Glucose-induced insulin secretion from islets of fasted rats: modulation by alternate fuel and neurohumoral agonists

W S Zawalich and K C Zawalich

Yale University School of Nursing, 100 Church Street South, New Haven, Connecticut 06536-0740, USA

(Requests for offprints should be addressed to W S Zawalich; Email: Walter.Zawalich@Yale.Edu)

Abstract

Islets from fed and 24-h-fasted rats were studied immediately after collagenase isolation. (1) After a 24-h fast, the insulin secretory responses to 8 mM glucose measured during perfusion were reduced by more than 90% from islets of fasted donors. (2) Increasing glucose to 11 or 27.5 mM resulted in enhanced insulin secretion from islets of fasted animals. (3) Fasting did not reduce islet insulin content. (4) Responses to 8 or 27.5 mM glucose were not affected if fatty acid-free albumin was used during the perfusion. (5) Inclusion of α -ketoisocaproate (5 mM), monomethyl succinate (10 mM) or carbachol (10 μ M) significantly amplified insulin release from fasted islets in the simultaneous presence of 8 mM glucose. (6) Phospholipase C activation by glucose, carbachol or their combination was not adversely affected by fasting. (7) The response to the protein kinase C activator, phorbol 12-

myristate 13-acetate (500 nM), was reduced by about 60% after fasting. (8) Extending the fast to 48 h resulted in a severe decline in response to 11 mM glucose; however, the further addition of 10 μ M carbachol still enhanced release from these islets. The results confirm that caloric restriction impairs islet sensitivity to glucose stimulation and that protein kinase C may be involved in the reduction of glucose-induced insulin release from these islets. The activation of phospholipase C by cholinergic stimulation may contribute to the maintenance of insulin secretion from calorically restricted animals. These results also demonstrate that free fatty acids are not essential for glucose to evoke secretion from isolated islets of fasted donors.

Journal of Endocrinology (2000) **166**, 111–120

Introduction

The capacity of the pancreatic β -cells to adapt their insulin secretory responses to caloric availability is well established. During times of excess caloric intake that occur, for example, during the genesis of obesity, their responses to glucose stimulation are enhanced significantly (Perley & Kipnis 1967, Kipnis 1972, Cunningham *et al.* 1983, Curry & Stern 1985, Atef *et al.* 1991). During caloric restriction, on the other hand, the glucose responsiveness of the β -cell to stimulation is significantly reduced, particularly to modest increments in the prevailing glucose level (Wolters *et al.* 1977, Zawalich *et al.* 1979, Carpinelli *et al.* 1986). Most recently, it has been proposed that circulating free fatty acids are essential for efficient glucose-induced insulin release from islets of 18- to 24-h-fasted animals (Stein *et al.* 1996). This conclusion was based primarily on the findings made with animals fitted with carotid artery and jugular vein catheters exteriorized in the posterior neck. These results (Stein *et al.* 1996) suggested that the interaction between glucose and fatty acids was much greater than previously appreciated. In subsequent work from the same group, it was concluded that 'were it not for the

raised plasma NEFA (nonesterified free fatty acids), the pancreas would be largely blind to glucose when the fast is terminated' (McGarry & Dobbins 1999).

Standing in apparent opposition to the idea that free fatty acids are essential for efficient coupling of glucose to insulin release are a number of early studies using islets isolated from rats fasted for various times. In these experiments, while islets from fasted donors were less sensitive to modestly stimulatory glucose in the range of 8–10 mM, their sustained secretory responses to higher levels (20–30 mM) of glucose were appreciable and only slightly, if at all, reduced when compared with the responses of islets from fed donors (Pagliara *et al.* 1975, Wolters *et al.* 1977, Grill *et al.* 1978, Zawalich *et al.* 1979). A possible contributory factor to this apparent divergence between these early studies (Pagliara *et al.* 1975, Wolters *et al.* 1977, Grill *et al.* 1978, Zawalich *et al.* 1979) and those most recently published (Stein *et al.* 1996, McGarry & Dobbins 1999) was the possible presence of free fatty acids in the albumin used to make up the perfusion medium. In the present series of studies we explored in more detail insulin secretion from rat islets isolated from 24- to 48-h-fasted donors. We repeated earlier experiments using stimulatory

glucose alone or in the presence of other fuel or neuro-humoral agonists. We also conducted experiments using fatty acid-free albumin. These results demonstrate that under the experimental conditions employed in these studies islets from fasted animals are not blind to stimulatory glucose, to other nutrient regulators of insulin secretion or to cholinergic stimulation.

Materials and Methods

Islet isolation

The detailed methodologies employed to assess insulin output from collagenase-isolated islets have been described previously (Zawalich & Zawalich 1988). Male Sprague-Dawley rats (body weights at time of study 376–475 g) were purchased from Charles River (Raleigh, NC, USA). All animals were treated in a manner which complied with the NIH Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 85–23, revised 1985). The control animals had food available *ad libitum*. The fasted animals were deprived of food but not water for 24–48 h prior to the islet studies. All experiments were initiated at 0800 h. After Nembutal (pentobarbital sodium, 50 mg/kg; Abbott, North Chicago, IL, USA)-induced anesthesia, islets were isolated by collagenase digestion and hand-picked, using a glass loop pipette, under a stereo microscope. They were free of exocrine contamination.

Perfusion studies

Groups of 14–18 isolated islets were loaded onto nylon filters (Tetko, Inc., Briarcliff Manor, NY, USA) and perfused in a Krebs–Ringer bicarbonate (KRB) buffer at a flow rate of 1 ml/min for 30 min in 3 mM glucose to establish basal and stable insulin secretory rates. After this 30-min stabilization period, they were then perfused with the appropriate agonist or agonist combinations as indicated in the figure legends and Results section. Perfusate solutions were gassed with 95% O₂/5% CO₂ and maintained at 37 °C. Insulin released into the medium was measured by radioimmunoassay (Albano *et al.* 1972). Islet insulin content was determined after the perfusion.

Islet labeling for inositol phosphate (IP) studies

After isolation, groups of 18–26 islets were loaded onto nylon filters, placed in a small glass vial and incubated for 3 h in a myo-[2-³H]inositol-containing KRB solution made up as follows: 10 µCi myo-[2-³H]inositol (specific activity 16–23 Ci/mM) were placed in a 10 mm × 75 mm culture tube. To this aliquot of tracer, 250 µl of warmed (to 37 °C) and oxygenated (KRB) medium supplemented with 5.0 mM glucose were added. After mixing, 240 µl of this solution were gently added to the vial with islets. The

vial was capped with a rubber stopper, gassed for 10 s with 95% O₂/5% CO₂ and incubated at 37 °C. The vials were again gently oxygenated after 90 min. After the labeling period, the islets still on nylon filters were washed with 5 ml fresh KRB.

IP measurements

After washing, the islets on nylon filters were placed in small glass vials. Added gently to the vial were 400 µl KRB supplemented with 10 mM LiCl to prevent IP degradation, and the appropriate agonists as indicated. The vials were capped and gently gassed for 5 s with 95% O₂/5% CO₂. After 30 min, the generation of IPs was stopped by adding 400 µl 20% perchloric acid. Total IPs formed were then measured using Dowex columns as described previously (Berridge *et al.* 1983, Zawalich & Zawalich 1992).

Reagents

Hank's solution was used for the islet isolation. The perfusion medium consisted of 115 mM NaCl, 5 mM KCl, 2.2 mM CaCl₂, 1 mM MgCl₂, 24 mM NaHCO₃, and 0.17 g/dl bovine serum albumin. The ¹²⁵I-labeled insulin used for the insulin assay was purchased from New England Nuclear (Boston, MA, USA). The labeled myo-[2-³H]inositol was purchased from Amersham (Arlington Heights, IL, USA). Bovine serum albumin (BSA; RIA grade), fatty acid-free BSA, glucose, α-ketoisocaproate, carbachol, phorbol 12-myristate 13-acetate (PMA) and the salts used to make the Hank's solution and perfusion medium were purchased from Sigma (St Louis, MO, USA). Monomethyl succinate (methyl hydrogen succinate) was purchased from Pfaltz and Bauer (Waterbury, CT, USA). Rat insulin standard (lot #615-ZS-157) was the generous gift of Dr Gerald Gold, Eli Lilly Co. (Indianapolis, IN, USA). Collagenase (Type P) was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA).

Because of the possible contribution of free fatty acids which might complicate interpretation of the data, the level of free fatty acids in both the RIA-grade albumin and the fatty acid-free albumin were determined spectrofluorometrically. In both instances, the concentration of free fatty acids was below the limits of assay detection which is approximately 50 µM.

Statistics

Statistical significance was determined using the Student's *t*-test for unpaired data or analysis of variance in conjunction with the Newman-Keuls test for unpaired data. A

P value ≤ 0.05 was taken as significant. Values presented in the figures and Results represent means $\pm$ S.E. of at least three observations.

Results

Body weights

The body weights of control animals with food available *ad libitum* averaged 423 ± 8 g at the time of islet isolation. The body weights of animals fasted for 24 h were initially 423 ± 7 g. After 24-h food deprivation, their weights decreased on average 27.6 g, a loss of 6.8%. Animals fasted for 48 h lost 9.5% of their initial body weight.

Insulin secretory responses to glucose stimulation

In agreement with prior islet or perfused pancreas studies (Wolters *et al.* 1977, Grill *et al.* 1978, Zawalich *et al.* 1979, Stein *et al.* 1996) using fasted donor rats, islet responses to glucose were altered when compared with those measured from fed animals. Most dramatic were the reductions to an 8 mM glucose stimulus (Fig. 1, top). For example, 35–40 min after the onset of stimulation, the insulin response from control animals' islets averaged 140 ± 13 pg/islet/min ($n=13$) while that from fasted animals' islets averaged only 46 ± 5 pg/islet/min ($n=4$) at this time. Subtracting prestimulatory insulin secretion rates of 45 ± 5 pg/islet/min (fed controls) or 38 ± 3 pg/islet/min (fasted animals) in the presence of 3 mM glucose, the decrease in response to 8 mM glucose from fasted islets was greater than 90% when compared with the responses from fed control animals.

Increasing the glucose level to 11 mM resulted in a significant biphasic insulin stimulatory response from islets of fed control animals (Fig. 1, middle). Peak first phase release averaged 163 ± 39 pg/islet/min ($n=12$). Insulin secreted into the final perfusate sample collected 35–40 min after the onset of stimulation averaged 605 ± 59 pg/islet/min. After a 24-h fast, peak first phase release in response to 11 mM glucose stimulation averaged 57 ± 9 pg/islet/min ($n=6$). Release rates measured 35–40 min after the onset of 11 mM glucose stimulation averaged 313 ± 67 pg/islet/min from these islets. Correcting for prestimulatory secretory rates measured in the presence of 3 mM glucose, peak first phase secretion and second phase release rates measured 35–40 min after the onset of stimulation were reduced by approximately 85% and 50% respectively.

In response to 27.5 mM glucose, peak first phase release from islets of fed control donors averaged 240 ± 15 pg/islet/min (Fig. 1, bottom, $n=9$). Release rates measured 35–40 min after the onset of 27.5 mM glucose stimulation averaged 801 ± 46 pg/islet/min. After a 24-h fast, peak first phase release rates now averaged 94 ± 29 pg/islet/

min ($n=5$). Release rates measured 35–40 min after the onset of stimulation averaged 659 ± 87 pg/islet/min (Fig. 1, bottom). Correcting for prestimulatory secretory rates measured in the presence of 3 mM glucose, peak first phase secretion and second phase release rates measured 35–40 min after the onset of stimulation were reduced by approximately 70% and 18% respectively. During the final 10 min of the perfusion, there was no significant difference between the responses of islets from fed or 24-h-fasted animals.

Insulin contents measured after the perfusion averaged 125 ± 12 , 107 ± 6 or 106 ± 6 ng/islet from islets of fed donors perfused with 8 mM, 11 mM or 27.5 mM glucose respectively. Insulin content in islets of fasted animals measured after the perfusion averaged 155 ± 20 , 172 ± 19 or 138 ± 22 ng/islet from islets perfused with 8 mM, 11 mM or 27.5 mM glucose respectively.

Based on an earlier report (Stein *et al.* 1996), it might be suggested that the profound stimulatory effect of 27.5 mM glucose on insulin secretion from 24-h-fasted rat islets might be a consequence of free fatty acids, an impurity in the RIA grade albumin routinely used in our perfusion studies. To test this possibility, additional studies were conducted using fatty acid-free BSA. Similar secretory profiles were observed in response to 8 mM (results not shown) or 27.5 mM glucose stimulation (Fig. 2) from islets of 24-h-fasted animals irrespective of the type of albumin used. At no time point during the perfusion were significant differences observed.

Insulin secretion from islets of fed and fasted donors in response to alternate nutrient agonists

Based on an earlier study (Stein *et al.* 1996) and reinforced in a recent article (McGarry & Dobbins 1999) was the concept that the presence of free fatty acids was essential in order for glucose to evoke insulin secretion during and after the recovery from a fast. We next tested, in islets isolated from fasted donors, whether compounds not related to free fatty acids but with established stimulatory effects on the pancreatic β -cell, could augment secretion in the presence of 8 mM glucose, a level of the hexose virtually devoid of insulinotropic activity after a 24-h fast. As shown in Fig. 3, the inclusion of the metabolic fuels α -ketoisocaproate (5 mM, Fig. 3, top), or monomethyl succinate (10 mM, Fig. 3, bottom) together with 8 mM glucose markedly enhanced insulin secretion from islets of 24-h-fasted donors. When compared with the responses of islets isolated from fed donors, the kinetics of the secretory profile were altered from islets of fasted donors. Release rates rose slowly from these islets and significantly smaller responses were observed throughout most of the perfusion. However, peak release rates comparable to the responses of fed control islets were achieved during the final minutes of the perfusion with each combination of agonists.

Islets responses to cholinergic stimulation after a 24–48 h fast

While a previous study (Stein *et al.* 1996) suggested that free fatty acids were largely responsible for the maintenance of islet glucose sensitivity during and after food deprivation, we considered the possibility that cholinergic stimulation of the β -cell, a normal physiological component of nutrient-regulated insulin secretion (Louis-Sylvestre 1976,

Figure 2 Influence of fatty acid-free BSA on islet responses to 27.5 mM glucose. Two groups of islets were isolated from fasted donors. One group ($n=5$) was stimulated for 40 min with 27.5 mM glucose contained in a medium supplemented with RIA grade BSA (open circles, solid line; this is the same data indicated in Fig. 1). The other group of islets ($n=5$) (open circles, dashed line) was stimulated for 40 min with 27.5 mM glucose contained in a fatty acid-free BSA medium.

1978, Rohner-Jeanrenaud *et al.* 1983), may play a role as well. To further explore this concept, islets were isolated from fed controls or donor rats after a 24-h period of food deprivation. Similar to the findings made when alternative fuel molecules were provided to islets from 24-h-fasted donors, a significant enhancement of insulin secretion was noted in response to 8 mM glucose plus 10 μ M carbachol irrespective of the nutritional status of the animals (Fig. 4). However, the response to this combination of agonists was reduced when compared with islets isolated from fed animals.

In additional studies, the period of caloric restriction was increased to 48 h and the glucose level used to stimulate the islets increased to 11 mM. Similar to the situation observed when islets isolated from 24-h-fasted donors were stimulated with 8 mM glucose (see Fig. 1), the insulin secretory responses to 11 mM glucose alone were profoundly impaired after a 48-h fast (Fig. 5). However,

Figure 1 Insulin secretion from islets of fed or 24-h-fasted donor rats. Groups of 14–18 islets were isolated from fed (closed circles) or fasted (open circles) donors and perifused. For the initial 30 min, the islets were maintained with 3 mM glucose (G3) to establish basal and stable insulin secretory rates. They were then perifused (indicated by the vertical line) for 40 min with 8 mM glucose (G8), 11 mM glucose (G11) or 27.5 mM glucose (G27.5). Mean values $\pm$ S.E. are given. This and subsequent perifusion figures have not been corrected for the dead space in the perifusion apparatus, 2.5 ml or 2.5 min with a flow rate of 1 ml/min. Note change in insulin release scale for the different graphs. The asterisks indicate significant ($P<0.05$) differences between responses of fed versus fasted animals.

Figure 3 Insulin secretion from islets of fed or 24-h-fasted donor rats in response to additional nutrient stimulants. Groups of 14–18 islets were isolated from fed (closed circles, $n=7$) or fasted (open circles) donors and perfused. For the initial 30 min the islets were maintained with 3 mM glucose (G3) to establish basal and stable insulin secretory rates. They were then perfused (indicated by the vertical line) for 40 min with 8 mM glucose (G8) supplemented with 5 mM α -ketoisocaproate (KIC, top panel, $n=5$) or for 40 min with 8 mM glucose (G8) supplemented with 10 mM monomethyl succinate (MMSucc, bottom panel, $n=4$). The asterisks indicate significant ($P<0.05$) differences between responses of fed versus fasted animals.

the additional presence of 10 μ M carbachol exerted a significant restorative effect on insulin secretion from islets isolated from 48-h-fasted donors (Fig. 5).

IP accumulation in islets from fed and fasted donors

A variety of structurally distinct agonists, including glucose, α -ketoisocaproate, monomethyl succinate and carbachol, activate phospholipase C (PLC) in islets (Axen *et al.*

Figure 4 Insulin secretion from islets of fed or 24-h-fasted donor rats in response to carbachol stimulation. Groups of 14–18 islets were isolated from fed (closed circles, $n=7$) or fasted (open circles, $n=5$) donors and perfused. For the initial 30 min the islets were maintained with 3 mM glucose (G3) to establish basal and stable insulin secretory rates. They were then perfused (indicated by the vertical line) for 40 min with 8 mM glucose (G8) supplemented with 10 μ M carbachol. The asterisks indicate significant ($P<0.05$) differences between responses of fed versus fasted animals.

1983, Best & Malaisse 1983, Best 1986, Zawalich 1988b, MacDonald *et al.* 1989, Zawalich *et al.* 1989, Vadakekalam *et al.* 1996). We next explored the potential influence of caloric restriction on PLC activation, monitored by IP accumulation in response to agonist addition. After a 3-h incubation period with [3 H]inositol to label their phosphoinositide (PI) pools, islets were stimulated with 8 mM glucose, 10 μ M carbachol or their combination. When compared with the IP levels observed in the absence of any added stimulant, 8 mM glucose alone modestly increased IP accumulation in islets of fed donors (Table 1). A more appreciable effect was observed with 10 μ M carbachol alone. However, the combination of 8 mM glucose plus carbachol markedly enhanced IP accumulation, an observation previously reported (Kelley *et al.* 1995). Islets isolated from fasted donors responded to stimulation with these agonists in a comparable manner. The response to 10 μ M carbachol alone was, however, significantly greater in islets from 24-h-fasted animals.

Effects of fasting on islet responses to the phorbol ester tetradecanoyl phorbol acetate (PMA)

While the activation of PLC by several agonists including glucose was similar in islets isolated from fed or fasted donors, we next explored the possible contribution of protein kinase C (PKC) activation to the reduction in insulin secretion observed after fasting. In these studies, islets from fed or fasted donors were stimulated with 500 nM PMA in the presence of 3 mM glucose. A

Figure 5 Insulin secretion in response to 11 mM glucose with (+) or without carbachol. Three groups of islets were studied. The first group of islets (closed circles, $n=12$) were isolated from fed control animals and, after a 30-min stabilization period with 3 mM glucose (G3), they were stimulated with 11 mM glucose for 40 min (this is the same data as in Fig. 1). The second group of islets (open circles, solid line, $n=6$) was isolated from 48-h-fasted donors and treated similarly to the first group. The third group of islets (open circles, dashed line, $n=4$) was isolated from 48-h-fasted animals. They were stimulated during the final 40 min of the perfusion with the combination of 11 mM glucose plus 10 μ M carbachol. The asterisks indicate significant ($P<0.05$) differences between the 48-h-fasted islet responses to 11 mM glucose with or without carbachol (the two open circles groups).

significant impairment in PMA-induced secretion occurred as a result of fasting (Fig. 6). For example, while islets from fed controls released insulin at rates of 182 ± 15 pg/islet/min 35–40 min after the onset of stimulation, the response from fasted donor islets was only 92 ± 13 pg/islet/min at this time. Correcting for pre-stimulatory secretory rates measured in the presence of 3 mM glucose, release rates measured 35–40 min after the onset of stimulation were reduced by approximately 60–65%.

Figure 6 Insulin secretion from islets of fed or 24-h-fasted donor rats in response to the protein kinase activator, PMA. Groups of 14–18 islets were isolated from fed (closed circles, $n=9$) or fasted (open circles, $n=5$) donors and perfused. For the initial 30 min the islets were maintained with 3 mM glucose (G3) to establish basal and stable insulin secretory rates. They were then perfused (indicated by the vertical line) for 40 min with 500 nM PMA in the continued presence of 3 mM glucose. The asterisks indicate significant ($P<0.05$) differences between responses of fed versus fasted animals.

Discussion

The effects of fasting on the secretory responsiveness of the pancreatic β -cell has been a topic of investigation for over 30 years (Malaisse *et al.* 1967). In spite of this, the biochemical events that initiate the decline in β -cell sensitivity to stimulation have yet to be completely elucidated. What has been established is that glucose usage is modestly reduced (Wolters *et al.* 1977, Zawalich *et al.* 1979), possibly as a result of a decline in glucokinase activity (Malaisse *et al.* 1976). Whether altered glucose metabolism is the initial lesion in islets from fasted donors and thus responsible for the decline in glucose-induced secretion is debatable. In two reports (Wolters *et al.* 1977, Zawalich *et al.* 1979), the decline in glucose usage rates

Table 1 Effects of caloric restriction on IP accumulation in rat islets. Results are means $\pm$ S.E. At least 4 experiments were performed under each condition

Treatment	IP accumulation (c.p.m./40 islets)	
	Fed	Fasted
No added stimulant	4284 $\pm$ 440	4536 $\pm$ 214
Carbachol (10 μ M)	8084 $\pm$ 534	11 879 $\pm$ 1189*
Glucose (8 mM)	8090 $\pm$ 793	8816 $\pm$ 1120
Glucose (8 mM) + carbachol (10 μ M)	18 162 $\pm$ 1584	19 691 $\pm$ 2861

Groups of rat islets were isolated from fed or 24-h fasted donors and incubated for 3 h in a 3 H-inositol-containing Krebs–Ringer bicarbonate buffer supplemented with 5 mM glucose. After washing to remove unincorporated label, the islets were incubated for 30 min as indicated under Treatment. Lithium chloride (10 mM) was also included to inhibit the phosphatases responsible for IP metabolism. * $P<0.05$ compared with fed animals under the same treatment.

was not evident after a 24-h period of food deprivation, a time when impaired insulin secretory responses to 8–10 mM glucose were already established (Wolters *et al.* 1977, Zawulich *et al.* 1979). Other investigators have reported, however, a more rapid decline in glucose usage rates that parallel secretion (Burch *et al.* 1981).

Most recently, an essential role for free fatty acids in the maintenance of β -cell responsivity to glucose has been proposed (Stein *et al.* 1996). This concept was based primarily, but not exclusively, on studies performed with rats fitted with carotid and jugular vein catheters prior to study. Based on their findings, the authors posit (Stein *et al.* 1996) that 'the high circulating concentration of FFA (free fatty acids) that accompanies food deprivation is a *sine qua non* for efficient GSIS (glucose-stimulated insulin secretion) when a fast is terminated.' This concept elevates free fatty acids to the status of a major contributor to the maintenance of glucose homeostasis both during and after food deprivation. One potential problem with this idea are the numerous studies showing that while glucose-induced insulin release to modest glucose concentrations may be significantly impaired by fasting, the responses to higher concentrations are left largely intact (Malaisse *et al.* 1967, Wolters *et al.* 1977, Grill *et al.* 1978, Zawulich *et al.* 1979).

A potential explanation for the maintenance of glucose-responsiveness from fasted islets is that the BSA used during islet studies may be contaminated with free fatty acids in amounts capable of influencing secretion. In the present series of studies, we repeated experiments using islets from fed and 24-h-fasted donors. Islets were isolated and immediately perfused to monitor the dynamics of insulin secretion. In some experiments fatty acid-free BSA was employed to eliminate the possible contribution of this fuel substrate to the secretory responses measured. Further comment on our findings is appropriate.

In most studies examining the influence of caloric restriction on β -cell responses to stimulation, the animals are simply deprived of food prior to study (Wolters *et al.* 1977, Grill *et al.* 1978, Zawulich *et al.* 1979, Burch *et al.* 1981). When subjected to a 24-h fast, the male rats used in these studies lost about 6–7% of their body weight, and this agrees with previous work (Burch *et al.* 1981). In the studies reported by Stein and coworkers (1996), animals were subjected to major surgical procedures prior to fasting. The potential contribution of this manipulation to the results has to be considered. Unfortunately, based on the results presented in this report (Stein *et al.* 1996), the recovery and normalization of body weights and food intake prior to the hyperglycemic clamp procedures cannot be assessed. In other words, the effects of an 18–24 h fast on β -cell responsiveness in a normal, healthy non-surgically manipulated rat may be quite different from those in a rat fitted with jugular and carotid artery catheters prior to the fast.

A biphasic pattern of insulin secretion was evoked from islets of fed control animals when the glucose level was

increased from 3 mM to 8, 11 or 27.5 mM during a dynamic perfusion. Most dramatic were the second phase responses measured during the later stages of the perfusion, particularly to the higher glucose levels used in these experiments. If one takes into consideration the fact that islets maintained during the entire 70-min perfusion with 3 mM glucose release insulin at rates of about 20–30 pg/islet/min during the last 5 min of the perfusion (results not shown), the response to 27.5 mM glucose amounts to an approximately 30-fold increase in the secretory rate. This response compares favorably with results obtained using the perfused pancreas preparation (Gerich *et al.* 1974, O'Connor *et al.* 1980, Curry 1986) and indicate that the physiological integrity of the islets has been maintained after collagenase isolation.

When islets isolated from 24-h-fasted donors were perfused, a marked deviation from control islet responses was most evident when 8 mM glucose was used to stimulate them. The response to this hexose level was reduced by more than 90% after fasting. However, increasing the stimulatory glucose to 11 or 27.5 mM enhanced insulin secretion significantly, especially during the sustained second phase response. In fact, peak second phase release rates to 27.5 mM glucose were comparable to those measured from fed animals' islets. The observation that supraphysiological glucose markedly enhances secretion from the islets of fasted animals is compatible with previous reports (Wolters *et al.* 1977, Grill *et al.* 1978, Zawulich *et al.* 1979).

We considered the possibility that the inadvertent contamination of the perfusion medium with free fatty acids contained in the RIA-grade BSA routinely used for the perfusions may be responsible for the marked enhancing effect of high glucose on release from islets of fasted donors. To circumvent this potential problem, additional studies were conducted with fatty acid-free BSA. Glucose (27.5 mM)-induced release of insulin from islets of 24-h-fasted animals could not be attributed to the presence of free fatty acids since comparable insulin release rates were measured irrespective of whether RIA grade or fatty acid-free albumin was used during the perfusion.

While the essentiality of free fatty acids in the maintenance of the *in vivo* responses to glucose has been suggested (Stein *et al.* 1996) and cannot be excluded by our *in vitro* studies, we considered the possibility that other compounds with established secretory effects may act in concert with or be capable of substituting for free fatty acids. To explore this issue further, islets from fasted donors were perfused with additional agonists in the simultaneous presence of 8 mM glucose, a level of glucose virtually devoid of any secretory effect after a 24-h fast. In the presence of 8 mM glucose, significant insulin secretory responses to the further addition of 5 mM α -ketoisocaproate, 10 mM monomethyl succinate or 10 μ M carbachol were elicited from these islets. Even after a more prolonged 48-h period of food deprivation, the

insulin secretory response to 11 mM glucose plus 10 μ M carbachol was retained, albeit reduced when compared with the responses from fed animals' islets. The reduction in secretion from islets of fasted donors, particularly during the initial minutes of the perfusion with fuel combinations, may be a consequence of reduced islet sensitivity to either the hexose itself or to the alternate fuel. It was not the goal of these studies to determine the specificity of the lesion in fasted islets but to determine if other secretagogues might substitute for fatty acids and support secretion in the additional presence of glucose. Our results with these alternate agonists do not support the suggestion that free fatty acids are essential in the stimulation of secretion from islets of fasted donors.

We have previously proposed that the activation of information flow in the PLC/PKC signaling cascade plays a pivotal role in the biphasic insulin secretory response stimulated by glucose from rat islets (Zawalich 1996). We based this concept, in part, on the following considerations. First, after an appropriate incubation period with [3 H]inositol to label their PI pools, the addition of a stimulatory glucose level to these islets results in a dramatic enhancement of labeled inositol phosphate accumulation or [3 H]inositol efflux from these islets (Axen *et al.* 1983, Best & Malaisse 1983, 1984, Zawalich & Zawalich 1988, Yamaguchi *et al.* 1989, Vadakekalam *et al.* 1996, Zawalich 1996, Zawalich & Zawalich 1996), findings indicative of PLC activation (Berridge *et al.* 1982, 1983, Rhee *et al.* 1989, Rhee & Choi 1992, Rhee & Bae 1997). Secondly, glucose stimulation promotes the translocation of PKC α from a predominantly cytosolic compartment to a membrane one (Ganesan *et al.* 1990, 1992, Yedovitzky *et al.* 1997) and the hexose increases the phosphorylation state of an established PKC substrate (Calle *et al.* 1992). Thirdly, in contrast to rat islets, mouse islets are minimally responsive to glucose stimulation alone (Zawalich *et al.* 1995), they fail to develop a rising second phase insulin secretory response to glucose (Lenzen 1979, Berglund 1980, Zawalich *et al.* 1995) and they are poorly responsive to glucose in terms of PLC activation (Zawalich *et al.* 1995). These considerations led us to examine the effect of a 24-h period of food deprivation on information flow in the PLC/PKC system.

Our results demonstrate that glucose- or carbachol-induced IP accumulation were comparable in islets irrespective of their prior nutritional status. Most importantly perhaps, from a physiological perspective at least, the combination of fuel and neurohumoral stimulation significantly augmented IP accumulation. On the basis of these results we conclude that the retained activation of PLC plays a potentially important role in the maintenance of insulin release during nutritional deprivation and that the prompt response of this family of isozymes generates the appropriate second messenger molecules to stimulate secretion, albeit at somewhat reduced rates when compared with islet responses of fed controls. However, if the

generation of second messenger molecules with the capacity to activate downstream effector proteins including PKC is normal, why is insulin release to 8 mM glucose so profoundly impaired? A possible resolution to this issue may be provided by our studies where islets were stimulated with PMA.

It has been reported previously that the phorbol ester, PMA, whose only established intracellular target is the activation of PKC (Castagna *et al.* 1982), stimulates insulin release (Malaisse *et al.* 1980, Zawalich *et al.* 1983, 1984). Release by the phorbol ester duplicates, from a qualitative perspective, the slowly rising second phase of glucose-induced release. Based on this and other observations (Zawalich 1988a, 1990, Zawalich & Zawalich 1996), we have proposed that PKC activation contributes to the regulation of glucose-induced insulin release from islets whose physiological responsiveness to stimulation is comparable to that noted with the perfused pancreas preparation (Curry 1986, Gerich *et al.* 1974, Grill *et al.* 1978, O'Conner *et al.* 1980). In addition, PMA (Niki *et al.* 1988, Zawalich *et al.* 1991) and a wide variety of agonists that activate PLC possess the capacity to prime or sensitize islets to subsequent restimulation (Zawalich & Zawalich 1987, Zawalich *et al.* 1988, 1989). Also referred to as time-dependent potentiation (Grill *et al.* 1978), the major effect of priming is a heightened first phase secretory response to glucose stimulation. When islets from fasted donors are stimulated with 500 nM PMA, sustained rates of secretion are reduced about 60%. Consistent with its possible involvement in the physiological regulation of stimulated insulin secretion (Zawalich 1988a, Rasmussen *et al.* 1990, Zawalich *et al.* 1990, Zawalich 1996), the altered activation of PKC may contribute to the reduction in islet sensitivity observed after caloric restriction as well.

Several conclusions appear warranted by these observations. First, the observed effect of fasting on glucose-induced insulin secretion is markedly dependent on the glucose level used to stimulate the islet. Altered sensitivity manifested by an increase in K_m but an apparently normal V_{max} for glucose-stimulated insulin release results from short term caloric restriction. Islets are not, however, blind to glucose stimulation, as evidenced from the data in this and previous reports (Wolters *et al.* 1977, Grill *et al.* 1978, Zawalich *et al.* 1979). Secondly, the kinetics of the secretory response to glucose and other agonists are altered. Thirdly, fasting has no adverse effect on islet insulin content, a finding that confirms a previous study (Malaisse *et al.* 1967). Thus, impaired release is not a consequence of reduced insulin stores in these islets. Fourthly, a number of agonists not directly related to fatty acids or their metabolism are capable of a significant amplification of release in the presence of 8 mM glucose. Thus, the absolute essentiality of fatty acids in the maintenance of glucose-induced release should be reconsidered. Fifthly, the contribution of cholinergic stimulation to glucose-stimulated insulin secretion after the termination

of a fast should be taken into consideration. Mediated by the activation of PLC (Best & Malaisse 1983, 1984), a response that remains intact after 24 h of caloric restriction, the concept that cholinergic stimulation markedly influences release is in accord with the established physiological action of the neurotransmitter during meal ingestion (Loubatieres-Mariani *et al.* 1973, Louis-Sylvestre 1976, 1978, Zawalich *et al.* 1989). Finally, the activation of PKC is reduced after a short-term fast. The observations made with islets isolated from fed or fasted donor animals support the concept that PKC activation may also regulate, at least in part, insulin secretory responses under varying nutritional circumstances.

Acknowledgements

The authors would like to thank Ralph Jacobs and Jennifer Morgen for performing the fatty acid measurements in the BSA. These studies were supported by NIH grant # 41230 and by a grant from the American Diabetes Association.

References

- Albano JDM, Ekins RP, Maritz G & Turner RC 1972 A sensitive, precise radioimmunoassay of serum insulin relying on charcoal separation of bound and free hormone moieties. *Acta Endocrinologica* **70** 487–509.
- Atef N, Brule C, Bihoreau M & Ktorza A 1991 Enhanced insulin secretory response to acetylcholine by perfused pancreas of 5-day-old preobese Zucker rats. *Endocrinology* **129** 2219–2224.
- Axen KV, Schubart UK, Blake AD & Fleischer N 1983 Role of Ca^{2+} in secretagogue-stimulated breakdown of phosphatidylinositol in rat pancreatic islets. *Journal of Clinical Investigation* **72** 13–21.
- Berglund O 1980 Different dynamics of insulin secretion in the perfused pancreas of the mouse and rat. *Acta Endocrinologica* **93** 54–60.
- Berridge MJ, Downes CP & Hanley MR 1982 Lithium amplifies agonist-dependent phosphatidylinositol responses in brain and salivary glands. *Biochemical Journal* **206** 587–595.
- Berridge MJ, Dawson RMC, Downes CP, Heslop JP & Irvine RP 1983 Changes in the levels of inositol phosphates after agonist-dependent hydrolysis of membrane phosphoinositides. *Biochemical Journal* **212** 473–482.
- Best L 1986 A role for calcium in the breakdown of inositol phospholipids in intact and digitonin-permeabilized pancreatic islets. *Biochemical Journal* **238** 773–779.
- Best L & Malaisse WJ 1983 Stimulation of phosphoinositide breakdown in pancreatic islets by glucose and carbamylcholine. *Biochemical and Biophysical Research Communications* **116** 9–16.
- Best L & Malaisse WJ 1984 Nutrient and hormone-neurotransmitter stimuli induce hydrolysis of polyphosphoinositides in rat pancreatic islets. *Endocrinology* **115** 1820–1831.
- Burch PT, Trus MD, Berner DK, Leontire A, Zawalich KC & Matschinsky FM 1981 Adaptation of glycolytic enzymes. Glucose use and insulin release in rat pancreatic islets during fasting and refeeding. *Diabetes* **30** 923–928.
- Calle R, Ganesan S, Smallwood JI & Rasmussen H 1992 Glucose-induced phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) in isolated rat pancreatic islets. *Journal of Biological Chemistry* **267** 18723–18727.
- Carpinelli AR, Mathias PCF, Leclercq-Meyer V & Malaisse WJ 1986 Fasting-induced dissociation of cationic and secretory events in pancreatic islets. *Cell Biochemistry and Function* **4** 123–130.
- Castagna M, Takai Y, Kaibuchi K, Sano K, Kikkawa U & Nishizuka Y 1982 Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *Journal of Biological Chemistry* **257** 7847–7851.
- Cunningham J, Calles J, Eisikowitz L, Zawalich W & Felig P 1983 Increased efficiency of weight gain and altered cellularity of brown adipose tissue in rats with impaired glucose tolerance during diet-induced overfeeding. *Diabetes* **32** 1023–1027.
- Curry DL 1986 Insulin content and insulinogenesis by the perfused rat pancreas: effects of long term glucose stimulation. *Endocrinology* **118** 170–175.
- Curry DL & Stern JS 1985 Dynamics of insulin hypersecretion by obese Zucker rats. *Metabolism* **34** 791–796.
- Ganesan S, Calle R, Zawalich K, Smallwood JI, Zawalich WS & Rasmussen H 1990 Glucose-induced translocation of protein kinase C in rat pancreatic islets. *PNAS* **87** 9893–9897.
- Ganesan S, Calle R, Zawalich K, Greenawalt K, Zawalich W, Shulman GI & Rasmussen H 1992 Immunocytochemical localization of α -protein kinase C in rat pancreatic β -cells during glucose-induced insulin secretion. *Journal of Cell Biology* **119** 313–324.
- Gerich JE, Charles MA & Grodsky GM 1974 Characterization of the effects of arginine and glucose on glucagon and insulin release from the perfused rat pancreas. *Journal of Clinical Investigation* **54** 833–841.
- Grill V, Adamson U & Cerasi E 1978 Immediate and time-dependent effects of glucose on insulin release from rat pancreatic tissue. *Journal of Clinical Investigation* **61** 1034–1043.
- Kelley GG, Zawalich KC & Zawalich WS 1995 Synergistic interaction of glucose and neurohumoral agonists to stimulate islet phosphoinositide hydrolysis. *American Journal of Physiology* **269** E575–E582.
- Kipnis DM 1972 Nutrient regulation of insulin secretion in human subjects. *Diabetes* (Suppl 2) **21** 606–616.
- Lenzen S 1979 Insulin secretion by isolated perfused rat and mouse pancreas. *American Journal of Physiology* **236** E391–E400.
- Loubatieres-Mariani MM, Chapal J, Alric R & Loubatieres A 1973 Studies of the cholinergic receptor involved in the secretion of insulin using isolated perfused rat pancreas. *Diabetologia* **9** 439–476.
- Louis-Sylvestre J 1976 Preabsorptive insulin release and hypoglycemia in rats. *American Journal of Physiology* **230** 56–60.
- Louis-Sylvestre J 1978 Relationships between the two stages of prandial insulin release in rats. *American Journal of Physiology* **235** E103–E111.
- MacDonald MJ, Fahien LA, Mertz RJ & Rana RS 1989 Effects of esters of succinic acid and other citric acid cycle intermediates on insulin release and inositol phosphate formation by pancreatic islets. *Archives of Biochemistry and Biophysics* **269** 400–406.
- McGarry JD & Dobbins RL 1999 Fatty acids, lipotoxicity and insulin secretion. *Diabetologia* **42** 128–138.
- Malaisse WJ, Malaisse-Lagae F & Wright PH 1967 Effect of fasting upon insulin secretion in the rat. *American Journal of Physiology* **213** 843–848.
- Malaisse WJ, Sener A & Levy J 1976 The stimulus-secretion coupling of glucose-induced insulin release. Fasting-induced adaptation of key glycolytic enzymes in isolated islets. *Journal of Biological Chemistry* **251** 1731–1737.
- Malaisse WJ, Sener A, Herchuelz A, Carpinelli AR, Poloczek P, Winand J & Castagna M 1980 Insulinotropic effect of the tumor promoter 12-O-tetradecanoyl phorbol-13-acetate in rat pancreatic islets. *Cancer Research* **40** 3827–3831.
- Niki I, Tamagawa J, Niki H, Niki A, Koide T & Sakamoto N 1988 Possible involvement of diacylglycerol-activated Ca^{2+} -dependent glucose protein kinase in memory of rat pancreatic β -cell. *Acta Endocrinologica* **118** 204–208.

- O'Connor MDL, Landahl H & Grodsky GM 1980 Comparison of storage- and signal-limited models of pancreatic insulin secretion. *American Journal of Physiology* **238** R378–R389.
- Pagliara AS, Stillings SN, Haymond MW, Hover BA & Matschinsky FM 1975 Insulin and glucose as modulators of the amino acid-induced glucagon release in the isolated pancreas of alloxan and streptozotocin diabetic rats. *Journal of Clinical Investigation* **55** 244–255.
- Perley MJ & Kipnis DM 1967 Plasma insulin responses to oral and intravenous glucose: studies in normal and diabetic subjects. *Journal of Clinical Investigation* **46** 1954–1962.
- Rasmussen H, Zawulich KC, Ganesan S, Calle R & Zawulich WS 1990 Physiology and pathophysiology of insulin secretion. (Review). *Diabetes Care* **13** 655–666.
- Rhee SG & Choi KD 1992 Regulation of inositol phospholipid-specific phospholipase C isozymes. *Journal of Biological Chemistry* **267** 12393–12396.
- Rhee SG & Bae YS 1997 Regulation of phosphoinositide-specific phospholipase C isozymes. *Journal of Biological Chemistry* **272** 15045–15048.
- Rhee SG, Suh R-G, Ryu S-H & Lee SY 1989 Studies of inositol phospholipid-specific phospholipase C. *Science* **244** 540–550.
- Rohner-Jeanrenaud F, Bobbioni E, Ionescu E, Sauter JF & Jeanrenaud B 1983 Central nervous system regulation of insulin secretion. *Advances in Metabolic Disorders* **10** 193–220.
- Stein DT, Esser V, Stevenson BE, Lane KE, Whiteside JH, Daniels MB, Chen S & McGarry JD 1996 Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. *Journal of Clinical Investigation* **97** 2728–2735.
- Vadakekalam J, Rabaglia M, Chen Q-H & Metz SA 1996 Glucose-induced phosphoinositide hydrolysis. A requirement for GTP in calcium-induced phospholipase C activation in pancreatic islets. *American Journal of Physiology* **271** E85–E95.
- Wolters GHJ, Konijnndijk W & Bouman PR 1977 Effects of fasting on insulin secretion, islet glucose metabolism, and the cyclic adenosine 3',5'-monophosphate content of rat pancreatic islets *in vitro*. *Diabetes* **26** 530–536.
- Yamaguchi T, Kanatsuka A, Makino H, Ohsawa H & Yoshida S 1989 Dual mechanism involved in the hydrolysis of polyphosphoinositides in rat pancreatic islets. *Endocrinology* **124** 1870–1874.
- Yedovitzky M, Mochly-Rosen D, Johnson JA, Gray MO, Ron D, Abramovitch E, Cerasi E & Nesher R 1997 Translocation inhibitors define specificity of protein kinase C isoenzymes in pancreatic B-cells. *Journal of Biological Chemistry* **272** 1417–1420.
- Zawalich WS 1988a Modulation of insulin secretion from beta-cells by phosphoinositide-derived second-messenger molecules. (Review). *Diabetes* **37** 137–141.
- Zawalich WS 1988b Time-dependent potentiation of insulin release induced by alpha-ketoisocaproate and leucine in rats: possible involvement of phosphoinositide hydrolysis. *Diabetologia* **31** 435–442.
- Zawalich WS 1990 Multiple effects of increases in phosphoinositide hydrolysis on islets and their relationship to changing patterns of insulin secretion. *Diabetes Research* **13** 101–111.
- Zawalich WS 1996 Regulation of insulin secretion by phosphoinositide-specific phospholipase C and protein kinase C activation. *Diabetes Reviews* **4** 160–176.
- Zawalich WS & Zawulich KC 1987 Prior cholecystokinin sensitizes islets to glucose stimulation. *Diabetes* **36** 16–22.
- Zawalich WS & Zawulich KC 1988 Phosphoinositide hydrolysis and insulin release from isolated perfused rat islets. Studies with glucose. *Diabetes* **37** 1294–3000.
- Zawalich WS & Zawulich KC 1992 Biochemical mechanisms involved in monomethyl succinate-induced insulin secretion. *Endocrinology* **131** 649–654.
- Zawalich WS & Zawulich KC 1996 Regulation of insulin secretion by phospholipase C. *American Journal of Physiology* **271** E409–E416.
- Zawalich WS, Dye ES, Pagliara AS, Rognstad R & Matschinsky FM 1979 Starvation diabetes in the rat: onset, recovery, and specificity of reduced responsiveness of pancreatic beta-cells. *Endocrinology* **104** 1344–1351.
- Zawalich W, Brown C & Rasmussen H 1983 Insulin secretion: combined effects of phorbol ester and A23187. *Biochemical and Biophysical Research Communications* **117** 448–455.
- Zawalich W, Zawulich K & Rasmussen H 1984 Insulin secretion: combined tolbutamide, forskolin and TPA mimic action of glucose. *Cell Calcium* **5** 551–558.
- Zawalich WS, Diaz VA & Zawulich KC 1988 Role of phosphoinositide metabolism in induction of memory in isolated perfused rat islets. *American Journal of Physiology* **254** E609–E616.
- Zawalich WS, Zawulich KC & Rasmussen H 1989 Cholinergic agonists prime the β -cell to glucose stimulation. *Endocrinology* **125** 2400–2406.
- Zawalich WS, Zawulich KC & Rasmussen H 1990 Control of insulin secretion: a model involving Ca^{2+} , cAMP and diacylglycerol. *Molecular and Cellular Endocrinology* **70** 119–137.
- Zawalich WS, Zawulich KC, Ganesan S, Calle R & Rasmussen H 1991 Effects of the phorbol ester phorbol 12-myristate 13-acetate on islet-cell responsiveness. *Biochemical Journal* **278** 49–56.
- Zawalich WS, Zawulich KC & Kelley GG 1995 Regulation of insulin release by phospholipase C activation in mouse islets: differential effects of glucose and neurohumoral stimulation. *Endocrinology* **136** 4903–4909.

Received 29 September 1999

Revised manuscript received 3 February 2000

Accepted 9 March 2000