Comparative effects of amino acids and glucose on insulin secretion from isolated rat or mouse islets

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Abstract

Glucose and the combination of leucine and glutamine were used to stimulate insulin secretion from rat islets during a dynamic perfusion and the responses obtained were compared with those elicited from mouse islets under identical conditions. In rat islets, glucose (15 mM) or the amino acid combination of 10 mM glutamine plus 20 mM leucine were most efficacious and peak second-phase insulin release responses were 20- to 30-fold above prestimulatory rates. In contrast to rat islet responses, sustained second-phase insulin secretory responses to the same agonists were minimally increased 1- to 2-fold from mouse islets. Parallel studies demonstrated that phospholipase C (PLC) was markedly activated in rat, but not mouse, islets by both high glucose concentrations and the amino acid combination.

Additional studies documented that glucose and amino acid responses of both rat and mouse islets were amplified by carbachol or forskolin. However, wortmannin, a phosphatidylinositol 3-kinase inhibitor, amplified only the responses to glucose leaving the responses to the amino acid mixture unaltered. These observations support the concept that mitochondrial metabolism alone is minimally effective in stimulating insulin secretion from islets. The activation of the supplementary second messenger systems (PLC and/or cAMP) appears essential for the emergence of their full secretory potential. The mechanism regulating the potency and specificity of wortmannin's impact on glucose-induced secretion remains to be identified; however a unique mechanism is supported by these findings.

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Introduction

In response to sustained square-wave stimulation by high glucose concentrations, rat pancreatic islets studied using the perfused pancreas preparation or perfused islets exhibit a robust, biphasic insulin secretory response (Grodsky 1972, Gerich *et al.* 1974, Henquin & Lambert 1976, Grill *et al.* 1978, O'Connor *et al.* 1980, Curry 1986, Zawalich 1990, Zawalich & Zawalich 1996a). Qualitatively and quantitatively similar responses have been noted during *in vivo* hyperglycemic clamp studies in humans (Van Haefen *et al.* 1990, Elahi 1996) suggesting that the same biochemical processes operative in rat islets also play an important role in human islets.

In contrast to rat and human islets, the responses from mouse islets to glucose are notably different. Most dramatic is the smaller flat but sustained secretory response to glucose stimulation observed in studies using the perfused pancreas or isolated perfused mouse islets (Lenzen 1979, Berglund 1980, Ma *et al.* 1995, Zawalich *et al.* 1995, Shiota *et al.* 2002). We have attributed this species difference, at least in part, to the under expression of a

nutrient-activated phospholipase C (PLC) isozyme in mouse islets when compared with rat islets (Zawalich *et al.* 1995, 2000). The failure of stimulatory glucose to provide sufficient phosphoinositide-derived second messenger molecules in mouse islets may account for their anomalous secretory behavior to high glucose when compared with rat islets. The observation that carbachol, a potent activator of PLC, markedly amplifies glucose-induced release from mouse islets supports this concept (Zawalich *et al.* 1995, Zawalich & Zawalich 1996a, Shiota *et al.* 2002).

Most recently, the combination of the amino acids leucine and glutamine has been employed to evoke biphasic insulin secretion from rat islets (Liu *et al.* 2003). Maximal sustained second-phase release rates comparable with those evoked by high glucose were reported. The present series of experiments were designed to: (1) establish whether the efficacy of these amino acids on the secretory process in rat islets also extends to mouse islets; (2) determine the effects of PLC activation and elevated cAMP on amino acid-induced secretion in both species; (3) determine whether the phosphatidylinositol 3-kinase (PI3K) inhibitor wortmannin, a compound that markedly

amplifies glucose-induced secretion (Hagiwara *et al.* 1995, Nunoi *et al.* 2000, Zawalich & Zawalich 2000, Eto *et al.* 2002, Zawalich *et al.* 2002), exerts a similar effect on amino-acid-induced release in these species. Our findings emphasize the complexity and species differences in agonist-induced secretion from perfused rodent islets and the uniqueness of wortmannin's potentiating effect on glucose-stimulated secretion.

Experimental procedures

The detailed methodologies employed to assess insulin output from collagenase-isolated islets have been described previously (Zawalich & Zawalich 1988a, Zawalich *et al.* 1989a, 1991). Sprague–Dawley rats (weighing 300–475 g) and CD-1 mice (7–14 weeks of age) were purchased from Charles River Labs (Raleigh, NC, USA) and used in all studies. Animals were treated in a manner that complied with the NIH Guidelines for the Care and Use of Laboratory Animals. The animals were fed *ad libitum*. After Nembutal (pentobarbital sodium, 50 mg/kg; Abbott, North Chicago, IL, USA)-induced anesthesia, islets were isolated by collagenase digestion and handpicked, using a glass loop pipette, under a stereomicroscope into Krebs–Ringer bicarbonate (KRB) supplemented with 3 mM glucose. They were largely free of visual exocrine contamination.

Perfusion studies

Islets were loaded onto nylon filters (Sefar America Inc., Kansas City, MO, USA) and perfused in a KRB buffer at a flow rate of 1 ml/min for 30 min in the presence of 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 labeling for inositol phosphate (IP) studies

Groups of 18–22 islets were loaded onto nylon filters 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 25 Ci/mmol) were placed in a 10 mm×75 mm culture tube. To this aliquot of tracer, 255 µl warmed (to 37 °C) and oxygenated KRB medium supplemented with 5.0 mM glucose were added. After mixing, 240 µl of this solution was 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 placed in a metabolic shaker at 37 °C. After the labeling period, the

islets were washed with 5 ml fresh KRB and used for IP measurements. After washing to remove free labeled inositol, the islets on nylon filters were placed in small glass vials. Added gently to the vial was 400 µl warmed (to 37 °C) KRB supplemented with 10 mM LiCl, to prevent IP degradation, and the appropriate agonists as indicated. The vials were capped and after 30 min the generation of IPs was stopped by adding 400 µl 20% perchloric acid. Total IPs were then measured using Dowex columns as described previously (Berridge *et al.* 1983, Zawalich *et al.* 1987).

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 for the insulin assay and the *myo*-[2-³H]-inositol, were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Bovine serum albumin (RIA grade), glucose, leucine, glutamine, wortmannin, carbachol and the salts used to make the Hank's solution and perfusion medium were purchased from Sigma. Forskolin was purchased from Calbiochem (LaJolla, CA, USA). Rat insulin standard (lot number A52-AWK-001) was the generous gift of Dr Gerald Gold, Eli Lilly Co. Collagenase (Type P) was obtained from Roche Diagnostics.

Statistics

Statistical significance was determined using Student's *t*-test for unpaired data or analysis of variance in conjunction with Newman–Keuls test for unpaired data. A *P* value ≥ 0.05 was taken as significant. Values presented in the Figures and results are the mean ± S.E. of at least three observations.

Results

Insulin secretory responses to amino acids and glucose

In the first series of studies we confirmed (Liu *et al.* 2003) that in rat islets the combination of 20 mM leucine plus 10 mM glutamine in the simultaneous presence of 3 mM glucose evokes an insulin secretory response comparable with that observed to high glucose. Prior to the onset of stimulation and in the presence of 3 mM glucose, basal rates of insulin output averaged approximately 20 pg/islet per min from rat islets. The addition of leucine plus glutamine resulted in a secretory response that was most notable for a large sustained output of the hormone (Fig. 1a). Release rates of 472 ± 28 pg/islet per min (*n* = 8) were measured 35–40 min after the onset of stimulation, an increase of about 25-fold over prestimulatory secretion rates.

Figure 1 Comparative effects of the amino acid combination of leucine plus glutamine and 15 mM glucose on insulin secretion from perfused rat and mouse islets. Groups of 14–16 rat (○) or mouse (●) islets were perfused for 30 min with 3 mM glucose (G3) to establish basal, stable rates of insulin output. (a) The islets were then stimulated, indicated by the vertical line, with the combination of 20 mM leucine plus 10 mM glutamine (in the continued presence of 3 mM glucose) for 40 min. The perfusion of mouse islets was continued for an additional 20 min during which time they were stimulated with 20 mM glucose plus 50 μ M carbachol. This data is not shown. (b) The islets were then stimulated, indicated by the vertical line, with 15 mM glucose (G15) for 40 min. The asterisks indicate a significant ($P < 0.05$) difference between release values at this time for the different species. This and subsequent perfusion figures have not been corrected for the dead space in the perfusion apparatus, 2.5 ml or 2.5 min with a flow rate of 1 ml/min. In this and subsequent figures values presented are means $\pm$ S.E.

The magnitude of sustained insulin release from rat islets to 15 mM glucose (Fig. 1b) was similar to the amino acid combination.

Similar studies were conducted using isolated perfused mouse islets. When stimulated with leucine plus glutamine, an initial rapid release of insulin was noted (Fig. 1a). During the initial 3 min of stimulation release rates to this combination from mouse islets were significantly greater than from rat islets. However, unlike rat islets, release rates remained flat as the perfusion progressed and averaged only 34 ± 6 pg/islet per min ($n=8$) when measured 35–40 min after the onset of stimulation. The functional integrity of these islets was established after stimulation with the amino acid mixture by perfusing these same islets with 20 mM glucose plus 50 μ M carbachol for 20 min. Release rates increased dramatically in response to the hexose plus the cholinergic agonist combination. They averaged 584 ± 97 pg/islet per min during the final 5 min of stimulation. When compared with insulin release rates prior to glucose plus carbachol stimulation, this amounted to a 15- to 20-fold increase (data not shown).

A similar dichotomy between these species was observed in response to 15 mM glucose stimulation (Fig. 1b). Again, most dramatic was the failure of mouse islets, when compared with rat islets, to display a large rising second-phase response to 15 mM glucose. Release rates averaged only 47 ± 8 pg/islet per min ($n=5$) during the final 5 min of the perfusion. In contrast, rat islet responses at this time averaged 513 ± 113 pg/islet per min ($n=3$).

Induction of time-dependent sensitization (TDP)

A characteristic of rat islets is their capacity to become sensitized to subsequent stimulation by prior brief exposure to a variety of agonists including glucose. Referred to as TDP, priming or sensitization, it can be induced by a large number of agonists that have in common the capacity to activate PLC or protein kinase C (PKC) (Sorensen 1986, Zawalich & Diaz 1987, Niki *et al.* 1988, Zawalich *et al.* 1988a, 1989b, 1991, Zawalich & Zawalich 1996b, 1998). We next examined how prior short-term exposure to the amino acid combination of leucine and glutamine influenced the subsequent rat secretory responses to glucose. Comparative studies using 15 mM glucose to induce TDP were also conducted in rat islets. As shown in Fig. 2a, prior short-term exposure of rat islets to either the amino acid combination or to 15 mM glucose resulted in an augmented secretory response to a subsequently applied 10 mM glucose stimulus. Most dramatic was the increase in peak first-phase release. While it averaged 34 ± 7 pg/islet per min ($n=5$) in control, unprimed, naïve islets, it increased to 208 ± 65 pg/islet per min ($n=6$) or 231 ± 66 pg/islet per min ($n=4$) after a prior 15 min exposure to the amino acid mixture or 15 mM glucose respectively. Peak second-phase release rates were, however, comparable in all groups of islets (Fig. 2a).

Additional studies were conducted with mouse islets (Fig. 2b). Since we (Zawalich *et al.* 1998) and others (Berglund 1987) reported previously that high glucose

Figure 2 Induction of TDP in rat but not mouse islets. Groups of rat or mouse islets were perfused for 30 min with 3 mM glucose (G3). (a) Rat islets were then stimulated for 15 min with either 15 mM glucose alone (○, solid line) or the combination of 20 mM leucine plus 10 mM glutamine in the continued presence of 3 mM glucose (○, dashed line). Control, unprimed rat islets (△) were maintained with 3 mM glucose alone during this time. This was followed by a 15 min washout period with 3 mM glucose alone. After this all three groups of islets were stimulated for 30 min with 10 mM glucose (G10) and this period is shown here. (b) Mouse islets were then stimulated for 15 min with the combination of 20 mM leucine plus 10 mM glutamine in the continued presence of 3 mM glucose (●, dashed line). Control, unprimed mouse islets (▲) were maintained with 3 mM glucose alone during this time. This was followed by a 15 min washout period with 3 mM glucose alone. After this both groups of islets were stimulated for 30 min with 15 mM glucose (G15) and this period is shown here. The asterisks indicate a significant ($P<0.05$) difference between release values for the primed islets or the control, naïve islets.

stimulation is incapable of inducing TDP in mouse islets, we focused our attention on the amino acid mixture using conditions that proved effective in inducing TDP in rat islets. Since 10 mM glucose alone is only minimally effective in stimulating secretion from mouse islets, the concentration of glucose used to assess the induction of TDP was increased to 15 mM. After a prior 15 min exposure to the amino acid combination, there was a tendency for the onset of secretion in response to 15 mM glucose to occur earlier in the amino-acid-pretreated mouse islets and the release of insulin was significantly

greater during the first several minutes of stimulation (Fig. 2b). However, prior exposure to the amino acid combination had no significant effect on the magnitude of the first-phase response to 15 mM glucose. For example, while peak first-phase release from control, naïve islets averaged 81 ± 17 pg/islet per min ($n=7$) in response to a subsequently administered 15 mM glucose stimulus, the comparable responses from amino-acid-primed islets was only 99 ± 14 pg/islet per min ($n=12$), an insignificant increase.

Impact of leucine plus glutamine on PLC activation: IP accumulation in islets

In an attempt to explore the potential biochemical mechanism responsible for the species dichotomy to amino acid stimulation, additional studies were performed. Islet phosphoinositide pools were prelabeled with ^3H -inositol for 3 h. They were subsequently stimulated with the amino acid combination or 15 mM glucose. The results are given in Table 1. Basal IP levels in the presence of 3 mM glucose alone averaged 4489 ± 484 c.p.m./40 islets per 30 min ($n=4$) in rat islets. When stimulated with amino acids, IP levels increased to 16724 ± 1300 c.p.m./40 islets per 30 min ($n=7$). Stimulation of rat islets with 15 mM glucose also resulted in a comparable increment in IP levels.

In contrast to rat islet responses to the amino acid mixture, but consistent with their minimal stimulatory effect on insulin secretion, the amino acid mixture failed to increase IP accumulation in mouse islets (Table 1) above that observed in response to 3 mM glucose alone. A small (25%) but significant increase in IP levels was observed in response to 15 mM glucose.

Impact of carbachol, forskolin or wortmannin on agonist-induced secretion

We next determined the impact of several established potentiators of insulin secretion on amino acid- or glucose-induced secretion. Three compounds were studied: carbachol, forskolin and wortmannin. Carbachol activates PLC, increases IP accumulation but has little, if any, sustained effect on release in the presence of substimulatory glucose (Best & Malaisse 1983, 1984, Henquin *et al.* 1988, Zawalich *et al.* 1989b, Gilon & Henquin 2001). Forskolin activates adenylate cyclase (Seamon & Daly 1981) and potentiates insulin secretion in response to a large number of compounds (Wiedenkeller & Sharp 1983, Henquin *et al.* 1987, Rasmussen *et al.* 1990, Zawalich *et al.* 1990). Like carbachol, however, it is impotent at low glucose concentrations (Wiedenkeller & Sharp 1983). Wortmannin inhibits the insulin signaling enzyme PI3K and markedly enhances glucose-induced secretion (Hagiwara *et al.* 1995, Nuno *et al.* 2000, Zawalich & Zawalich 2000, Eto *et al.* 2002, Zawalich *et al.* 2002).

Table 1 Inositol phosphates responses in rat and mouse islets

	IP accumulation (c.p.m./40 islets/30 min)	
	Rat	Mouse
1. G3	4489 ± 484 (n=4)	1936 ± 137 (n=3)
2. G3+LEU+GLN	16 724 ± 1300 (n=7)	1998 ± 134 (n=3)
3. G15	17 150 ± 1494 (n=4)	2545 ± 250 (n=3)
4. G3+LEU+GLN+CARB	22 501 ± 1923 (n=3)	16 506 ± 2762 (n=3)

Groups of rat or mouse islets were incubated for 3 h in the presence of ^3H -inositol to label their phosphoinositide pools. After washing to remove unincorporated labels they were incubated for 30 min with the following compounds as indicated above: G3, 3 mM glucose; G3 plus 20 mM leucine (LEU) plus 10 mM glutamine (GLN); G15, 15 mM glucose; G3 plus LEU plus GLN plus CARB (carbachol, 50 μM). The labeled IPs generated during a 30 min incubation period were measured as described in the Methods section. Statistical analyses are as follows. Rat: line 1 vs lines 2, 3 and 4, significant; line 2 vs line 3, not significant (NS). Mouse: line 1 vs line 2, NS; line 1 vs line 3, significant; lines 1, 2 and 3 vs line 4, significant.

Similar to both carbachol and forskolin, its impact on secretion is glucose dependent (Zawalich & Zawalich 2000).

First, in the presence of 3 mM glucose alone, carbachol (50 μM), forskolin (10 μM) or wortmannin (50 nM) have no sustained stimulatory effect on insulin secretion from mouse islets (results not shown). However, the inclusion of carbachol or forskolin, but not wortmannin, together with leucine plus glutamine converts their minimal impact on release into a robust secretory response. Most dramatic was the response to carbachol addition to the amino acid mixture (Fig. 3a). Release was biphasic in nature and when measured 35–40 min after the onset of stimulation, rates averaged 545 ± 66 pg/islet per min ($n=10$). Forskolin addition to the amino acid mixture also significantly potentiated secretion (Fig. 3b). Like the response to the mixture alone, however, release rates tended to fall as the perfusion progressed. During the final 5 min of stimulation, release rates averaged 233 ± 24 pg/islet per min ($n=5$). In contrast to carbachol or forskolin, wortmannin had no potentiating effect on amino-acid-induced insulin secretion from mouse islets (Fig. 3b). However, the PI3K inhibitor markedly potentiated the secretory response of mouse islets to 15 mM glucose (Fig. 4).

The observation that wortmannin had no effect on mouse islet responses to the amino acid mixture prompted additional studies using rat islets. Our goal was to ascertain if wortmannin, known to potentiate glucose-induced secretion from both rat and mouse islets (Nunoi *et al.* 2000, Zawalich & Zawalich 2000, Eto *et al.* 2002, Lee *et al.* 2003) fails to potentiate amino-acid-induced release from rat islets as well. Since the impact of wortmannin on glucose-induced release from rat islets is best observed at modest stimulatory levels of glucose (Zawalich & Zawalich 2000), lower levels (7–8 mM) of the hexose were utilized and the amino acid concentrations were also reduced. For comparative purposes, and for a positive control, forskolin (1 μM) was utilized in additional studies. The results are depicted in Figs 5 and 6.

At a hexose concentration of 7 mM, glucose-induced insulin release from rat islets was modest and comparable with that observed in response to the combination of 2.5 mM leucine plus 2.5 mM glutamine (Fig. 5a and b, ○). Irrespective of the nutrient agonist employed, forskolin (●) significantly amplified their effectiveness on the secretory process. The addition of 50 nM wortmannin (▲) also markedly amplified the response to 7 mM glucose from rat islets, particularly during the latter stages of the perfusion. For example, 55–60 min after the onset of stimulation release rates in the presence of 7 mM glucose alone averaged 49 ± 16 pg/islet per min ($n=8$). The inclusion of 50 nM wortmannin increased the response at this time to 206 ± 37 pg/islet per min ($n=6$). In sharp contrast to its impact on glucose-induced release, wortmannin had no significant potentiating effect on an approximately equipotent amino acid mixture (Fig. 5b).

We considered the possibility that the level of the amino acid mixture used was too low and that a positive effect of wortmannin might be observed if we elevated the levels of amino acids to 5 mM each. Similar experiments were conducted with an approximately equipotent 8 mM glucose stimulus. The results are shown in Fig. 6. Despite a greater impact on secretory rates, we corroborated what we observed with the lower levels of glucose or amino acid combination. Wortmannin (50 nM) significantly potentiated 8 mM glucose-induced secretion from rat islets (Fig. 6a) but had no effect on an approximately equipotent 5 mM amino acid mixture (Fig. 6b). Forskolin (1 μM), however, amplified secretion irrespective of the stimulant used.

Discussion

The present studies conducted with rat and mouse islets had three primary objectives. The first was to establish if the combination of the amino acids leucine and glutamine was equally effective in both species. Secondly, we wanted

Figure 3 Amplifying effect of carbachol or forskolin on amino-acid-induced insulin secretion from perfused mouse islets. Groups of mouse islets were perfused for 30 min with 3 mM glucose (G3). (a) For an additional 40 min a control group (●, $n=8$) was perfused for 40 min with 20 mM leucine plus 10 mM glutamine in the continued presence of 3 mM glucose (these are the same data presented in Fig. 1a). The second group (●, dashed line, $n=4$) was perfused for 40 min with 20 mM leucine plus 10 mM glutamine plus 50 μ M carbachol in the continued presence of 3 mM glucose. (b) The third group (○, $n=5$) was perfused for 40 min with 20 mM leucine plus 10 mM glutamine plus 1 μ M forskolin in the continued presence of 3 mM glucose. The fourth group (▲, $n=3$) was perfused for 40 min with 20 mM leucine plus 10 mM glutamine plus 50 nM wortmannin. The asterisks indicate a significant ($P<0.05$) difference between release values at this time.

to establish the impact of two established, but mechanistically different, potentiators of secretion – i.e. carbachol and forskolin – on secretory response to the amino acids.

Figure 4 Effect of wortmannin on 15 mM glucose-induced insulin release from mouse islets. Groups of mouse islets were perfused for 30 min with 3 mM glucose (G3). For an additional 40 min a control group (○, $n=7$) was perfused for 40 min with 15 mM glucose (G15). The second group (▲, $n=4$) was stimulated for 40 min with 15 mM glucose plus 50 nM wortmannin. The asterisks indicate a significant ($P<0.05$) difference between release values at this time.

Finally, we wanted to determine if wortmannin, a PI3K inhibitor that potentiates glucose-induced secretion from rat islets, exerted a similar effect if amino acids are used to stimulate the islets. Further comments on our findings are warranted.

In response to 15 mM glucose or to the combination of 20 mM leucine plus 10 mM glutamine our isolated perfused rat islet preparation responded with a brisk insulin secretory response. The onset of stimulated secretion was comparable under both stimulatory conditions and the functional integrity of our preparation was established by the approximately 25-fold increase in release rates that occurred during the peak second-phase response. These observations are consistent with those made previously by Liu *et al.* (2003). We also documented, in agreement with previous studies (Grill 1978, Grill *et al.* 1978, O'Connor *et al.* 1980, Zawalich 1988, Zawalich *et al.* 1988b, Bliss & Sharp 1992), that prior short-term exposure of rat islets to 15 mM glucose induced TDP of insulin secretion, a phenomenon most noticeable in our studies during the initial response to a subsequently administered 10 mM glucose stimulus. The combination of leucine plus glutamine proved as effective an inducer of TDP as 15 mM glucose alone. We have previously suggested that events associated with PLC/PKC signaling in islets are intimately involved in supporting both a large rising second-phase secretory response in rat islets and in the induction of TDP (Zawalich 1996, Zawalich & Zawalich 1996a). Consistent

Figure 5 Effects of forskolin and wortmannin on glucose or amino-acid-induced insulin secretion. Groups of rat islets were isolated and perfused for 30 min with 3 mM glucose (G3). (a) One group (○, $n=8$) was stimulated for 60 min with 7 mM glucose (G7) alone. The second group (●, $n=6$) was stimulated for 60 min with 7 mM glucose plus 1 μM forskolin. The third group (▲, $n=6$) was stimulated for 60 min with 7 mM glucose plus 50 nM wortmannin. (b) One group (○, $n=11$) was stimulated for 60 min with the combination of 2.5 mM leucine plus 2.5 mM glutamine. The second group (●, $n=7$) was stimulated for 60 min with the combination of 2.5 mM leucine plus 2.5 mM glutamine plus 1 μM forskolin. The third group (▲, $n=11$) was stimulated for 60 min with the combination of 2.5 mM leucine plus 2.5 mM glutamine plus 50 nM wortmannin. The asterisks indicate a significant ($P<0.05$) difference in secretion at this time between control islets stimulated with 7 mM glucose or the amino acid combination versus those perfused in the additional presence of either wortmannin or forskolin.

with this concept we observed in these studies that both high glucose and the amino acid mixture induced a significant activation of PLC, monitored by IP accumulation in ^3H -inositol-prelabeled islets. These observations reinforce our contention that this biochemical pathway plays a major role in these rat islet response patterns.

In contrast to what was observed using rat islets, perfused mouse islet responses to 15 mM glucose alone or to the amino acid combination were most notably different from rat islets in their failure to mount a robust rising

Figure 6 Effects of forskolin and wortmannin on glucose or amino-acid-induced insulin secretion. Groups of rat islets were isolated and perfused for 30 min with 3 mM glucose (G3). (a) One group (○, $n=6$) was stimulated for 60 min with 8 mM glucose (G8) alone. The second group (●, $n=4$) was stimulated for 60 min with 8 mM glucose plus 1 μM forskolin. The third group (▲, $n=6$) was stimulated for 60 min with 8 mM glucose plus 50 nM wortmannin. (b) One group (○, $n=11$) was stimulated for 60 min with the combination of 5 mM leucine plus 5 mM glutamine. The second group (●, $n=7$) was stimulated for 60 min with the combination of 5 mM leucine plus 5 mM glutamine plus 1 μM forskolin. The third group (▲, $n=10$) was stimulated for 60 min with the combination of 5 mM leucine plus 5 mM glutamine plus 50 nM wortmannin. The asterisks indicate a significant ($P<0.05$) difference in secretion at this time between control islets stimulated with 8 mM glucose or the amino acid combination versus those perfused in the additional presence of either wortmannin or forskolin.

second-phase response to these compounds. During the first few minutes of stimulation, however, insulin release responses to the amino acid combination were significantly greater from mouse islets than from rat islets. Whether this reflects more rapid permeation of the amino acids into the islet, a difference in their initial rate of metabolism or the more rapid but transient generation of second messenger signals is not clear from this type of analysis. Despite this initial response, release rates fell rapidly as the perfusion progressed. While it might be

argued that mouse islets are more vulnerable than rat islets to any potential deleterious effect of the collagenase isolation procedure, the secretory integrity of our islet preparation was confirmed by subsequent stimulation with 20 mM glucose plus the PLC activator carbachol. Under this condition robust and sustained insulin secretion was observed.

Two other deviant response patterns of mouse islets when compared with rat islets were also noted. First, while prior exposure to the amino acid combination was capable of sensitizing rat islets to subsequent stimulation with glucose, no such priming impact was observed with perfused mouse islets. For example, peak first-phase responses to restimulation with high glucose were increased about 600% from rat islets. In mouse islets peak first-phase release to glucose was insignificantly increased by about 20%. Second, the amino acid mixture also failed to activate PLC in mouse islets. For example, the accumulation of IPs in response to the amino acid mixture was increased 3- to 4-fold in rat islets. In mouse islets, no stimulatory effect was observed in response to the amino acid combination.

The next set of experiments focused on two known potentiators of insulin secretion that work by activating PLC or by increasing cAMP. Information flow in both systems is activated by glucose and they appear integral for the maintenance of islet responsiveness (Henquin *et al.* 1987, Zawalich *et al.* 1990). The studies with carbachol were based on our hypothesis that the activation of PLC provides those signals that play an important role in the regulation of several β -cell response patterns, including a rising second phase of insulin release and the induction of TDP, in rat islets. We predicted that carbachol should have a major restorative impact on second-phase secretion from mouse islets on the basis of the following considerations. First, the deficient activation of PLC is responsible, at least in part, for the aberrant mouse islet secretory responses reported here and elsewhere (Berglund 1980, 1987, Ma *et al.* 1995, Zawalich *et al.* 1995) to both glucose and the amino acid mixture. Secondly, since there are no data to suggest the contrary, we assumed that comparable amounts of a mitochondrial signal are generated in both rat and mouse islets by the amino acid mixture. However, this signal alone, in the absence of significant PLC activation, is insufficient to induce a large rising second-phase response from mouse islets. Thirdly, carbachol activates PLC and generates PI-derived second messenger molecules (Best & Malaisse 1984, Zawalich *et al.* 1989b, Gilon & Henquin 2001). However, it fails to augment secretion because it fails to generate the appropriate mitochondrial signal provided by fuel agonists (Zawalich *et al.* 1989b, Kelley *et al.* 1994). We reasoned that carbachol inclusion together with the amino acid mixture should, if our hypothesis is correct, now result in a large rising insulin secretory response. This prediction was confirmed. Moreover, the failure of the amino acid mixture to stimulate

secretion from mouse islets cannot be ascribed to a reduction in the generation of the mitochondrial fuel signal. Sufficient amounts of this signal must be produced since the addition of carbachol, a compound without any significant effect on metabolism or insulin release in the absence of fuel, markedly potentiates secretion to the amino acid combination. If the lesion in mouse islets is at the level of fuel signal generation, carbachol addition should have little if any restorative or potentiating impact. This was clearly not the case.

Because both the PLC/PKC and the PKA/cAMP second messenger system serve to amplify nutrient-induced secretion (Henquin *et al.* 1987, Zawalich *et al.* 1990), we probed the effect of elevating cAMP levels using the diterpene forskolin. This pharmacological tool is a direct activator of adenylate cyclase that in the absence of added nutrient has little, if any, independent stimulatory effect (Wiedenkeller & Sharp 1983, Yamada *et al.* 2002). The addition of forskolin to the amino acid mixture significantly augmented insulin secretion from mouse islets. Since no stimulatory effect to forskolin alone was observed, these results also reinforce our contention that the amino acid mixture is generating the appropriate metabolic signals. The provision of the second messenger cAMP allows expression of their secretory effect.

The final set of experiments was conducted exclusively with rat islets and was designed to assess the impact of wortmannin on amino-acid-induced release from this species. Comparable studies were conducted using equipotent glucose levels as well. Wortmannin has been used in many studies to inhibit the enzyme PI3K, a major insulin signaling protein in a number of tissues (Acaro & Wymann 1993, Okada *et al.* 1994, Yeh *et al.* 1995). We reported previously that wortmannin potentiates glucose-induced secretion from lean but not obese rodent islets and have suggested further that impaired insulin signaling, mediated via defective PI3K activation, contributes to the islets hyperresponsiveness seen in obesity. The fact that wortmannin potentiates insulin secretion has been noted by at least four other groups (Hagiwara *et al.* 1995, Nunoi *et al.* 2000, Eto *et al.* 2002, Lee *et al.* 2003).

We confirmed that in the presence of 7–8 mM glucose, 50 nM wortmannin is a potentiator of hexose-induced insulin secretion. This effect on release was also duplicated by forskolin which elevates cAMP in islets (Wiedenkeller & Sharp 1983). Not surprisingly, forskolin also amplified amino-acid-induced release. However, secretion in response to the amino acid combination was immune to wortmannin. If, as has been suggested, wortmannin amplifies secretion by elevating cAMP (Nunoi *et al.* 2000), it should have mimicked the effect of forskolin on amino acid-induced secretion. This finding suggests that the impact of wortmannin on glucose-induced secretion is not mediated exclusively via cAMP and that other potential mechanisms should be considered.

While the specificity of wortmannin's actions remains to be clarified, a reasonable suggestion is that metabolic events unique to glucose utilization are involved. Since both classes of nutrients are metabolized within the mitochondrion, these studies suggest that PI3K interacts with glucose-derived glycolytic or pentose cycle signals, metabolic pathways unique to glucose but not amino acid metabolism. It is interesting to note that, more than 25 years ago, Ammon and coworkers (Akhtar *et al.* 1977) reported that insulin, presumably via PI3K, regulates the pentose cycle in rat islets. The impact of wortmannin on pentose cycle activation and NAD(P)H/NAD(P) ratios should be explored in future studies.

In attempting to provide a unifying working hypothesis to explain the failure of both high glucose concentrations and the amino acid mixture to evoke a rising and sustained second-phase insulin secretory response from mouse islets, and its restoration by carbachol and forskolin, one additional point should be taken into consideration. It might be reasonably argued that the anomalous behavior of mouse islets is the result of two separate but independent lesions: failure to activate the adenylate cyclase/cAMP and the PLC/PKC signaling systems. In rat islets, glucose is known to increase both the activation of PLC (Axen *et al.* 1983, Best & Malaisse 1983, Zawalich & Zawalich 1988b, Vadakekalam *et al.* 1996) and adenylate cyclase (Charles *et al.* 1973, Zawalich *et al.* 1975). Might the generation of one of these second messengers increase the generation of the other? This issue has been addressed in other studies using a number of tissues including islets. First, to the best of our knowledge there are no data suggesting that the activation of adenylate cyclase results in the activation of PLC. In fact, cAMP appears to restrain the activation of PLC, a type of negative feedback (Zawalich *et al.* 1988a, Rhee *et al.* 1989, Zawalich & Zawalich 1990, Rhee & Choi 1992). In contrast there are numerous reports demonstrating that the activation of PKC results in the activation of adenylate cyclase (Malaisse *et al.* 1980, Audinot *et al.* 1991, Böhl *et al.* 1997, Tian & Laychock 2001). These findings suggest, perhaps, that the failure of nutrients to activate PLC may secondarily result in a failure to increase cAMP (Ma *et al.* 1995). In any case, the provision of either a PLC or adenylate cyclase activator should exert a significant restorative effect, which is indeed the case.

When taken in their entirety, these experiments emphasize the inherent complexity of stimulus-response coupling in rodent islets. The factors that limit secretion may differ among different species. Converging or parallel signaling pathways may compensate for any anomalous biochemical response patterns as observed in the activation of PLC by nutrients in rat versus mouse islets. For example, the failure of the fuel molecule glucose to significantly activate PLC in mouse islets may be compensated for by receptor-mediated activation of the enzyme by cholinergic input to the islet. Moreover, other signaling

molecules, such as cholecystokinin, which also activates PLC (Zawalich *et al.* 1987), and the incretins glucagon-like peptide or gastric inhibitory polypeptide that elevate cAMP, may augment the secretory response as well (Zawalich *et al.* 1989c, Zawalich & Zawalich 1996b). These findings suggest caution in the extrapolation of signal transduction biochemical data between species unless accompanied by supporting insulin secretory responses as well. These also serve to emphasize the potential significance of phosphoinositide-derived signals to the evocation of a robust rising second-phase secretory response and the induction of TDP. Even though important metabolic signals are generated in response to a variety of fuels, their transduction into an effective secretory response may depend, at least in part, on the provision of the appropriate phospholipid-derived signals. Finally, these experiments also serve to emphasize the potential involvement of PI3K in the regulation of glucose-induced secretion. Considering its possible clinical importance, the potential contribution of this enzyme to the β -cell adaptation that occurs during obesity, diabetes and other insulin-resistant states should be rigorously investigated. In conclusion, establishing the identity of the biochemical pathways uniquely activated by glucose and that underlie the emergence of robust biphasic insulin secretion may allow us to identify the lesion that impairs this response in manifest diabetes. Species studies such as those presented here may facilitate achieving this objective.

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