

Evidence for a sustained increase in clonal β -cell basal intracellular Ca^{2+} levels after incubation in the presence of newly diagnosed Type-1 diabetic patient sera. Possible role in serum-induced inhibition of insulin secretion

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Abstract

We have previously reported that newly diagnosed Type-1 diabetic patient sera potently suppressed insulin secretion from a clonal rat pancreatic β -cell line (BRIN BD11) but did not alter cell viability. Here, we report that apoptosis in BRIN BD11 cells incubated in various sera types (fetal calf serum (FCS), normal human serum and Type-1 diabetic patient) was virtually undetectable. Although low levels of necrosis were detected, these were not significantly different between cells incubated in sera from different sources. ATP levels were reduced by approximately 30% while nitrite production increased twofold from BRIN BD11 cells incubated for 24 h in the presence of Type-1 diabetic patient sera compared with normal human sera. Additionally, ATP levels were reduced by approximately 40% and DNA fragmentation increased by more than 20-fold in BRIN BD11 cells incubated in FCS in the presence of a pro-inflammatory cytokine cocktail (interleukin-1 β , tumour necrosis factor- α and interferon- γ), compared with cells incubated

in the absence of cytokines. Nitric oxide production from BRIN BD11 cells was markedly increased (up to 10-fold) irrespective of sera type when the cytokine cocktail was included in the incubation medium.

Type-1 diabetic patient sera significantly ($P < 0.001$) raised basal levels of intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) in BRIN BD11 cells after a 24-h incubation. The alteration in $[\text{Ca}^{2+}]_i$ concentration was complement dependent, as removal of the early complement components C1q and C3 resulted in a significant reduction ($P < 0.01$) of sera-induced $[\text{Ca}^{2+}]_i$ changes. We propose that the mechanism of Type-1 diabetic patient sera-induced inhibition of insulin secretion from clonal β -cells may involve complement-stimulated elevation of $[\text{Ca}^{2+}]_i$ which attenuates the nutrient-induced insulin secretory process possibly by desensitizing the cell to further changes in Ca^{2+} .

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Introduction

Type-1 diabetes, also known as insulin-dependent diabetes mellitus (IDDM), is a disease involving immune-mediated damage and destruction of the insulin-secreting β -cells of the pancreatic islets of Langerhans. Diabetes susceptibility genes as well as environmental factors may be involved in disease aetiology. Auto-immunity is normally associated with an initial production of antibodies against β -cell proteins such as glutamic acid decarboxylase, insulin, or IA-2 (also known as 40 kDa antigen or islet cell

antibody (ICA) 512, a protein tyrosine phosphatase-like molecule; Nepom 1995) although auto-antibodies themselves are not thought to be destructive at least in the early stages of disease pathogenesis. β -Cell loss is thought to be mediated by T-lymphocytes and macrophages *in vivo* (Mauricio & Mandrup-Poulsen 1998). The component of the immune system that is ultimately responsible for the destruction of β -cells in humans is unknown. However, it is now generally accepted that β -cells become defective and fail to secrete insulin appropriately before destruction occurs *in vivo* and prior to clinical diagnosis of Type-1

diabetes. The component(s) which precipitate β -cell dysfunction *in vivo* are thought to be immune in nature and a number of pro-inflammatory cytokines have been implicated including interleukin- 1β (IL- 1β), tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) (Comens *et al.* 1987, Di Matteo *et al.* 1997, Hadjivassiliou *et al.* 1998, Mauricio & Mandrup-Poulsen 1998). These cytokines are thought to induce dysfunction and, under appropriate conditions, stimulation of apoptosis via generation of nitric oxide (Sandler *et al.* 1987, Southern *et al.* 1990, Dunger *et al.* 1996). Nutrients such as glucose and some amino acids initiate insulin secretory responses primarily by depolarizing the β -cell plasma membrane. This results in an influx of extracellular Ca^{2+} through voltage-dependent Ca^{2+} channels (VDCC, Ashcroft & Rorsman 1989, Satin & Smolen 1994). This initiating or triggering event (Henquin 2000) is followed by a potentiating or amplifying mechanism which involves mitochondrial metabolism and possibly activation of the protein kinases PKA and PKC. An elevation in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) and stimulation of protein kinase activity (especially Ca^{2+} /calmodulin kinase II; Easom 1999) results in fusion of insulin-containing granules with the plasma membrane and subsequent release of insulin. However, there appears to be a desensitization mechanism in place which becomes operative if Ca^{2+} levels remain high, as in the case of β -cells permeabilised in the presence of $10\ \mu\text{M}$ Ca^{2+} (Harris *et al.* 2000). In these cells Ca^{2+} /calmodulin kinase II activity is reduced, resulting in a decreased rate of insulin secretion.

Activation of the complement system can result in a sustained increase in $[\text{Ca}^{2+}]_i$ (Newsholme *et al.* 1999). It is possible that Type-1 diabetic patient serum-induced inhibition of clonal β -cell insulin secretion, which is partially complement mediated (Conroy *et al.* 2000), may well be mediated by increased basal levels of $[\text{Ca}^{2+}]_i$ thus desensitizing the cells to nutrient stimulation. In this paper, we have investigated the effects of Type-1 diabetic patient serum on β -cell viability (by determining levels of necrosis and apoptosis), ATP concentration, nitrite production and $[\text{Ca}^{2+}]_i$. Additionally, we have explored the effect of an apoptosis-inducing pro-inflammatory cytokine cocktail (IL- 1β , TNF- α and IFN- γ) on these parameters for comparative purposes. The use of a robust clonal β -cell line, BRIN BD11, which is resistant to serum-induced apoptosis (Conroy *et al.* 2000) has allowed investigation of serum-induced β -cell dysfunction in the absence of apoptosis.

Materials and Methods

Human sera samples for insulin secretion experiments

Samples were obtained by venepuncture from 9 consenting newly diagnosed Type-1 diabetic patients and 9 age-matched control subjects without a history of diabetes

and confirmed normal glucose tolerance. The Type-1 diabetic patients consisted of 5 males and 4 females, aged 18–34 years. Serum samples were obtained between 5 days and 1 month post-diagnosis and at least 11 h after the last insulin injection. The 9 age-matched control subjects consisted of 5 males and 4 females. The serum samples were obtained between 0830 and 1030 h after an overnight fast. Blood samples were collected in silicone-coated evacuated blood tubes and allowed to clot for 20 min at room temperature. Samples were then centrifuged at $400\ \text{g}$ for 10 min and the supernatant was stored at $-70\ ^\circ\text{C}$. Sera samples were subsequently freeze/thawed only once before use. Each experiment reported in this paper was designed so that cells were incubated in sera from at least three different individuals unless otherwise stated.

The basal insulin concentration of the Type-1 diabetic group was $8.58 \pm 3.53\ \text{mU/l}$. The range in normal subjects was 1.0 – $17.5\ \text{mU/l}$. The fasting blood glucose concentration was between 4.5 and $6.9\ \text{mM}$ for all subjects.

Autoantibody assay

All Type-1 diabetic patient sera samples used in this study were positive for at least two auto-antibodies. Anti-glutamic acid decarboxylase (GAD) and insulin autoantibodies (IAA) were assessed in serum samples by using an indirect ELISA as previously described (Conroy *et al.* 2000). ICA were detected by immunofluorescence as previously described (Conroy *et al.* 2000).

Culture of BRIN BD11 cells

Clonal insulin secreting BRIN BD11 cells were maintained in RPMI-1640 tissue culture medium with 10% (v/v) fetal calf serum (FCS), 0.1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and $11.1\ \text{mM}$ D-glucose, pH 7.4. The cells were maintained at $37\ ^\circ\text{C}$ in an atmosphere of 5% CO_2 and 95% air using a Forma Scientific incubator. The cells were cultured in 20–50 ml tissue culture medium in 75-cm^2 sterile tissue culture flasks. Cells were subsequently seeded in 24-well multiplates (of 2.5×10^5 cells per well) for culture in medium with and without supplementation with 10% human sera as described elsewhere (Conroy *et al.* 2000).

Determination of cell viability, necrosis and apoptosis

Cell viability was determined by measuring internal lactate dehydrogenase ($[\text{LDH}]_i$) i.e. the enzyme activity associated with intact cells after 24-, 48- and 72-h incubation in the presence of the various sera types. To determine cell viability, BRIN-BD11 cells were plated at a density of 2.5×10^5 cells per well and after 12 or 24 h culture medium was removed and $100\ \mu\text{l}$ lysis buffer (20 mM EDTA, 10 mM Tris-HCl, 0.5% Triton-X 100, pH 8.0)

were added to each well. Cell lysates were stored at -70°C until enzyme activity determination. LDH was measured by an adaption of a method described previously (Conroy *et al.* 2000) in which NADH formation is coupled to p-iodonitrotetrazolium (INT) to form formazan, the increase in absorbance is then determined at 490 nm.

In some experiments Hoechst propidium iodide (HPI) staining was used to determine levels of apoptotic or necrotic cell death. Cell nuclei were stained simultaneously with DNA binding dyes Hoechst 33342 and propidium iodide (20 μg Hoechst 33342/ml and 100 μg PI/ml). The cells were viewed using a fluorescence microscope with a broad-band filter (excitation wavelength 365 nm). Viable cells were identified by their intact nuclei with blue fluorescence (H33342), necrotic cells by their intact nuclei with pink fluorescence (positive for both H33342 and PI) and apoptotic cells by their fragmented nuclei, fluorescence either blue (early phase) or pink (H33342+PI positive, late phase with secondary necrosis).

ATP determination

ATP was determined by the use of a Sigma (Dublin, Ireland) ATP bioluminescent assay kit according to the manufacturer's instructions.

Determination of DNA fragmentation

Determination of DNA fragmentation was achieved by the use of a Cell death detection ELISA^{plus} (Roche Diagnostics, Lewes, East Sussex, UK) and used in accordance with the manufacturer's instructions. Fragmented DNA was proportional to A_{405} .

Nitrite determination

The conventional method for nitrite determination is the Griess reaction, a method described over 100 years ago. However, the detection level for nitrite by this method is only 1 μM . The method used here was a modified version of the Griess reaction which allowed greater sensitivity for nitrite detection (to 0.1 μM). To 100 μl cell incubation medium, 50 μl ice-cold dapsone (14 mM 4,4'-diaminodiphenylsulphone in 2 M HCl) were added. This was followed by 50 μl NED (4 mM N-(1-naphthyl)-ethylenediamine in H_2O). The plate was incubated at room temperature for 5 min and the absorbance read at 550 nm. The concentration of nitrite in the test samples was determined from a nitrite standard curve.

Ca^{2+} determination

$[\text{Ca}^{2+}]_i$ measurements were performed using Fura 2-AM, a Ca^{2+} selective fluorescence dye (excitation wavelength 340/380 nm, Em 510 nm) as described by Juntti-

Berggren *et al.* 1993. Cells were resuspended in Hanks balanced salt solution (HBSS) at a final concentration of 1×10^7 cells/ml with 5 μM Fura2-AM and were incubated in the dark at 37°C for 40 min in the absence of sera. Cells were washed free of extracellular dye by centrifugation. Cells were then resuspended in HBSS containing 0.1% BSA at a concentration of 1×10^6 cells/ml and CaCl_2 was added to a final concentration of 1 mM (in the dark). Cells were transferred to a 3 ml quartz cuvette for $[\text{Ca}^{2+}]_i$ determination using a Perkin Elmer Luminescence Spectrometer LS50B.

Removal of complement components C1q and C3 from serum

Anti-C1q or anti-C3 antibodies (Sigma Chemical Company, Poole, Dorset, UK) were diluted 100-fold in 10 mM phosphate buffer, containing 145 mM NaCl, pH 7.2 (from 44 mg/ml anti-human C1q or 38 mg/ml anti-human C3 stock solutions). Diluted anti-C1q antibodies (100 μl) were added to columns 1–6, wells A–H of a 96-well microtitre plate, and 100 μl diluted anti-C3 were added to columns 7–12, wells A–H. After incubation at 40°C overnight, the buffer was removed and the wells were washed 3 times with 10 mM phosphate buffer containing 500 mM NaCl, 0.1% w/v Tween 20 (pH 7.2). Sera (100 μl) were then added to column 1, wells A–H and the plate was incubated at room temperature for 40 min. After this time, the sera samples were removed and transferred to column 2, wells A–H where they were incubated for 40 min and then transferred to column 3, wells A–H, and so on. By the time the sera samples reached column 12 they were complement depleted (C1q and C3) as assessed by cell viability studies. Control plates were set up with 0.1% BSA replacing anti-C1q and anti-C3. The sera obtained from the control plates had complement activation profiles similar to that not added to control plates.

Determination of Ca^{2+} /calmodulin-dependent kinase II activity

Cell extracts were prepared and Ca^{2+} /calmodulin-dependent kinase II activity determined as previously described (Jones & Persaud 1998).

Informed consent and Ethics Committee approval

All patients and controls gave informed consent. Ethical approval was obtained from the Research and Ethics Committees in participating hospitals (St James's and St Vincent's Hospitals, Dublin 8 and Dublin 4 respectively, Ireland).

Statistics

The results are presented as means \pm S.E.M. unless stated otherwise. Groups of data were compared using unpaired

Table 1 Viability of BRIN BD11 cells incubated for 12 or 24 hours in various sera types (10%). BRIN BD11 cell viability was determined by measurement of internal LDH activity as described in Materials and Methods. Results were normalized to cell LDH activity at the start of the incubation period and are expressed as % \pm s.d. of this value

	12 Hours			24 Hours		
	FCS	Normal human serum	Type-1 diabetic patient serum	FCS	Normal human serum	Type-1 diabetic patient serum
No cytokines	95.0 \pm 1.9	101.4 \pm 2.8	98.2 \pm 3.4	119.6 \pm 7.7	117.4 \pm 10.4	111.1 \pm 7.2
Plus cytokines	105.0 \pm 3.4	99.5 \pm 2.1	91.2 \pm 2.6	120.2 \pm 7.7	84.2 \pm 16.0	59.3 \pm 6.2*

The viability of BD11 cells was significantly reduced ($*P < 0.001$) in cells incubated in Type-1 diabetic sera plus a pro-inflammatory cytokine cocktail compared with cells incubated in the absence of the cytokines.

or paired Students *t*-test as appropriate. Differences were considered significant if $P < 0.05$.

Results

Effects of sera and a pro-inflammatory cytokine cocktail on cell viability

BRIN BD11 cell viability, determined by measurement of internal LDH activity (Table 1), was not significantly different for cells cultured in 10% (v/v) FCS, Type-1 patient or normal human serum after 12 and 24 h of incubation. However, if a pro-inflammatory cytokine cocktail of 2×10^{-11} M IL-1 β , 10^{-10} M TNF- α and 5 U/ml IFN- γ was added to the culture medium for the time periods indicated, significant ($P < 0.001$) loss in viability was observed after 24 h of incubation in cells incubated in Type-1 diabetic patient sera and the pro-inflammatory cytokine cocktail compared with the absence of the cytokine cocktail (Table 1).

Effects of sera and a pro-inflammatory cytokine cocktail on necrosis or apoptosis

The level of apoptosis or necrosis in BRIN BD11 cells after incubation in various sera was determined after 24 h by Hoechst dye uptake and propidium iodide uptake. Levels of apoptosis in BRIN BD11 cells was negligible in all sera types tested, whereas levels of necrosis were higher, but not significantly different between cells incubated in the various sera types (Fig. 1). In contrast, a significant number (7–8%, $P < 0.05$ compared with absence of cytokines) of BRIN BD11 cells incubated in FCS supplemented with a pro-inflammatory cytokine cocktail (2×10^{-11} M IL-1 β , 10^{-10} M TNF- α and 5 U/ml IFN- γ) for 24 h were found to be apoptotic (results not shown).

Effects of sera and a pro-inflammatory cytokine cocktail on cellular ATP concentration

BRIN BD11 cells were incubated in the presence of 10% (v/v) Type-1 patient sera, normal human sera or FCS for

4, 8 and 24 h. ATP concentration in cells exposed to Type-1 diabetic patient or normal human sera was significantly reduced ($P < 0.001$) compared with cells incubated in FCS (Fig. 2). However, there was a significant difference in ATP concentration between BRIN BD11 cells incubated in Type-1 diabetic patient sera compared with normal human sera after 24 h of incubation ($P < 0.001$). When BRIN BD11 cells were incubated in FCS, normal human sera, or Type-1 diabetic patient sera and the pro-inflammatory cytokine cocktail (2×10^{-11} M IL-1 β , 10^{-10} M, TNF- α and 5 U/ml IFN- γ), the ATP levels were significantly reduced in all conditions tested compared with the incubation which did not contain the cytokine cocktail (Fig. 2).

Effects of sera and a pro-inflammatory cytokine cocktail on DNA fragmentation

One of the hallmarks of apoptotic death is early DNA fragmentation. It is possible to detect histone-associated DNA fragments (mono and oligonucleosomes) released from the fragmenting nuclei of cells undergoing apoptosis by an ELISA method. This technique revealed no significant difference in DNA fragmentation in BRIN BD11 cells exposed to 10% Type-1 diabetic patient sera, normal human sera or FCS for 4, 8 or 24 h (Fig. 3). Similar results were obtained in cells exposed to 10% sera in the presence of a pro-inflammatory cytokine cocktail (2×10^{-11} M IL-1 β , 10^{-10} M TNF- α and 5 U/ml IFN- γ) for 4, 8 or 24 h. However, the proportion of total cellular DNA that was fragmented in the presence of cytokines was significantly greater ($P < 0.01$) than in BRIN BD11 cells incubated in the absence of cytokines (Fig. 3).

Effects of sera and a pro-inflammatory cytokine cocktail on nitrite production

We have previously demonstrated that cytokine-induced changes in islet insulin secretion rates, DNA damage and apoptosis are dependent, in part, on nitric oxide generation (Hadjivassiliou *et al.* 1998). We therefore determined whether sera alone or in combination with a pro-inflammatory cytokine cocktail can alter rates of nitrite

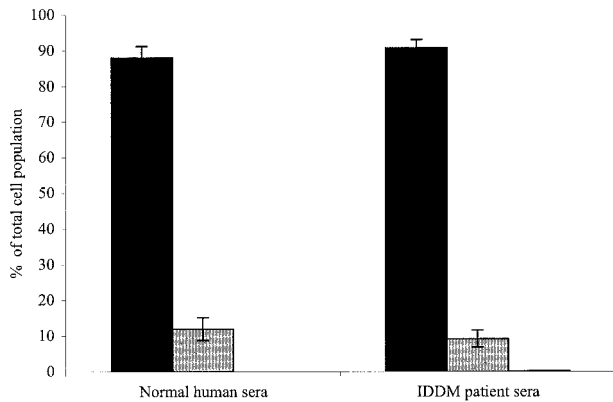


Figure 1 Effect of sera on BRIN BD11 necrosis or apoptosis following 24-h incubation. BRIN BD11 cells (1×10^5) were incubated in 10% normal human or Type-1 diabetic patient (IDDM) serum for 24 h. The number of viable (solid bars), necrotic (grey bars) and apoptotic cells present were then determined via the HPI staining method (see Materials and Methods). No apoptotic cells were detected following the 24-h incubation period. Four separate normal human and Type-1 patient serum samples were tested in triplicate and the results expressed as means \pm S.E.M.

production in BRIN BD11 cells. Nitrite is a stable end-product of nitric oxide oxidation. BRIN BD11 cells cultured in normal human sera or FCS produced measurable quantities of nitrite after 12 or 24 h. There were no significant differences in nitrite produced by cells incubated in either of these two sera types after 24 h. However, cells incubated in Type-1 diabetic patient sera displayed a small increase in nitrite production compared with normal human sera or FCS incubated cells after 12 ($P < 0.001$) and 24 h ($P < 0.001$) (Table 2). BRIN BD11 cells incubated in the presence of a pro-inflammatory cytokine cocktail (2×10^{-11} M IL- 1β , 10^{-10} M TNF- α and 5 U/ml IFN- γ) produced a large increase in nitrite production compared with cells incubated in the absence of cytokines (up to 10-fold increase), irrespective of the sera in the incubation medium, after 24 h ($P < 0.001$) (Table 2).

Effects of sera and a pro-inflammatory cytokine cocktail on basal and alanine-stimulated $[Ca^{2+}]_i$

The mechanism by which nutrients stimulate insulin secretion includes an elevation in $[Ca^{2+}]_i$ (Wollheim & Pozzan 1984). After culture in normal human or Type-1 diabetic patient sera for 24 h, the concentration of basal and alanine-stimulated $[Ca^{2+}]_i$ were determined in BRIN BD11 cells following a 20-min incubation in the presence of 1.1 mM glucose or 1.1 mM glucose plus 10 mM L-alanine. The $[Ca^{2+}]_i$ in cells which had been incubated in normal human sera for 24 h was increased almost threefold on addition of L-alanine (Fig. 4). However, those cells which had been cultured in Type-1 diabetic patient

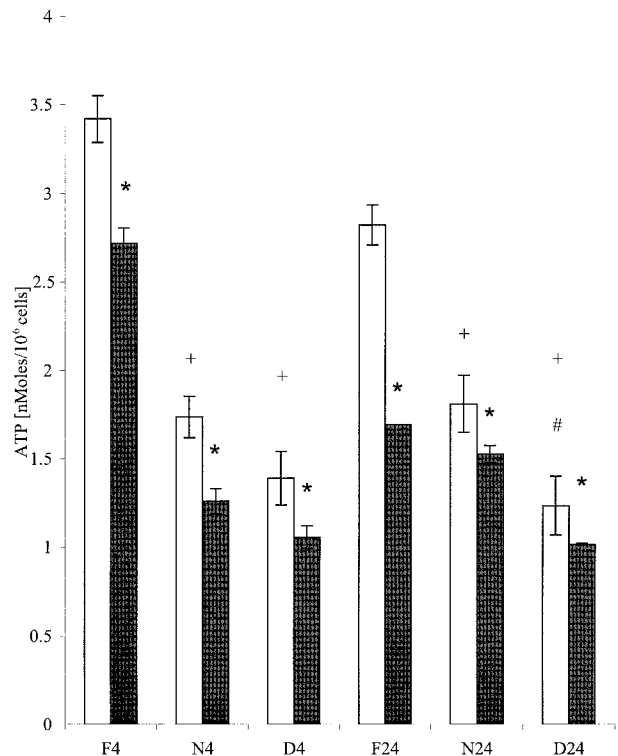


Figure 2 Effect of sera and a pro-inflammatory cytokine cocktail on BRIN BD11 ATP concentration (serum only, open bars; serum plus cytokines, grey bars). BRIN BD11 cells (1×10^5) were incubated in 10% FCS (F), normal human (N) or Type-1 diabetic patient (D) serum for 4 or 24 h at which time cells were harvested and cellular ATP content determined (see Materials and Methods). Results are expressed as means \pm S.E.M. of at least 3 separate experiments containing 3 different diabetic or normal sera samples in each experiment. ATP concentrations in cells incubated in normal or Type-1 patient serum were significantly less ($+P < 0.001$) than in cells incubated in FCS. After 24 h, ATP concentrations were significantly less in cells incubated in Type-1 patient serum compared with normal human serum ($\#P < 0.01$). Addition of a pro-inflammatory cytokine cocktail (2×10^{-11} M IL- 1β , 10^{-10} M TNF- α and 5 U/ml IFN- γ) resulted in a significant reduction ($*P < 0.05$) in BD11 ATP concentrations.

sera had significantly elevated basal levels of $[Ca^{2+}]_i$ compared with those cultured in normal human serum (approximately twofold, $P < 0.001$) which were not further elevated on addition of L-alanine (Fig. 4).

The L-type voltage-activated Ca^{2+} channel antagonist, nifedipine, significantly attenuated L-alanine-stimulated elevations of $[Ca^{2+}]_i$ in cells incubated in normal human serum but not in cells incubated in Type-1 diabetic sera (the alanine-induced increase in $[Ca^{2+}]_i$ was poor in the latter cells, Fig. 4). However, nifedipine partially, but not significantly, lowered the Type-1 patient sera-induced elevation in basal $[Ca^{2+}]_i$ (Fig. 4).

It has previously been reported that rapid complement dependent increases in $[Ca^{2+}]_i$ are observed on addition of

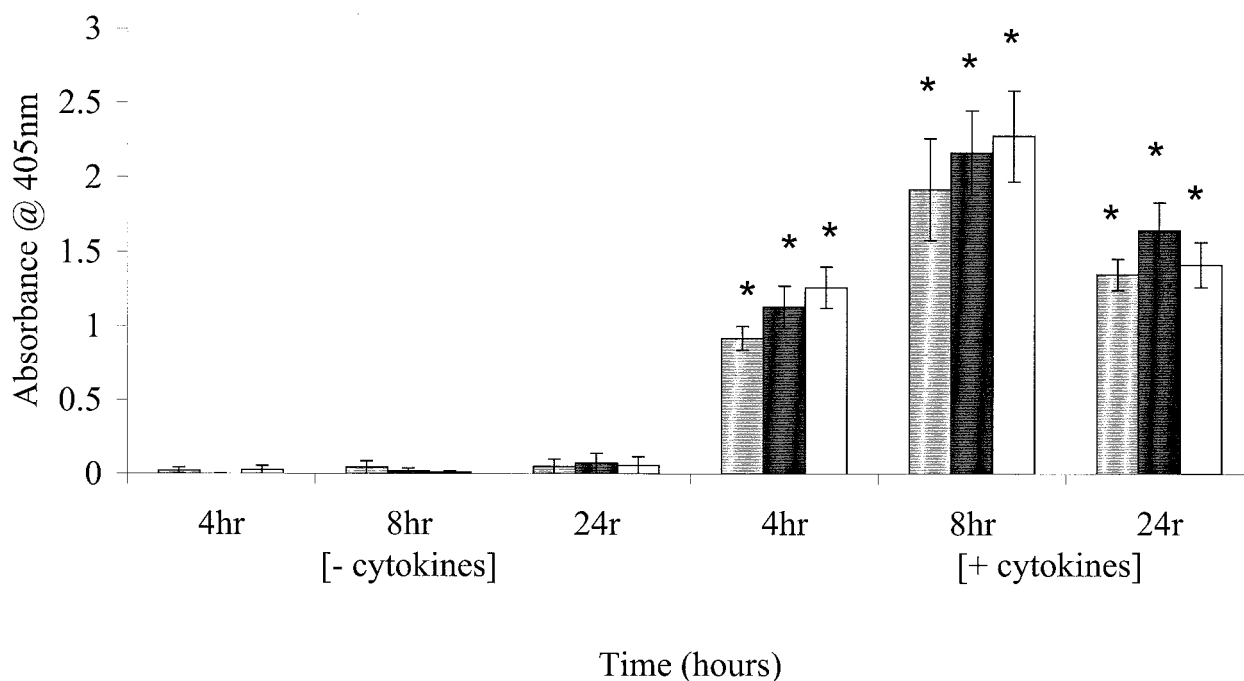


Figure 3 Effects of sera and a pro-inflammatory cytokine cocktail on BRIN BD11 DNA fragmentation. BRIN BD11 cells (1×10^5) were incubated in 10% FCS (light grey bars), normal human serum (dark grey bars) or Type-1 diabetic patient serum (open bars) for 4, 8 or 24 h at which time cells were lysed. Fragmented DNA in the cell lysates was subsequently determined using an ELISA based kit (see Materials and Methods). Results are expressed as means \pm S.E.M. of at least 3 separate experiments containing 3 different diabetic or normal sera samples in each experiment. The level of fragmented DNA was significantly greater ($*P < 0.01$) in cells exposed to a pro-inflammatory cytokine cocktail (2×10^{-11} M IL- 1β , 10^{-10} M TNF- α and 5 U/ml IFN- γ) compared with cells incubated in the absence of the cytokines.

Table 2 Nitrite concentration in BD11 culture medium in the presence or absence of a pro-inflammatory cytokine cocktail. BD11 cells were incubated in various sera types (10%) for 12 or 24 h in the presence or absence of a pro-inflammatory cytokine cocktail. At the end of the incubation period an aliquot of media was removed for determination of nitrite concentration. Results are given as μM nitrite \pm S.D. in the culture medium

	12 Hours			24 Hours		
	FCS	Normal human serum	Type-1 diabetic patient serum	FCS	Normal human serum	Type-1 diabetic patient serum
No cytokines	0 ± 0.27	5.37 ± 0.70	$10.19 \pm 0.49^*$	4.63 ± 0.45	6.48 ± 0.34	$16.48 \pm 0.40^*$
Plus cytokines	1.67 ± 1.03	7.69 ± 1.61	9.35 ± 0.84	$43.33 \pm 0.85^\dagger$	$38.06 \pm 1.34^\dagger$	$46.11 \pm 1.79^\dagger$

BD11 cells incubated in Type-1 diabetic sera produced significantly higher concentrations of nitrite compared to normal human sera after 12 and 24 hours of incubation ($*P < 0.001$). BD11 cells incubated in the presence of a pro-inflammatory cytokine cocktail produced nitrite at significantly higher levels than cells incubated in the absence of cytokines after 24 h of incubation ($\dagger P < 0.001$) irrespective of the sera type in which they were incubated.

serum to various cell types (Campbell & Luzio 1981, Newsholme *et al.* 1993). Addition of normal human or Type-1 diabetic patient sera to BRIN BD11 cells incubated in a cuvette in the spectrometer resulted in a rapid increase in $[\text{Ca}^{2+}]_i$. However, the increase in $[\text{Ca}^{2+}]_i$ mediated by normal human serum appeared to be transient in nature (results not shown). The addition of a proinflammatory cytokine cocktail (2×10^{-11} M IL- 1β , 10^{-10} M TNF- α and 5 U/ml IFN- γ) did not significantly

alter the level of the serum-stimulated elevation of $[\text{Ca}^{2+}]_i$ (results not shown).

Effect of C1q and C3 depletion from Type-1 diabetic patient or normal human sera on $[\text{Ca}^{2+}]_i$

The increase in basal $[\text{Ca}^{2+}]_i$ mediated by Type-1 diabetic patient sera over a 24-h culture was complement dependent as removal of complement components C1q and

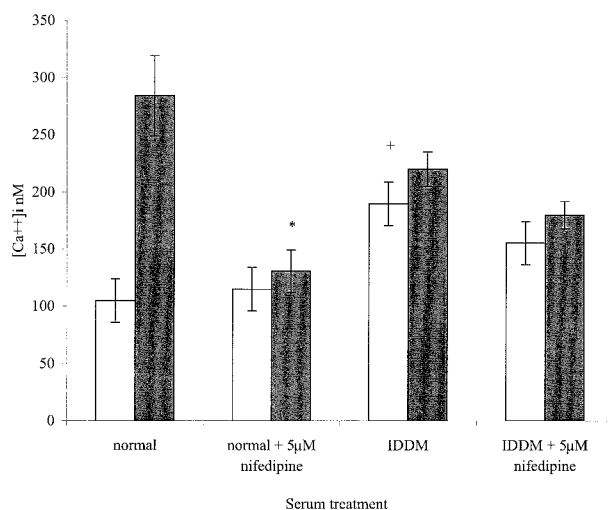


Figure 4 Effects of sera on basal and alanine-stimulated $[\text{Ca}^{2+}]_i$. BRIN BD11 cells (2×10^7) were incubated in 10% normal human or Type-1 diabetic patient serum (IDDM) for 24 h in the presence or absence of $5 \mu\text{M}$ nifedipine. Cells were then exposed either to 1.1 mM glucose (open bars) or to 1.1 mM glucose + 10 mM alanine (grey bars) for 20 min after which $[\text{Ca}^{2+}]_i$ was determined (see Materials and Methods). Results are expressed as means \pm S.E.M. of at least 3 separate experiments containing 3 different diabetic or normal sera samples in each experiment. Nifedipine significantly reduced the alanine stimulated increase in $[\text{Ca}^{2+}]_i$ ($*P < 0.01$) in cells incubated in normal human sera but not in cells incubated in Type-1 diabetic patient sera. Cells which had been cultured in Type-1 patient sera had a significantly greater concentration of basal $[\text{Ca}^{2+}]_i$ compared with cells cultured in normal human sera ($+P < 0.001$).

C3 from the Type-1 diabetic patient sera resulted in a significantly lower increase in basal $[\text{Ca}^{2+}]_i$ levels compared with untreated sera ($P < 0.01$, Fig. 5).

Effect of sera on Ca^{2+} /calmodulin kinase II activity

BRIN BD11 cells were incubated in the presence of 10% (v/v) Type-1 patient sera, normal human sera or FCS for 24 h. Cell extracts were subsequently prepared and Ca^{2+} /calmodulin kinase II activity determined as described in Materials and Methods. The activity determined for BRIN BD11 cells cultured in FCS was 0.55 pmol/min per 10^6 cells. This activity was reduced by approximately 50% in cells incubated in normal human sera and by approximately 60% in cells incubated in Type-1 diabetic patient sera (results not shown).

Discussion

Insulin secretion

Glucose or amino acids such as alanine and leucine stimulate insulin secretion by generating a specific and co-ordinated set of signals. Glucose enters the cell by

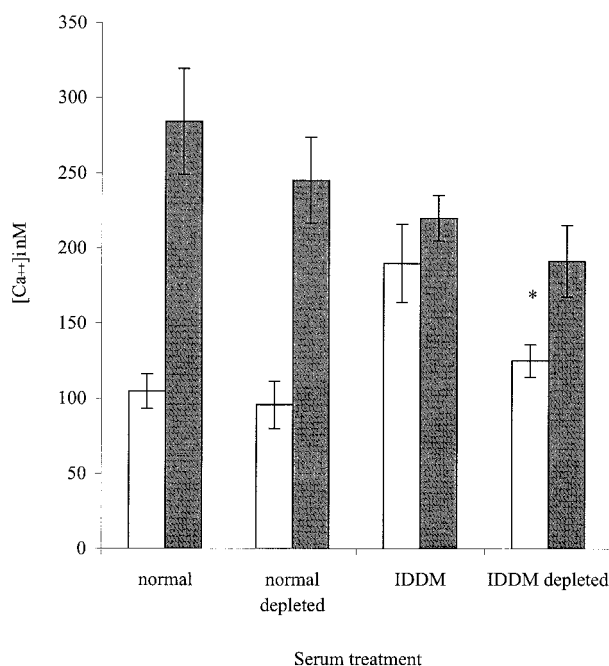


Figure 5 Effect of C1q and C3 depletion from Type-1 diabetic patient serum or normal human serum on $[\text{Ca}^{2+}]_i$. BRIN BD11 cells (2×10^7) were incubated in 10% normal human, Type-1 diabetic patient (IDDM), complement depleted normal human or complement depleted Type-1 diabetic patient serum for 24 h. Cells were then exposed to either 1.1 mM glucose (open bars) or to 1.1 mM glucose + 10 mM alanine (grey bars) for 20 min after which $[\text{Ca}^{2+}]_i$ was determined (see Materials and Methods). Results are expressed as means \pm S.E.M. of at least 3 separate experiments containing 3 different diabetic or normal sera samples (depleted or non-depleted) in each experiment. Removal of C1q and C3 from Type-1 diabetic patient sera resulted in a significantly lower ($*P < 0.01$) increase in basal $[\text{Ca}^{2+}]_i$ compared with untreated Type-1 diabetic patient sera.

facilitated diffusion, and amino acids via specific transport proteins which are usually Na^+ -dependent. Metabolism of glucose by glycolysis, alanine via transamination to pyruvate, and leucine to acetyl-CoA will result in subsequent oxidation in the TCA cycle. This results in an increase in the ATP/ADP ratio, closure of ATP-sensitive K^+ channels, membrane depolarisation, opening of voltage-activated Ca^{2+} channels, Ca^{2+} influx, a rise in $[\text{Ca}^{2+}]_i$ and activation of the exocytotic machinery. The opening of Ca^{2+} channels is intermittent, oscillating with the membrane potential therefore resulting in oscillations of $[\text{Ca}^{2+}]_i$ (Santos *et al.* 1991, Gilon & Henquin 1992) that in turn trigger oscillations of insulin secretion (Gilon *et al.* 1993). Attenuation in oscillating $[\text{Ca}^{2+}]_i$ concentration, for example by exposure to a high extracellular concentration of glucose (27 mM) for extended periods (e.g. 48 h), results in attenuation of normal patterns of insulin secretion (Bjorklund *et al.* 2000).

We have demonstrated here that Type-1 diabetic patient sera provoked a sustained elevation in basal levels

of $[\text{Ca}^{2+}]_i$, as well as an attenuation in L-alanine-induced elevation of $[\text{Ca}^{2+}]_i$ by a complement dependent mechanism. Specifically, removal of the complement components C1q and C3 from the Type-1 patient sera resulted in a significant loss of the sustained increase of basal $[\text{Ca}^{2+}]_i$ induced by the Type-1 patient sera. Furthermore, the L-type Ca^{2+} channel antagonist, nifedipine, partially but not significantly attenuated the elevation of basal $[\text{Ca}^{2+}]_i$ mediated by Type-1 diabetic patient sera, suggesting that $[\text{Ca}^{2+}]_i$ was elevated via a mechanism partially independent of L-type Ca^{2+} channels.

Previous studies using a related β -cell line, RINm5F, have clearly demonstrated that Type-1 diabetic patient sera induced activation of Ca^{2+} currents, mainly through L-type channels, an effect that initiated cell death via apoptosis (Juntti-Berggren *et al.* 1993). The authors described in detail the kinetics in terms of current–time and current–voltage relationships. As the authors also confirmed the existence of similar sera-induced currents in primary β -cells, we did not explore Ca^{2+} channel properties further in the study reported here. However, we have reported in this paper that the sera-induced effect on BRIN BD11 cell $[\text{Ca}^{2+}]_i$ was dependent upon complement activation, did not induce apoptosis, but may have resulted in an inhibition of insulin secretion as previously reported (Conroy *et al.* 2000).

Since sustained levels of intracellular Ca^{2+} concentration have been documented to desensitise β -cells to subsequent insulin secretory signals (Bjorklund *et al.* 2000) we would like to propose that the mechanism by which Type-1 diabetic patient sera inhibits insulin secretion from clonal BRIN BD11 β -cells is dependent upon the following events: (i) complement activation after binding of β -cell specific antibodies present in diabetic patient serum; (ii) complement dependent sustained Ca^{2+} influx via L-type and non-L-type channels; (iii) desensitisation of the β -cell to subsequent nutrient stimuli. The desensitisation process may involve reduction in the activity of Ca^{2+} /calmodulin kinase II, an enzyme that is intimately involved in regulating the later stages of the insulin secretory process and which is known to be inhibited by sustained increased $[\text{Ca}^{2+}]_i$ (Harris *et al.* 2000). The desensitisation process may also involve decreased rates of nutrient metabolism, which would result in a reduction in the ATP/ADP ratio. We have reported a significant reduction in BRIN BD11 Ca^{2+} /calmodulin kinase II activity and ATP concentration after incubation for 24 h in Type-1 diabetic patient sera (Fig. 2).

Cell death

Complement activation and cellular attack is known to involve a rapid elevation of $[\text{Ca}^{2+}]_i$ within the target cell (Campbell *et al.* 1981, Morgan & Campbell 1985, Newsholme *et al.* 1993, 1999) which may result in

induction of death via apoptosis or necrosis (Juntti-Berggren *et al.* 1993, Caraher *et al.* 1999), or result in activation of recovery mechanisms (Morgan & Campbell 1985). Application of serum from Type-1 diabetic patients to rat islets and clonal β -cells clearly results in complement activation and disturbances in insulin secretion (Conroy *et al.* 2000 and references therein). Previous studies have shown that serum from Type-1 diabetic patients elevated $[\text{Ca}^{2+}]_i$ in rat islets and RINm5F β -cells which resulted in eventual death of the cells (Juntti-Berggren *et al.* 1993). However, in this paper we clearly demonstrate that the viability of clonal BRIN BD11 β -cells was not significantly different after incubation in FCS, normal human sera or Type-1 diabetic patient sera. Pro-inflammatory cytokines mediate loss in viability through a number of different signal transduction pathways which will result in induction of apoptosis. Indeed, the pro-inflammatory cytokines used in this study (TNF- α , IL-1 β and IFN- γ) have been shown to cause cell death in islets and other β -cell lines, when added either individually or together (Dunger *et al.* 1996, Eizirik *et al.* 1996, Delaney *et al.* 1997, Di Matteo *et al.* 1997, Hadjivassiliou *et al.* 1998). As reported in this paper, the clonal β -cell line, BRIN BD11, appears to respond similarly to these cytokines compared with other cell lines and islet cells with an elevated rate of production of nitric oxide, a significant reduction in the level of ATP, elevated levels of DNA fragmentation, ultimately resulting in apoptosis. However, it is known that ATP concentration must fall substantially before apoptosis is triggered directly by this mechanism. For example, the concentration of ATP was reduced by 90–95% in rat islet cells incubated in Type-1 diabetic patient serum, before cell death occurred by apoptosis (Caraher *et al.* 1999), while apoptosis was induced in insulin-producing pituitary cells overexpressing GLUT-2 and glucokinase after exposure to 20 mM glucose, a condition which also reduced ATP concentration by approximately 80% (Faradji *et al.* 2001). The latter paper also reported a drop in ATP concentration of approximately 40% in cells overexpressing glucokinase only, after exposure to 20 mM glucose, a condition which did not result in apoptosis of the cells. Therefore, apoptosis may be induced in the BRIN BD11 cell line by cytokines, via a mechanism that is only partially dependent upon a fall in ATP concentration. Thus we have demonstrated here that sera from Type-1 diabetic patients do not significantly alter ATP concentration, DNA fragmentation and levels of apoptosis in BRIN BD11 cells compared with normal human sera. There was a small but significant elevation in nitric oxide generation in BRIN BD11 cells incubated in Type-1 diabetic compared with control sera. However, the concentrations of nitric oxide produced under these conditions was only 30–50% of that produced in the presence of the pro-inflammatory cytokine cocktail.

The mechanisms underlying the pathogenesis of the disease of Type-1 diabetes are thought to involve

immune-mediated β -cell destruction (Eisenbarth 1986, Bach 1988, Castano & Eisenbarth 1990, Atkinson & Maclaren 1994). The dysfunctional state of the β -cell, in which the mechanisms of nutrient-stimulated insulin secretion are compromised, is generally accepted to be associated only with Type-2 diabetes. However, we suggest that normal patterns of nutrient-induced β -cell insulin secretion may be altered in the early stages of Type-1 diabetes, possibly via an auto-antibody/non-lethal complement activation response, resulting in a β -cell dysfunctional state. This dysfunctional state may result in an enhanced auto-immune response, leading eventually to β -cell destruction and the clinical appearance of the disease.

Our previous results have shown that Type-1 diabetic patient sera-derived complement activation resulted in inhibition of nutrient-induced insulin secretion (Conroy *et al.* 2000). In this paper we provide evidence that a sustained elevation in basal $[Ca^{2+}]_i$ occurred as a consequence of complement activation. In contrast, pro-inflammatory cytokines appear to inhibit insulin secretion via a mechanism involving elevated NO levels but not $[Ca^{2+}]_i$. Any possible contribution of NO to the mechanism(s) by which Type-1 diabetic sera inhibits insulin secretion appears to be minor.

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References

Ashcroft FM & Rorsman P 1989 Electrophysiology of the pancreatic β -cell. *Progress in Biophysics and Molecular Biology* **54** 87–143.
 Atkinson MA & Maclaren NK 1994 The pathogenesis of insulin-dependent diabetes mellitus. *New England Journal of Medicine* **331** 1428–1436.
 Bach J-F 1988 Mechanisms of autoimmunity in insulin-dependent diabetes mellitus. *Clinical and Experimental Immunology* **72** 1–8.
 Bjorklund A, Lansner A & Grill VE 2000 Glucose-induced $[Ca^{2+}]_i$ abnormalities in human pancreatic islets. Important role of overstimulation. *Diabetes* **49** 1840–1848.
 Campbell AK & Luzio JP 1981 Intracellular free calcium as a pathogen in cell damage initiated by the immune system. *Experientia* **37** 1110–1112.
 Campbell AK, Daw RA, Hallet MB & Luzio JP 1981 Direct measurement of the increase in intracellular free calcium ion concentration in response to the action of complement. *Biochemical Journal* **194** 551–560.

Caraher EM, Conroy SJ & Newsholme P 1999 Evidence for enhanced rates of complement activation in newly diagnosed IDDM patient sera exposed to rat islet cells and complement dependent induction of islet cell apoptosis. *Journal of Endocrinology* **162** 143–153.
 Castano L & Eisenbarth GS 1990 Type 1 diabetes: a chronic autoimmune disease of human, mouse, and rat. *Annual Review of Immunology* **8** 647–679.
 Comens P, Wolf BA, Unanue ER, Lacy PE & McDaniel ML 1987 Interleukin-1 is a potent modulator of insulin secretion from isolated rat islets of Langerhans. *Diabetes* **36** 963–970.
 Conroy SJ, Abdel-Wahab YHA, Caraher EM, Byrne PM, Flatt PR & Newsholme P 2000 Evidence for complement-dependent and independent inhibition of insulin secretion from clonal β -cells incubated in the presence of newly diagnosed IDDM patient sera. *Journal of Endocrinology* **164** 139–147.
 Delaney CA, Pavlovic D, Hoorens AD, Pipeleers G & Eizirik DL 1997 Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* **138** 2610–2614.
 Di Matteo MA, Loweth AC, Thomas S, Mabley JG, Morgan NG, Thorpe JR & Green IC 1997 Superoxide, nitric oxide, peroxynitrite and cytokine combinations all cause functional impairment and morphological changes in rat islets of Langerhans and insulin-secreting cell lines but dictate cell death by different mechanisms. *Apoptosis* **2** 164–177.
 Dunger AM, Cunningham JM, Delaney CA, Lowe JE, Green MHL, Bone AJ & Green IC 1996 Tumour necrosis factor- α and interferon- γ inhibit insulin secretion and cause DNA damage in unweaned-rat islets. Extent of nitric oxide involvement. *Diabetes* **45** 183–189.
 Easom RA 1999 CaM kinase II: a protein kinase with extraordinary talents germane to insulin exocytosis. *Diabetes* **48** 675–684.
 Eisenbarth GS 1986 Type 1 diabetes mellitus: a chronic autoimmune disease. *New England Journal of Medicine* **315** 1360–1368.
 Eizirik DL, Delaney CA, Green MHL, Cunningham JM, Thorpe JR, Pipeleers DG, Hellerström C & Green IC 1996 Nitric oxide donors decrease the function and survival of human pancreatic islets. *Molecular and Cellular Endocrinology* **118** 71–83.
 Faradji RN, Havari E, Chen Q, Gray J, Tornheim K, Corkey BE, Mulligan RC & Lipes MA 2001 Glucose-induced toxicity in insulin-producing pituitary cells that coexpress GLUT-2 and glucokinase. *Journal of Biological Chemistry* **276** 36695–36702.
 Gilon P & Henquin J-C 1992 Influence of membrane potential changes on cytoplasmic Ca^{2+} concentration in an electrically excitable cell, the insulin-secreting pancreatic β -cell. *Journal of Biological Chemistry* **267** 20713–20720.
 Gilon P, Shepherd RM & Henquin J-C 1993 Oscillations of secretion driven by oscillations of cytoplasmic Ca^{2+} as evidenced in single pancreatic islets. *Journal of Biological Chemistry* **268** 22265–22268.
 Hadjivassiliou V, Green MHL, James RFL, Swift SM, Clayton HA, & Green IC 1998 Insulin secretion DNA damage and apoptosis in human and rat islets of Langerhans following exposure to nitric oxide, peroxynitrite and cytokines. *Nitric Oxide* **2** 429–441.
 Harris TE, Persaud SJ, Squires PE & Jones PM 2000 Depolarizing stimuli reduce Ca^{2+} /calmodulin-dependent protein kinase II activity in islets of Langerhans. *Biochemical and Biophysical Research Communications* **270** 1119–1123.
 Henquin J-C 2000 Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **49** 1751–1760.
 Jones PM & Persaud SJ 1998 Ca^{2+} -induced loss of Ca^{2+} /calmodulin-dependent protein kinase II activity in pancreatic β -cells. *American Journal of Physiology* **274** E708–E715.
 Juntti-Berggren L, Larsson O, Rorsman P, Ammal K, Bokvist K, Wahlander K, Nicotera P, Dypbukt J, Orrenius S, Hallberg A & Berggren P-O 1993 Increased activity of L-type Ca^{2+} channels exposed to serum from patients with type-1 diabetes. *Science* **261** 86–90.

- Mauricio D & Mandrup-Poulsen T 1998 Apoptosis and the pathogenesis of IDDM: a question of life and death. *Diabetes* **47** 1537–1543.
- Morgan BP & Campbell AK 1985 The recovery of human polymorphonuclear leucocytes from sublytic complement attack is mediated by changes in intracellular free calcium. *Biochemical Journal* **231** 205–208.
- Nepom GT 1995 Glutamic acid decarboxylase and other autoantigens in IDDM. *Current Opinion in Immunology* **7** 825–830.
- Newsholme P, Adogu AA, Soos MA & Hales CN 1993 Complement-induced Ca^{2+} influx in cultured fibroblasts is decreased by the calcium channel antagonist nifedipine or by some bivalent inorganic cations. *Biochemical Journal* **295** 773–779.
- Newsholme P, Ashford MLJ & Hales CN 1999 Identification of a novel complement-dependent serum elicited inward current in the *Xenopus* oocyte provoking Ca^{2+} influx and subsequent activation of Cl^{-} channels. *Biochemical Pharmacology* **57** 491–501.
- Sandler S, Andersson A & Hellerström C 1987 Inhibitory effects of IL-1 on insulin secretion, insulin biosynthesis and oxidative metabolism of isolated rat pancreatic islets. *Endocrinology* **121** 42–44.
- Santos RM, Rosario LM, Nadal A, Garcia-Sancho J, Soria B & Valdeolmillos M 1991 Widespread synchronous $[Ca^{2+}]$ oscillations due to bursting electrical activity in single pancreatic islets. *Pflugers Archiv* **418** 417–422.
- Satin LS & Smolen PD 1994 Electrical bursting in β -cells of the pancreatic islets of Langerhans. *Endocrine* **2** 677–687.
- Southern C, Schulster D & Green IC 1990 Inhibition of insulin secretion by interleukin 1β and tumour necrosis factor- α by an L-arginine-dependent nitric oxide generating mechanism. *FEBS Letters* **276** 42–44.
- Wollheim CB & Pozzan T 1984 Correlation between cytosolic free Ca^{2+} and insulin release in an insulin-secreting cell line. *Journal of Biological Chemistry* **259** 2262–2267.

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