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Characterisation of the Insulinotropic Activity of an Aqueous Extract of *Gymnema Sylvestre* in Mouse β-Cells and Human Islets of Langerhans

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Key Words
Islets of Langerhans • Insulin secretion • Plant-derived secretagogues

Abstract
Leaves of the *Gymnema sylvestre* (GS) plant have been used to treat diabetes mellitus for millennia, but the previously documented insulin secretagogue effects of GS extracts *in vitro* may be non-physiological through damage to the β-cells. We have now examined the effects of a novel GS extract (termed OSA) on insulin secretion from the MIN6 β-cell line and isolated human islets of Langerhans. Insulin secretion from MIN6 cells was stimulated by OSA in a concentration-dependent manner, with low concentrations (0.06-0.25mg/ml) having no deleterious effects on MIN6 cell viability, while higher concentrations (≥0.5mg/ml) caused increased Trypan blue uptake. OSA increased β-cell Ca^{2+} levels, an effect that was mediated by Ca^{2+} influx through voltage-operated calcium channels. OSA also reversibly stimulated insulin secretion from isolated human islets and its insulin secretagogue effects in MIN6 cells and human islets were partially dependent on the presence of extracellular Ca^{2+}. These data indicate that low concentrations of the GS isolate OSA stimulate insulin secretion *in vitro*, at least in part as a consequence of Ca^{2+} influx, without compromising β-cell viability. Identification of the component of the OSA extract that stimulates regulated insulin exocytosis, and further investigation of its mode(s) of action, may provide promising lead targets for Type 2 diabetes therapy.

Introduction

The majority of individuals with diabetes have Type 2 diabetes, which occurs under conditions of insulin resistance coupled with pancreatic β-cell failure. While public health programmes directed at reducing both physical inactivity and the rising epidemic of obesity are obviously critically important in diabetes management, it is also clear that development of novel diabetes-targeted therapies is required to ensure effective treatment. Pharmaceutical companies have risen to the challenge of providing additional agents to complement the established therapies of sulphonylureas and biguanides. Thus, in the last decade drugs to minimise insulin resistance such as PPARγ agonists and agents that stimulate insulin secretion such as meglitinides, the

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GLP-1 analogue exenatide and DPPIV inhibitors have been marketed to provide increased choice for clinicians treating Type 2 diabetes reviewed in [1]. However, there is growing concern that some of these novel drugs may not be ideal. Thus, the PPARγ agonist rosiglitazone has been associated with increased incidence of bone fractures in women [2] and increased risk of heart attack [3], there are concerns that elevated GLP-2 levels with DPPIV inhibitors may increase the risk of tumour growth and metastasis [4] and exenatide requires patient-unfriendly subcutaneous administration.

Medicinal herbs have been used to treat diabetes for many hundreds of years, and they offer a natural resource of antidiabetic products in Asia and Africa where the plants grow abundantly. There have been many reports of insulinotropic actions of such plant extracts *in vitro* demonstrating direct stimulatory effects on β-cells reviewed in [5]. One plant that has been studied previously is *Gymnema sylvestre* (GS), and aqueous extracts of GS leaves are reported to stimulate insulin secretion and increase glucose uptake *in vitro* and *in vivo* [6-11]. The main chemical constituents of GS are a group of triterpenoid saponins known as gymnemic acids, and these are considered to be the active compounds responsible for the anti-diabetogenic effects of the extracts [12]. We have previously investigated the capacity of a GS extract (GS4) to stimulate insulin release from rodent insulin-secreting cell lines and isolated rat islets and found that the presence of high levels of gymnemic acids led to unregulated insulin release by exerting deleterious effects on the β-cell plasma membrane rather than regulated, reversible insulin secretion [11]. The anecdotal and published reviewed in [13] accounts of the glucoregulatory effects of GS prompted us to re-examine whether its insulinotropic activity could be isolated from the membrane perturbing components. We have now used an aqueous extract of GS leaves (termed OSA) prepared by a molecular weight cut-off isolation procedure which allows exclusion of the majority of low molecular weight saponin constituents.

The aim of the current study was therefore to identify whether OSA stimulates insulin secretion from the mouse MIN6 β-cell line without exerting deleterious effects on β-cell viability. In addition, the potentially insulinotropic effects of GS extracts have not been previously identified using isolated human islets of Langerhans, which is a pre-requisite if these agents are to be ultimately used therapeutically for the treatment of Type 2 diabetes, so we have also determined whether OSA stimulates insulin secretion from human islets of Langerhans. Physiological insulin secretory responses are dependent on influx of extracellular Ca^{2+} into β-cells with a concomitant increase in cytosolic Ca^{2+}, so we have also examined the calcium-dependence of the insulin secretagogue effects of OSA in MIN6 cells and human islets.

Materials and Methods

Materials

The MIN6 mouse β-cell line was a kind gift from Dr. Y. Oka and Professor J.-I. Miyazaki (then at University of Tokyo, Japan). All reagents and chemicals were purchased from Sigma Chemical Co (Dorset, UK) unless otherwise stated.

Plant material and extraction

The extract used in this study (OSA) was prepared by aqueous alcoholic extraction of botanically pure, fresh *Gymnema sylvestre* leaves according to the protocols described in US Patents 6949261 and 6949151. In brief, a liquid extract was prepared by soaking *Gymnema sylvestre* leaves in aqueous ethanol (40% v/v) for at least 4 hours, after which the ethanol was removed by distillation and acid-insoluble salts were precipitated out in the presence of 1M sulphuric acid and removed by filtration. The aqueous extract was neutralised with dilute sodium hydroxide, deionised by passing through an ion exchange column, then filtered through a membrane having a molecular weight cut-off of 3kDa. The material retained by the membrane, having a molecular weight of at least 3kDa, was collected and lyophilised, and was termed OSA. OSA in powder form was kindly provided by Dr. Arun Chatterji (Ayurvedic Life International, Wisconsin, USA). It was freshly prepared for experiments as a 200mg/ml stock in a physiological salt solution [14] and diluted as appropriate in this buffer. GS4 was prepared as described previously [11].

Insulin secretion from MIN6 cells and measurements of MIN6 cell membrane integrity

MIN6 β-cells were maintained in culture at 37°C under a 5% CO_2 atmosphere in DMEM supplemented with 10% fetal calf serum (FCS) and 2mM glutamine, 100U/ml penicillin/0.1 mg/ml streptomycin. They were seeded at a density of 30,000 cells per well in 96-well plates and left to adhere for 1-2 days before use. After a 2 hour pre-incubation in a physiological salt solution [14] in the presence of 2mM glucose, cells were incubated for 30 minutes in the absence or presence of OSA, and insulin secreted into the supernatant was measured by radioimmunoassay [15]. In some experiments $CaCl_2$ was omitted from the salt solution, which was supplemented with 0.1mM EGTA to maintain very low levels of extracellular Ca^{2+}. The effects of OSA on MIN6 cell membrane integrity were assessed by exposure of MIN6 cells in suspension to 0.06-2mg/ml OSA at 2mM and 20mM glucose for 30 minutes followed by incubation of cells in a Trypan blue solution (0.1% w/v) for 15 minutes. Cells to which the dye had gained access (blue) and non-stained cells (white) were counted on a

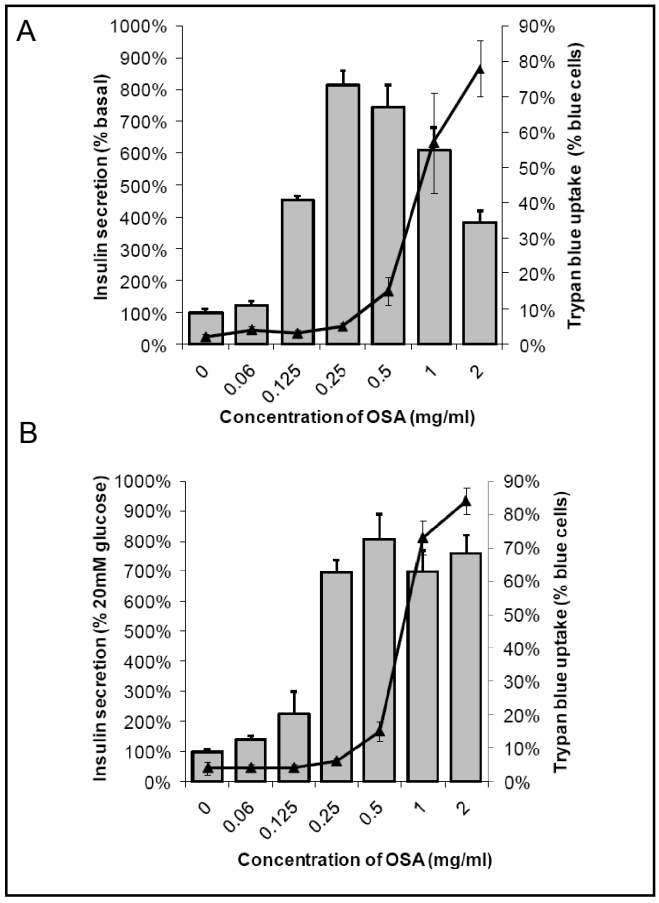


Fig. 1. Effect of OSA on insulin secretion and cell viability. MIN6 β-cells were incubated for 30 minutes in the presence of 0.06-2mg/ml OSA at 2mM glucose (panel A) or 20mM glucose (panel B) and insulin secretion (bars) was measured by radioimmunoassay. MIN6 cell membrane integrity (▲) was measured in parallel by exposing MIN6 cells to 0.06-2mg/ml OSA at 2mM and 20mM glucose for 30 minutes and quantifying Trypan blue-stained cells using a haemocytometer. Secretion data are means±SEM, n=8-10 separate wells of cells, representative of three separate experiments. Trypan blue uptake data are means±SEM of 4 separate MIN6 cell samples, with an average of 268 and 283 cells analysed per sample at 2mM and 20mM glucose respectively. P<0.0001 by ANOVA at 2mM and 20mM glucose, for both insulin secretion and Trypan blue uptake.

haemocytometer by light microscopy.

Calcium microfluorimetry

The effects of OSA on changes in intracellular Ca^{2+} ($[Ca^{2+}]i$) were determined by seeding 50,000 MIN6 cells onto glass coverslips, and loading adherent cells with 5μM of the Ca^{2+}-fluorophore Fura-2/AM (30 minutes, 37°C). The coverslips were placed in a steel chamber that was mounted into a heating platform on the stage of an Axiovert 135 Research Inverted microscope and changes in intracellular Ca^{2+} were determined as described previously [16].

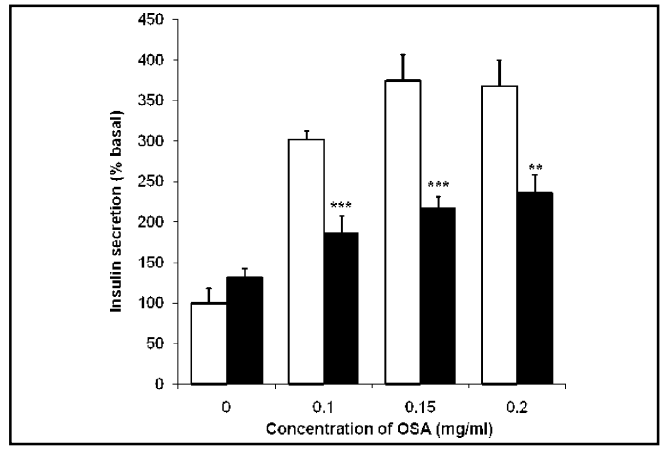


Fig. 2. Requirement of extracellular Ca^{2+} for OSA-stimulated insulin secretion. MIN6 β-cells were incubated in the presence of 0.1-0.2mg/ml OSA at 2mM glucose in the presence (open bars) or absence (filled bars) of 2mM extracellular Ca^{2+} Data are means±SEM, n=5-6, P<0.0001 and P<0.01 by ANOVA in the presence and absence of Ca^{2+} respectively; **P<0.001; ***P<0.005 versus appropriate control in the presence of Ca^{2+}.

Insulin secretion from human islets of Langerhans and measurements of islet cell membrane integrity

Human islets were isolated as described previously [17] and maintained in culture in CMRL medium supplemented with 10% FCS, 100U/ml penicillin/0.1 mg/ml streptomycin and 2mM glutamine. Five separate batches of human islets, ranging from 60% to 85% purity as assessed by dithizone staining [17], were used for the experimental data generated in this paper. All islet batches had viability of 90% or greater, and islets from all batches showed enhanced insulin output in response to elevations in extracellular glucose concentration. The effects of OSA on insulin secretion from human islets were examined by perifusion, where the rate of onset and reversibility of responses can be readily monitored. In these experiments islets were transferred to chambers containing 1μm pore-size nylon filters and perifused with a physiological buffer [14] containing 2mM glucose at a flow rate of 0.5ml/min at 37°C in a temperature-controlled environment. Any islet cells that may be dissociated from the islets during the course of the experiments are retained on the filters and do not pass through into the perifusate. Perifusate during a one hour pre-perifusion period was discarded, and then islets were perifused with buffers containing 2 or 20mM glucose, either in the absence or presence of OSA (0.125 or 0.25mg/ml). Perifusate fractions were collected every two minutes and insulin content was determined by radio-immunoassay [15]. Trypan blue uptake by human islet cells after a 30 minute exposure to 0.25mg/ml OSA or 0.25mg/ml GS4, the saponin-rich GS extract, was assessed by incubation of islets in Trypan blue (0.1% w/v) for 15 minutes followed by visualisation by light microscopy.

Statistical analysis

Secretion and calcium data are expressed as means ± SEM. Statistical analyses were performed using ANOVA or Student's *t*-tests as appropriate and P<0.05 was considered significant.

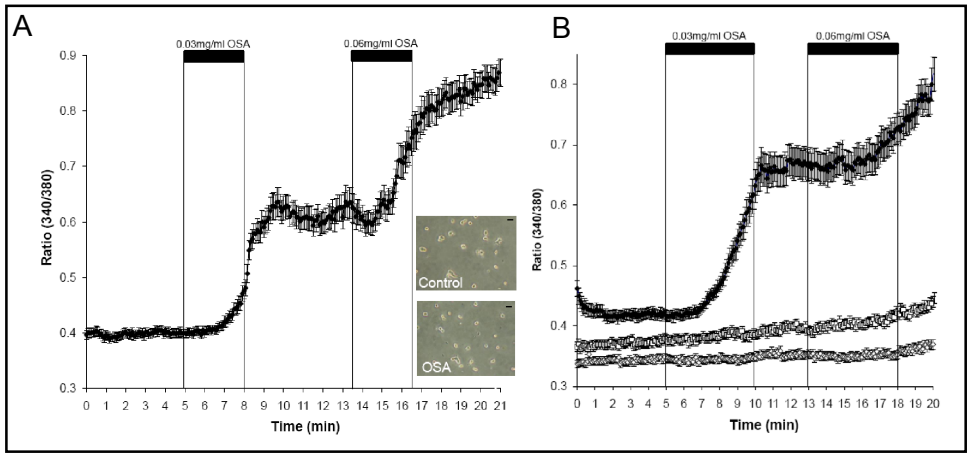


Fig. 3. Effect of OSA on intracellular Ca^{2+} levels in MIN6 β-cells. Fura-2-loaded MIN6 β-cells were perifused with buffers supplemented with 2mM glucose and 0.03 and 0.06mg/ml OSA (as shown by the bars) in the presence (♦) or absence (◇) of 2mM extracellular Ca^{2+}, or in the presence of 10μM nifedipine (□). Changes in $[Ca^{2+}]i$ were determined by single cell microfluorimetry, and expressed as 340/380nm ratiometric data. OSA significantly ($P<0.001$) increased $[Ca^{2+}]i$ in the presence of extracellular Ca^{2+} but had no effect in the absence of extracellular Ca^{2+} or in the presence of nifedipine. Data are means±SEM, n=27-31 cells for each experimental treatment. The micrographs inset in panel A show MIN6 cells on coverslips that had been perifused with buffer supplemented with 2mM glucose in the absence or presence of 0.06mg/ml OSA, after staining with 0.1% (w/v) Trypan blue for 15 minutes. Scale bars show 10μm.

Results

Insulin secretion from MIN6 cells

Static incubation experiments with MIN6 β-cells indicated that OSA (0.06-2mg/ml) significantly stimulated insulin secretion at 2mM glucose (Figure 1A) and potentiated 20mM glucose-stimulated insulin secretion (Figure 1B), with maximal stimulatory effects at 0.25-0.5mg/ml. Figure 1 also indicates that >90% of cells restricted entry of Trypan blue after 30 minutes exposure to ≤0.25mg/ml OSA at both 2mM and 20mM glucose, but higher concentrations of OSA caused progressive reductions in cell viability. Thus, for further experiments ≤0.25mg/ml OSA was used to stimulate insulin secretion without any significant increases in Trypan blue uptake.

The requirement of extracellular Ca^{2+} for MIN6 cell insulin secretory responses to OSA was investigated by performing experiments in buffers lacking $CaCl_2$ and supplemented with 0.1mM EGTA. In these experiments OSA (0.1-0.2mg/ml) induced a concentration-dependent stimulation of insulin secretion at 2mM glucose in both the presence (open bars) and absence (solid bars) of extracellular Ca^{2+}, but its stimulatory effect was significantly reduced by removal of Ca^{2+} (Figure 2).

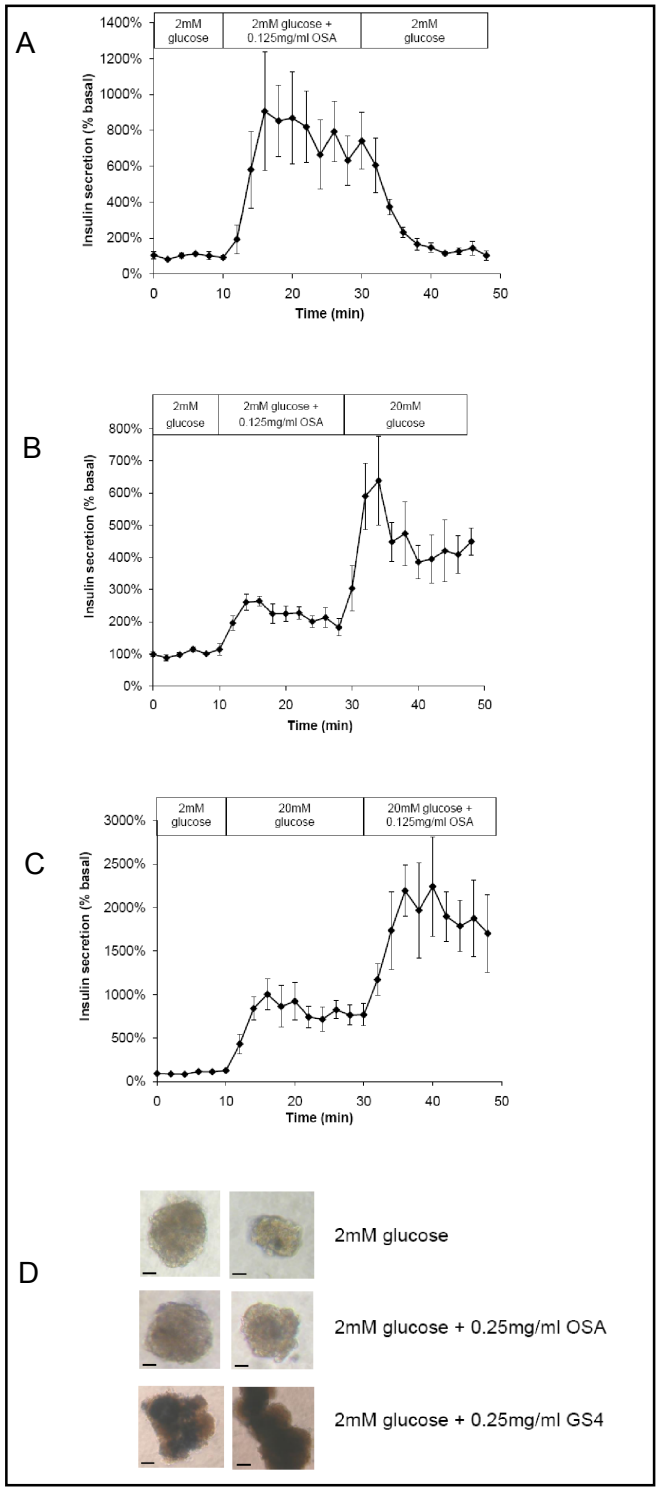
Changes in β-cell $[Ca^{2+}]i$

Concentrations of OSA as low as 0.03mg/ml stimulated marked and sustained increases in $[Ca^{2+}]i$ in Fura-2-loaded MIN6 β-cells. The response to 0.03mg/ml OSA was delayed in onset, with a lag of approximately 2 minutes before Ca^{2+} levels began to rise and $[Ca^{2+}]i$ remained elevated after removal of the OSA and perifusion for over 5 minutes with a basal buffer supplemented with 2mM glucose alone (Figure 3A). Exposure to 0.06mg/ml OSA caused a further increase in $[Ca^{2+}]i$ above the new baseline obtained after exposure to 0.03mg/ml OSA, and this was also irreversible upon removal of OSA. The MIN6 cells remained viable with a maintained membrane potential after exposure to 0.06mg/ml OSA since they were able to show increases in Ca^{2+} in response to 50μM tolbutamide and also to 100μM ATP (basal to peak amplitude, 340/380nm: 0.055±0.006 and 0.145±0.014 respectively; means±SEM, n=27 cells) and there was no increase in Trypan blue uptake when MIN6 cells on coverslips were stained at the end of the microfluorimetry experiments (Figure 3A, inset micrographs). It can be seen from Figure 3B that the stimulatory effect of OSA on $[Ca^{2+}]i$ was completely abolished by perifusing the cells in the absence of extracellular Ca^{2+}. The requirement of Ca^{2+} influx through voltage-operated calcium channels (VOCCs) for the sustained increases in $[Ca^{2+}]i$ in response to OSA was demonstrated by the use of the VOCC inhibitor nifedipine, which also abolished OSA-induced stimulation of $[Ca^{2+}]i$ (Figure 3B).

Insulin secretion from human islets

OSA also stimulated insulin secretion from isolated human islets of Langerhans *in vitro*. Thus, it can be seen from Figure 4A that the profile of insulin release from human islets at 2mM glucose in response to 0.125mg/ml OSA was rapid in onset, sustained, and fully reversible upon the withdrawal of OSA. Figure 4B demonstrates that human islets showed a significant further increase in

Fig. 4. Effect of OSA on insulin secretion from human islets. Isolated human islets were perifused with buffers supplemented with 0.125mg/ml OSA at 2mM glucose (panels A and B) or 20mM glucose (panel C), as indicated. Fractions were collected every 2 minutes and insulin release was determined by radioimmunoassay. Insulin secretion was significantly stimulated in the presence of 0.125mg/ml OSA at 2mM and 20mM glucose ($P<0.05$ for all panels). Data are means±SEM, n=3. Panel D shows micrographs of Trypan blue-stained human islets after incubation for 30 minutes in control buffer (2mM glucose; top) or in buffers supplemented with 0.25mg/ml OSA (middle) or 0.25mg/ml GS4 (bottom). Two separate fields of view are shown for each condition. Control and OSA-treated islets showed minimal Trypan blue uptake, with staining restricted to cells on the islet periphery, whereas GS4-treated islets showed extensive uptake of Trypan blue by cells throughout the islets. Scale bars show 50µm.



insulin secretion in response to 20mM glucose following a sustained secretory response to 0.125mg/ml OSA at 2mM glucose. The stimulatory effects of OSA in human islets at 20mM glucose are shown in Figure 4C where insulin secretion was stimulated approximately 10-fold when the glucose concentration was increased from 2mM to 20mM, and this glucose-induced secretory response was potentiated in a sustained manner in the presence of 0.125mg/ml OSA. Human islets maintained in the presence of 0.25mg/ml OSA for 30 minutes did not show increased Trypan blue staining, but incubation with 0.25mg/ml GS4, the GS extract used in earlier studies [11], led to marked Trypan blue uptake by human islet cells (Figure 4D).

The requirement of extracellular calcium for OSA-stimulated insulin secretion from human islets was determined by carrying out perifusion experiments in the absence of $CaCl_2$ and presence of 0.1mM EGTA. It can be seen from Figure 5 that 0.125mg/ml OSA significantly stimulated insulin secretion both in the presence and absence of extracellular Ca^{2+}, but in the absence of Ca^{2+} the secretory response was not maintained and returned towards basal levels, despite the continued presence of OSA. The data shown in Figure 6 also support a role for extracellular Ca^{2+} in OSA-stimulated insulin secretion from human islets. Thus, inhibition of Ca^{2+} influx by nifedipine-induced blockade of voltage-operated Ca^{2+} channels caused a significant inhibition, but not abolition, of the secretory response to OSA, and this was partially reversible when nifedipine was removed. Consistent with these observations, exposure of human islets to 10µM nifedipine during a sustained secretory response to OSA resulted in a rapid but incomplete inhibition of OSA-stimulated insulin secretion.

Discussion

Oral administration of aqueous GS leaf extracts to rats and humans is reported to decrease blood glucose levels [6-9] without improving insulin sensitivity [10], and an insulin secretagogue function was ascribed to GS following observations that its administration led to

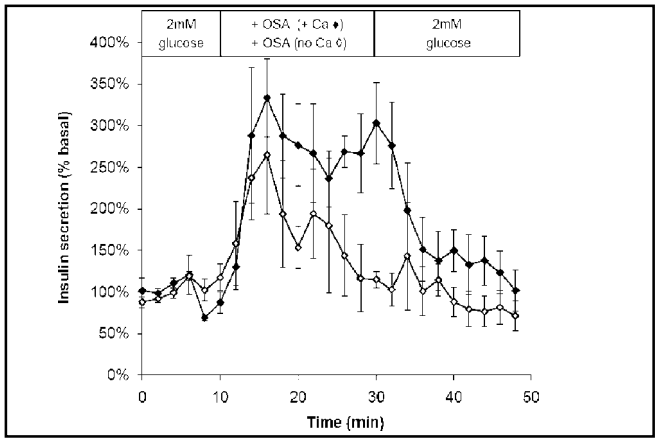


Fig. 5. Requirement of extracellular Ca^{2+} for OSA-stimulated insulin secretion from human islets. In the presence of extracellular calcium 0.125mg/ml OSA (♦) stimulated a sustained, reversible increase in insulin secretion (P<0.001 versus 2mM glucose). The magnitude and maintenance of the insulin secretory response to 0.125mg/ml OSA was significantly (P<0.05) diminished in the absence of extracellular Ca^{2+} (◇), but OSA still caused a significant (P<0.05) Ca^{2+}-independent elevation in insulin secretion. Data are means±SEM, n=4.

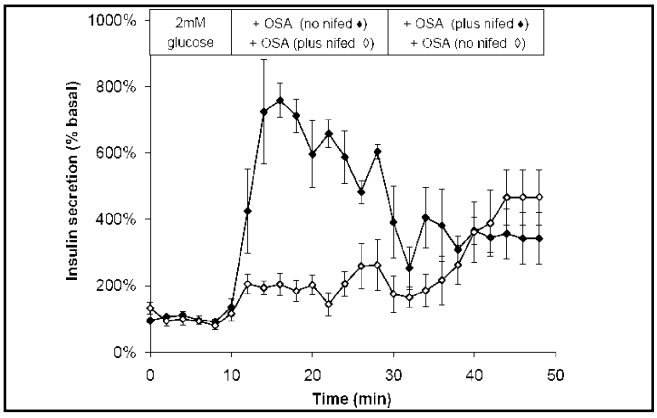


Fig. 6. Effect of nifedipine on OSA-stimulated insulin secretion from human islets. 0.25mg/ml OSA stimulated a sustained increase in insulin secretion (P<0.001) from human islets in the absence of nifedipine, and this was partially inhibited by nifedipine (♦). Islets showed a significantly (P<0.01) reduced secretory response to 0.25mg/ml OSA when it was administered in the presence of nifedipine, but OSA still caused a significant (P<0.01) stimulation of insulin secretion, and there was a further increase in insulin output when nifedipine was removed (◇). Data are means±SEM, n=4.

elevations in serum insulin levels in individuals with Type 2 diabetes [6]. However, it is difficult to identify the mode(s) of action of glucose lowering agents by *in vivo* approaches, so we have carried out experiments *in vitro* where potential effects of GS extracts to stimulate insulin secretion by direct action at β-cells can be investigated.

Gymnema sylvestre leaves contain numerous saponin components, all of which may solubilise mammalian cell membrane proteins in much the same way as digitonin, which is used experimentally to permeabilise plasma membranes [18]. Our earlier studies examining the insulinotropic effects of GS4, a GS extract prepared by ethanol and sulphuric acid extraction, indicated that its stimulatory effect on insulin release from MIN6 β-cells at 0.25mg/ml was accompanied by 98% of cells being stained with the membrane impermeant dye Trypan blue [11]. Thin layer chromatography analysis of GS4 in that study indicated that the major component was the triterpenoid saponin gymnemic acid VIII, which was most likely responsible for the loss of membrane integrity, and it was therefore not possible to establish whether GS4 could stimulate regulated insulin secretion or whether all of the insulin released was due to leakage from damaged β-cells.

The major saponins present in *Gymnema sylvestre* leaves have been identified as gymnemic acids, gymnemasins and gymnemosides [19], all of which have low molecular weights (<1kDa). The molecular weight filtration cut-off extraction procedure used in the current studies yielded a fraction, termed OSA, with a molecular weight of at least 3kDa. That this resulted in a saponin content considerably lower than that of GS4 and other *Gymnema sylvestre* extracts used in earlier studies was borne out by the observations that ≤0.25mg/ml OSA stimulated insulin secretion *in vitro* without adverse effects on cell viability. Higher concentrations of OSA did increase β-cell plasma membrane permeability, and at the highest concentration tested (2mg/ml) maximally 84% of β-cells were stained by Trypan blue. These observations suggest that although membrane perturbing components are present in OSA, their concentrations are much lower than in GS4, allowing investigation of the insulin secretagogue activity of OSA distinct from its adverse effects on membrane integrity. Trypan blue is an established test of cell viability that allows rapid visual readout of compromised plasma membrane integrity, and in this study increased dye uptake was not directly coupled to leakage of insulin. Thus, maximum Trypan blue uptake by MIN6 β-cells at 2mM glucose was observed at 2mg/ml OSA, but this was associated with less insulin output than obtained with 0.125mg/ml OSA, which caused only 3% of β-cells to take up Trypan blue. Further viability tests were therefore performed by determining the reversibility of the stimulatory effects of OSA on insulin secretion and the capacity of β-cells to respond to 20mM glucose after previous exposure to OSA.

The insulin secretagogue effects of OSA were not confined to a mouse insulin-secreting cell line, since it also stimulated insulin secretion from isolated human islets. Thus, perifusion experiments showed that initiation of insulin secretion by OSA at 2mM glucose was rapid in onset, with peak insulin output 4-6 minutes after exposure to OSA. The magnitude of insulin secretion in response to 0.125mg/ml OSA at 2mM glucose varied from approximately 3-fold to 10-fold in different experiments, which most likely represented biological variability between islets obtained from different donors. The observation of variabilities in the magnitude of insulin secretion from human islets is not limited to OSA and is also seen in response to other insulin secretagogues. For example, we have previously reported that 20mM glucose stimulated insulin secretion from different batches of human islets by approximately 3-fold [20] and 10-fold [17]. The insulin secretory response to OSA was sustained for the duration of exposure and it was readily reversible upon removal of OSA from the perifusion buffer, confirming that the increased release of insulin was not a consequence of OSA-induced cytotoxicity and insulin leakage from damaged β-cells. Furthermore, the capacity of islets to show a rapid, sustained secretory response to 20mM glucose after an initial response to OSA indicated that human β-cells maintained their membrane integrity and that cell polarity was not disrupted after exposure to OSA, and it did not compromise β-cell metabolic activity or the downstream pathways coupled to regulated exocytosis. OSA was also an effective potentiator of insulin secretion from human islets, and it induced a robust, sustained amplification of insulin secretion at 20mM glucose. The ability of OSA to further stimulate insulin secretion above the maximal 20mM glucose-induced response demonstrated that OSA is not merely acting as a nutrient, nor does it act by enhancing glucose metabolism within the β-cells. These measurements of islet function confirmed that 0.125 and 0.25mg/ml OSA did not increase insulin output through a constitutive, uncontrolled mechanism and there was no detectable increase in Trypan blue uptake in human islets after 30 minutes exposure to 0.25mg/ml OSA. This was in marked contrast to the large increases in Trypan blue staining observed after 30 minutes exposure of human islets to 0.25mg/ml of the saponin-containing GS4 extract, consistent with our earlier observations of GS4-induced Trypan blue uptake by isolated rat islet cells [11].

Some insulin secretagogues increase insulin secretion by allowing Ca^{2+} entry into the cells through depolarisation of the plasma membrane and activation of Ca^{2+}-dependent processes. This is the main mode of action of glucose and the sulphonylurea family of drugs that are used for therapy in Type 2 diabetes reviewed in [1]. We therefore examined the capacity of OSA to stimulate increases in $[Ca^{2+}]i$ in Fura-2-loaded MIN6 β-cells. In these experiments OSA stimulated increases in $[Ca^{2+}]i$ that were sustained even after its removal, and these OSA-induced elevations in Ca^{2+} were completely inhibited by removal of extracellular Ca^{2+} or by blockade of VOCCs with nifedipine. These observations indicate that OSA increases $[Ca^{2+}]i$ in β-cells by influx of extracellular Ca^{2+} through VOCCs, and the possibility that this was the mechanism by which it stimulates insulin secretion was addressed by examining the effect of OSA on insulin secretion in the absence of extracellular calcium or presence of nifedipine, thus circumventing potential effects of OSA on regulated Ca^{2+} influx. In these experiments OSA-stimulated insulin secretion from MIN6 cells and/or human islets was significantly inhibited, but not abolished, by the removal of extracellular Ca^{2+} or the presence of 10μM nifedipine.

Taken together, these observations provide evidence that OSA directly stimulates increases in Ca^{2+} influx in β-cells through VOCCs and that these elevations in Ca^{2+} may contribute to the enhanced secretory output. The ability of OSA to cause small increases in insulin secretion under conditions where its effects on Ca^{2+} influx were completely abolished imply that part of its insulin secretagogue effect is independent of increases in Ca^{2+}. Furthermore, our data suggest that the increases in Ca^{2+} may not be responsible for the onset of insulin secretion in response to OSA since elevations in Ca^{2+} were delayed by 2-3 minutes whereas OSA-stimulated insulin secretion was evident within the first 2 minutes of exposure to OSA. Furthermore, there are other data indicating a dissociation of OSA effects on $[Ca^{2+}]i$ and insulin secretion. Thus, 0.03mg/ml OSA stimulated substantial increases in $[Ca^{2+}]i$, but had no effect on insulin secretion; the effects of OSA on insulin release were readily reversible but it effects on $[Ca^{2+}]i$ were sustained; and an initial secretory response to OSA still occurred in the absence of extracellular Ca^{2+}.

The ability of OSA to stimulate insulin secretion at sub-stimulatory glucose concentrations is shared by sulphonylureas and meglitinide analogues that are currently used for the treatment of Type 2 diabetes. The mode of action of these agents has been established as being depolarisation of β-cells consequent to closure of K_{ATP} channels reviewed in [1] and this may account for some of the insulin secretagogue effects of OSA, but it can also stimulate insulin secretion independently of Ca^{2+}

influx. In this context it shares some similarities with arachidonic acid, an insulin secretagogue that is generated in β-cells by PLA_2-mediated hydrolysis of membrane phospholipids, which can stimulate insulin secretion in the presence of an inhibitor of VOCCs [21].

In summary, these data provide convincing evidence that low concentrations of the *Gymnema sylvestre* isolate OSA stimulate insulin secretion from mouse β-cells and isolated human islets *in vitro*, without compromising β-cell viability. These observations also indicate that OSA stimulates increases in Ca^{2+} influx in β-cells through VOCCs and that these elevations in Ca^{2+} may contribute to the enhanced secretory output. The ability of OSA to cause small increases in insulin secretion under conditions where its effects on Ca^{2+} influx were abolished imply that at least part of its insulin secretagogue effect is independent of increases in Ca^{2+}. Our demonstration that OSA has direct insulin secretagogue effects in human islets validates its development for clinical use, and identification of the OSA component(s) that stimulates regulated insulin exocytosis may provide promising lead targets for Type 2 diabetes therapy.

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