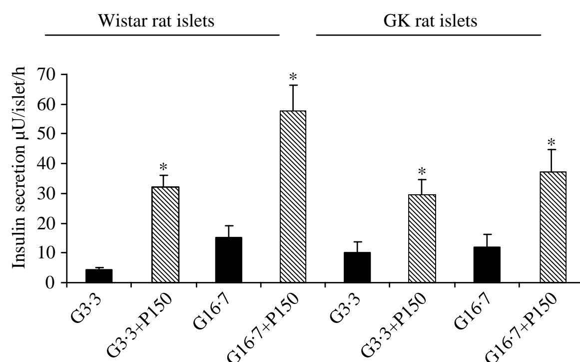

Figure 1 Figure1. Effects of phanoside in Wistar and GK rat islets incubated in KRB at 3·3 mM glucose (G3·3) or 16·7 mM glucose (G16·7) with or without 150 μM phanoside (P150). Results of insulin release (μU/islet per h) are the mean ± S.E.M. ($n=9$). *$P<0·001$ when compared with control group with no addition.

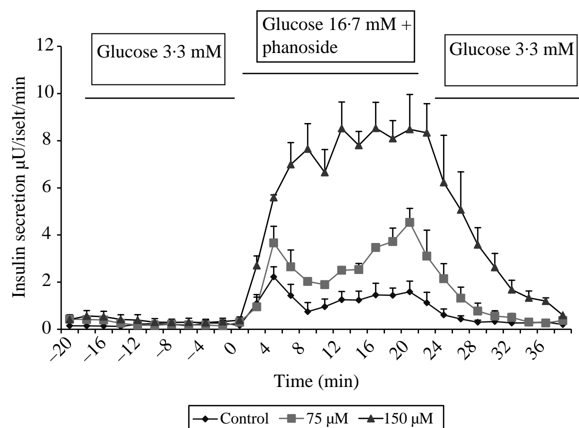

Figure 2 Effect of phanoside on kinetics of insulin secretion of Wistar rat islets. Batches of 30 isolated Wistar rat islets were perifused with medium containing 3·3 mM glucose for 20 min. After this time ($t=-20$ min), perifusion medium was collected in fractions every 2 min to establish the basal insulin secretion rate at 3·3 mM glucose. From min 0 to 20, the glucose concentration was increased to 16·7 mM glucose and then decreased to 3·3 mM. Phanoside (75 or 150 μM) was added from min 0 to 20. Aliquots of the medium were collected and their insulin content determined by RIA. Results represent mean values ± S.E.M. from three to five separate experiments.

Effects of phanoside on insulin secretion in W rat islets with K-ATP channel opened by diazoxide

At 3·3 mM glucose, diazoxide (0·25 mM) did not affect basal insulin release or insulin response to phanoside (Table 1). At 16·7 mM glucose, diazoxide abolished the glucose-induced insulin release, and decreased insulin response to phanoside by almost 50% ($P<0·01$).

Table 1 Effect of phanoside with or without diazoxide and potassium chloride on glucose-stimulated insulin secretion from isolated Wistar rat islets. Results of insulin release (μU/islet per h) are the mean ± S.E.M. of six to seven batch incubations at each condition

	Glucose	
	3·3 mM	16·7 mM
Addition to the medium		
None	3·5 ± 0·4	14·5 ± 1·0
Phanoside (150 μM)	27·2 ± 3·0*	61·2 ± 6·6*
Diazoxide (0·25 mM)	3·1 ± 0·7	4·7 ± 0·4*
Diazoxide (0·25 mM) + KCl (50 mM)	24·1 ± 3·2*	101·1 ± 9·9*,†
Phanoside 150 (μM) + diazoxide (0·25 mM)	29·1 ± 2·5*	33·7 ± 4·6*,‡
Phanoside 150 (μM) + diazoxide (0·25 mM) + KCl (50 mM)	55·3 ± 5·1*,†	94·3 ± 8·0*,†

*$P<0·001$ when compared with control group with no addition. †$P<0·001$, ‡$P<0·01$, when compared with group with only phanoside.

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Effects of phanoside on insulin secretion in W rat islets depolarized by KCl

At 3·3 and 16·7 mM glucose, depolarization of W rat islet B-cells by exposure to 50 mM KCl+0·25 mM diazoxide increased insulin release 6·9- and 6·1-fold respectively ($P<0·001$ for both; Table 1). When islets were incubated at 3·3 mM glucose with 50 mM KCl, 0·25 mM diazoxide and 150 μM phanoside, insulin secretion was 2·0-fold higher than when islets were incubated in 150 μM phanoside alone ($P<0·001$) and 2·3-fold higher than when islets were incubated in 50 mM KCl+0·25 mM diazoxide ($P<0·001$; Table 1). At 16·7 mM glucose, however, the insulin response to 50 mM KCl, 0·25 mM diazoxide and 150 μM phanoside was higher than to 150 μM phanoside ($P<0·001$) but not significantly different than to 50 mM KCl+0·25 mM diazoxide. Also at 27 mM glucose, the insulin response at depolarizing conditions was within a similar range (112·5±14·1 μU/islet per h).

Effect of nimodipine on phanoside-induced insulin secretion from isolated W rat islets

When using nimodipine to block L-type Ca^{2+} channels in membrane of B-cell, the phanoside-induced insulin secretion of islets was not affected at 3·3 mM glucose (Table 2). However, at 16·7 mM glucose, insulin secretion was decreased by nimodipine from 16·0±0·8 to 3·8±1·0 μU/islet per h ($P<0·001$). In addition, nimodipine decreased phanoside-induced insulin release from 58·5±8·0 to 34·2±3·7 μU/islet per h ($P<0·01$; Table 2).

Effects of PKA and PKC inhibition on phanoside-induced insulin release

When W rat islets were incubated, at 3·3 and 16·7 mM glucose, with phanoside plus the PKA inhibitor, H89, or the PKC inhibitor, calphostin-C, there were no differences in insulin secretion compared with the release from islets incubated with phanoside alone (Table 3). However, H89 and calphostin-C, although not suppressing the insulin response to 16·7 mM glucose, inhibited insulin secretion elicited by forskolin and carbachol respectively (Table 3).

Table 2 Effect of nimodipine on phanoside-induced insulin secretion from isolated Wistar rat islets. Results of insulin release (μU/islet per h) are the mean±S.E.M. of six to seven batch incubations at each condition.

	Glucose	
	3·3 mM	16·7 mM
Addition to the medium		
None	3·5±0·4	16·0±0·8
Phanoside (150 μM)	34·5±2·9*	58·5±8·0*
Nimodipine (5 mM)	2·4±0·1	3·8±1·0*
Phanoside 150 (μM) ++nimodipine (5 mM)	28·3±2·3*	34·2±3·7*,†

*$P<0·001$ when compared with control group with no addition. †$P<0·01$ when compared with group with only phanoside.

Table 3 Effects of a PKA inhibitor, H89, and a PKC inhibitor, calphostin C, on insulin secretion induced by phanoside as well as forskolin and carbachol from isolated Wistar rat islets. Results of insulin release (μU/islet per h) are the mean±S.E.M. of seven to eight batch incubations at each condition

	Glucose	
	3·3 Mm	16·7 mM
Addition to the medium		
None	5·6±0·8	15·7±2·5
H89 (10 μM)	4·8±0·6	19·5±2·4
Calphostin C (1·5 μM)	5·2±0·5	17·0±1·9
Phanoside (150 μM)	22·1±2·9	40·8±5·2
Phanoside (150 μM)+H89 (10 μM)	17·4±3·8	35·5±5·8
Phanoside 150 (μM)+calphostin C (1·5 μM)	17·0±2·6	37·8±5·4
Forskolin (5 μM)	Not tested	91·1±10·8
Forskolin (5 μM)+H89 (10 μM)	Not tested	24·1±2·7*
Carbachol (50 μM)	Not tested	40·5±3·8
Carbachol (50 μM)+calphostin C (1·5 μM)	Not tested	25·8±3·1†

*$P<0·001$ when compared with forskolin alone, †$P<0·01$ when compared with carbachol alone, at 16·7 mM glucose.

Effect of pertussis toxin on insulin releasing effect of phanoside in W rats islets

Without pretreatment of islets with pertussis toxin, 150 μM phanoside stimulated release of insulin in W rat islets from 3·6±0·3 μU/islet per h in the control group to 31·1±3·9 μU/islet per h ($P<0·001$) at 3·3 mM glucose and from 16·2±0·8 to 71·4±6·5 μU/islet per h ($P<0·001$) at 16·7 mM glucose (Fig. 3). When islets were pretreated with pertussis toxin, insulin response to 3·3 mM glucose was similar but the response to 16·7 mM glucose was greatly

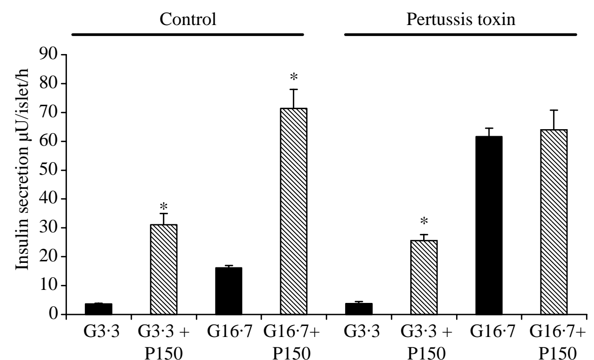

Figure 3 Effects of phanoside on insulin secretion of Wistar rat islets with or without exposure overnight to pertussis toxin (G3·3, glucose 3·3 mM; G16·7, glucose 16·7 mM; P150, phanoside 150 μM). Results of insulin release (μU/islet per h) are the mean±S.E.M. ($n=5$). *$P<0·001$ when compared with group without phanoside.

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increased to 61·6±3·0 μU/islet/h/islet/h. The insulin responses to 150 μM phanoside were 25·5±2·1 μU/islet per h at 3·3 mM glucose ($P<0{\cdot}001$) and 63·9±6·9 μU/islet per h at 16·7 mM glucose (Fig. 3).

Cells viability assay

Exposure of islet cells to phanoside for 60 min did not significantly affect the number of cells to which trypan blue dye gained access, with 9·6±1·7% of the cells taking up the dye at 150 μM phanoside and 6·2±1·0% in the control group ($P=0{\cdot}0878$, $n=11$). According to the measurements of LDH release from islets, the percentage of dead islet cells after 60-min incubation with 150 μM phanoside was 8·7±1·3%.

Discussion

We have recently demonstrated that phanoside, isolated from the plant *G. pentaphyllum*, reduces blood glucose in normal rats and the effect is accounted for by stimulation of insulin secretion (Norberg *et al.* 2004). Our present results confirm and extend these observations by showing that phanoside stimulates insulin secretion in islets not only of normal W rats but also of diabetic GK rats, and this effect is exerted mainly on the exocytotic machinery.

Phanoside is a gypenoside, related to saponins that may be cytotoxic (Persaud *et al.* 1999). Previously, the herbal extract of *Gymnema sylvestre*, containing several saponins or surfactants, was shown to induce insulin release from rat islets and several pancreatic B-cell lines by increased membrane permeability (Persaud *et al.*1999). The number of cells to which trypan blue dye gained access was 98% of MIN6 cells, 95% of RINm5F cells, and 88% of HIT-T15 at 0·25 mg/ml GS4 – a compound extracted from *G. sylvestre* that stimulated insulin secretion. Thus, a similar mechanism could explain phanoside-induced insulin release. However, several observations speak against such an explanation and rather favor a specific effect of phanoside on the B-cell secretion. First of all, phanoside at concentrations used in islet incubations only slightly increased the release of LDH from islets exposed to the compound for 60 min (<10%) and did not increase uptake of trypan blue. Secondly, in the perifusion experiments, insulin secretion returned to basal levels when phanoside was omitted from the perifusate, indicating that exposure to the compound did not cause leakage of insulin from the islets. Finally, if there was a cytotoxic effect by phanoside inducing insulin leakage, it is not likely that the insulin secretion of islets incubated with phanoside could have been blocked by nimodipine, or diazoxide at high glucose concentration.

When exploring the mechanism of phanoside-induced insulin release, we first considered the K-ATP channel-dependent mechanism for glucose-stimulated insulin secretion. A rise in circulating glucose concentration increases intracellular ATP and decreases intracellular ADP, thereby closing ATP-sensitive K^+ (K-ATP) channels in the B-cells. This results in membrane depolarization, opening of voltage-dependent Ca^{2+} channels and a rise in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$), which triggers insulin secretion. (Straub & Sharp 2002, MacDonald & Wheeler 2003). When the K-ATP channels are kept open by diazoxide, glucose-induced insulin secretion is decreased (Trube *et al.* 1986). Phanoside stimulated insulin secretion at both 3·3 and 16·7 mM glucose, but in the presence of diazoxide the insulin response to phanoside was decreased at 16·7 mM, but not at all at 3·3 mM glucose. Thus, the effect of phanoside did not seem to involve the closure of B-cell K-ATP channels. In the presence of a high concentration of K^+ and diazoxide, the B-cells are depolarized, leading to increased cytosolic Ca^{2+} concentration and insulin release (Quynh *et al.* 2005). At 3·3 mM glucose, phanoside stimulated insulin secretion from depolarized islets suggesting that the effect of phanoside resides in the distal part of the B-cell stimulus-secretion coupling for glucose, i.e in the exocytotic machinery. However, at 16·7 mM glucose, phanoside did not further enhance insulin secretion from depolarized islets, suggesting that islets have a near-maximal exocytosis of insulin under the conditions.

L-type Ca^{2+} channels play an important role in insulin secretion. By using nimodipine, an L-type Ca^{2+} channel blocker, the Ca^{2+} channels will be closed and thus the insulin secretion due to influx of Ca^{2+} from outside the cell is blocked (Keahey *et al.* 1989, Hellman *et al.* 1994*b*, Chow *et al.* 1995, Straub & Sharp 2002). In our experiments, phanoside-induced insulin secretion in the presence of nimodipine at 3·3 mM glucose was not blocked, indicating that the effect of phanoside does not involve L-type Ca^{2+} channels. At 16·7 mM glucose, the insulin secretion of islets incubated with nimodipine plus phanoside was lower than that of the islets incubated with phanoside alone, but still higher than that of the islets incubated with 16·7 mM glucose. Thus, it is likely that nimodipine blocks glucose-induced insulin secretion but does not affect the phanoside effect.

An increase in intracellular Ca^{2+} in the B-cell in response to insulin secretagogs, including glucose, directly triggers exocytosis of the insulin granules (Hellman *et al.* 1994*a*). Second messengers, such as cAMP and DAG, increase insulin release through protein phosphorylation events mediated by PKA (Thams *et al.* 2005) and PKC respectively (Jones *et al.* 1991). Using the PKA inhibitor, H89 (Thams *et al.* 2005) and the PKC inhibitor, calphostin C (Thams & Capito 2001), it was not possible to block the insulin-stimulating effect of phanoside. This indicates that phanoside does not exert its effect on B-cells involving the PKA or PKC systems.

GTP-binding proteins (G-proteins) play functional roles in the process of signal transduction for hormone release (Robertson *et al.* 1991). Some G-proteins are inhibited by pertussis toxin such as G_i (the protein that mediates inhibition of adenylcyclase) and G_e (which is directly coupled with exocytosis; Sontag *et al.* 1991, Komatsu *et al.* 1993). In the pancreatic B-cell, G_e-proteins have been functionally linked

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to insulin exocytosis (Komatsu *et al.* 1993). In our study, pre-treatment of islets with pertussis toxin increased glucose-induced insulin secretion. This effect can be explained by the fact that pertussis toxin treatment of islets reverses the inhibition of insulin secretion by e.g. epinephrine and somatostatin via G_i-protein. Phanoside-induced insulin secretion was not suppressed by pertussis toxin; thus, the mechanism by which phanoside modulates insulin secretion seems not to involve exocytotic G_e-proteins. An alternative explanation would be that phanoside, similar to pertussis toxin, suppresses G_i-proteins and thereby induces enhanced secretion of insulin.

In conclusion, phanoside stimulated insulin secretion from W and GK rat islets. This effect seems to be exerted distal to K-ATP channels and L-type Ca^{2+} channels, which is on the exocytotic machinery of the B-cells. Thereby, the mechanism behind phanoside's effect on the B-cells differs from that of sulfonylurea that acts by closing the K-ATP channels (Sturgess *et al.* 1985). However, similar to sulfonylureas, the effect of phanoside is not glucose-dependent.

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