cAMP-mediated signaling normalizes glucose-stimulated insulin secretion in uncoupling protein-2 overexpressing β-cells

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Abstract

We investigated whether an increase in cAMP could normalize glucose-stimulated insulin secretion (GSIS) in uncoupling protein-2 (UCP2) overexpressing (*ucp2*-OE) β-cells. Indices of β-cell (β-TC-6f7 cells and rodent islets) function were measured after induction of *ucp2*, in the presence or absence of cAMP-stimulating agents, analogs, or inhibitors. Islets of *ob/ob* mice had improved glucose-responsiveness in the presence of forskolin. Rat islets overexpressing *ucp2* had significantly lower GSIS than controls. Acutely, the protein kinase A (PKA) and *epac* pathway stimulant forskolin normalized insulin secretion in *ucp2*-OE rat islets and β-TC-6f7 β-cells, an effect blocked by specific PKA inhibitors but not mimicked by *epac* agonists. However, there was no effect of *ucp2*-OE on cAMP concentrations or PKA activity. In *ucp2*-OE islets, forskolin inhibited ATP-dependent potassium (K_{ATP}) channel currents and $^{86}Rb^{+}$ efflux, indicative of K_{ATP} block. Likewise, forskolin application increased intracellular Ca^{2+}, which could account for its stimulatory effects on insulin secretion. Chronic exposure to forskolin increased *ucp2* mRNA and exaggerated basal secretion but not GSIS. In mice deficient in UCP2, there was no augmentation of either cAMP content or cAMP-dependent insulin secretion. Thus, elevating cellular cAMP can reverse the deficiency in GSIS invoked by *ucp2*-OE, at least partly through PKA-mediated effects on the K_{ATP} channel.

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Introduction

Previously, uncoupling protein-2 (UCP2) was identified in islets and shown to be a negative regulator of insulin secretion (Chan *et al.* 1999, 2001, Hong *et al.* 2001, Zhang *et al.* 2001). *Ucp2* mRNA and protein expressions are increased in obese, diabetic rodents (Zhang *et al.* 2001) and may contribute to the development of insulin insufficiency. In addition, genetic studies in humans show an association between carriers of −866A/A allele, reduced glucose-stimulated insulin secretion (GSIS) and type-2 diabetes (D'Adamo *et al.* 2004, Sasahara *et al.* 2004). A potential strategy for improving insulin secretion in diabetic patients is to directly lower UCP2 activity. However, another strategy might be to find conditions that indirectly negate the effects of UCP2 overexpression.

The enzyme adenylate cyclase catalyzes the conversion of ATP to cAMP, an important second messenger involved in potentiating rather than initiating insulin secretion (Prentki & Matschinsky 1987). Deficient cAMP production has been implicated in streptozotocin diabetes (Dachicourt *et al.* 1996), while increasing cAMP with forskolin improved GSIS in Goto-Kakizaki (GK) rats (Abdel-Halim *et al.* 1996). It is unknown whether enhancing cAMP production can ameliorate insulin secretion in *ob/ob* mice or other models where UCP2 is upregulated.

cAMP is proposed to influence insulin secretion at multiple steps via two distinct pathways, one mediated by protein kinase A (PKA) and the other by cAMP-regulated guanine nucleotide exchange factors (cAMP-GEFs, also known as *epac*; Holz 2004). Two important cAMP-dependent steps that also require adequate intracellular ATP are exocytosis, including regulation and replenishment of the readily releasable pool of granules (Kashima *et al.* 2001, Eliasson *et al.* 2003) and ATP-dependent potassium (K_{ATP}) channel inactivation (MacDonald *et al.* 2002).

We tested the hypothesis that elevation of cAMP subsequent to upregulation of UCP2 expression will normalize insulin secretion because cAMP is an important potentiator of GSIS, acts at many steps in stimulus-secretion pathways of β-cells and has been shown to ameliorate defective insulin secretion in other models.

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Materials and Methods

Isolation of rat and mouse islets

All animals used in the present research were cared for in accordance with the Guidelines of the Canadian Council on Animal Care. The protocols were approved by animal use committees from the University of Toronto and the University of Prince Edward Island. Rats were anesthetized with sodium pentobarbital (0·1 ml/100 g). Islets were harvested from 10-week-old male and female lean Zucker rats (Charles River Laboratories, Boston, MA, USA and AVC born) and from 8-week-old lean and *ob/ob* mice (Jackson Labs, Bar Harbour, ME) and cultured as described (Kibenge & Chan 1995).

Adenoviral infection of rodent islets

Islets were infected with adenovirus encoding the full-length human or rat cDNA for *ucp2* (Ad*ucp2*) as described (Chan *et al.* 1999, 2001, Joseph *et al.* 2004) for 48 h. Control islets received no treatment and were simply cultured in DMEM (Ducbecco's modified Eagle medium; Invitrogen) for 48 h, because non-specific adenoviral infection had not been shown to affect insulin secretion (Chan *et al.* 1999). In some experiments, isolated rat islets were cultured overnight in DMEM supplemented with 10 μM forskolin (Sigma). Overexpression of *ucp2* was confirmed by quantitative reverse transcriptase (RT)-PCR as described (Joseph *et al.* 2002).

Perfused mouse pancreas

Control (wild type, WT) and *ucp2* knockout (KO) mice were raised in a colony at the University of Toronto. Generation of the KO line has been described previously (Zhang *et al.* 2001). The pancreases from 4-month-old male *ucp2* KO mice were perfused after an overnight fast. The anesthetic was i.p. sodium pentobarbital (80 mg/kg). Details of the perfusion protocol are given elsewhere (Joseph *et al.* 2002). In these experiments, the glucose infusion was changed from 1·4 to 13·4 mM after 5 min, then returned to 1·4 mM at 25 min. Exendin-4 (1 nM, Bachem, Bubendorf, Switzerland), which acts as an agonist at GLP-1 receptors (Raufman *et al.* 1992) was added in some experiments.

Cell culture and transient transfection of βTC-6f7 cells

Cultures of βTC-6f7 (β-TC) cells were grown in RPMI-1640 media (Invitrogen), pH 7·4 as described (Chan *et al.* 2001). For transient gene transfection, β-TC cells were plated at a density of $2{\cdot}5\times10^5$ cells/cm^2 and cultured overnight. Plasmids containing either *ucp2* or enhanced green fluorescent protein (*egfp*) cDNA were produced as described (Chan *et al.* 2001) and transfection carried out as per the instructions of the manufacturers of the transfection agent (Lipofectamine^{2000}, Invitrogen). The degree of *ucp2* overexpression (*ucp2*-OE) induced was assessed by quantitative RT-PCR, as for rat islets (Joseph *et al.* 2002).

cAMP content

Control and *ucp2*-OE islets were exposed to 0–10 μM forskolin (Sigma) or exendin-4 (1 nM) in the presence of 2·8 or 11 mM glucose in KRBB–HEPES for 15 min at 37 °C. The medium was then removed and 70% ethanol (0·5 ml/well) was added to the cell pellets. Cell lysates were frozen at −20 °C until assayed by RIA using the acetylation protocol provided in the kit for increased sensitivity (Biomedical Technologies, Inc., Stoughton, MA, USA).

Insulin secretion from cell lines or islets

Control and *ucp2*-OE β-TC cells or islets were prepared as described for cAMP formation and incubated for 2 h at 37 °C. Where indicated in the Results, additional test compounds were added to islet incubations. The PKA inhibitors, H89 and KT5720 (Sigma), were added to test wells 30 min prior to the addition of forskolin for a further 90 min. The medium was aspirated and placed in clean 1·5 ml microcentrifuge tubes, which were then centrifuged briefly to remove cellular debris. To each cell pellet, 1 ml 3% acetic acid was added to extract the total remaining insulin. All samples were frozen at −20 °C until assayed. Concentrations of insulin were determined by RIA.

Protein kinase A activity

The effects of *ucp2*-OE on PKA activity were assessed in rat islets using a colorimetric assay (Stressgen, Victoria, BC, Canada). Control and *ucp2*-OE islets (100 per condition) were incubated with 1 μM forskolin ± 10 μM H89 (Calbiochem, La Jolla, CA, USA) for 30 min, and washed in 0·5 ml PBS (pH 7·4). The islet cytosol was reserved following the cell lysis as recommended in the manufacturer's instructions. The lysate (60 islet equivalents in 30 μl aliquots) was incubated for 30 min with the ATP substrate. Detection of phosphorylated proteins was facilitated by incubation with phosphoprotein-specific antibody. Although the assay was performed with crude extracts, the ability to inhibit most of the activity with H89 suggests that the majority of kinase activity was due to PKA.

ATP content

ATP and ADP were quantified in *ucp2* KO mouse islets by the method of Schultz *et al.* (1993).

Assessment of K_{ATP} channel activity

Efflux of radiolabeled $^{86}Rb^+$ was quantified to estimate changes in K^+ channel activity in islets as described previously (Chan & MacPhail 1996). Islets were loaded with ^{86}RbCl

(Amersham) for 90 min at 37 °C, during which time the radiolabel was taken up into the islet cells. After washing twice, the islets were resuspended in KRBB–HEPES containing low (2·8 mM) or high (11 mM) glucose to determine what degree of K channel activity was glucose metabolism-dependent and therefore reflective of K_{ATP} channels. In some tubes, 1 μM forskolin ± 1 μM H89 were also added. After 20 min, the supernatant buffer and the islet pellets were transferred to clean vials for scintillation counting. Results were expressed as the fractional efflux of $^{86}Rb^+$/20 min.

Electrophysiological assessment of K_{ATP} channel activity was performed using patch clamping of intact islets infected with control (AdEmpty) or Ad*ucp2* virus. Islets were held in the recording chamber with the assistance of a suction pipette. K_{ATP} current was measured in the perforated-patch configuration during 200 ms 20 mV incremental step-depolarizations from −140 to −60 mV from a holding potential of −70 mV. Mean outward current at each potential was measured during the last 50 ms of depolarization. The pipette solution contained (mM) K_2SO_4 28·4, KCl 63·7, NaCl 11·8, $MgCl_2$ 1, HEPES 20·8, EGTA 0·5, pH 7·2 with KOH + 0·1 mg/ml amphotericin B (Sigma). The bath solution contained (mM) NaCl 115, $CaCl_2$ 3, KCl 5, $MgCl_2$ 2, glucose 11·1, HEPES 10, pH 7·2 with NaOH. Islets were held in a static bath maintained at 34 °C by an automatic temperature controller (TC-324B, Warner Instrument Corp., Hamden, CT, USA). Current recordings were amplified, digitized, and analyzed using pClamp9.0 software (Axopatch 200B, Axon Instruments, Union City, CA, USA).

Calcium flux measurement

β-TC cells or β-cells from dispersed islets were plated on glass coverslips (about 80% confluency) and transfected with plasmid encoding *ucp2* or *egfp* (control) as described in Chan *et al.* (2001). Fluorescent measurements were conducted 48 h after transfection. Changes in intracellular Ca^{2+} concentrations were assessed using Fura 2-AM. The cells were loaded with 5 μM Fura 2-AM for 50 min in a medium containing (in millimolar) 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 $NaHCO_3$, 10 HEPES, pH 7·4 at 37 °C. The cells were then washed, transferred to an open chamber, placed on an Olympus BX51W1 fluorescent microscope stage and perfused with the same medium at 1 ml/min. All experiments were performed at 36–37 °C using a TC-324B Heater Controller (Warner Instruments). For excitation, a xenon lamp-based DeltaRam high-speed monochromator (Photon Technology International, London, ON, Canada) was used. Cells were excited by dual excitation at 340/380 nm and emission was detected by a 510 nm band pass filter. For control of the monochromator and videocamera, as well as for fluorescent imaging and collecting of data the ImageMaster 3 software (Photon Technology International) was used. The basal intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was calculated according to the equation $[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min})/(R - R_{max})$, where R is the fluorescence ratio at 340 and 380 nm excitations (F_{340}/F_{380}), R_{min} and R_{max} are the fluorescence ratios at Ca^{2+}-free and Ca^{2+}-saturating conditions, K_d is the dissociation constant (= 224 nM), and $\beta = F_{380}$ (Ca^{2+}-free)/F_{380} (Ca^{2+}-saturating). Saturating and Ca^{2+}-free conditions were obtained by the addition of 10 μM ionomycin (Sigma) to cells incubated with 5 mM Ca^{2+}, and by addition of 10 mM EGTA to cells incubated in Ca^{2+}-free medium.

Statistical analysis

Data are presented as means ± S.E.M. Results were compared by unpaired *t*-test, one- or two-way ANOVA, where applicable, and $P < 0·05$ was considered significant. Prism 3.0 software (GraphPad, San Diego, CA, USA) was used for the statistical analysis.

Results

Glucose- and forskolin-stimulated insulin secretion in ob/ob *mouse islets*

To determine if forskolin could restore GSIS in a model of insulin insufficiency and documented overexpression of UCP2 (Zhang *et al.* 2001), islets were obtained from lean and *ob/ob* mice. In these experiments, *ob/ob* islets had a ~twofold increase in *ucp2* mRNA expression compared with age-matched lean mouse islets (Table 1). In lean islets, glucose alone increased insulin secretion by ~twofold over basal. Forskolin potentiated the response to higher glucose concentrations (Fig. 1A). Basal insulin secretion was elevated in *ob/ob* mice and there was no response to glucose. Addition of 10 μM forskolin significantly increased the insulin secretion at glucose concentrations from 5·5 to 22·0 mM (Fig. 1B).

Forskolin-stimulated cAMP formation and insulin secretion

Infection of rat islets with Ad*ucp2* induced an 19-fold increase in *ucp2* mRNA (Table 1). In these experiments, the ability of forskolin, an adenylate cyclase activator, to stimulate insulin secretion was tested. Both UCP2 expression ($P < 0·0001$) and forskolin concentration ($P < 0·0001$) influenced insulin secretion. A significant two-way interaction ($P < 0·005$) revealed that *ucp2*-OE cells responded differently compared with control islets to forskolin. Insulin secretion from Ad*ucp2*-infected cells was lower than control cells at 0 and 0·1 μM forskolin ($P < 0·03$) but was not different at 1 and 10 μM forskolin (Fig. 2A). Thus, at higher doses, forskolin normalized insulin secretion in Ad*ucp2*-infected rat islets. Similar effects were observed in clonal β-TC cells (not shown; Chan *et al.* 2001).

To determine if the effects of forskolin on insulin secretion were dependent on PKA activation, islets were preincubated with 10 μM H89 or 1 μM KT5720 for 30 min prior to the addition of forskolin. Figure 2B shows that inhibition of PKA by H89 and KT5720 blocked the stimulatory effects of

Table 1 Induction of UCP2 in various experimental models

	Ratio of UCP2 mRNA expression to GAPDH mRNA	Fold increase
Experimental model		
ob/ob Mouse islets (4)	0·13±0·01*	1·86
Lean mouse islets (5)	0·069±0·020	
β-TC+*ucp2* plasmid (3)	1·82±0·21†	28·2
β-TC (3)	0·064±0·007	
Rat islets+AdUCP2 (7)	0·21±0·08†	19·4
Rat islets (9)	0·011±0·007	

Expression data are means±S.E.M. *$P<0·05$ and †$P<0·001$ compared with respective controls.

forskolin on insulin secretion in both *ucp2*-OE and control islets. That *ucp2*-OE had no effect on PKA activity in islets is verified in Fig. 2C. Moreover, the increase in PKA activity, as measured by the production of phosphoproteins, was totally negated in the presence of 10 μM H89.

cAMP concentrations were measured following exposure to forskolin in rat islets that had normal or elevated *ucp2* mRNA expression. In the presence of 11 mM glucose, a similar concentration response to forskolin was seen in control and Ad*ucp2*-infected islets (Fig. 2D, $P<0·0001$). No differences between control and Ad*ucp2*-infected β-cells were observed at any forskolin concentration.

cAMP analogs and insulin secretion

The ability of cell-permeant cAMP analogs 8-Br-cAMP (0·1 mM) and Sp-cAMPs (0·1 mM) to stimulate insulin secretion in *ucp2*-OE islets was compared with forskolin (1 μM). Both compounds increased insulin secretion in control and *ucp2*-OE islets (Fig. 2E). To examine PKA-independent effects mediated by *epac*, 8-pCPT-2-*O*-Me-cAMP (0·3 mM) was used. Significant effects on control but not *ucp2*-OE islets were observed (Fig. 2E).

Stimulation of insulin secretion after bypassing K_{ATP} channels

The K_{ATP}-independent pathway of insulin secretion can be studied by opening K_{ATP} channels with diazoxide and depolarizing the β-cells with KCl. This treatment resulted in the stimulation of insulin secretion in both control and *ucp2*-OE islets (Fig. 2F). Addition of 1 μM forskolin caused additional stimulation of insulin secretion in both groups ($P<0·01$).

K_{ATP} channel activity

In the presence of 11 mM glucose, β-cells from *ucp2*-OE islets tended to have greater K_{ATP} current through K_{ATP} channels than control islets ($P=0·15$, $n=5$–6; Fig. 3A–C). Increased variability in the *ucp2*-OE group likely reflects different infection efficiencies between preparations. Because β-cell K_{ATP} channels are largely inhibited at 11 mM glucose (Ashcroft & Rorsman 1989, Cook & Ikeuchi 1989) and, thus, the K_{ATP} currents are very small, statistical significance was not achieved. Forskolin abrogated this enhancement of K_{ATP} current in *ucp2*-OE islets, diminishing K_{ATP} current amplitude to a level similar to that observed in control islets.

Rubidium efflux from β-cells was measured as an index of the effects of forskolin on K^+ channel activity. Increasing glucose from 2·8 to 11 mM reduced $^{86}Rb^+$ efflux in control rat islets by ~20% (Fig. 3C), but had no significant effect in *ucp2*-OE islets (Fig. 3D). Addition of 1 μM forskolin significantly inhibited

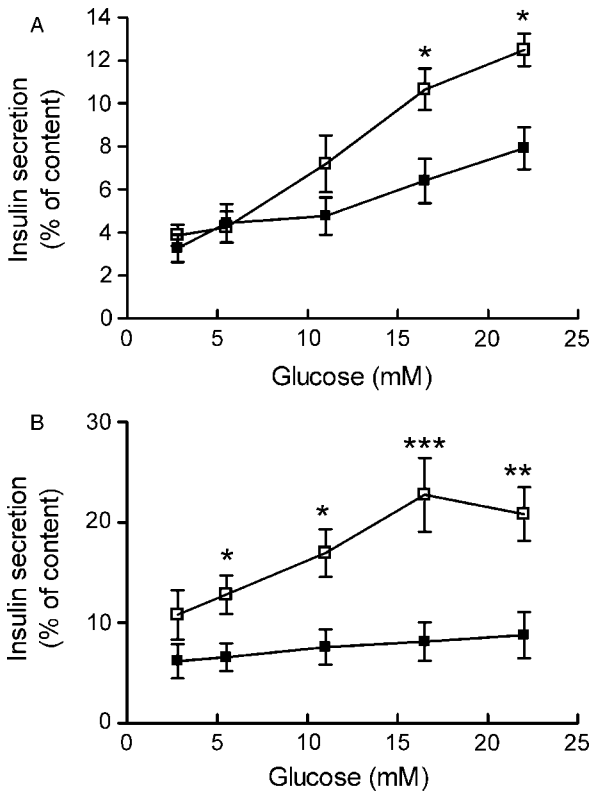

Figure 1 Effects of forskolin (□) on glucose (2·8–22·0 mM)-stimulated insulin secretion of islets isolated from (A) lean ($n=14$), and (B) *ob/ob* mice ($n=10$). Control islets from each genotype are shown by ■. *$P<0·05$, **$P<0.01$ and ***$P<0.001$ compared with controls.

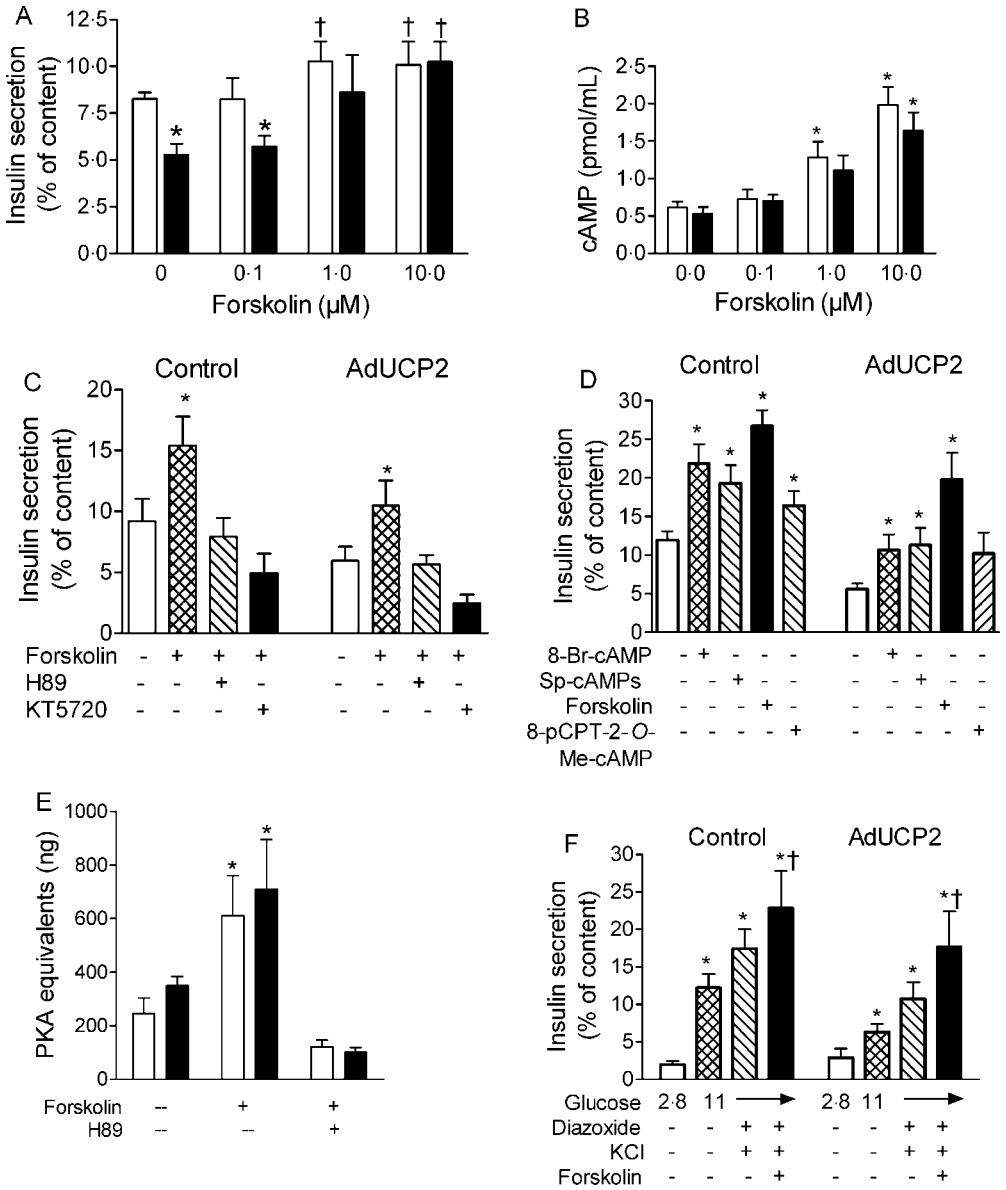

Figure 2 (A) Effects of *ucp2*-OE (solid bars) compared with controls (open bars) on forskolin-stimulated insulin secretion in the presence of 11 mM glucose in isolated rat islets. $n=8$ or greater. *Significant ($P<0{\cdot}05$) effect of *ucp2*-OE. †Significant ($P<0{\cdot}05$) effect of forskolin. (B) Effects of *ucp2*-OE (solid bars) on forskolin-stimulated cAMP generation in rat islets exposed to 11 mmol/l glucose. Control islets are represented by open bars. $n=5$–8. *Significant effect of forskolin ($P<0{\cdot}05$). (C) Effects of specific PKA inhibitors H89 (10 μM) and KT5720 (1 μM) on forskolin (1·0 μM)-stimulated insulin secretion in *ucp2*-OE rat islets. *$P<0{\cdot}05$ compared with 11 mM glucose (n=at least 9 for all groups). (D) Effects on insulin secretion of cAMP analogs that activate PKA and *epac* (0·1 mM 8-Br-cAMP, 0·1 mM Sp-cAMPs or 1 μM forskolin) and an *epac*-specific analog 8-pCPT-2-*O*-Me-cAMP (0·3 mM). *$P<0{\cdot}05$ compared with 11 mM glucose. $n=6$–10. (E) Activity of PKA in islets overexpressing *ucp2* (solid bars, $n=5$) compared with controls (open bars, $n=6$) under basal conditions (0), stimulated with 1 μmol/l forskolin (Forsk) ± H-89 (10 μM). *$P<0{\cdot}05$ compared with basal PKA activity. (F) Insulin secretion via K_{ATP}-independent pathway in control and *ucp2*-OE islets. Islets were held in a depolarized state by addition of 0·1 mM diazoxide and 20 mM KCl in the presence of 11 mM glucose. Forskolin (1 μM) was added to the depolarized islets. *$P<0{\cdot}05$ compared with 2·8 mM glucose, †$P<0.05$ compared with 11 mM glucose. $n=6$–10 for all groups.

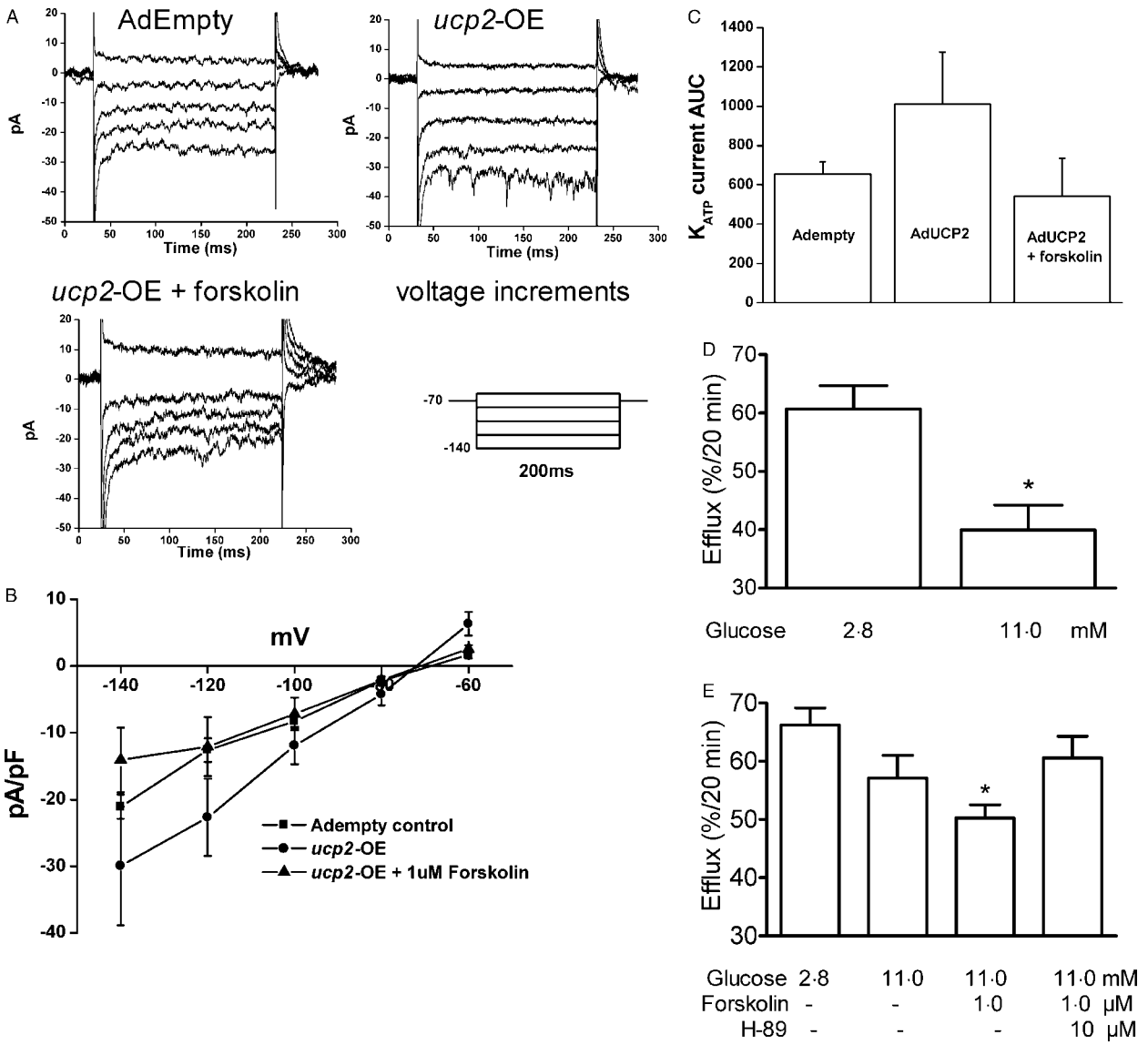

Figure 3 K_{ATP} channel activity. (A) Representative K_{ATP} current recordings, current tracings from control (AdEmpty) and *ucp2*-OE rat islets in the absence and presence of 1 µM forskolin as the clamp voltage is raised from −140 to −60 mV. Glucose concentration in the bath was 11 mM. (B) I/V curves of grouped K_{ATP} current data. Summary of K_{ATP} channel current data normalized to cell size (pA/pF) at selected voltages (n=5–6). (C) Area under the curve (AUC) of current-voltage relationship shown in (B) showing increased K_{ATP} current in *ucp2*-OE islets, and attenuation of this by 1 µmol/l forskolin (n=5–6). (D) Glucose-induced inhibition of $^{86}Rb^+$ efflux (% per 20 min), as a marker of K^+ flux, in control rat islets, indicating closure of K_{ATP} channels. Islets were exposed to either 2·8 or 11·0 mmol/l glucose. (E) Effects of *ucp2*-OE in rat islets on $^{86}Rb^+$ efflux (% per 20 min) in the presence of 2·8 mM glucose (baseline), 11 mM glucose, 11 mM glucose +1 µM forskolin (all n=15), or 11 mM glucose+1 µM forskolin+10 µM H-89 (n=8). *P<0·05 compared with 2·8 mM glucose.

$^{86}Rb^+$ efflux from *ucp2*-OE islets (Fig. 3D). However, in the presence of the PKA inhibitor H89 (10 µM), the inhibitory effects of forskolin on $^{86}Rb^+$ efflux in the *ucp2*-OE islets were reversed.

Calcium flux

Influx of Ca^{2+} through voltage-dependent Ca^{2+} channels occurs secondary to the inactivation of K_{ATP} channels. Representative traces showing changes in intracellular Ca^{2+} in β-TC cells transfected with *ucp2* plasmid compared with control cells (Fig. 4A and B) and islet β-cells (Fig. 4C) are shown. A summary of the results is presented in Table 2. The basal $[Ca^{2+}]_i$ was significantly higher in *ucp2*-OE than control β-TC cells. Addition of 10 mM glucose invoked an increase in $[Ca^{2+}]_i$ that was only 14% of that observed in control cells, consistent with the failure to alter $^{86}Rb^+$ efflux. The increase

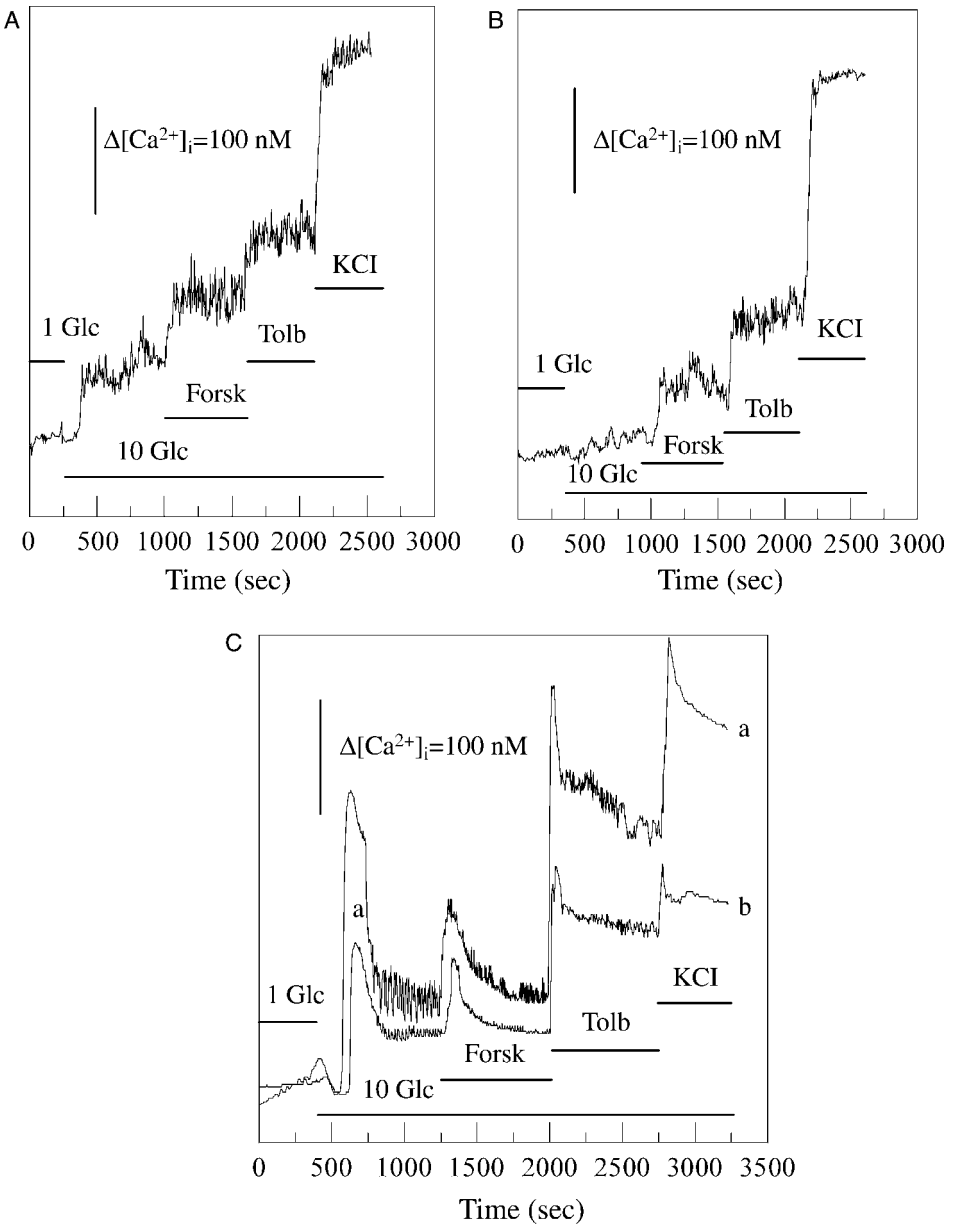

Figure 4 Representative results of Ca^{2+} flux measurements in (A) control and (B) *ucp2*-OE β-TC cells. (C) Ca^{2+} flux measurements in dispersed β-cells from (a) control and (b) *ucp2*-OE mouse islets. Coverslips with cells or islets loaded with Fura-2 AM were transferred to an open chamber, placed on the microscope stage and perfused at 37 °C at a flow rate of 1 ml/min (see Materials and Methods). Horizontal bars indicate the addition of stimulants as follows: Basal $[Ca^{2+}]_i$ was measured in the presence of 1 mM glucose (Glc). The responses to 10 mM glucose and 10 mM glucose+forskolin (Forsk, 25 μM), tolbutamide (Tolb, 300 μM), or KCl (50 mM) were measured sequentially. The vertical bar indicates the magnitude of change in $[Ca^{2+}]_i$. Summary results of replicate experiments are reported in Table 2.

in $[Ca^{2+}]_i$ was identical in *ucp2*-OE and control β-TC cells when forskolin was applied (~23%) and the magnitude of the response was similar to that seen when the K_{ATP} channels were inactivated with the sulphonylurea tolbutamide. Likewise, exposure of the cells to 50 mM KCl evoked a strong increase in $[Ca^{2+}]_i$ of >250 nM in both control and *ucp2*-OE conditions. In islet β-cells, the increase in $[Ca^{2+}]_i$ evoked by glucose was reduced by ~50% in *ucp2*-OE but the response to forskolin was ~75% of control. The difference probably reflects lower transfection efficiency in the primary cells.

Table 2 $[Ca^{2+}]_i$ (nM) responses to glucose and other stimuli in UCP2-OE β-TC cells

	Basal $[Ca^{2+}]_i$	$\Delta[Ca^{2+}]_i$ 10 mM glucose	$\Delta[Ca^{2+}]_i$ 25 μM forskolin	$\Delta[Ca^{2+}]_i$ 300 μM tolbutamide	$\Delta[Ca^{2+}]_i$ 50 mM KCl
Control	73·5±2·18 (14)*	43·8±9·1 (14)	22·7±8·0 (4)	37·7±6·0 (10)	266±20 (14)
UCP2-OE	106·2±2·38 (20)	6·0±3·0 (20)*	23·7±4·2 (9)	33·4±2·9 (19)	255±9·5 (20)

Data are means±S.E.M. (*n*), where (*n*) is the total number of replicates performed from four independent transfections. $\Delta[Ca^{2+}]_i$ represents the amplitude of the change (in nM) following the treatment indicated. *$P<0·01$ compared with *ucp2*-OE cells.

Effects of chronic forskolin exposure on ucp2 *expression and insulin secretion*

It was previously reported (Lameloise *et al.* 2001) that culturing clonal β-cells in the presence of high concentrations of forskolin for ∼18 h could inhibit the expression of *ucp2*, possibly representing an alternative mechanism for its ability to improve GSIS. To determine if effects on gene expression could contribute to improved insulin secretion, in addition to its acute stimulatory effects, rat islets were cultured overnight in the presence of 10 μM forskolin (Fig. 5A). Treatment with forskolin increased *ucp2* mRNA expression by ∼30% ($P<0·05$). Basal insulin secretion was increased ∼fivefold in islets cultured in forskolin and 16·5 mM glucose failed to elicit any further augmentation of secretion (Fig. 5B). In contrast, GSIS was threefold basal in the control islets.

Glucose and exendin-4 stimulated insulin secretion in UCP2 knockout mice

To determine how null expression of *ucp2* affects cAMP-potentiated insulin secretion, *ucp2* KO mice were utilized. The results of pancreas perfusion experiments are shown in Fig. 6A (WT) and B (*ucp2* KO). In the presence of 1·4 mM glucose (1–5 min from Fig. 6A and B), *ucp2* KO mice had threefold higher insulin secretion than WT mice. Higher (13·4 mM) glucose evoked a twofold higher response in *ucp2* KO than WT mice (Fig. 6C). In WT mice, 1 nM exendin-4 stimulated insulin secretion by fivefold compared with 13·4 mM glucose alone (Fig. 6A and C). Exendin-4 also enhanced insulin secretion from *ucp2* KO mouse islets, but by only 1·8-fold (Fig. 6B,C), such that there was no longer any difference in insulin secretion between *ucp2* KO and WT mice.

ATP and cAMP content of UCP2 knockout mouse islets

cAMP, cATP, and cADP contents were measured in freshly isolated *ucp2* KO and WT mouse islets. No genotype-dependent difference was observed when cAMP was measured in the presence of glucose alone, 1 nM exendin or 1 μM forskolin (Fig. 6D). However, in the presence of both low and high glucose concentrations, islets from *ucp2* KO mice had a higher ATP:ADP ratio than WT mice (Table 3).

Discussion

The findings in the present study support the hypothesis that increasing cellular cAMP can partially ameliorate the effects of low ATP in islets overexpressing UCP2 either endogenously (*ob/ob* mice) or after genetic manipulation (*ucp2*-OE). Restoration of the insulin-secretory response to glucose in *ucp2*-OE islets was largely PKA-dependent and did not appear to involve the non-PKA-dependent cAMP signaling pathway acting through *epac*.

To study the mechanisms through which cAMP exerts its effects, an abundant source of β-cells was required. Normal

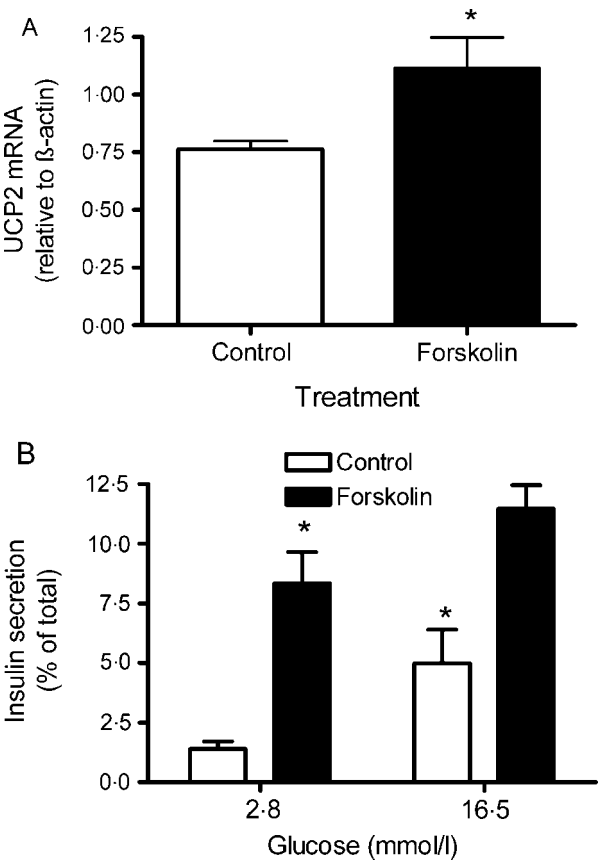

Figure 5 Effect of culturing rat islets overnight in medium containing 10 μM forskolin on (A) *ucp2* expression and (B) insulin secretion stimulated by 2·8 or 16·5 mM glucose. $n=8$ for all experiments; *$P<0·05$.

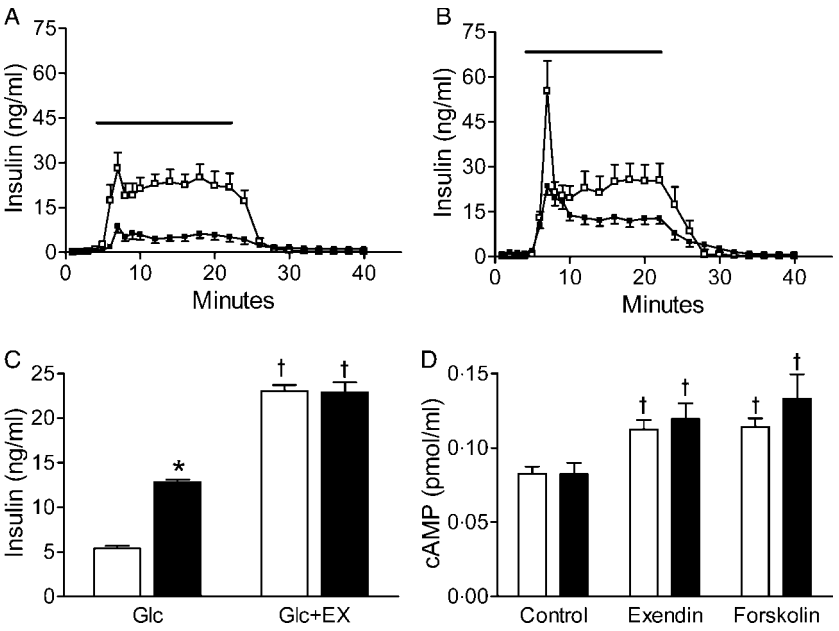

Figure 6 Effects on insulin secretion of glucose (■) versus glucose+1 nM exendin-4 (□) in isolated pancreases from WT (A, $n=10$ each) and *ucp2* KO mice (B, $n=10$ each). Pancreases were perfused with 1·4 mM glucose for 1–5 min, 13·4 mM glucose± exendin-4 for 6–25 min (indicated by the horizontal bar), and 1·4 mM glucose for 26–40 min. (C) Mean insulin secretion following glucose (Glc) or glucose+exendin-4 (Glc+EX) treatment during 12–20 min (WT open bars, *ucp2* KO solid bars) from the data in A and B. This time frame was chosen to cover the second phase of insulin secretion. (D) cAMP content in isolated islets from WT (open bars, $n=8$) and *ucp2* KO mice (solid bars, $n=10$) incubated with 13·4 mM glucose alone or with 1 nM exendin-4 or 1 μM forskolin. *$P<0·05$ compared with WT, †$P<0·05$ compared with glucose alone, within genotype.

rat islets or β-TC cells overexpressing UCP2 (Chan *et al.* 1999, 2001) were utilized. The quantitative PCR results suggest that *ucp2* mRNA induction in both models was approximately 20-fold; however, previous work by Joseph *et al.* (2004) has shown discordance between transcription and translation levels, such that the actual induction of protein is actually two- to fivefold using these transfection or infection methods.

When β-cell ATP is decreased by *ucp2*-OE, the ability of glucose to inactivate K_{ATP} channels is attenuated (this study and Chan *et al.* 2001). Similar results were obtained with *ucp1*-OE in β-cells (Nakazaki *et al.* 2002). In contrast, in *ucp2* KO mouse islets, glucose-stimulated Ca^{2+} influx is enhanced (Joseph *et al.* 2005), reflecting increased K_{ATP}-dependent depolarization. Therefore, treatments that normalize K_{ATP} channel activity and Ca^{2+} influx should overcome the suppressive effects of UCP2 on GSIS. Here, forskolin reduced K_{ATP} currents measured by patch clamping and $^{86}Rb^{+}$ efflux in *ucp2*-OE islets and normalized glucose-stimulated Ca^{2+} influx in response to glucose. The effects of forskolin were largely mediated by PKA because the specific inhibitors H89 and KT5720 (Yaekura *et al.* 1996) blocked both K_{ATP} channel inhibition and insulin secretion, whereas the *epac* pathway-specific agonist 8-pCPT-2′-*O*-Me-cAMP (Holz 2004) did not enhance insulin secretion in *ucp2*-OE islets. Even in control islets, the *epac* agonist 8-pCPT-2-*O*-Me-cAMP had only modest effects on insulin secretion compared with forskolin, suggesting that this pathway 'fine tunes' insulin

Table 3 ATP and ADP content of islets isolated from UCP2 KO and WT mice

	Genotype	**ATP** (pmol/islet)	**ADP** (pmol/islet)	**ATP/ADP**
Glucose (mM)				
2·8	UCP2 KO	26·63±2·53	2·89±0·46	10·27±1·46*
	WT	20·8±1·94	4·20±0·58	5·19±0·49
16·7	UCP2 KO	32·56±2·52	2·30±0·23	14·41±0·69*
	WT	25·42±1·24	2·64±0·26	10·23±1·46

Data are means±S.E.M. $n=5$ for all experiments. *$P<0·05$ comparing phenotypes at the same glucose concentrations.

secretion through mechanisms not sufficient to over-ride other deficits. cAMP exerts its effects at least partially via K_{ATP} channels (Light *et al.* 2002). Using β-cell lines, Light *et al.* (2002) showed that H89 completely blocked the inhibition of K_{ATP} currents by GLP-1. The results presented here support the hypothesis that an increase in cAMP can ameliorate insulin secretion despite low cellular ATP via actions on the K_{ATP} channel.

These studies also support the potential effects of cAMP on distal events related to exocytosis (Takahashi *et al.* 1999). Forskolin enhanced insulin secretion by the K_{ATP}-independent pathway in both control and *ucp2*-OE islets. The magnitude of potentiation in depolarized islets was about 30%, whereas total potentiation in non-depolarized islets was 50–100%, suggesting that a significant fraction of cAMP-mediated effects were exerted on the exocytotic machinery. Exocytosis of insulin can be positively modulated by both PKA and *epac* (Holz 2004). Since 8-pCPT-2′-*O*-Me-cAMP was ineffectual in *ucp2*-OE islets, we speculate that PKA-dependent steps, for example, replenishment of the readily releasable granule pool were contributing to the enhancement of GSIS under these conditions.

An unexpected finding of these studies was the higher basal $[Ca^{2+}]_i$ in *ucp2*-OE ß-cells. The cause of this observation was not investigated but we speculate that lower cellular ATP may lead to sluggish activity of Ca-ATPases that normally sequester Ca^{2+} in the endoplasmic reticulum or pump it out of the cell. Interestingly, *ucp2* KO mice, which have elevated cellular ATP, did not have lower basal $[Ca^{2+}]_i$ but after exposure to palmitic acid, which impairs the ability of WT but not *ucp2* KO mouse islets to generate ATP, the $[Ca^{2+}]_i$ was lower in the *ucp2* KO islets (Joseph *et al.* 2004).

Changes in UCP2 expression did not alter cAMP production in β-cells. The lack of difference is consistent with kinetic analyses of adenylate cyclase, which showed that the K_m for ATP is ∼350 μM (Dessauer & Gilman 1997). Resting ATP concentrations in β-cell cytosolic and plasma membrane domains have been estimated to be ∼1000 μM, approximately doubling after high glucose exposure (Kennedy *et al.* 1999). Thus, even a 50% reduction in ATP should not affect cAMP generation except under exceptional circumstances. Moreover, as the cAMP content increased after forskolin stimulation, the insulin secretion by *ucp2*-OE islets was normalized. Because PKA activity was similar in control and *ucp2*-OE islets, it is unlikely that upregulation of this enzyme contributed to the effect. Results from the *ob/ob* mouse islets showed that in the presence of forskolin a robust insulin response to glucose was generated. Thus, adequate cAMP levels were able to 'rescue' energy-deficient islets. Likewise, cAMP is shown to partially relieve the impairment in GSIS observed in the hyperglycemic GK rat (Abdel-Halim *et al.* 1996). The gene for *ucp2* maps within the quantitative trait locus of chromosome 1 for glucose intolerance in the GK rat (Kaisaki *et al.* 1998), but whether β-cell *ucp2* expression is elevated is unclear. One study showed that the treatment with insulin to normalize glycemia reduced UCP2 protein expression on GK rats fed a high fat diet (Briaud *et al.* 2002). In neonatal streptozotocin diabetic rats, agents that induce β-cell cAMP also enhance GSIS, although not to control levels (Dachicourt *et al.* 1996). Our data, together with other published reports, support the development of new treatment modalities for type-2 diabetes mellitus that target cAMP, such as long-acting stimulants of cAMP production or phosphodiesterase inhibitors (Furman *et al.* 2004). However, caution against chronic amplification of cAMP-mediated effects is warranted in light of the upregulation of *ucp2* mRNA observed in this study and the accompanying exaggerated basal insulin secretion. Agonists that increase cAMP also increased UCP2 expression in muscle cells (Nagase *et al.* 2001) and activation in dopamine-secreting cells (Yamada *et al.* 2003), presumably via a cAMP response element in the *ucp2* promoter (Yoshitomi *et al.* 1999). Interestingly, a previous report indicated that 18-h forskolin treatment of rat insulinoma cells (INS-1) reduced *ucp2* expression (Lameloise *et al.* 2001). Differences from our study might be due to the experimental model (transformed cells versus rat islets) and the concentration of forskolin used (fivefold higher than the present study).

Previous studies showed that *ucp2* KO mice have an increased insulin-secretory response to both basal and stimulatory glucose concentrations (Zhang *et al.* 2001, Joseph *et al.* 2004), a finding confirmed here. When WT mouse pancreases were perfused with stimulatory glucose plus exendin-4, a glucagon-like peptide analog that activates adenylate cyclase to elevate cAMP, insulin secretion was increased by ∼fourfold. In comparison, although exendin-4 potentiated GSIS in the *ucp2* KO mice, the increment was only ∼twofold; thus, the difference in insulin-secreting capacity between the WT and *ucp2* KO mice was negated. These data suggest that an abundance of ATP does not lead to any exceptional potentiation of GSIS by cAMP. Rather, the situation appears to resemble that found in the Zucker fatty rat, where a decrease in the K_m for glucokinase (Chan 1993, Chan *et al.* 1995) leads to a left shift in the responsiveness to glucose at both the level of the K_{ATP} channel (Chan & MacPhail 1996) and insulin secretion (Chan *et al.* 1995, 1996). The enhanced inhibition of the K_{ATP} channel was presumed due to a greater increment in ATP permitted by the increased glucose flux through glucokinase. Thus, these rats do not display any increased sensitivity to cAMP generating incretins, such as GIP (Chan *et al.* 1984). Similarly in the diabetic sand rat, any increase in response to incretins or forskolin was attributed to an increase in sensitivity to glucose (Nesher *et al.* 2001). Of interest, however, was the relatively greater enhancement of the first phase than the second phase of insulin secretion by exendin in *ucp2* KO mice. The magnitude of the first phase depends upon the number of granules in the readily releasable pool (Barg *et al.* 2002) and, in the intact, perfused pancreas, the total β-cell mass, as well as K_{ATP} channel inactivation. K_{ATP} channel closure may be enhanced in *ucp2* KO mice because of the relatively higher ATP/ADP at low glucose (Joseph *et al.* 2004). Also, in *ucp2* KO mice, β-cell mass is increased by 50%, but alterations in granule priming and docking in the presence of elevated cAMP cannot be ruled out.

In conclusion, when β-cell ATP is increased or decreased by manipulating UCP2 expression, different effects on ATP-dependent pathways are observed. Whereas the ability of the K_{ATP} channel to close in response to glucose is impaired, cAMP formation is unaltered. This supports the theory that various elements of the secretory pathway have differing energy requirements (Detimary *et al.* 1994). However, when cAMP concentrations in the islet are elevated, insulin secretion in the ATP-deficient state can be normalized, largely through PKA-dependent mechanisms.

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