Cephalic phase insulin secretion is K_{ATP} channel independent

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Abstract

Glucose-induced insulin secretion from pancreatic β-cells critically depends on the activity of ATP-sensitive K^+ channels (K_{ATP} channel). We previously generated mice lacking *Kir6.2*, the pore subunit of the β-cell K_{ATP} channel (*Kir6.2*$^{-/-}$), that show almost no insulin secretion in response to glucose *in vitro*. In this study, we compared insulin secretion by voluntary feeding (self-motivated, oral nutrient ingestion) and by forced feeding (intra-gastric nutrient injection via gavage) in wild-type (*Kir6.2*$^{+/+}$) and *Kir6.2*$^{-/-}$ mice. Under *ad libitum* feeding or during voluntary feeding of standard chow, blood glucose levels and plasma insulin levels were similar in *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice. By voluntary feeding of carbohydrate alone, insulin secretion was induced significantly in *Kir6.2*$^{-/-}$ mice but was markedly attenuated compared with that in *Kir6.2*$^{+/+}$ mice. On forced feeding of standard chow or carbohydrate alone, the insulin secretory response was markedly impaired or completely absent in *Kir6.2*$^{-/-}$ mice. Pretreatment with a muscarine receptor antagonist, atropine methyl nitrate, which does not cross the blood–brain barrier, almost completely blocked insulin secretion induced by voluntary feeding of standard chow or carbohydrate in *Kir6.2*$^{-/-}$ mice. Substantial glucose-induced insulin secretion was induced in the pancreas perfusion study of *Kir6.2*$^{-/-}$ mice only in the presence of carbamylcholine. These results suggest that a K_{ATP} channel-independent mechanism mediated by the vagal nerve plays a critical role in insulin secretion in response to nutrients *in vivo*.

Key Words
- K_{ATP} channel
- cephalic phase insulin secretion
- acetylcholine

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Introduction

Insulin secreted from pancreatic β-cells plays a pivotal role in the maintenance of glucose homeostasis. Insulin secretion is regulated by various factors including nutrients such as glucose, amino acids, and fatty acids; gastrointestinal hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP); and neuronal (sympathetic and parasympathetic) inputs to β-cells (Nishi *et al.* 1987, Fehmann *et al.* 1995, Ahrén 2000, Gilon & Henquin 2001, Bratanova-Tochkova *et al.* 2002, Gautam *et al.* 2006, Nolan *et al.* 2006). Among these factors, glucose is unique in that it can trigger insulin secretion by itself, while the other factors stimulate or potentiate insulin only in the presence of glucose (Pagliara *et al.* 1975, Hedeskov 1980, Weir *et al.* 1989, Henquin 2000). Glucose

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is transported into the β-cells by glucose transporters and then metabolized, leading to an increase in the ATP concentration (ATP:ADP ratio), closure of the ATP-sensitive K^+ (K_{ATP}) channels, depolarization of the β-cell membrane, and opening of the voltage-dependent Ca^{2+} channels (VDCCs), which allows Ca^{2+} influx that triggers insulin secretion. The K_{ATP} channel plays an essential role in this process by linking metabolic alteration to electrical activity of the cells.

We previously generated mice lacking *Kir6.2*, the pore subunit of the β-cell K_{ATP} channel (*Kir6.2*$^{-/-}$) (Miki *et al.* 1998), and found that *Kir6.2*$^{-/-}$ mice elicit no significant increase in insulin secretion in response to glucose using perifusion of isolated pancreatic islets (Miki *et al.* 1998) and perfusion of pancreas (Miki *et al.* 2005), both of which were performed under denervated, *in vitro* condition. In *Sur1*$^{-/-}$ mice, which lack the regulatory subunit of the channel, only a very small amount of insulin in response to glucose is triggered, as assessed by perfusion of pancreas (Shiota *et al.* 2002) and batch incubation of isolated pancreatic islets (Doliba *et al.* 2004). Recently, in pancreatic β-cell-specific *Kir6.2* mutant mice, only a small amount of insulin in response to glucose was induced (Girard *et al.* 2009, Remedi *et al.* 2009). Intra-gastric glucose injection via gavage resulted in no or a very small insulin secretion in both *Kir6.2*$^{-/-}$ and *Sur1*$^{-/-}$ mice (Shiota *et al.* 2002, Miki *et al.* 2005). In addition to glucose, many other factors increase insulin secretion. Among these, GLP-1 and GIP potentiate insulin secretion in a glucose-dependent manner. We previously reported that there is an insulinotropic effect of GLP-1 even under K_{ATP} channel-deficient condition, suggesting a K_{ATP} channel-independent mechanism of GIIS; however, in *Kir6.2*$^{-/-}$ mice, GIIS induced by GLP-1 is markedly attenuated when compared with that in the *Kir6.2*$^{+/+}$ mice (Fujimoto *et al.* 2009). In addition, acetylcholine stimulation was shown to increase intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) in β-cells of both *Kir6.2*$^{-/-}$ and *Sur1*$^{-/-}$ mice *in vitro* (Miki *et al.* 1998, Doliba *et al.* 2004). *In vivo*, the insulin secretory response to mixed liquid meal via gastric gavage is delayed but is induced to some extent in *Kir6.2*$^{-/-}$ mice (Miki *et al.* 2005). However, *Kir6.2*$^{-/-}$ mice fail to develop diabetes in an *ad libitum*-fed state with standard chow. In addition, we incidentally found that plasma insulin levels did not differ in *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice in an *ad libitum*-fed state. These findings suggest that the insulin secretory response to glucose alone is defective in *Kir6.2*$^{-/-}$ mice, while that to other nutrition or vagal nerve stimulation may be maintained. In this study, we demonstrate that cephalic phase insulin secretion is mediated by neuronal action through the K_{ATP} channel-independent pathway.

Materials and methods

Mice

Kir6.2$^{-/-}$ mice were generated as described previously (Miki *et al.* 1998). As the *Kir6.2*$^{-/-}$ mice had been backcrossed to the C57BL/6 mouse strain for more than five generations, wild-type (*Kir6.2*$^{+/+}$) C57BL/6 mice were used as control mice. The mice had free access to food and water for maintenance breeding. Prior to the voluntary feeding experiments, the mice were individually caged and trained for 3 days to immediately eat the test nutrients presented on the floor of the cage at mealtimes. All animal experiments were performed in accordance with the guidelines of the Animal Care Committee of Kobe and Chiba University.

Blood sampling

Blood glucose levels and plasma insulin levels were measured at 0, 15, and 60 min after nutrients were delivered.

Materials

Standard rodent normal chow diet, Racol (Otsuka Pharmaceutical Co, Ltd, Tokushima, Japan), Twinline (Otsuka Pharmaceutical Co, Ltd), and dextrin were used. Standard normal chow (CE-2, CLEA Japan, Inc., Tokyo, Japan) contains 3.5 kcal/g calorie in total (protein 28.7%, carbohydrate 58.6%, and fat 12.7% kcal/g). A clinically used polymeric formula (Racol) mainly consists of casein from milk protein, soy protein isolate, maltodextrin, and purified white soft sugar and fat from oils and tricaprylin. It contains 5.06 g/dl protein, 16.2 g/dl carbohydrate, and 1.96 g/dl fat (1 kcal/ml calorie in total). The oligomeric formula (Twinline) comprises mostly di- and tri-peptides as nitrogen source, maltodextrin as carbohydrate, and safflower oil and tricaprylin as fat. It contains 4.05 g/dl protein, 14.7 g/dl carbohydrate, and 2.78 g/dl fat (1 kcal/ml calorie in total). Dextrin hydrate was purchased from Wako, Osaka, Japan. Racol, Twinline, or 30 g/dl dextrin were made into solid food by adding 4 g/dl agar.

Oral tolerance test

Into the stomach of mice, 1.5 ml dextrin liquid (containing dextrin hydrate 0.3 g) or 1.5 ml normal chow liquid (containing standard chow 0.3 g) were injected.

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Voluntary feeding (re-feeding) and forced feeding experiment

Both *Kir6.2*$^{-/-}$ mice and *Kir6.2*$^{+/+}$ mice were trained to eat the materials (standard chow, dextrin, Racol, or Twinline) as soon as they were put on the floor of the cage. After a 16-h fast, both *Kir6.2*$^{-/-}$ mice and *Kir6.2*$^{+/+}$ mice were given either free access to the materials for voluntary feeding or they were injected directly into the stomach by gavage for the forced feeding experiment.

Atropine experiment

Sixty minutes before the mice ate standard chow or dextrin, either 2.5 mg/kg atropine methyl nitrate (Sigma; Havel *et al.* 1993) or saline was administered intraperitoneally.

Perfusion

Perfusion experiments using mouse pancreata were performed as described previously (Miki *et al.* 2005). Perfusion protocols began with a 10-min equilibration period with the same buffer used in the initial step. The insulin levels in the perfusate were measured by an ELISA kit (Mesacup Insulin Test) from BML, Inc. (Nagoya, Japan). Carbachol (carbamylcholine chloride) was from Wako.

Serum assay

Plasma insulin was measured using commercially available kits (Morinaga Institute of Biological Science, Yokohama, Japan).

Statistical analysis

Data are expressed as mean$\pm$S.E.M. Significance of the differences between two groups was measured by Student's *t*-test. ANOVA was used for multiple comparisons with a control group. A probability level of $P<0.05$ was considered statistically significant.

Results

Blood glucose and plasma insulin levels in voluntary feeding and forced feeding

Blood glucose and plasma insulin levels in fasted and *ad libitum*-fed states were similar in *Kir6.2*$^{+/+}$ mice and *Kir6.2*$^{-/-}$ mice (Fig. 1a), indicating normal regulation of insulin secretion in physiological conditions *in vivo*. To evaluate the insulin response by voluntary feeding, we measured plasma insulin levels before and 15 and 60 min after the initiation of voluntary feeding of standard chow (CE-2). Plasma insulin levels in *Kir6.2*$^{-/-}$ mice were increased at 15 and 60 min to levels comparable to those of *Kir6.2*$^{+/+}$ mice (Fig. 1b). These results show that the secretory response to voluntary feeding of standard chow in *Kir6.2*$^{-/-}$ mice is maintained.

We then administered powdered, water-suspended, standard chow directly into the stomach of *Kir6.2*$^{+/+}$ mice or *Kir6.2*$^{-/-}$ mice by gavage. After forced feeding, insulin secretion in *Kir6.2*$^{-/-}$ mice at 15 min after loading was significantly lower than that in *Kir6.2*$^{+/+}$ mice (62.6 $\pm$10.2 pmol/l, $n=4$, 164$\pm$34.7 pmol/l, $n=4$, $P<0.05$) and the glucose level of *Kir6.2*$^{-/-}$ mice (33.0$\pm$2.85 mmol/l, $n=4$) was significantly higher than that of *Kir6.2*$^{+/+}$ mice (21.0$\pm$2.38 mmol/l, $n=4$, $P<0.05$) at 60 min (Fig. 1c).

To assess the effects of feeding behavior (taste, mastication, and swallowing) on insulin secretion, we administered *Kir6.2*$^{-/-}$ mice dextrin, a partially hydrolyzed product of starch, by forced intra-gastric ingestion (0.3 g/body) or by voluntary feeding (23% (wt/vol) dextrin jellied by 4% agar). Dextrin is digested in the oral cavity by amylase and its derivative maltose gives rise to sweet sensation in the mouth (Ninomiya *et al.* 1998). Thus, voluntary feeding of dextrin is likely to evoke insulin secretion through sensing of sweetness in the cephalic phase of insulin secretion (Berthoud *et al.* 1980). Interestingly, a small but significant increase in insulin secretion was induced by voluntary feeding of carbohydrate only in *Kir6.2*$^{-/-}$ mice (Fig. 1d). As expected, no significant increase in insulin secretion in *Kir6.2*$^{-/-}$ mice was detected by forced feeding of dextrin (carbohydrate alone) (Fig. 1e). In addition, we also found that voluntary feeding of dextrin augmented insulin secretion in *Kir6.2*$^{-/-}$ mice even at 60 min after dextrin ingestion, suggesting that cephalic regulation modulates both early (cephalic phase) and late (gut phase) phases of insulin secretion.

Participation of the parasympathetic nerve in insulin response to voluntary feeding

We then examined parasympathetic stimulation of GIIS in K_{ATP} channel-deficient β-cells. We performed perfusion experiments in *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice (Fig. 2a). In *Kir6.2*$^{+/+}$ mice, 50 μM carbachol failed to stimulate insulin secretion in the presence of glucose (2.8 mM), but insulin secretion was markedly enhanced by switching

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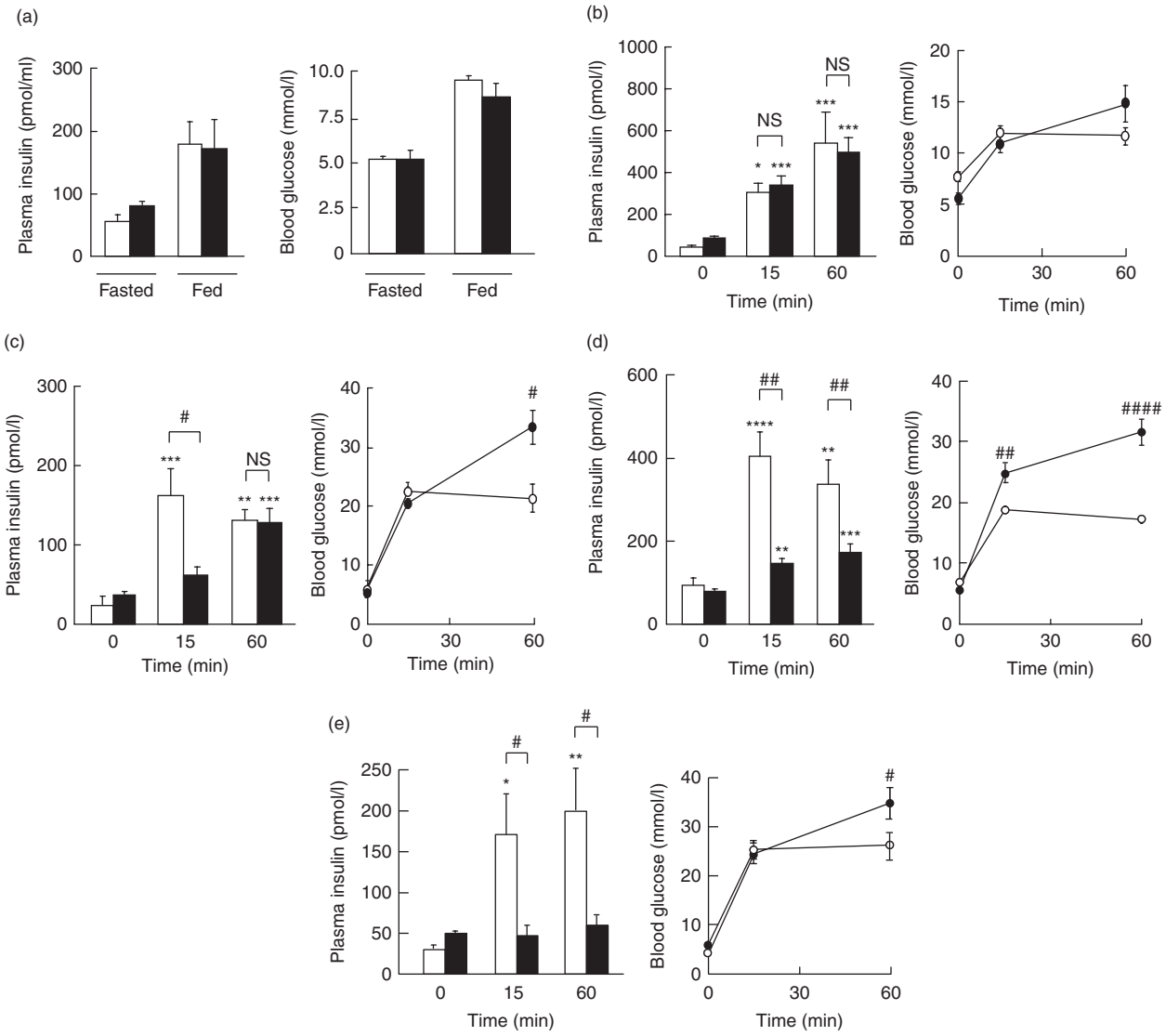

Figure 1
Plasma insulin and blood glucose levels. (a) Fasted and *ad libitum*-fed *Kir6.2*$^{+/+}$ (white bar; $n=7$–11) and *Kir6.2*$^{-/-}$ (black bar; $n=7$–11) mice. (b) Voluntary feeding of standard chow. *Kir6.2*$^{+/+}$ (open circle and white bar; $n=8$) and *Kir6.2*$^{-/-}$ (solid circle and black bar; $n=8$). (c) Forced feeding of standard chow in *Kir6.2*$^{+/+}$ (open circle and white bar; $n=4$) and *Kir6.2*$^{-/-}$ (solid circle and black bar; $n=4$) mice. (d) Voluntary feeding of dextrin in *Kir6.2*$^{+/+}$ (open circle and white bar; $n=10$) and *Kir6.2*$^{-/-}$ (solid circle and black bar; $n=9$) mice. (e) Forced feeding of dextrin in *Kir6.2*$^{+/+}$ (open circle and white bar; $n=5$) and *Kir6.2*$^{-/-}$ (solid circle and black bar; $n=5$) mice ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$) relative to 0 min ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#\#}P<0.0001$) compared *Kir6.2*$^{+/+}$ mice with *Kir6.2*$^{-/-}$ mice at the indicated time points.

from 2.8 to 16.7 mM glucose (Fig. 2a, open circles). By contrast, in *Kir6.2*$^{-/-}$ mice, 50 μM carbachol significantly stimulated insulin secretion in the presence of 2.8 mM glucose, probably because the Ca^{2+} concentration in the presence of 2.8 mM glucose is already high in pancreatic β-cells of *Kir6.2*$^{-/-}$ mice (Miki *et al.* 1998). Switching from 2.8 to 16.7 mM glucose stimulated insulin secretion significantly in pancreatic β-cells of *Kir6.2*$^{-/-}$ mice (Fig. 2a, solid circles). We then investigated involvement of the parasympathetic nerve in the insulin response to voluntary feeding in *Kir6.2*$^{-/-}$ mice using the muscarine receptor antagonist atropine, which does not cross the blood–brain barrier. We analyzed only mice eating more than 0.2 g standard chow within 15 min after initiation of voluntary feeding of standard chow. Pretreatment with atropine almost completely blocked the insulin response to voluntary feeding of standard chow in both *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice (Fig. 2b). Pretreatment with

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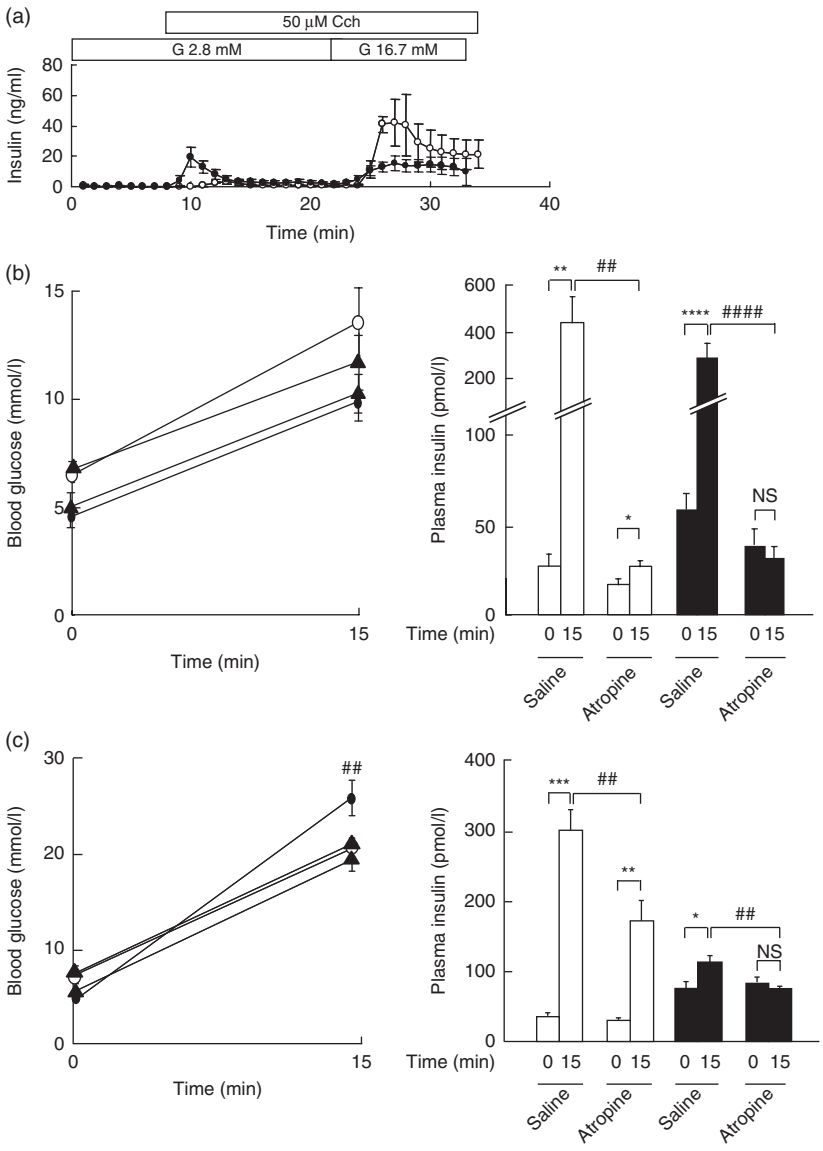

Figure 2

(a) Insulin secretion from perfused pancreas of *Kir6.2*$^{+/+}$ and *Kir6.2*$^{-/-}$ mice in response to 50 μmol/l carbachol with 2.8 or 16.7 mmol glucose. *Kir6.2*$^{+/+}$ (open circle; $n=3$) and *Kir6.2*$^{-/-}$ (solid circle; $n=3$). (b) Effect of atropine methyl nitrate on voluntary feeding of standard chow in *Kir6.2*$^{+/+}$ mice treated with saline (open circle and white bar; $n=13$) or atropine (open triangle and white bar; $n=6$) and in *Kir6.2*$^{-/-}$ mice treated with saline (solid circle and black bar; $n=10$) or atropine (solid triangle and black bar; $n=5$). (c) Effect of atropine methyl nitrate on voluntary feeding of dextrin in *Kir6.2*$^{+/+}$ mice treated with saline (open circle and white bar; $n=7$) or atropine (open triangle and white bar; $n=6$) and in *Kir6.2*$^{-/-}$ mice treated with saline (solid circle and black bar; $n=12$) or atropine (solid triangle and black bar; $n=9$) ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$) relative to 0 min ($^{\#\#}P<0.01$, $^{\#\#\#\#}P<0.0001$) compared saline-treated mice with atropine methyl nitrate-treated mice at the indicated time points.

atropine partially blocked the insulin response to voluntary feeding of dextrin in these *Kir6.2*$^{+/+}$ mice and completely blocked the insulin response to voluntary feeding of dextrin in these *Kir6.2*$^{-/-}$ mice (Fig. 2c). These findings demonstrate that parasympathetic neural input to β-cells is critical for the insulin response to voluntary feeding independent of the K_{ATP} channel *in vivo*.

Effects of Twinline and Racol and insulin secretion

We then investigated the effects of nutrients other than carbohydrate on insulin secretion *in vivo*: the effects of voluntary feeding of Twinline jelled by 4% agar, which contains not only carbohydrate but also various lipids and amino acids, and Racol jelled by 4% agar, which contains carbohydrate, lipids, and polypeptides (Fig. 3a and b).

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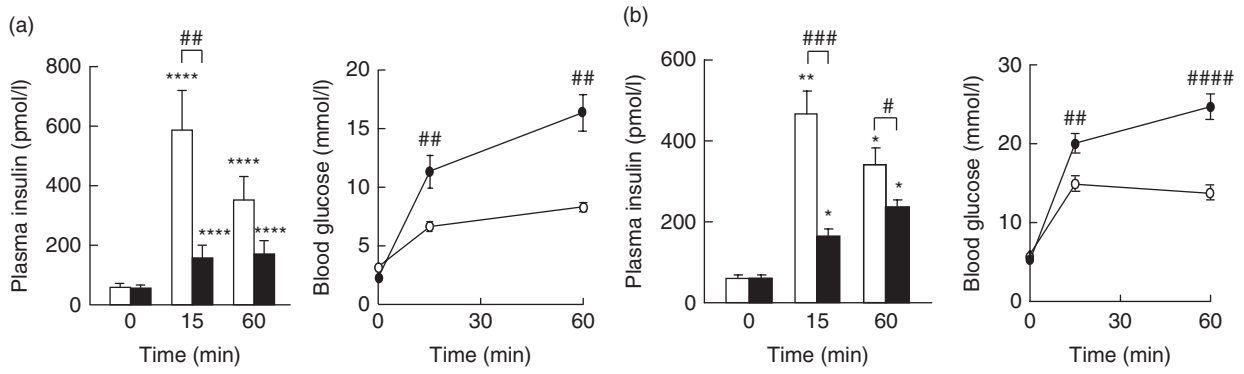

Figure 3
Plasma insulin and blood glucose levels. (a) Voluntary feeding of Twinline in *Kir6.2*$^{+/+}$ (open circle and white bar; $n=9$) and *Kir6.2*$^{-/-}$ (solid circle and black bar; $n=7$). (b) Voluntary feeding of Racol in *Kir6.2*$^{+/+}$ (open circle and white bar; $n=10$) and *Kir6.2*$^{-/-}$ (solid circle and black bar; $n=10$) (*$P<0.05$, **$P<0.01$, ****$P<0.0001$) relative to 0 min ($^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, $^{\#\#\#\#}P<0.0001$) comparing *Kir6.2*$^{+/+}$ mice with *Kir6.2*$^{-/-}$ mice at the indicated time points.

Voluntary feeding of either Racol or Twinline in *Kir6.2*$^{-/-}$ mice induced insulin secretion to a level similar to that induced by dextrin alone (Fig. 3a and b compared with Fig. 1d). These results indicate that only voluntary feeding of standard chow induces insulin secretion in *Kir6.2*$^{-/-}$ mice to a level similar to that of *Kir6.2*$^{+/+}$ mice *in vivo*.

Nutrient and food intake

We found that intake of dextrin in voluntary feeding was greater in *Kir6.2*$^{-/-}$ mice than that in *Kir6.2*$^{+/+}$ mice (*Kir6.2*$^{-/-}$, 3.52 ± 0.223 g/120 min, $n=11$, *Kir6.2*$^{+/+}$, 2.69 ± 0.154 g/120 min, $n=10$, $P<0.01$) (Fig. 4a). However, intake of standard chow by voluntary feeding in *Kir6.2*$^{-/-}$ mice was the same as that in *Kir6.2*$^{+/+}$ mice. On the other hand, intake of Racol and Twinline in *Kir6.2*$^{-/-}$ mice was increased about 30% compared with that in *Kir6.2*$^{+/+}$ mice, which is an increase similar to that by dextrin (Racol, $37.3\pm6.38\%$, $n=5$–6, $P<0.01$; Twinline, $24.3\pm5.07\%$, $n=6$–7, $P<0.001$).

We then evaluated the K_{ATP} channel-independent insulin response to voluntary feeding in the cephalic phase. We compared the increment of insulin secretion from 0 to 15 min in *Kir6.2*$^{-/-}$ mice with that in *Kir6.2*$^{+/+}$ mice during voluntary feeding (Fig. 4b) and forced feeding (Fig. 4c). There was no difference in the insulin response to voluntary feeding of standard chow between *Kir6.2*$^{+/+}$ mice and *Kir6.2*$^{-/-}$ mice, indicating that almost all of this insulin secretory response in *Kir6.2*$^{-/-}$ mice occurs in a K_{ATP} channel-independent manner. By contrast, the insulin response to voluntary feeding of Racol, Twinline, and dextrin was ~80% of that by the K_{ATP} channel-dependent pathway (Fig. 4b), and the insulin response to forced feeding of standard chow in *Kir6.2*$^{-/-}$ mice was decreased to ~20% in *Kir6.2*$^{+/+}$ mice (Fig. 4c). Thus, the major pathway of cephalic phase insulin secretion by voluntary feeding of standard chow is K_{ATP} channel independent and regulated by parasympathetic nerves.

Discussion

The brain has long been known to respond to meal and elicit insulin secretion from β-cells through the CNS (Bergman & Miller 1973, Strubbe & Steffens 1975, Henderson *et al.* 1976, Louis-Sylvestre 1976, 1978, Steffens 1976, Powley 1977). The brain triggers a rapid but small increase in insulin secretion through autonomic input to β-cells. This response is followed by a long-lasting (up to 1 h), substantial secretory response mediated by nutrients in the gut lumen (Berthoud *et al.* 1980, 1981, Bellisle *et al.* 1985, Berthoud & Powley 1990). Cephalic phase insulin secretion can be evaluated only within an ~7-min period after meal ingestion because it is soon masked by the gut response to glucose.

We previously found that *Kir6.2*$^{-/-}$ mice almost completely lack GIIS (Miki *et al.* 1998). We show in this study that *Kir6.2*$^{-/-}$ mice retain normal insulin secretion to voluntary feeding of standard mouse chow but exhibit markedly attenuated insulin secretion by forced ingestion (Fig. 1b and c). In addition, the insulin secretory response to voluntary feeding of standard chow in *Kir6.2*$^{-/-}$ mice is completely blocked by atropine (Fig. 2b). We first considered the possibility that the atropine treatment inhibits gastrointestinal movement and digestion in the gut and therefore does not induce insulin secretion. However, blood glucose in atropine-treated

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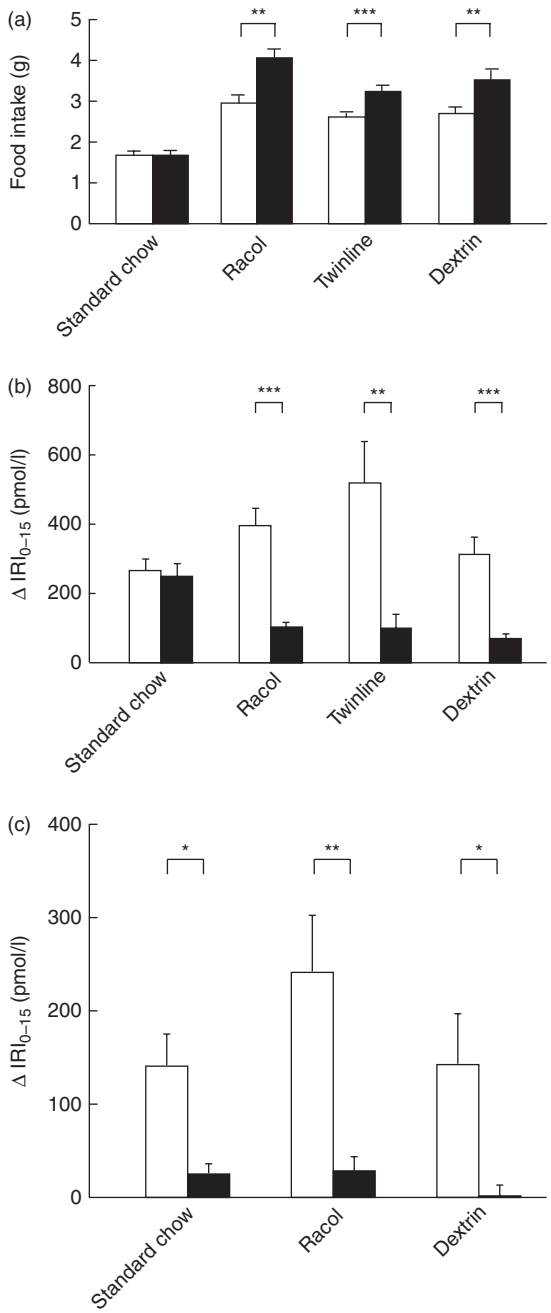

Figure 4

(a) Differences in food intake of various nutrients between *Kir6.2*$^{+/+}$ mice (white bar) and *Kir6.2*$^{-/-}$ mice (black bar) (n=5–11). The amount of food intake was calculated for 120-min refeeding after 16-h fasting. Asterisk (*) indicates a significant difference between *Kir6.2*$^{+/+}$ mice and *Kir6.2*$^{-/-}$ mice (**P<0.01, ***P<0.001). (b) The increment of insulin secretion from 0 to 15 min in *Kir6.2*$^{+/+}$ mice and *Kir6.2*$^{-/-}$ mice during voluntary feeding. *Kir6.2*$^{+/+}$ mice (white bar) and *Kir6.2*$^{-/-}$ mice (black bar) (**P<0.01, ***P<0.001). (c) The increment of insulin secretion from 0 to 15 min in *Kir6.2*$^{+/+}$ mice and in *Kir6.2*$^{-/-}$ mice during forced feeding. *Kir6.2*$^{+/+}$ mice (white bar) and *Kir6.2*$^{-/-}$ mice (black bar) (*P<0.05, **P<0.01).

Kir6.2$^{-/-}$ mice reached a level similar to that in saline-treated *Kir6.2*$^{-/-}$ mice. Thus, insulin secretion during voluntary feeding of standard chow is mediated by parasympathetic input to β-cells. However, the insulin secretion induced by voluntary feeding of dextrin was partially blocked in *Kir6.2*$^{+/+}$ mice in contrast to *Kir6.2*$^{-/-}$ mice in which the insulin response was completely blocked. This indicates that the effect of dextrin-induced insulin secretion by voluntary feeding is mediated almost entirely through vagal nerve activity in *Kir6.2*$^{-/-}$ mice whereas it is mediated only partially through vagal nerve activity in *Kir6.2*$^{+/+}$ mice. This finding also indicates that various components of food induce insulin secretion through neural inputs to different degrees. Racol and Twinline contain fats and casein or amino acids in addition to carbohydrates. Voluntary feeding of Racol or Twinline in *Kir6.2*$^{-/-}$ mice also did not elicit a sufficient amount of insulin secretion (Figs 3a, b, and 4b) (~20% of that in *Kir6.2*$^{+/+}$ mice). Which factor in voluntary feeding of standard chow is the major determinant of insulin secretion is not known at present. Taste, smell, mastication, and visual information (Powley & Berthoud 1985, Suzuki *et al.* 2005) are implicated in normal insulin secretion in response to voluntary feeding of normal chow diet in *Kir6.2*$^{-/-}$ mice.

A muscarine receptor subtype M3 is expressed in pancreatic β-cells and plays an important role in cholinergically mediated insulin secretion (Gautam *et al.* 2006). The cholinergic signal activates phospholipase C, which produces diacylglycerol and inositol 1,4,5-trisphosphatase, thereby leading to insulin secretion through a rise in the intracellular Ca^{2+} concentration and protein kinase C activation in β-cells (Ahrén 2000, Gilon & Henquin 2001). This mechanism operates even in the K_{ATP} channel-deficient state *in vivo*. This accords with our previous *in vitro* finding that acetylcholine increases $[Ca^{2+}]_i$ to stimulate insulin secretion in *Kir6.2*$^{-/-}$ islets (Miki *et al.* 1998).

In summary, there has been no good animal model in which cephalic phase insulin secretion can readily be distinguished from gut phase insulin secretion. *Kir6.2*$^{-/-}$ mice exhibit no insulin secretion in response to gastric loading of standard chow, dextrin, or glucose but do exhibit insulin secretion in response to voluntary feeding of nutrients, which is completely blocked by atropine. In addition, insulin secretion in *Kir6.2*$^{-/-}$ mice persists during voluntary feeding. These findings suggest that *Kir6.2*$^{-/-}$ mice can be used to unmask neural involvement during gut phase insulin secretion.

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Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
Y S, T M, and S S contributed to the study design and data analyses. Y S, T M, W F, E Y L, and Y T performed the research. Y S, T M, K M, Y O, and S S contributed to discussion. Y S, T M, and S S wrote the manuscript.

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