Relief from glucose-induced over-stimulation sensitizes the adenylate cyclase–cAMP system of rat pancreatic islets

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Abstract

Hyperglycemia impairs β -cell function. This effect is partly exerted by β -cell over-stimulation by mechanisms that are not completely clarified. We have presently investigated whether over-stimulation alters the responsiveness of the islet adenylate cyclase–cAMP system. Effects of over-stimulation were assessed from comparisons in rat pancreatic islets after stimulation by culture for 22 h with high (27 mM) glucose or after the additional presence of diazoxide which reversibly blocks secretion. Islet ATP levels were similar under both conditions. Forskolin increased islet cAMP levels dose-dependently after culture under both conditions; however, the cAMP responses to forskolin were enhanced by the previous co-presence of diazoxide: by 354, 183 and 168% respectively in the presence of 0.1, 1.0 and 25 μ M forskolin ($P < 0.05$) or less for the effect of diazoxide. Enhancement was not diminished by Ca^{2+} omission during final incubations, nor by

blocking Gi proteins with pertussis toxin (0.1 μ g/ml). Enhancement was dependent on the glucose concentration during culture, i.e. co-culture with diazoxide at a non-stimulatory concentration of glucose (6.0 mM) failed to affect the subsequent cAMP response to forskolin. Acute administration of glucose (16.7 mM) failed to increase islet cAMP content after culture at high glucose only, whereas a modest (about 20%) but significant stimulation was seen after co-culture with diazoxide. Co-culture with diazoxide left-shifted the insulin dose-response to a cAMP analogue 5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole-3',5'-cyclic monophosphorothioate. We conclude that over-stimulation importantly modifies the generation of cAMP, and also affects the insulin-releasing effect of the cyclic nucleotide.

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Introduction

A diabetic state impairs β -cell function both in animals (Leahy *et al.* 1992) and in man (Yki-Järvinen 1992). Both hyperglycemia (Leahy *et al.* 1992, Yki-Järvinen 1992) and elevated free fatty acids (Sako & Grill 1990a, Zhou & Grill 1994) are involved in this process. One effect of hyperglycemia is a rapidly (within 24 h) induced β -cell insensitivity to glucose. This effect of glucose is indirect. It can thus be protected against by inhibiting the insulin-releasing effect of glucose during hyperglycemia (Sako & Grill 1990b). Such inhibition can be achieved with diazoxide. Diazoxide antagonizes the closing effect of glucose on ATP-dependent potassium channels (Trube *et al.* 1986), thereby inhibiting glucose-induced insulin secretion and simultaneously protecting against β -cell insensitivity (Sako & Grill 1990b, Björklund & Grill 1993). Over-stimulation is likely to be important in human diabetes, since ' β -cell rest' is beneficial in patients with type 1 diabetes with residual β -cell function (Björk *et al.* 1996, DCCT Study 1998) and in patients with overt

type 2 diabetes (Greenwood *et al.* 1976, Garvey *et al.* 1985, Hollenbeck & Reaven 1987, Yki-Järvinen *et al.* 1988).

It is not known to what extent over-stimulation affects different second messenger systems of the β -cell. In this study we have used diazoxide as a probe to examine the effects of over-stimulation on the adenylate cyclase–cAMP system. Increased knowledge of the function of this system in β -cells during high glucose conditions could be important clinically, since cAMP-raising agents, such as glucagon-like peptide 1 (GLP-1), are currently being tested for insulin-releasing potency in type 2 diabetic patients (Holst 1996). Our experimental design was to induce a state of relative over-stimulation by culturing islets at a high glucose concentration (Björklund & Grill 1993, Björklund *et al.* 1997) and then to compare the functioning of the islet adenylate–cAMP system with that after co-culture with diazoxide. Specifically, the aims of the present study were (1) to test for effects of over-stimulation on cAMP responses to forskolin, a potent stimulator of adenylate cyclase, (2) to test for effect of

over-stimulation on a cAMP response to glucose and (3) to assess whether the insulin response to cAMP or a cAMP analogue is affected by over-stimulation.

Materials and Methods

Animals

Male Sprague–Dawley rats were obtained from B&K Universal AB, Sollentuna, Sweden. At the time of experiments they weighed between 220 and 420 g. All rats had free access to tap water and a standard pelleted diet (Rat and Mouse Standard Diet; B&K Universal AB). The pellets contained, on a weight basis, 60.3% carbohydrate, 20% protein, 4.4% fat, 3.3% supplements (vitamins and minerals), 3% water and 4.1% ashes. A 12-h light:12-h darkness cycle (0600–1800 h) was enforced. The protocols used were approved by the Stockholm Ethics Committee for Animal Experiments.

Materials

Forskolin (dissolved in dimethyl sulfoxide) was from Sigma Chemical Co., St Louis, MO, USA, (Sp)5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole-3',5'-cyclic monophosphorothioate (cBIMPS) from Biolog Life Science Institute, Bremen, Germany, and diazoxide (Hyperstat) from Schering-Plough, Labo N.V., Heist-op-den-Berg, Belgium. Bovine serum albumin, fraction V (BSA) and pertussis toxin were obtained from Sigma.

Isolation and culture of islets

Pancreatic islets were isolated by digestion with collagenase (Boehringer–Mannheim, Mannheim, Germany) as described (Lacy & Kostianovsky 1967). Digestion and sedimentation of islets were carried out in Hanks' medium containing 5.5 mM glucose. Islets were then selected under a stereo microscope and transferred to Petri dishes (Sterilin, Teddington, Middx, UK). The islets were cultured for 20–22 h at 37 °C, in a gas phase of 5% CO₂ in air. Each dish contained 4.5 ml RPMI 1640 (Statens veterinär medicinska anstalt, Uppsala, Sweden) with 27 mM glucose or (in some experiments) 6 mM glucose and 10% heat inactivated newborn calf serum (Sigma). The concentration of diazoxide, when present, was 325 μ M. This concentration was chosen so as to provide at least 85% inhibition of glucose-induced insulin secretion (Björklund & Grill 1993, Björklund *et al.* 1997).

Measurements of cAMP

Islets cultured as above were placed in batches of three into 'baskets' as previously described (Grill *et al.* 1977). The 'baskets' were prepared by cutting microcentrifuge

plastic tubes (Beckman Ultra-Micro System, Spinco (Labora, Stockholm, Sweden)). The hollow cylinders thus obtained were capped with nylon gauze (70 threads/cm² gauze, 36% open space). For preincubations, 'baskets' were immersed 0.5 cm in a Petri dish containing Krebs–Ringer bicarbonate (KRB) buffer (Cohen 1957) with 0.05% BSA and 3.3 mM glucose for a period of 30 min at 37 °C. Final incubations were started by transferring each 'basket' to a test tube with 300 μ l KRB which contained 3.3 mM glucose, 16.7 mM glucose or 3.3 mM glucose together with a forskolin concentration which was varied as specified below. Incubations were carried out for 5 or 15 min in a shaking water bath at 37 °C. Three to four 'baskets' were run for each experimental condition. To extract cAMP these final incubations were terminated by transferring 'baskets' into new tubes containing 200 μ l 50 mM sodium acetate buffer. The 'baskets' were kept at 90–100 °C in the sodium acetate-containing tubes for 5 min. The remaining KRB media of the 15-min incubations were saved for later determinations of insulin.

Islet contents of cAMP were determined in 0.15 ml of boiled islet extracts using a kit (Amersham International plc, Amersham, Bucks, UK) in which acetylation was performed with acetic anhydride and triethylamine in volumes of 1:2 before adding [¹²⁵I]cAMP and antiserum. The antibody-bound fraction was separated by centrifugation and counted in a gamma scintillation counter. The sensitivity of the assay was 1.0 fmol and the range of concentrations assayable from the standard curve was 2–128 fmol/tube. The intra-assay coefficient of variation (C.V.) was 4–14% and the interassay C.V. was 5.4–7.0%.

Insulin release

Following culture, islets were preincubated in 5 ml KRB (Cohen 1957) medium containing 10 mM Hepes, 0.2% BSA and 3.3 mM glucose for 30 min at 37 °C. Islets were then transferred to tubes containing 300 μ l KRB with additions dictated by each protocol. (When Ca²⁺ was omitted, CaCl₂ was substituted with NaCl in an equimolar concentration.) Three to four tubes with three islets each were run for each experimental condition. Final incubations were carried out for 15 or 60 min at 37 °C in a shaking water bath. At the end of incubations a sample of the medium was secured for insulin assay. Immunoreactive insulin was measured using rat insulin as standard and insulin antibodies which had been raised in our laboratory against porcine insulin. Bound and free insulin were separated after charcoal addition (Herbert *et al.* 1965).

ATP

ATP was measured by the firefly lantern luciferase method (Idahl 1979). After culture, batches of five islets were put into Beckman tubes containing an NaOH solution (0.04 M NaOH, 2 mM EDTA) then frozen immediately

on a mixture of ethanol/dry ice and stored at -70°C . A 10^{-5} M solution of ATP was prepared in Hepes buffer containing 25 mM Hepes, 10 mM MgSO_4 , 2 mM EDTA, 1 mg/ml BSA, pH=7.5 and stored at -70°C . From this stock solution, standard solutions (2, 1, 0.5, 0.25 and 0.125 pmol ATP/20 μl) in Hepes buffer were prepared shortly before they were required. Before assay, islets were homogenized and incubated at 60°C in a waterbath for 10 min. The islet extracts were mixed with Hepes buffer containing luciferin, and luciferase (kindly provided by Professor Hans Löw) was added. Standard and blanks were treated identically to samples. Duplicate measurements were made from each batch of islets. Luminiscence was measured in a Packard scintillation counter.

Expression of results

Results are expressed as means $\pm$ S.E.M. Significance testing was performed using Student's *t*-test for unpaired and paired data as appropriate and indicated in Tables, Figures and text. A *P* value <0.05 was considered significant.

Results

Islet cAMP contents under basal conditions

Contents of cAMP during non-stimulatory conditions (i.e. in the presence of 3.3 mM glucose) were assessed by pooling data from 42 experiments (some of which were used in other experiments not related to this study). Islets cultured without diazoxide contained 12.3 ± 0.7 pmol/islet and islets cultured with diazoxide 12.8 ± 0.7 pmol/islet ($P>0.20$). Thus, previous use of diazoxide did not alter cAMP contents in rat islets during non-stimulatory conditions.

Effects of 0.1–25 μM forskolin on islet cAMP

In islets pre-cultured with 27 mM glucose alone, forskolin increased cAMP by 2.0 ± 1 , 28.0 ± 9 and 171 ± 21 fmol/islet at forskolin concentrations of 0.1, 1.0 and 25 μM (Fig. 1A). These effects of forskolin were obtained in the co-presence of 3.3 mM glucose.

The stimulatory effects of forskolin were significantly increased in diazoxide-precultured islets. The cAMP responses were thus 7.1 ± 0.8 , 51 ± 14 and 288 ± 41 fmol/islet respectively, $P<0.025$ or less for the effects of diazoxide vs 27 mM glucose alone (Fig. 1A). The

Figure 1 Effects of forskolin on cAMP levels. Islets were pre-cultured for 20–22 h in (A) 27 mM glucose or (B) 6.0 mM glucose in the presence (solid bars) or absence (open bars) of 325 μM diazoxide. Data are means $\pm$ S.E.M. for (A) six or (B) four experiments. * $P<0.025$ or less for effect of diazoxide.

Table 1 Effects of calcium omission on islet cAMP. Islets were cultured for 20–22 h in 27 mM glucose in the absence or presence of 325 μM diazoxide. Ca²⁺ was omitted during 30-min preincubations and 15-min final incubations. Data are means ± S.E.M. fmol/islet per 15 min; there were seven experiments

Final incubations			Culture conditions	
Glucose (mM)	Forskolin (μM)	Calcium added (2.5 mM)	Glucose (27 mM)	Glucose (27 mM) and diazoxide (325 μM)
3.3	—	+	13.0 ± 1.1	13.9 ± 2.0
3.3	25	+	227 ± 43	430 ± 79*
3.3	—	—	15.8 ± 1.8	13.9 ± 2.0
3.3	25	—	293 ± 47	487 ± 88*

*P<0.025 for effect of diazoxide.

amplifying effect of previous diazoxide amounted to 354, 183 and 168% of the cAMP responses in islets not pretreated with diazoxide.

It was important to determine whether the effects of diazoxide were due to inhibition of glucose stimulation rather than to an effect of the drug *per se*. To this end, islets were cultured for 20 h at a non-stimulatory concentration of glucose (6.0 mM) in the absence or presence of diazoxide. During these conditions diazoxide pretreatment did not affect forskolin-induced increases in cAMP (Fig. 1B).

Effects of calcium omission on the forskolin-induced rise in islet cAMP

Forskolin is known to directly stimulate adenylate cyclase; however, such stimulation could possibly be potentiated by a calcium-dependent and calmodulin-mediated enhancement of adenylate cyclase activity (Sharp 1979, Valverde *et al.* 1979). In the present work, calcium omission during preincubation and final incubations failed to inhibit the rise in cAMP evoked by 25 μM forskolin; instead calcium omission tended to increase cAMP levels (Table 1). Importantly, calcium omission failed to affect the enhancement by diazoxide of forskolin-stimulated cAMP contents. Similar results were obtained when final incubations contained a low concentration (0.1 μM) of forskolin (*n*=6, results not shown).

Effect of pertussis toxin on the forskolin-induced rise in cAMP

Specific G proteins (Gi) inhibit adenylate cyclase activity and this effect is blocked by pertussis toxin. We used pertussis toxin (0.1 μg/ml) to investigate whether increased Gi activity mediates the over-stimulation effects on cAMP. Such a notion would be supported if pertussis toxin abolished or attenuated the difference in forskolin-induced cAMP between non-diazoxide- and diazoxide-

Figure 2 Effect of pertussis toxin (0.1 μg/ml) during preincubation and final incubations for 15 min on the forskolin-induced cAMP response. The concentration of forskolin was 25 μM in the presence of 3.3 mM glucose. Islets were pre-cultured for 20–22 h in 27 mM glucose in the presence (solid bars) or absence (open bars) of 325 μM diazoxide. Data are means ± S.E.M. of three experiments. *P<0.05 or less for effect of diazoxide.

pretreated islets. Such findings were not obtained; in contrast, pertussis toxin tended to increase the difference in forskolin-induced cAMP between non-diazoxide- and diazoxide-pretreated islets. The effect of diazoxide was thus an increase from 163 to 360 fmol/islet (2.2-fold) in islets treated with pertussis toxin as compared with an increase from 87 to 143 fmol/islet (1.6-fold) in islets not treated with the toxin (Fig. 2).

Effects of 11 mM glucose on the forskolin-induced rise in cAMP

Interactions between forskolin and glucose on cAMP levels were investigated by comparing the effects of 0.1 μM forskolin at a stimulatory and a non-stimulatory concentration of glucose (Table 2). Glucose (11 vs 3.3 mM glucose, in combination with forskolin) failed to significantly affect cAMP contents. At both combinations of glucose and forskolin, the cAMP contents were lower in non-diazoxide- than in diazoxide-pretreated islets.

Effects of 16.7 mM glucose on islet cAMP

Stimulation with 16.7 mM glucose in the final incubations failed to induce an islet cAMP response in islets pre-cultured at high glucose (Fig. 3). However, islets that had been cultured in the concomitant presence of diazoxide were responsive. In these islets, 16.7 mM glucose

Table 2 Effects of 11 mM glucose on forskolin-induced rise in cAMP. Islets were cultured for 20–22 h in 27 mM glucose in the absence or presence of 325 μ M diazoxide. Data are means $\pm$ S.E.M. fmol/islet per 15 min; there were eight experiments

Final incubations		Culture conditions	
Glucose (mM)	Forskolin (μ M)	Glucose (27 mM)	Glucose (27 mM) and diazoxide (325 μ M)
3:3	—	15.4 $\pm$ 0.6	16.1 $\pm$ 0.5
3:3	0.1	19.3 $\pm$ 0.9	22.9 $\pm$ 1.5*
11	—	16.0 $\pm$ 0.5	17.2 $\pm$ 0.7
11	0.1	20.0 $\pm$ 1.3	23.7 $\pm$ 1.5*

* $P < 0.025$ for effect of diazoxide.

increased cAMP levels modestly but significantly (+21% after 5-min and +17% after 15-min final incubations, $P < 0.05$, Fig. 3).

ATP

Because ATP is the substrate for adenylate cyclase we determined islet contents of ATP after culture with 27 mM glucose without and with diazoxide. Previous diazoxide failed to affect islet contents of ATP (12.0 $\pm$ 2.6 pmol/islet without and 11.2 $\pm$ 1.4 after culture with diazoxide, $n = 4$).

Insulin responses to forskolin and to glucose

At low (3.3 mM) glucose, forskolin induced only modest insulin responses (Fig. 4A).

Forskolin-induced insulin responses were greatly potentiated by increasing the glucose concentration to 11 mM (compare Fig. 4A and B). Enhancement by glucose was less marked in islets cultured without than in those cultured with diazoxide. The after-effect of diazoxide was further enhanced in final incubations performed for 60 compared with 15 min (compare Fig. 4B and C). The difference was not due to an effect of diazoxide *per se* since previous diazoxide was without effect when the culture medium contained 6.0 rather than 27 mM glucose (results not shown)

Effect of 0.01–0.1 mM cBIMPS on insulin release

In order to further evaluate the effects of cAMP on insulin secretion during conditions of over-stimulation, an analogue of cAMP (cBIMPS) was added to intact islets. This compound has shown increased lipophilicity compared with 8-(p-chlorophenylthio)-cAMP (Sandberg *et al.* 1991). It also potently and specifically activates cAMP-dependent protein kinase, and is resistant to hydrolysis by

Figure 3 Effects of 16.7 mM glucose on islet cAMP content and insulin release (insert). Islets were pre-cultured for 20–22 h in 27 mM glucose in the presence (solid bars) or absence (open bars) of 325 μ M diazoxide. Data are means $\pm$ S.E.M. of six or seven experiments. * $P < 0.05$ or less for effect of glucose.

cyclic nucleotide phosphodiesterases (Sandberg *et al.* 1991). In non-diazoxide-treated islets, only the highest concentration (0.1 mM) of cBIMPS (tested at 6.0 mM glucose) increased insulin release (Fig. 5). After pretreatment with diazoxide the lower concentrations of 0.025 and 0.05 mM cBIMPS were also stimulatory (Fig. 5, $P < 0.025$ for effect of diazoxide pretreatment). With 0.1 mM cBIMPS, insulin secretion in diazoxide-cultured islets was not significantly higher than from islets without diazoxide pretreatment ($P < 0.15$, Fig. 5).

Figure 4 Forskolin-induced insulin release at (A) 3.3 or (B and C) 11 mM glucose. (A and B) 15-min final incubation and (C) 60-min final incubation. Islets were pre-cultured for 20–22 h in 27 mM glucose in the presence (solid bars) or absence (open bars) of 325 μM diazoxide. Data are means $\pm$ S.E.M. of six experiments. * $P < 0.05$ or less for effect of diazoxide.

Figure 5 Effects of 0.01–0.1 mM cBIMPS in intact islets. Islets were pre-cultured for 20–22 h in 27 mM glucose in the presence (●) or absence (○) of 325 μM diazoxide. Data are means $\pm$ S.E.M. of seven experiments. * $P < 0.05$ or less for effect of diazoxide.

Discussion

Our study demonstrates, for the first time, that over-stimulation exerts several important effects on the adenylate cyclase–cAMP signal transduction pathway. These effects include, in intact islets, a decreased cAMP response to forskolin, a complete loss of a modest cAMP response to glucose and a right-shift of the insulin response to a cAMP analogue.

The dose–response experiments with forskolin demonstrate that diazoxide pretreatment augments the cAMP responses at all concentrations of forskolin. Since forskolin acts at the catalytic subunit of adenylate cyclase (Seamon & Daly 1981) our findings indicate a basic (not receptor-related) influence by over-stimulation on adenylate cyclase activity. This influence of over-stimulation seems not to be due to substrate (ATP) deficiency, since ATP contents were similar in diazoxide-pretreated and untreated islets. Furthermore, our results with pertussis toxin indicate that Gi proteins are not involved in the over-stimulation effect.

Our results also demonstrate a complete loss of a cAMP response to a high (16.7 mM) glucose concentration. Such a response is known to be modest in freshly isolated islets of the rat and was also so in islets cultured in the presence of diazoxide. Loss of a cAMP response to glucose during culture was reported many years ago (Rabinovitch *et al.* 1978) and more recently confirmed (Laychock 1995). Our results with diazoxide offer over-stimulation as a possible explanation for the loss of a glucose-induced cAMP response after culture.

Our results do not support a role of Ca^{2+} fluxes behind the effects of diazoxide on the forskolin-induced cAMP

response, since the enhancing effect of previous diazoxide was also upheld after Ca^{2+} omission. In this context, it is interesting that cAMP responses to forskolin were increased rather than decreased by Ca^{2+} omission. Such an enhancing effect could be secondary to activation of Ca^{2+} -inhibitable isoforms of adenylate cyclase which have been demonstrated in β -cells (Leech 1999).

The dose-response to different concentrations of a potent (Laychock 1993) cAMP analogue (cBIMPS) suggests that relief from over-stimulation by previous diazoxide left-shifted the potency of cAMP to release insulin. This notion is compatible with the insulin data obtained with forskolin at 3.3 mM glucose (Fig. 4A). However, at 11 mM glucose, a high forskolin concentration (25 μM) exerted a more marked insulin response in diazoxide-pretreated than in non-pretreated islets. Although the per cent increase due to diazoxide was smaller with 25 than with 0.1 μM forskolin, the last mentioned data nevertheless indicate an effect on maximal capacity for secretion. This could, in turn, be secondary to a well-documented twofold difference in islet insulin content (Björklund & Grill 1993). It seems possible that relief from over-stimulation has two effects: one that leads by unknown mechanisms to a left-shift in potency of cAMP for insulin secretion, another that affects maximal capacity for secretion because of higher islet insulin content. Further studies are needed to assess the relative roles of these putative mechanisms.

It is interesting to note that the adenylate cyclase stimulators glucagon and GLP-1 can induce insulin secretion in type 2 diabetic patients (Gutniak *et al.* 1992, Holst 1996, Nauck 1998) and that such stimulation is observed under conditions of over-stimulation and β -cell insensitivity to glucose (Castillo *et al.* 1996). Our finding that the potential for large cAMP responses at high concentrations of forskolin is retained after high glucose culture may offer a molecular basis for the potency of the cAMP-raising secretagogues when used in pharmacological concentrations.

In summary, our study has shown, for the first time, that 'resting' β -cells from intense and prolonged glucose-induced stimulation enhance the adenylate cyclase-cAMP transduction pathway, both with regard to a cAMP response and to the effect of the second messenger on insulin secretion. The enhancing effect on forskolin-induced cAMP responses is not dependent on Ca^{2+} inflow, nor on substrate (ATP) availability, nor on interactions with Gi proteins. As to insulin responses to cAMP, our results also indicate that enhancement is due, at least in part, to a left-shift in the dose-response for cAMP.

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