**Gymnema sylvestre** stimulates insulin release *in vitro* by increased membrane permeability

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**Abstract**

To determine whether extracts of *Gymnema sylvestre* may have therapeutic potential for the treatment of non-insulin-dependent diabetes mellitus (NIDDM), we examined the effects of an alcoholic extract of *G. sylvestre* (GS4) on insulin secretion from rat islets of Langerhans and several pancreatic β-cell lines. GS4 stimulated insulin release from HIT-T15, MIN6 and RINm5F β-cells and from islets in the absence of any other stimulus, and GS4-stimulated insulin secretion was inhibited in the presence of 1 mM EGTA. Blockade of voltage-operated Ca²⁺ channels with 10 μM isradipine did not significantly affect GS4-induced secretion, and insulin release in response to GS4 was independent of incubation temperature. Examination of islet and β-cell integrity after exposure to GS4, by trypan blue exclusion, indicated that concentrations of GS4 that stimulated insulin secretion also caused increased uptake of dye. Two gymnemic acid-enriched fractions of GS4, obtained by size exclusion and silica gel chromatography, also caused increases in insulin secretion concomitant with increased trypan blue uptake. These results confirm the stimulatory effects of *G. sylvestre* on insulin release, but indicate that GS4 acts by increasing cell permeability, rather than by stimulating exocytosis by regulated pathways. Thus the suitability of GS4 as a potential novel treatment for NIDDM can not be assessed by direct measurements of β-cell function *in vitro*.


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**Introduction**

Diabetes mellitus is an endocrine disorder in which glucose metabolism is impaired because of total loss of insulin after destruction of pancreatic β-cells (insulin-dependent diabetes mellitus; IDDM), inadequate release of insulin from the pancreatic β-cells, or insensitivity of target tissues to insulin (non-insulin-dependent diabetes mellitus; NIDDM). NIDDM accounts for up to 90% of the UK diabetic population, and there is an increasing drive to develop novel methods for its treatment, to improve either the insulin output of β-cells or the sensitivity of peripheral tissues to circulating insulin. Of the currently available therapies for NIDDM, only the sulphonylureas are used to stimulate β-cells to secrete more insulin. The others, such as biguanides, α-glucosidase inhibitors and thiazolidinediones, reduce hyperglycaemia independently of increases in circulating insulin.

Sulphonylureas act at a proximal stage in the β-cell stimulus–secretion coupling cascade: they close plasma membrane ATP-sensitive K⁺ channels and the consequent decrease in K⁺ efflux depolarises the cells, leading to Ca²⁺ influx via voltage-operated Ca²⁺ channels (Henquin 1980, Sturgess et al. 1985). Increases in intracellular Ca²⁺ are sufficient to stimulate insulin release from β-cell secretory granules (Jones et al. 1985). Novel drugs that act at downstream sites, perhaps directly on the exocytotic release of insulin, would be useful adjuncts to sulphonylureas in the treatment of NIDDM.

The therapeutic potential of *Gymnema sylvestre* R.Br., a woody climber of the Asclepiadaceae family, has been known for many years and it has a key place in Ayurvedic medicine. There are reports that *G. sylvestre* leaf extracts reduce hyperglycaemia in diabetic rabbits (Shanmugasundaram et al. 1983), rats (Srivastava et al. 1985) and humans (Khare et al. 1983, Baskaran et al. 1990), and these glucose-decreasing effects may be mediated by increases in insulin secretion (Shanmugasundaram & Panneerselvam 1981). There has been little systematic characterisation of the identities of the insulinotropic agents within the leaf extracts, but aqueous ethanolic extractions of the leaves provide two potentially active fractions, one containing conduritol A, an acid-soluble polyol-polyhydroxy cyclic compound (Miyatake et al. 1993), and the other containing a mixture of acid-insoluble triterpenoid saponins (gymnemic acids), designated GS3 and GS4 (Shanmugasundaram et al. 1990).
Conduritol A has been reported to have small stimulatory effects on basal insulin secretion through an undefined mechanism (Billington et al. 1994). GS3 and GS4, which would be devoid of conduritol, have been reported to reduce hyperglycaemia in diabetic rats, increase insulin release in vivo and in vitro, and increase β-cell number after streptozotocin-induced diabetes (Shanmugasundaram et al. 1990). GS3 is a fairly crude fraction prepared by acid precipitation from a hydroalcoholic extract of Gymnema sylvestre leaves. Most studies have been performed using GS4, which is purified from GS3 by reprecipitation with acid of GS3 solubilised in alkali (Shanmugasundaram et al. 1990). GS4 has been used clinically to treat NIDDM, and it was shown to increase serum insulin concentrations, normalise blood glucose concentrations and reduce the requirement for sulphonylurea (Baskaran et al. 1990). The potential use of GS4 in the treatment of diabetes is intriguing, and in the current study we have examined whether GS4, prepared according to a previously described method (Shanmugasundaram et al. 1990), exerts insulinotropic effects on β-cell lines and on isolated islets of Langerhans. We have also investigated its mode of action in vitro, to assess its potential for the treatment of NIDDM.

Materials and Methods

Dried leaves of Gymnema sylvestre were a gift from Cipla Ltd (Mumbai, India). They were authenticated by the Herbarium at the Royal Botanic Gardens, Kew. A voucher specimen (reference Gy11 12) is deposited in the Pharmacognosy Museum, Department of Pharmacy, King’s College London. Isradipine was purchased from Research Biochemicals International (Herts, UK). Tissue culture media, antibiotics and foetal calf serum were obtained from Life Technologies (Paisley, UK). All other reagents were of analytical grade from BDH (Poole, Dorset, UK) or Sigma Chemical Co. (Poole, Dorset, UK). Rats were supplied by King’s College London Animal Unit. HIT-T15 β-cells were purchased from the American Type Culture Collection (Rockville, MD, USA). MIN6 β-cells were kindly provided by Professor J-I Miyazaki (University of Tokyo, Japan) and RINm5F β-cells were from Professor A J Bone (University of Brighton, UK).

Tissue preparation

Islets of Langerhans were isolated from the pancreata of male Sprague–Dawley rats (200–250 g) by collagenase digestion as previously described (Jones et al. 1993). Approximately 400–500 islets were obtained from each pancreas and islets were used immediately after isolation for all experiments. Pancreatic β-cell lines were maintained in culture in DMEM (MIN6) or RPMI (HIT-T15, RINm5F) supplemented with 5–15% foetal calf serum, 100 μg/ml streptomycin and 100 U/ml penicillin, in a humidified atmosphere of 5% CO2.

Preparation of Gymnema fractions

GS4 was prepared as follows: dried Gymnema leaves (1 kg) were ground to a coarse powder in a coffee grinder. The powder was placed in a large beaker and aqueous ethanol (50%; 6 l) was added to cover the grounds. Steam, generated separately, was bubbled through the mixture for 3–4 h. After cooling, the material was filtered and ethanol removed from the filtrate by heating over a boiling water bath to leave a thick brown viscous fluid. Sulphuric acid (98%) was then added until the mixture reached pH 3. The precipitate that formed after the mixture was left at 4 °C overnight, corresponding to the GS3 fraction, was collected by filtration and then redissolved in aqueous potassium hydroxide (0·1 M, 400 ml). GS4 was precipitated from this solution by the addition of sulphuric acid to pH 3 and overnight cooling as before. The precipitate was collected by filtration and freeze-dried. Chlorophyll was removed from the methanol-soluble components of GS4 by size exclusion chromatography on Sephadex LH–20 gel using methanol as eluent, yielding a major fraction, termed F2. A major component of F2, termed F43, was isolated in semi-pure form by silica gel column chromatography and preparative thin layer chromatography (TLC) using chloroform–methanol–water mixtures. TLC analysis (data not shown) indicated that GS4 and F2 contained a complex mixture of compounds with chromatographic characteristics typical of triterpenoid saponins. The major component of GS4 and F2 was identified as gymnemic acid VIII by comparison of spectral data with values given in the literature (Yoshikawa et al. 1992). This compound was not available in sufficient quantities for testing in the present study. However, F43 contains a mixture of gymnemic acid VIII and at least one other compound, most likely to be a closely related gymnemic acid. GS4, F2 and F43 were dissolved directly in the aqueous incubation medium (Gey & Gey 1936) used for the insulin secretion and trypan blue uptake experiments.

Insulin secretion

Groups of three islets were incubated in 600 μl physiological salt solution (Gey & Gey 1936), in the absence or presence of GS4, for 1 h at 37 °C, after which time a sample of the supernatant was removed for the measurement of insulin release. Groups of 30 000 MIN6, 100 000 HIT-T15 and 200 000 RINm5F β-cells were seeded into 96-well plates, left to adhere overnight, then pre-incubated in a glucose-free medium for 2 h before a 1-h incubation in glucose-free medium in the absence or presence of GS4. The insulin content of the supernatants
was determined by radioimmunoassay (Jones et al. 1988). All experiments were performed with six to nine replicates per treatment group.

**Membrane integrity**

After incubation in the absence or presence of GS4, islets and β-cells were exposed to the membrane-impermeant dye, trypan blue (0·1% w/v) for 15 min at 4 °C or 37 °C. The presence of dye within cells was determined by light microscopy and the numbers of stained and unstained cells in a field were counted to obtain an estimate of the percentage of cells taking up the dye. MIN6 cells that had been incubated in the presence of the semi-purified GS4 extracts, F2 and F43, were also incubated with trypan blue as described above.

**Statistical analysis**

Data were analysed by one-way analysis of variance or Student’s t-tests, as appropriate. Differences between experimental and control samples were considered significant at P<0·05.

**Results**

**Isolation of GS4, F2 and F43**

GS4, a smooth greenish-brown powder, was obtained in 2·5% w/w yield from dried G. sylvestre leaves. Chromatography of 10 g GS4 yielded 5·9 g F2, and 88 mg F43 were obtained from 5·4 g F2. F2 and F43 were both obtained as light-brown powders.

**Effects of GS4 on insulin secretion from β-cells and islets**

Exposure of β-cells to GS4 resulted in a dose-related increase in insulin release (Table 1). In the case of MIN6 β-cells, insulin release was stimulated at concentrations as low as 31 µg/ml GS4 (252±15% basal, P<0·001). GS4 (0·2 mg/ml) caused significant (P<0·001) increases in the release of insulin from isolated rat islets at both sub-stimulatory (2 mM glucose: 0·18±0·02 ng/islet per h; +0·2 mg/ml GS4: 0·86±0·18 ng/islet per h, n=6) and stimulatory (10 mM glucose; 59±5% increase; 20 mM glucose: 259±43% increase, n=6) concentrations of glucose. Higher concentrations of GS4 caused progressively increased insulin release from islets such that in the absence of any other stimuli (2 mM glucose), the release rate at 2 mg/ml GS4 was 16±4±1·3 ng/islet per h (n=6, P<0·001 compared with 2 mM glucose).

**Mode of action of GS4**

As GS4 exerted stimulatory actions on all the β-cell lines tested and on whole islets, the HIT-T15 cell line was used as a representative β-cell population in which to examine the mechanisms through which GS4 increased insulin secretion. It was found that, when extracellular Ca2+ was chelated in the presence of 1 mM EGTA, the dose-dependent effects of GS4 were shifted to the right such that 0·25 mg/ml only had a small stimulatory effect, but 0·5 mg/ml still caused a large increase in insulin release (Fig. 1). However, EGTA significantly reduced insulin release at 0·5 mg/ml GS4 and at 0·25 mg/ml (P<0·01). Blockade of Ca2+ channels with the dihydropyridine blocker, isradipine, did not significantly inhibit the

Table 1 GS4-stimulated insulin release from β-cell lines. Cells were incubated in a physiological salt solution (zero glucose) either in the absence of GS4 or supplemented with 0·125–0·5 mg/ml GS4 for 1 h at 37 °C, and insulin released into the supernatant was measured by radioimmunoassay. Data are means±S.E.M., n=5–9.

<table>
<thead>
<tr>
<th>GS4 (mg/ml)</th>
<th>MIN6</th>
<th>RINm5F</th>
<th>HIT-T15</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22·3±1·35</td>
<td>0·24±0·02</td>
<td>2·5±0·58</td>
</tr>
<tr>
<td>0·125</td>
<td>63·9±0·92***</td>
<td>0·79±0·05*</td>
<td>5·11±0·86*</td>
</tr>
<tr>
<td>0·25</td>
<td>65·6±0·55***</td>
<td>4·07±0·21***</td>
<td>20·71±1·43***</td>
</tr>
<tr>
<td>0·5</td>
<td>8·06±0·48***</td>
<td>32·33±2·75***</td>
<td></td>
</tr>
</tbody>
</table>

*P<0·05, ***P<0·001, compared with secretion in the absence of GS4.

Figure 1 Effect of Ca2+ chelation on GS4-stimulated insulin release. HIT-T15 cells were incubated for 1 h at 37 °C in the absence (open bars) or presence (hatched bars) of 1 mM EGTA. Insulin released into the supernatant was measured by radioimmunoassay. Data are means±S.E.M., n=8. *P<0·05, ***P<0·001 compared with appropriate control in the absence of GS4; **P<0·01 compared with secretion in the absence of EGTA.
stimulatory effects of 0.5 mg/ml GS4 on insulin release from HIT cells (control: 29.4 ± 2.7 ng/10^6 cells per h; +10 µM isradipine: 24.0 ± 1.9 ng/10^6 cells per h; n=8, P>0.1). Incubation of HIT-T15 β-cells at a range of temperatures indicated that GS4 (0.125 mg/ml) stimulated insulin release even at temperatures as low as 4°C (37°C: 6.1 ± 0.75 ng/10^6 cells per h; 30°C: 5.1 ± 0.33 ng/10^6 cells per h; 24°C: 4.3 ± 0.67 ng/10^6 cells per h; 4°C: 5.7 ± 1.33 ng/10^6 cells per h; n=5–6).

Effects of GS4 on membrane integrity

Exposure of β-cells to GS4 resulted in a dose-related increase in the number of cells to which trypan blue dye gained access, with 98% of MIN6 cells, 95% of RINm5F cells and 88% of HIT-T15 cells taking up the dye at 0.25 mg/ml GS4. Islets incubated in the absence of GS4 excluded trypan blue, but those which had been incubated in the presence of 0.25 mg/ml GS4 showed substantial dye uptake, whether they were exposed to GS4 at 37°C or at 4°C (Fig. 2).

Effects of GS4 fractionation products on insulin release and membrane integrity

F2 and F43, fractions obtained by further purification of GS4, increased the rate of insulin release from MIN6 cells in the absence of any other stimulus (Table 2). However, F2 and F43 also caused a significant increase in the extent of trypan blue uptake by MIN6 cells (Table 2). In contrast, exposure of MIN6 cells to a maximal stimulatory combination of the nutrient secretagogue, 4α ketoisocaproate, and the protein kinase C activator, 4β phorbol myristate acetate, caused a significant increase in insulin secretion that was not accompanied by a loss of membrane integrity, as assessed by trypan blue uptake (Table 2).

Discussion

The results of the current study confirm previous observations that alcoholic extracts of the leaves of Gymnema sylvestre are capable of direct actions on pancreatic β-cells to increase the release of insulin (Shanmugasundaram et al. 1990). We found that GS4 caused a dose-related increase in insulin release from a variety of β-cell lines and rat islets in the absence of any other stimulus, and, although there were some minor differences in sensitivity to the extract, in all cases it caused a profound secretory response. In the case of islets, with which a concentration as high as 2 mg/ml was used, it was found that insulin release was in excess of 16 ng/islet per h. This high output, equivalent to ~50% of the islet insulin content, suggests that the effects of GS4 may not be physiological because, under these conditions, the insulin release would not be compensated for by sufficient insulin biosynthesis. Confirmation that the stimulatory effects of GS4 differed from those of other insulin secretagogues was provided by the observation that GS4 was able to stimulate insulin release at temperatures as low as 4°C, whereas regulated physiological insulin secretion only occurs at temperatures in excess of 30°C (Hedeskov 1980).

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Insulin release (ng/10^6 cells per h)</th>
<th>Cells taking up trypan blue (% total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 glucose</td>
<td>22.7 ± 3.3</td>
<td>5–10</td>
</tr>
<tr>
<td>0.5 mg/ml F2</td>
<td>85.4 ± 8.0***</td>
<td>95–100</td>
</tr>
<tr>
<td>0.5 mg/ml F43</td>
<td>41.4 ± 2.7***</td>
<td>50–60</td>
</tr>
<tr>
<td>0.5 mg/ml GS4</td>
<td>48.0 ± 4.7***</td>
<td>90–100</td>
</tr>
<tr>
<td>10 mM KIC</td>
<td>89.4 ± 7.3***</td>
<td>5–10</td>
</tr>
<tr>
<td>+500 nM PMA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

KIC, 4α ketoisocaproate; PMA, phorbol myristate acetate. ***P<0.001, compared with secretion at 0 glucose.
Evidence that GS4 was acting at a physical, rather than a physiological, level was provided by estimates of uptake of the membrane impermeant dye, trypan blue. Cells or islets that had been incubated in the absence of GS4 for 1 h showed low levels of trypan blue uptake, indicative of an intact plasma membrane capable of excluding dye entry. However, increasing concentrations of GS4 caused progressively more damage to the β-cells, such that, for all β-cell types, trypan blue gained access to virtually all cells when used in a concentration of 0.25 mg/ml. Similar results were obtained with whole islets, which consist of clusters of around 3–5000 cells, and dye uptake was observed at both 4 °C and at 37 °C. At 4 °C, the dye gained access to an outer mantle of cells, and at 37 °C more islet cells became permeable to trypan blue. The loss of membrane integrity after exposure to GS4 may have been mediated by gymnemic acids, a complex mixture of saponin glycosides, known to be present in the extract (Suttisn et al. 1995). Glycosides such as saponin and digitonin have long been used experimentally to permeabilise cellular membranes, including those of cells within islets of Langerhans (Biden et al. 1984, Colca et al. 1985), and it is known that their effects are independent of temperature (Schulz 1990) and that they cause loss of large cytosolic proteins when used in high concentrations (Ahnert-Hilger & Gratliz 1988). The likelihood that membrane damage resulted from the presence of glycosides was borne out experimentally using GS4 subfractions enriched in gymnemic acid saponins (F2 and F43), which also caused increased release of insulin from MIN6 cells concomitant with increased uptake of trypan blue dye.

Thus the ability of GS4 to stimulate insulin release at 4 °C and its effects on β-cell plasma membrane integrity are indicative of a mode of action in which GS4 causes insulin to leak from effectively permeabilised β-cells. There also appears to be a regulated component to the stimulatory effects of GS4, as the increase in insulin release was sensitive to chelation of extracellular Ca2⁺ by EGTA. However, the increase in insulin release still occurred after isradipine-induced channel blockade, indicating that it did not result from Ca2⁺ influx through voltage-operated Ca2⁺ channels. Moreover, in the presence of sufficiently high concentrations of GS4, there was still a substantial release of insulin, despite the presence of EGTA, suggesting that GS4 can affect the secretory apparatus independently of changes in Ca2⁺.

In summary, the current data suggest that GS4 increases insulin release in vitro by two mechanisms: 1) the major mode of action is through permeabilisation of the β-cell plasma membranes, most likely resulting from the high saponin glycoside content of the extract, leading to unregulated loss of insulin from the cells; 2) there is also a Ca2⁺-sensitive component, and at least part of this release of insulin may be dependent on channel-independent Ca2⁺ influx into the β-cells, perhaps through the pores formed by plasma membrane disruption. Thus, although extracts of G. sylvestre have been shown to reduce hyperglycaemia in vivo, the effects of GS4 on β-cells described herein suggest that its suitability as a potential novel treatment for NIDDM can not be assessed by direct measurements of islet and β-cell function in vitro. However, it is worth bearing in mind that the membrane-damaging effects of GS4 are unlikely to be observed in vivo, as sugars in the saponins will be hydrolysed off within the gastrointestinal tract, so islets are more likely to be exposed to the aglycone moiety. There is, as yet, no information on the effects of aglycones on insulin secretion in vitro or in vivo, but these components of G. sylvestre merit further investigation, particularly in light of the enhanced insulin concentrations observed in vivo after GS4 administration (Shanmugasundaram et al. 1990).

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