



In vitro insulin secretion by pancreatic tissue from infants with diazoxide-resistant congenital hyperinsulinism deviates from model predictions

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Congenital hyperinsulinism (CHI) is the major cause of persistent neonatal hypoglycemia. CHI most often occurs due to mutations in the *ABCC8* (which encodes sulfonyleurea receptor 1) or *KCNJ11* (which encodes the potassium channel Kir6.2) gene, which result in a lack of functional KATP channels in pancreatic β cells. Diffuse forms of CHI (DiCHI), in which all β cells are abnormal, often require subtotal pancreatectomy, whereas focal forms (FoCHI), which are characterized by localized hyperplasia of abnormal β cells, can be cured by resection of the lesion. Here, we characterized the in vitro kinetics of insulin secretion by pancreatic fragments from 6 DiCHI patients and by focal lesion and normal adjacent pancreas from 18 FoCHI patients. Responses of normal pancreas were similar to those reported for islets from adult organ donors. Compared with normal pancreas, basal insulin secretion was elevated in both FoCHI and DiCHI tissue. Affected tissues were heterogeneous in their secretory responses, with increased glucose levels often producing a rapid increase in insulin secretion that could be followed by a paradoxical decrease below prestimulatory levels. The KATP channel blocker tolbutamide was consistently ineffective in stimulating insulin secretion; conversely, the KATP channel activator diazoxide often caused an unanticipated increase in insulin secretion. These observed alterations in secretory behavior were similar in focal lesion and DiCHI tissue, and independent of the specific mutation in *ABCC8* or *KCNJ11*. They cannot be explained by classic models of β cell function. Our results provide insight into the excessive and sometimes paradoxical changes in insulin secretion observed in CHI patients with inactivating mutations of KATP channels.

Introduction

Congenital hyperinsulinism (CHI) is the major cause of persistent hypoglycemia in newborns and infants (1, 2). The underlying genetic etiology and biochemical mechanisms are multiple, resulting in heterogeneous clinical presentation from relatively mild, medically responsive forms to severe forms requiring near total pancreatectomy. The most common and severe cases of CHI are caused by inactivating mutations in *ABCC8* (90%) and *KCNJ11* (10%) genes, which are both located on chromosome 11p15 and respectively encode sulfonyleurea receptor 1 (SUR1) and Kir6.2, the regulatory and pore-forming subunits of ATP-sensitive K (K_{ATP}) channels in pancreatic β cells (2–6). More than 150 mutations in *ABCC8* and about 25 mutations in *KCNJ11* have been reported (6, 7), which cause defective channel trafficking to the plasma membrane or impairment of channel regulation by nucleotides.

Glucose control of insulin secretion is normally achieved through the interaction of triggering and amplifying pathways in β cells (reviewed in ref. 8). K_{ATP} channels play an essential role in the triggering pathway. When β cells sense a rise in blood glucose, their metabolism accelerates, causing an increase in ATP and

a decrease in Mg-ADP concentrations in the cytosol, which close K_{ATP} channels. As a consequence, the membrane depolarizes and voltage-gated Ca^{2+} channels open, allowing Ca^{2+} influx and leading to a rise in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) that triggers exocytosis of insulin granules. Simultaneously, glucose activates an amplifying pathway that does not elevate $[Ca^{2+}]_c$ further but augments the secretory response to high $[Ca^{2+}]_c$. Conversely, a fall in blood glucose is normally followed by opening of K_{ATP} channels with membrane hyperpolarization, lowering of $[Ca^{2+}]_c$, and cessation of insulin secretion.

Drugs that increase or lower β cell $[Ca^{2+}]_c$ independently of metabolism reproduce the secretory changes mediated by the triggering pathway (9). Antidiabetic sulfonyleureas augment insulin secretion by closing K_{ATP} channels, whereas diazoxide inhibits insulin secretion by opening the channels. This explains the use of diazoxide as primary medical treatment of CHI (1). This approach is successful except in most patients with a mutation of K_{ATP} channels, who therefore require other medical and dietary treatments and eventually surgical pancreatic resection in the most severe cases.

Histologically, 2 forms of diazoxide-resistant hyperinsulinism can be distinguished (10, 11). In diffuse forms of CHI (DiCHI), all β cells in all islets are affected. The genetic cause is a recessive (more rarely dominant) mutation in *ABCC8* or *KCNJ11* (2, 6, 7,

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Table 1
Clinical and genetic characteristics of patients

Case No.	Months at surgery	Post-op	Mutation Gene	Nucleotide level	Protein level	Impact on channel function (ref.)
FoCHI1	5	Cured	<i>KCNJ11</i> Exon 1 ^A	c.244_247dupAGCT	p.Trp83X	
FoCHI2	7.5	Cured	<i>ABCC8</i> Exon 4	c.560T>A	p.Val187Asp	Intracellular retention (58, 59) No K-ATP current (57)
FoCHI3	2	Cured	<i>ABCC8</i> Exon 4	c.560T>A	p.Val187Asp	Intracellular retention (58, 59) No K-ATP current (57)
FoCHI4	2	Cured	<i>ABCC8</i> Exon 4	c.560T>A	p.Val187Asp	Intracellular retention (58, 59) No K-ATP current (57)
FoCHI5	14	Cured: 2 op	<i>ABCC8</i> Intron 4	c.580-1G>C	p.?	
FoCHI6	7	Cured	<i>ABCC8</i> Exon 5	c.683G>A	p.Gly228Asp	Intracellular retention (60)
FoCHI7	11	Cured	<i>ABCC8</i> Intron 7	c.1176+2T>C	p.?	
FoCHI8	9	Cured: 3 op	<i>ABCC8</i> Exon 12	c.1792C>T	p.Arg598X	No K-ATP current (66)
FoCHI9	3.5	Cured	<i>ABCC8</i> Exon 12 ^A	c.1817G>A	p.?	
FoCHI10	6	Cured	<i>ABCC8</i> Exon 15	c.2064G>A	p.Trp688X	
FoCHI11	5	Cured	<i>ABCC8</i> Exon 22 ^A	c.2588_2591delATCT	p.His863fs	
FoCHI12	2	Cured: 2 op	<i>ABCC8</i> Intron 32	c.3991+2_3991+15del14	p.?	
FoCHI13	2.5	Cured	<i>ABCC8</i> Exon 36	c.4325delC	p.Pro1442fs	
FoCHI14	8	Cured	<i>ABCC8</i> Exon 36 ^A	c.4354T>C	p.Trp1452Arg	
FoCHI15	10	Cured: 2 op	<i>ABCC8</i> Exon 36 ^A	c.4359G>T	p.Glu1453Asp	
FoCHI16	10.5	Cured	<i>ABCC8</i> Exon 36	c.4408G>T	p.Gly1470Val	
FoCHI17	5	Cured	<i>ABCC8</i> Intron 36	c.4415-13G>A	p.?	
FoCHI18	6	Cured	Unknown	Unknown	Unknown	
DiCHI1	3	Diabetes	<i>ABCC8</i> Exon 5	c.683G>A; Homoz	p.Gly228Asp	Intracellular retention (60)
DiCHI2	5	Diabetes	<i>ABCC8</i> Exon 9	c.1420C>T; Homoz	p.Gln474X	
DiCHI3	2.5	Glucose intol.	<i>ABCC8</i> Intron 28 ^A	c.3561-3C>G; Homoz	p.?	No K-ATP current, but rescue by culture at 25°C (67)
DiCHI4	6	Diabetes: 2 op	<i>ABCC8</i> Exon 34	c.4160C>T	p.Ser1387Phe	84% membrane expression of defective channel (62)
DiCHI5	7	Hypos	<i>ABCC8</i> Exon 37	c.4480C>T; Homoz	p.Arg1494Trp	Intracellular retention (61)
DiCHI6	18	Diabetes	<i>ABCC8</i> Exons 28 ^A and 37	c.3559A>G; c.4481G>A	p.Arg1187Gly; p.Arg1494Gln	

The mutation nomenclature is based on the reference sequences *KCNJ11* NM_000525.3 and *ABCC8* NM_000352.3 corresponding to the L72808 isoform (1,582 amino acids), which incorporates the extra serine residue in exon 17 (6). All mutations identified at heterozygous state with the exception of DiCHI1, -2, -3, and -5 (homozygous), and DiCHI6 (compound heterozygous). ^AMutations believed to be novel. References to the others in refs. 6, 7. Post-op, post-operative; glucose intol., glucose intolerant; homoz, homozygous; op, operations.

12, 13). In focal forms (FoCHI), a localized hyperplasia of abnormal β cells is present within an otherwise normal pancreas. These focal lesions result from the coexistence of inherited and acquired events. Most often, the inherited event is a constitutional recessive paternal mutation in *ABCC8* or *KCNJ11* that would remain silent alone. The acquired event is a somatic deletion of the corresponding maternal segment of chromosome 11p15, which leads to loss of heterozygosity (with isodisomy or duplication of the paternal mutated allele) (14, 15). The same 11p15 region also contains several imprinted genes involved in cell replication: the maternally expressed *CDKN1C* (p57) and *H19*, and the paternally expressed *IGF-II*, which respectively inhibit and promote cell proliferation. The local β cell hyperplasia is attributed to the imbalance between these genes (16, 17). Distinction between these 2 forms has major therapeutic implications: whereas medically unresponsive DiCHI usually requires near total pancreatectomy, FoCHI can be cured by a selective localized resection of the lesion (18, 19).

Patch-clamp studies (reviewed in ref. 5) have shown that β cells, obtained after near total pancreatectomy of diazoxide-resistant DiCHI patients, have no or very few functional K_{ATP} channels in

their plasma membranes. They are depolarized, with variably elevated $[Ca^{2+}]_c$ in the presence of low glucose concentrations, and do not display changes in $[Ca^{2+}]_c$ upon application of high glucose concentrations, tolbutamide, or diazoxide (20, 21). Studies of 3 FoCHI cases showed that functional K_{ATP} channels are absent from β cells within the focal lesion and present and normally regulated in the adjacent pancreas (22). In vitro data on insulin secretion by CHI pancreas are scanty and sometimes controversial. Three studies were performed before the pathogenesis of the disease started to be understood. Islets isolated from 1 diazoxide-resistant CHI patient doubled insulin secretion in response to glucose (23). Islet cell clusters from 4 diazoxide-resistant DiCHI patients poorly responded to glucose but were stimulated by cAMP (24). Islet cell clusters from 4 DiCHI patients were stimulated by cAMP, were insensitive to high glucose, and variably responded to other fuels while maintained in culture for 1–4 weeks (25). More recently, insulin secretion was studied in islets from 3 DiCHI patients lacking functional K_{ATP} channels in their β cells because of a mutation in *ABCC8*. Tolbutamide and KCl were ineffective in low glucose conditions, but high glucose conditions were stimulatory. The effect

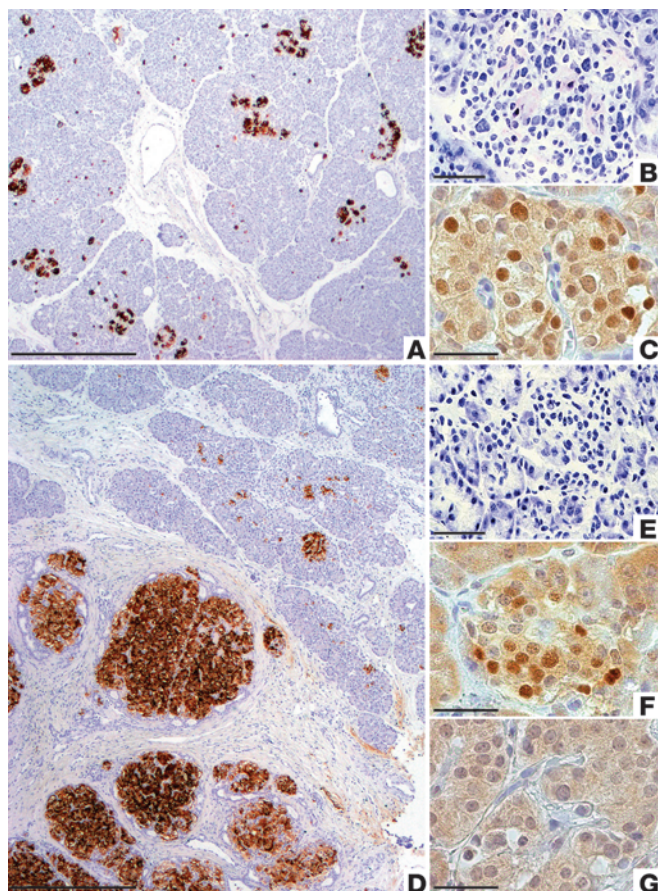


Figure 1

DiCHI and FoCHI pancreas: morphological aspect and *CDKN1C* expression in islets. The pancreas of representative cases of DiCHI (A) and FoCHI (D) patients were postoperatively immunostained for insulin. DiCHI does not show obvious changes in islet number or size, whereas FoCHI is characterized by a localized adenomatous hyperplasia of β cells. Peroperative diagnosis of DiCHI on frozen sections is based on the presence of β cells with a large nucleus and abundant clear cytoplasm (B, toluidine staining). In FoCHI, islets outside the lesion are small and contain β cells with a relatively small nucleus and scanty cytoplasm (E). *CDKN1C* (p57) peptide is present in several cells of all DiCHI islets (C, dark brown nuclei) and islets located outside the lesion in FoCHI (F), but it is absent from the focal lesion (G). Scale bars: 500 μ m (A and D); 25 μ m (B, C, E–G).

of glucose was Ca^{2+} dependent, but it is not known whether it was sensitive to diazoxide (21). Unfortunately, in none of the above studies was appropriate control tissue available for comparison.

In the present study, we used a perfusion system to characterize *in vitro* the kinetics of insulin secretion by the pancreas of 24 infants suffering from diazoxide-resistant FoCHI or DiCHI because of inactivating mutations of K_{ATP} channels in their β cells. In all cases, intraoperative microscopic examination of frozen sections established the differential diagnosis (19) and dictated the localization and extent of pancreatic resection. This also made it possible to select fragments of normal and pathologic tissue from the pancreas of patients with FoCHI and to compare their secretory behavior. Such a comparison is crucial for identification and correct interpretation of abnormalities in the focal lesion and in the whole pancreas from DiCHI patients.

Results

Morphological aspect of DiCHI and FoCHI pancreas. The 6 DiCHI and 18 FoCHI studied subjects (Table 1) underwent pancreatic surgery because of their clinical resistance to diazoxide treatment. In DiCHI pancreas, the number, size, and general appearance of the islets (Figure 1A) are similar to those in

the pancreas from control infants (11). Intraoperative diagnosis, based on the presence of β cells with an abnormally large nucleus and abundant cytoplasm in frozen specimens (Figure 1B), was subsequently confirmed on fixed specimens. The pattern is different in FoCHI pancreas. As shown in Figure 1D, focal lesions consist of an agglomerate of large islets. The size of the lesion is variable (usually < 10 mm in diameter), and its limits are not always as regular as in the figure. Outside the focal lesion, islets contain β cells with a small nucleus and relatively little cytoplasm, which give the impression of packed cells (Figure 1E and ref. 11). In DiCHI, *CDKN1C* is normally expressed, so that all islets contain cells positive for the *CDKN1C* (p57) protein (Figure 1C). In FoCHI pancreas, *CDKN1C* immunostaining is positive in islets outside the lesion (Figure 1F) but negative in the focal lesion (Figure 1G) because of the loss of maternal 11p15 (16, 17). These characteristics were present in all studied cases.

When intraoperative diagnosis of DiCHI was established, subtotal pancreatectomy was performed and an unselected fragment of the whole organ was saved for functional studies. In FoCHI, the resection was limited to the lesion and a zone of normal tissue at the periphery. The pieces of pathological and normal tissue destined for functional studies were selected by morphological examination of adjacent frozen samples, with a preference for the inconvenience of small size over the risk of overlapping zones.

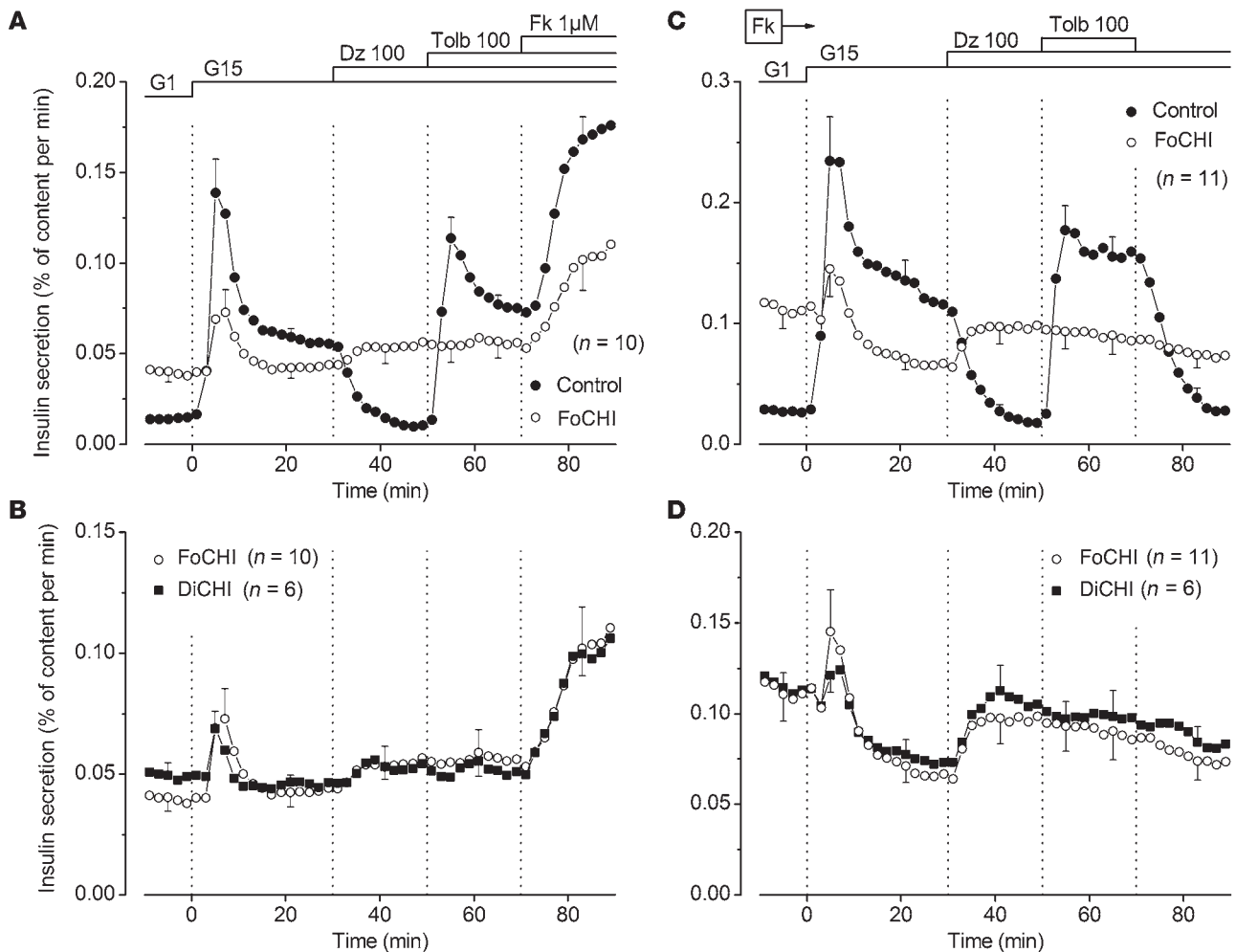
Insulin content and insulin secretion during culture. The initial insulin content of fresh pancreas was not directly measured but estimated by adding the total amount of insulin secreted during the experiments

Table 2

Insulin secretion during tissue culture and estimation of initial insulin concentration in the normal and pathological pancreas

	Initial insulin content (ng/mg)	Insulin secretion during culture (% of content in ~19 hours)
FoCHI pancreas (n = 17)		
Normal region	46.2 ± 6.6 (16–113)	21.5 ± 7.2 (9–38)
Lesion	515 ± 60 (204–1048)	29.5 ± 8.4 (15–40)
Ratio: lesion/normal	14.0 ± 2.5 (5.2–37.4)	1.40 ± 0.14 (0.7–2.6)
DiCHI pancreas (n = 6)		
Whole pancreas	38.6 ± 19.3 (11–64)	24.7 ± 11.5 (13–40)
Ratio: diffuse/normal focal	0.84	1.15

Insulin secretion was measured during the culture period (17–22 hours) preceding transfer of the tissue into perfusion chambers. It is expressed as a percentage of the initial insulin content of the tissue, which was calculated as described in Methods. Values are mean ± SEM with range in parentheses. Data are missing for 1 FoCHI pancreas.

**Figure 2**

Effects of glucose and drugs acting on K_{ATP} channels on insulin secretion by control, FoCHI, and DiCHI pancreas. (A and C) Comparison of insulin secretion from the focal lesion and the adjacent normal pancreas of the same subjects. (B and D) Comparison of insulin secretion from focal lesions and the pancreas of DiCHI subjects; results obtained in focal lesions are the same as those presented in the above panels, but are shown with another scale. The concentration of glucose (G) was increased from 1 to 15 mM, and 100 μ M diazoxide (Dz) and 100 μ M tolbutamide (Tolb) were added as indicated at the top of the figures. In A and B, 1 μ M forskolin (Fk) was added at 70 minutes. In C and D, 1 μ M forskolin was present throughout. Values are mean \pm SEM for the indicated number of subjects. Individual responses are shown in Supplemental Figures 1–4.

and the tissue content at the end of the experiments (see Methods). The normal region of FoCHI pancreas contained 46 ng insulin/mg on average and secreted 21.5% of this content during the approximately 19 hours of culture in the presence of 5 mM glucose (Table 2). In the focal lesion, the insulin content was 14-fold greater than in the normal region, but fractional insulin secretion during culture (29.5%) was not consistently larger (on average 1.4-fold, NS by paired *t* test). In DiCHI pancreas, insulin content (39 ng/mg) and fractional insulin secretion during culture (24.7%) were not different from those in the normal region of FoCHI pancreas (Table 2). The surprising similarity between fractional insulin secretion rates in FoCHI and DiCHI tissue and in normal pancreas is attributed to the stimulation of normal islets by the concentration of 5 mM glucose in culture medium (see below).

Acute effects of glucose on insulin secretion. Stimulation of the normal pancreas of FoCHI subjects by a rapid increase in glucose from 1 to 15 mM induced biphasic insulin secretion (Figure 2A).

Second phase was suppressed by diazoxide and restored by tolbutamide. At the end of the experiments, addition of forskolin to increase cAMP in β cells doubled insulin secretion (Figure 2A). The presence of forskolin during the whole experiment augmented the amplitude of both first and second phases of glucose-induced secretion, but did not alter the inhibitory and stimulatory effects of diazoxide and tolbutamide (compare Figure 2C with Figure 2A and note the different scales). These changes, here measured with partially digested pancreas, are superimposable on those observed with isolated islets from adult organ donors (26). At least one of the protocols shown in Figure 2, A and C, was performed with normal pancreas of each FoCHI patient and gave qualitatively similar results, attesting to the quality of the tested tissues. Supplemental Figures 1 and 2 (supplemental material available online with this article; doi:10.1172/JCI58400DS1) show all individual responses in the absence and presence of forskolin, respectively.

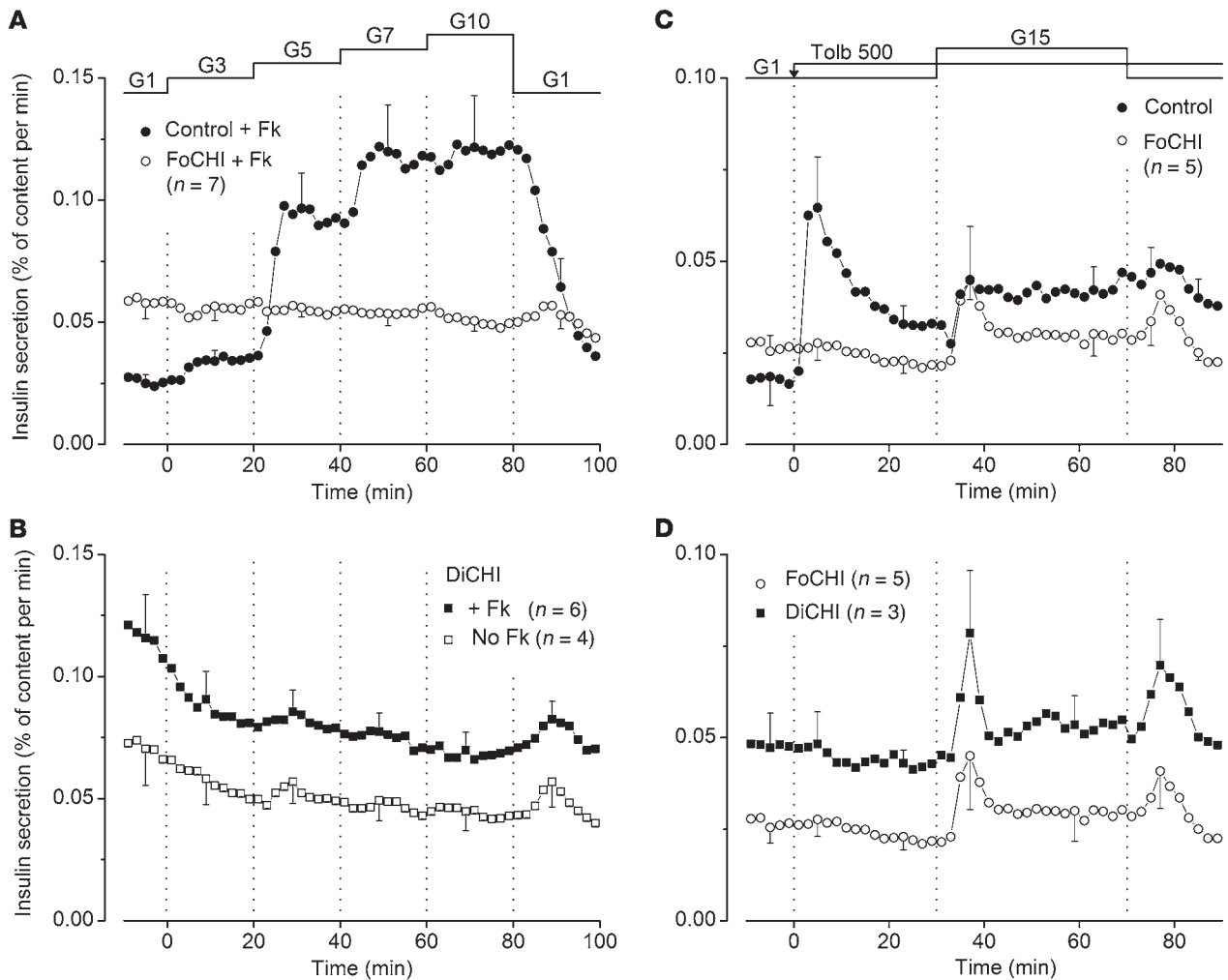


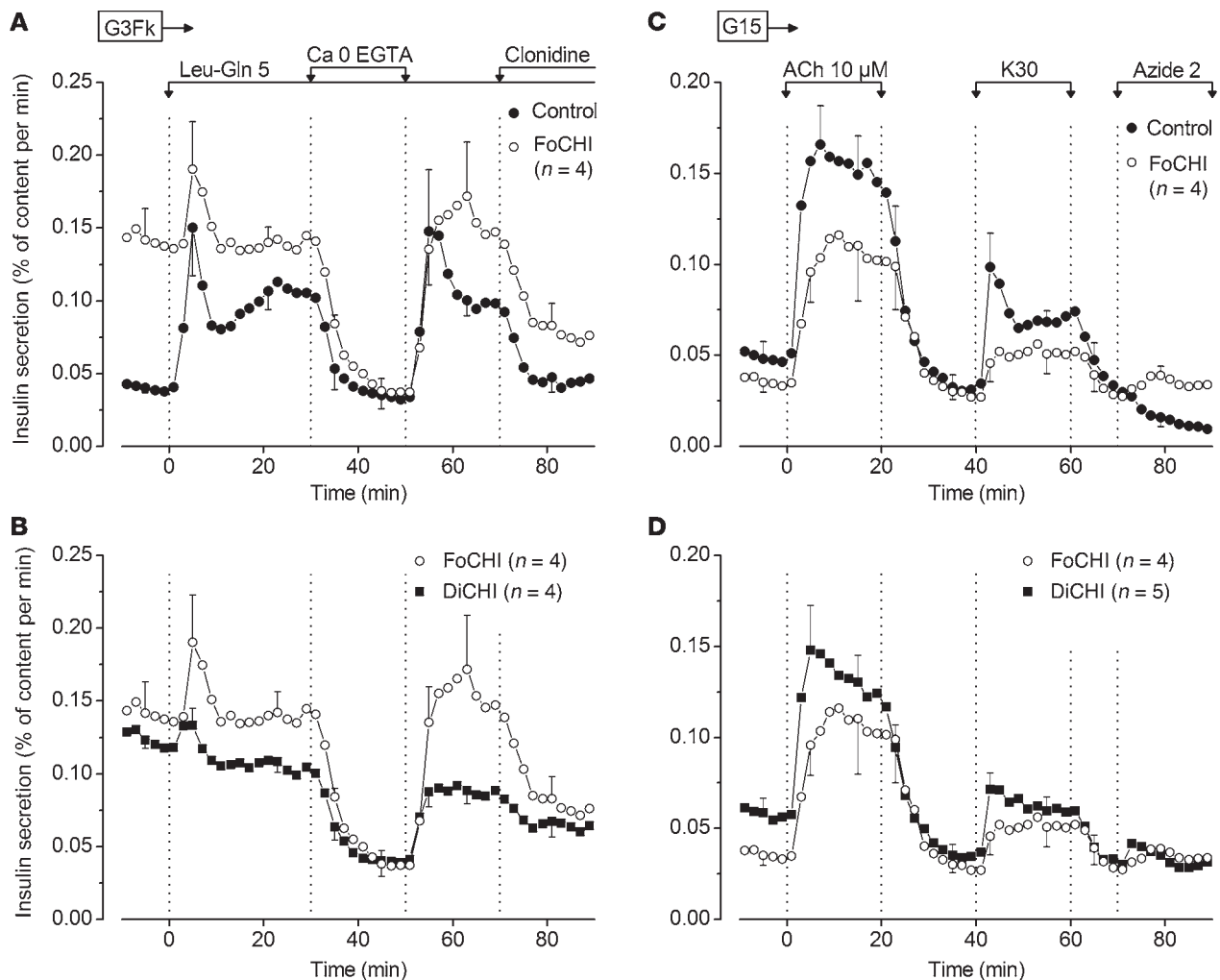
Figure 3 Effects of glucose and tolbutamide on insulin secretion by control, FoCHI, and DiCHI pancreas. (A and B) Effects of stepwise increases in glucose (in mM). (A) Responses of focal lesion and adjacent normal pancreas of the same subjects. The experiments were performed in the presence of 1 μ M forskolin throughout. (B) Responses of DiCHI pancreas in the presence and absence of forskolin. (C and D) Effects of high glucose in the presence of tolbutamide. (C) Responses of focal lesion and adjacent normal pancreas of the same subjects. (D) Responses of the same focal lesions and of DiCHI pancreas. Tolbutamide (500 μ M) was added, and the concentration of glucose was increased from 1 to 15 mM as indicated. The experiments were performed in the absence of forskolin. Values are mean \pm SEM for the indicated number of subjects.

The focal lesion of the same subjects was characterized by an elevated basal insulin secretion rate in 1 mM glucose (2.8-fold higher than in paired control pancreas; $P < 0.001$) (Figure 2A). Stimulation by 15 mM glucose induced a modest first phase of insulin secretion in 6 of 10 focal lesions ($P < 0.01$ for all responses), and a small second phase increase above the high prestimulatory values was detected in only 3 of 10 subjects. Diazoxide never inhibited insulin secretion but unexpectedly increased it in 3 of 10 subjects (Supplemental Figure 1), although the mean change was not significant (Figure 2A). Tolbutamide was always ineffective, and forskolin, added at the end of the experiments, approximately doubled the secretion rate (Figure 2A).

When forskolin was present from the start of the experiments, basal insulin secretion was augmented and the difference with paired control pancreas now averaged 4.1-fold ($P < 0.001$) (Figure 2C). Stimulation with 15 mM glucose induced a small first peak of secretion in 5 of 11 preparations ($P < 0.05$ for all responses). This small peak was

followed by a paradoxical decrease ($P < 0.01$) during second phase. We acknowledge that, in the absence of paired experiments in low glucose throughout, it is debatable whether the average decrease in insulin secretion rate (Figure 2C) was induced by high glucose or was spontaneous. However, examination of the individual responses (Supplemental Figure 2) suggests that an inhibition by glucose was real in 6 of 11 cases. Equally paradoxical was the increase in insulin secretion produced by diazoxide in 6 of 11 cases (Supplemental Figure 2) ($P < 0.05$ for all responses). In contrast, tolbutamide was always without effect (Figure 2B). In 3 other experiments, omission of CaCl_2 from the perfusion medium markedly inhibited the high rate of insulin secretion in FoCHI pancreas (not shown).

Mean insulin secretory responses of DiCHI pancreas were similar to those of focal lesions (Figure 2, B and D). Responses of individual DiCHI cases tested without and with forskolin are shown in Supplemental Figures 3 and 4. In the presence of forskolin throughout, 15 mM glucose induced an initial peak of insulin secretion in 4 of

**Figure 4**

Effects of various stimulators and inhibitors on insulin secretion by control, FoCHI, and DiCHI pancreas. **(A and B)** Effects of amino acids, CaCl_2 omission, and clonidine. **(A)** Responses of focal lesion and adjacent normal pancreas of the same subjects. **(B)** Responses of the same focal lesions and of DiCHI pancreas. The experiments were performed in the presence of 3 mM glucose and 1 μM forskolin throughout (G3 Fk). Leucine and glutamine (5 mM each) and clonidine (1 μM) were added, and extracellular CaCl_2 was omitted (with addition of 100 μM EGTA) as indicated. **(C and D)** Effects of acetylcholine, KCl, and azide. **(C)** Responses of focal lesion and adjacent normal pancreas of the same subjects. **(D)** Responses of the same focal lesions and of DiCHI pancreas. The experiments were performed in the presence of 15 mM glucose (G15) and absence of forskolin throughout. Acetylcholine (10 μM) and azide (2 mM) were added, and the concentration of KCl was raised to 30 mM (K30) as indicated. Values are mean \pm SEM for the indicated number of subjects.

6 subjects and a paradoxical decrease below initial values during second phase in 5 of 6 subjects ($P < 0.01$). The stimulatory effect of diazoxide was consistently observed (6 of 6) ($P < 0.01$). Pinacidil, a structurally unrelated K_{ATP} channel opener with greater affinity for SUR2 than SUR1, inhibits glucose-induced insulin secretion in normal islets (27) and, like diazoxide, is inactive in *Sur1*-knockout islets (28). Pinacidil also paradoxically increased insulin secretion in FoCHI lesion and DiCHI pancreas perfused with 3 mM glucose ($P = 0.01$), while having no effect in normal pancreas (Supplemental Figure 5).

Stepwise increases of the glucose concentration in the presence of forskolin induced a concentration-dependent increase in insulin secretion in the normal pancreas from FoCHI subjects (Figure 3A). A response was already evoked by 3 mM glucose in 4 of 7 cases ($P < 0.01$ for the whole group), and a maximum was reached at 7 mM glucose.

Excellent reversibility was observed on return to 1 mM glucose at the end of the experiments. Whereas the threshold at 3 mM was the same as in adult islets, the maximum effect of glucose was reached at a lower concentration (26). These progressive changes in glucose concentration were without significant effect on insulin secretion by focal lesions in the presence of forskolin (Figure 3A). They were also ineffective in DiCHI pancreas, both in the presence and absence of forskolin (Figure 3B).

In the presence of only 1 mM glucose, tolbutamide, used at the concentration of 500 μM to close all K_{ATP} channels, consistently stimulated insulin secretion from normal pancreas of FoCHI patients (Figure 3C). An initial peak was followed by a slow decline and stabilization at approximately 1.8-fold above baseline ($P < 0.05$). Subsequently, raising the concentration of glucose to 15 mM in the

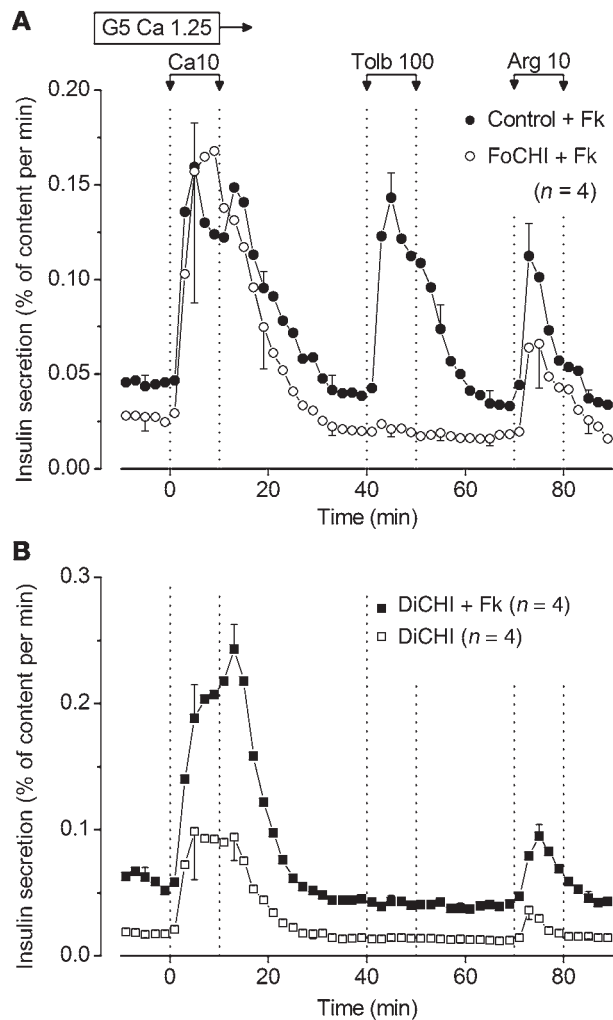


Figure 5

Effects of CaCl_2 , tolbutamide, and arginine on insulin secretion by control, FoCHI, and DiCHI pancreas. **(A)** Responses of focal lesion and adjacent normal pancreas of the same subjects. **(B)** Responses of DiCHI pancreas. The experiments were performed in the presence of 5 mM glucose (G5) throughout. The concentration of CaCl_2 was 1.25 mM except during 10 minutes when it was increased to 10 mM (Ca10). Tolbutamide (100 μM) and 10 mM arginine (Arg) were added as indicated. Forskolin (1 μM) was present in all experiments in **A** and in 1 series only in **B**. Values are mean \pm SEM for the indicated number of subjects.

presence of tolbutamide was followed by a slight increase in insulin secretion (4 of 5 subjects) ($P < 0.05$), an effect that is attributed to the amplifying pathway (8). In focal lesion, tolbutamide was ineffective in 1 mM glucose, but the subsequent increase in glucose induced a first peak of insulin secretion (4 of 5 subjects) ($P < 0.05$) and a minor and inconsistent (2 of 5) sustained elevation (NS for the whole group). A qualitatively similar response was observed in DiCHI pancreas (Figure 3D). In focal lesion and DiCHI pancreas, these effects of glucose on top of tolbutamide are not different from those of glucose alone (compare with Figure 2B).

Acute effects of other agents on insulin secretion. In the presence of 3 mM glucose and forskolin throughout, the combination of leucine and glutamine (5 mM each) induced biphasic insulin secretion from the normal pancreas of FoCHI patients (Figure 4A). The effect of the amino acids was reversibly abrogated by omission of extracellular CaCl_2 and markedly inhibited by activation of α_2 -adrenoceptors with clonidine. In focal lesion, the combination of amino acids evoked a first-phase insulin secretion in 4 of 4 preparations ($P = 0.05$) but no secondary elevation. CaCl_2 omission inhibited the high rate of secretion to basal values similar to those in control pancreas, i.e., well below the initial secretion rate in focal lesions. Clonidine caused partial inhibition (Figure 4A). In DiCHI pancreas, leucine and glutamine induced a first-phase insulin

secretion in only 2 of 4 preparations. Omission of CaCl_2 consistently suppressed secretion, while clonidine was inhibitory in 3 of 4 preparations (Figure 4B), as was epinephrine in a previous study (25). In mouse islets lacking K_{ATP} channels because of a knockout of *Sur1*, clonidine abrogated insulin secretion by lowering $[\text{Ca}^{2+}]_c$ and interfering with exocytosis (28). In a single case (DiCHI1), glutamine (2 mM) was tested alone and only had a very small effect on insulin secretion, in contrast with the large response induced by a mixture of amino acids (Supplemental Figure 6).

In the presence of 15 mM glucose, acetylcholine and KCl reversibly increased insulin secretion in the normal pancreas of FoCHI patients (Figure 4C). At the end of the experiments, addition of azide, a mitochondrial poison that lowers ATP levels in islets (29), abolished insulin secretion. In focal lesions, qualitatively similar but slightly smaller effects were produced by acetylcholine (4 of 4) and KCl (3 of 4). In contrast, azide did not inhibit insulin secretion (Figure 4C). The responses of DiCHI pancreas were similar to those of FoCHI pancreas (Figure 4D). We tentatively attribute the smaller effects of KCl and acetylcholine to a lesser stimulation of Ca^{2+} influx in depolarized CHI β cells. The suppression of glucose-induced insulin secretion by azide in control pancreas is attributed to the opening of K_{ATP} channels with subsequent lowering of $[\text{Ca}^{2+}]_c$. No such effect can occur in CHI β cells lacking functional K_{ATP} channels (29).

Acute effects of diagnostic agents on insulin secretion. Acute stimulation by CaCl_2 , tolbutamide, or arginine are commonly used tests in the diagnostic phase of CHI (30–33). In the presence of 5 mM glucose and forskolin, an increase in extracellular CaCl_2 from 1.25 to 10 mM evoked a large peak of insulin secretion in the normal pancreas of FoCHI patients, and so did 100 μM tolbutamide and 10 mM arginine (Figure 5A). In focal lesions, CaCl_2 and arginine also induced peaks of insulin secretion, whereas tolbutamide had no effect. In DiCHI pancreas, tolbutamide was consistently ineffective in contrast with CaCl_2 and arginine, the effects of which were markedly potentiated by forskolin (Figure 5B).

Discussion

Preparation. Because islet organization is altered in focal adenomatous lesions, we dispersed normal and pathological fragments in exactly the same way. Although morphological (Figure 1 and ref. 11) and functional (32) indices suggest that β cells are quiescent outside focal lesions, secretory responses by fragments of normal pancreas from FoCHI patients were similar to those by islets isolated from healthy adult organ donors (26). This means that any alterations of β cell function in vivo (e.g., by drug treatment or lack of stimulation) have been reversed by the initial 17–22 hours of culture in 5 mM glucose. However, 2 quantitative differences must be pointed out. First, whereas the low threshold of 3 mM for glucose-induced insulin secretion is similar in islets and fragments,



the maximum effect of glucose is reached at a much lower concentration (7 vs. 15 mM) in fragments of infant pancreas. Second, the increase in insulin secretion occurring when the concentration of glucose is raised after closure of K_{ATP} channels by tolbutamide (measure of the amplifying pathway) is of smaller magnitude than in adult islets (26). Because no similar studies with islets from normal young infants are available, it is unclear whether these differences are solely attributable to the age of the subjects. With this reservation, we are confident that our procedure did not distort β cell secretory function. Most importantly, results obtained with focal lesions were included in the study only when healthy pancreas from the same patient was available for comparison and displayed qualitatively normal responses to glucose, diazoxide, and tolbutamide (Supplemental Figures 1 and 2). This control is crucial to ascertain that abnormal responses of the lesion are not simply due to tissue damage. Normal pancreas from FoCHI patients was also used as control for the responses recorded in DiCHI that affects all islets from the patients.

Major abnormalities of insulin secretion in CHI pancreas. We first acknowledge that, in contrast to the good intersubject reproducibility of insulin secretion in normal pancreas, some alterations were inconsistently observed in focal lesion and DiCHI pancreas, which complicates their interpretation. We next emphasize that our study was performed in a subgroup of CHI infants requiring partial or subtotal pancreatectomy because of clinical resistance to diazoxide and other medical treatment. The abnormalities of insulin secretion would be different if the mutations led to expression of partially functional K_{ATP} channels or to defects in other pathways. In both focal lesion and DiCHI pancreas, insulin secretion rate in the presence of 1–3 mM glucose (with or without forskolin) was consistently elevated compared with that in normal pancreas. This is attributable to continuous depolarization of β cells (5) due to the lack of K_{ATP} channels and elevation of $[Ca^{2+}]_c$ even in low glucose concentrations (20). Thus, as previously reported (25), omission of extracellular $CaCl_2$ lowered insulin secretion by CHI pancreas to basal rates similar to those in normal pancreas.

The differences in insulin secretion rates between CHI and normal pancreas disappeared or were reversed at 5 mM glucose and above because the stimulatory action of glucose occurred in normal tissue only. The effects of glucose were indeed peculiar in CHI pancreas. Small stepwise increases in its concentration had no effect, while a larger step (1 to 15 mM) sometimes produced a rapid but small increase in insulin secretion above the already elevated initial level. Most often, the increase was transient and followed by a decrease of insulin secretion (particularly in the presence of forskolin) rather than by a sustained second phase elevation. A rapid and transient secretion of insulin also occurred in response to leucine plus glutamine, but this was not followed by a secondary decrease. In the absence of precise information on how $[Ca^{2+}]_c$ changes under these conditions, no mechanistic explanation is straightforward. In one single study of islets from 3 DiCHI patients (21), high glucose concentrations did not increase the surprisingly low basal $[Ca^{2+}]_c$. Cautious extrapolation to the present study would therefore suggest that the peak of insulin secretion produced by glucose is due to the amplifying pathway. Although recent work established that metabolic amplification is of rapid onset and contributes to both phases of glucose-induced insulin secretion in normal mouse islets (8, 34), it remains unclear why it was not sustained to produce a second phase increase in CHI pancreas (further discussion in next paragraph).

Whereas the consistent ineffectiveness of tolbutamide in CHI pancreas conforms to model predictions, the increase in insulin secretion that diazoxide produced in several cases is paradoxical and not predicted by any model. It is unlikely to reflect an indirect action via neighboring non- β cells, such as inhibition of somatostatin release (35), because the germinal mutation (DiCHI patients) also affects K_{ATP} channels in δ cells. Higher concentrations of diazoxide have been reported to affect mitochondrial membrane potential and slightly decrease ATP production (36), but such an effect is not expected to increase insulin secretion. Since pinacidil, another opener of K_{ATP} channels in normal β cells (27), reversibly increased insulin secretion in both focal lesion and DiCHI pancreas, a mere artefact of diazoxide is unlikely, but the cellular target remains unknown.

Comparisons with animal models. Unlike infants with diazoxide-resistant CHI, mice with a knockout of either *Sur1* or *Kir6.2*, thus lacking K_{ATP} channels in their β cells, spontaneously correct their postnatal hypoglycemia within a few days (37, 38), and only fasting can disclose mild hypoglycemia in adults (39–41). In vitro, their islets show Ca^{2+} -dependent elevation of insulin secretion in low glucose concentrations and complete insensitivity to tolbutamide and diazoxide (28, 37–39, 42–44). Notably, the paradoxical increase in insulin secretion produced by diazoxide in CHI pancreas has never been reported in mice. There is general agreement that glucose only weakly increases insulin secretion in the perfused pancreas or freshly isolated islets from these models (37–39, 42–45) except when the cAMP pathway (44, 46) or the protein kinase C pathway (44, 47) is activated. In CHI pancreas, cAMP (forskolin) augmented insulin secretion but did not induce glucose responsiveness. Similar observations have been made with long-term cultures of DiCHI pancreas challenged with phosphodiesterase inhibitors (24, 25). Acetylcholine similarly increased insulin secretion in CHI and normal pancreas in 15 mM glucose, but the experiments were not appropriate to detect whether CHI β cells are hyperresponsive to the neurotransmitter as are *Sur1*-knockout islets (44, 47).

We (43, 48) and others (49, 50) observed that overnight culture of *Sur1*- or *Kir6.2*-knockout islets influenced basal secretion rates in low glucose concentrations and unmasked strong sustained effects of glucose on insulin secretion, which could be attributed to a very efficient amplifying pathway and, more unexpectedly, to a K_{ATP} channel-independent control of Ca^{2+} influx (42, 43, 45). Although our fragments of CHI pancreas were studied after overnight culture in 5 mM glucose, no sustained stimulation of insulin secretion was observed. These discrepancies cannot be attributed to age because islets from 2-week-old *Sur1*- or *Kir6.2*-knockout mice behaved like islets from adult mice (43, 45). It seems that a lack of K_{ATP} channels does not lead to the same adaptation mechanisms in mouse and human β cells.

As already alluded to above, the poor efficacy of the amplifying pathway in CHI pancreas is intriguing. This may be one key to the phenotypic differences between the 2 species. In islets from one *Sur1*-knockout model (39), glutamine potentially increased insulin secretion in the presence of low glucose, an effect that was barely augmented by leucine, used to activate glutamate dehydrogenase (40, 51). These and other observations in normal islets prompted the suggestion that glutamine serves as coupling factor in metabolic amplification of exocytotic responses to Ca^{2+} (51, 52). It was also proposed that inhibition of the GABA shunt in *Sur1*-knockout β cells contributes to their hyposensitivity to glucose and hypersensitivity to amino acids (52). We cannot exclude that simi-



lar alterations underlie the poor responsiveness of CHI pancreas to glucose but wish to remain very cautious in such extrapolations between species. Thus, in our own studies using islets from the other *Sur1*-knockout model (38), glutamine alone had virtually no effect on insulin secretion in contrast with activation of glutamate dehydrogenase (42). We also point out that glutamine alone was poorly effective in the pancreas of a single DiCHI subject (Supplemental Figure 6). Finally, it is puzzling that the amplifying pathway is less efficient in normal pancreas from FoCHI patients than in normal adult islets. The question thus remains open.

Comparison between insulin secretion in vitro and in vivo. Insulin secretion has been studied in CHI infants before surgery, generally in an attempt to distinguish between FoCHI and DiCHI. The tolbutamide stimulation test only poorly distinguishes between the 2 forms for 2 reasons. First, some mutations do not completely suppress β cell sensitivity to sulfonylureas, and second, the normal pancreas in FoCHI does not always strongly respond to tolbutamide, perhaps because of a long-lasting resting state induced by ambient low glucose or drug treatment (32, 33, 53). This was not the case in our *in vitro* studies of FoCHI pancreas, in which tolbutamide was consistently inactive in focal lesion and active in normal tissue, possibly relieved from any inhibition by the overnight culture period. In cases of null mutations in *ABCC8* or *KCNJ11*, the test could still be helpful if performed after elevation of blood glucose levels to prime the normal pancreas.

In many patients with either form of CHI, an acute increase in plasma insulin levels was observed after *i.v.* glucose injection (32, 33, 53). While this increase was anticipated and attributed to the normal pancreas in FoCHI, it was paradoxical in DiCHI. Our *in vitro* results provide an explanation in showing that high glucose acutely stimulates insulin secretion from at least some focal lesions and DiCHI pancreas. The lack of a sustained second phase and of any stimulatory effect of a graded increase in glucose concentration *in vitro* is also compatible with *in vivo* observations in a single, nonoperated, older DiCHI patient (54).

The acute insulin response to calcium has been used to distinguish CHI due to K_{ATP} channel mutations from other forms (peripheral *i.v.* calcium injection with modest elevation of calcemia) and to localize a focal lesion in the pancreas by selective arterial calcium stimulation of the pancreas with hepatic venous sampling (ASVS) (30, 32, 33, 53). The test is based on the expectation of a larger calcium influx (hence of insulin secretion) in β cells that are depolarized because of the absence of K_{ATP} channels (5). Our *in vitro* data obtained with a high $CaCl_2$ concentration are relevant to the ASVS only. We observed that high $CaCl_2$ induced insulin secretion not only from CHI pancreas but also from normal pancreas, which is explainable by the fact that the physiological glucose concentration of 5 mM used in our *in vitro* experiments is stimulatory for normal β cells. At best, a quantitative difference in the responses could be expected *in vivo* when the normal pancreas is not stimulated by glucose.

Genotype-phenotype correlations. As already emphasized, insulin secretory responses of the normal pancreas from FoCHI subjects were qualitatively similar to those of normal adult islets. This means that an inactivating mutation of *ABCC8* (or *KCNJ11*) in only 1 allele does not alter β cell function. Our conclusion extends 2 *in vivo* studies showing that heterozygous carriers of an *ABCC8* mutation (one of which is Val187Asp as in our subjects FoCHI2, -3, and -4) normally secreted insulin in response to glucose or tolbutamide (54, 55). Half the number of functional K_{ATP} channels would thus seem sufficient to ensure normal control of insulin secretion.

The genetic basis of FoCHI and DiCHI is different. Notably, owing to the loss of maternal *CDKN1C* (p57) and *H19*, and unrestrained expression of paternal *IGF-II*, focal lesions are characterized by an increased proliferation rate, as shown by Ki-67 immunodetection in approximately 8% of β cells within the lesion as compared with approximately 3% in DiCHI or normal pancreas (56). In spite of these differences, the alterations of insulin secretion were similar in FoCHI and DiCHI pancreas.

The functional impact of 6 of the *ABCC8* mutations found in our patients has previously been evaluated by expression studies (Table 1). The mutation identified in subjects FoCHI2, -3, and -4 (57) abolishes K_{ATP} channel expression at the plasma membrane by causing SUR1 retention in the endoplasmic reticulum (57–59). In these 3 cases, the paradoxical decrease in insulin secretion in response to glucose and increase in response to diazoxide were not observed. However, cases with another mutation also causing intracellular channel retention (FoCHI6 and DiCHI1, and DiCHI5) (60, 61) did show the paradoxical response to diazoxide. Except for the fact that high glucose did not decrease insulin secretion in DiCHI1, the responses of all 6 DiCHI patients were similar in spite of different mutations. In one case (DiCHI4), the dominant mutation allows membrane expression (84%) of a quasi-nucleotide-insensitive K_{ATP} channel (62), but no difference in insulin secretion was observed with mutations causing complete inhibition of channel trafficking. Finally, no specific alteration of insulin secretion characterized the pancreas of the single patient with a mutation in *KCNJ11* (FoCHI1). With the limitations imposed by the small number of possible comparisons in our group comprising only diazoxide-resistant CHI patients, this *in vitro* study shows that the ultimate impact on insulin secretion is determined by the lack of functional K_{ATP} channels at the plasma membrane rather than the underlying genetic/cellular cause of this lack.

Conclusions. The sole presence of a paternal mutation in *ABCC8* or *KCNJ11* does not alter *in vitro* insulin secretion by the pancreas outside the lesion in FoCHI. Compared with normal pancreas, insulin content is very high in focal lesion and normal in DiCHI pancreas, but fractional insulin secretion is not particularly elevated in either pathological tissue at physiological glucose levels. However, qualitative perturbations are major and similar in the focal lesion and DiCHI pancreas despite their distinct genetic and cellular pathogeny. The *in vitro* insulin secretion rate is abnormally elevated in the presence of low glucose and virtually not increased by high glucose concentrations. Most surprising are the increase in secretion produced by diazoxide and the decrease sometimes caused by high glucose concentrations. Neither of these changes was reported in mouse islets lacking K_{ATP} channels, and neither can be explained by current models of β cells.

Methods

CHI subjects. The present study was conducted with the approval of and according to the regulations of the Commission d'Ethique Biomédicale of the University of Louvain Faculty of Medicine. Informed consent was obtained from the parents of the children.

The diagnosis of CHI was established on the basis of widely accepted criteria (1). The patients underwent partial or subtotal pancreatectomy because diazoxide and other medical measures proved inefficient to prevent hypoglycemia. Between 2000 and 2009, 30 cases of diazoxide-resistant CHI were included in the study, but only 18 FoCHI and 6 DiCHI cases could be retained in the final analysis (Table 1).



In FoCHI, mean age at surgery was 6.4 (range 2–11) months. All patients were cured after resection of the focal lesion, sometimes after 2 or 3 operations (Table 1). Four additional subjects were studied but could not be included in the present analysis. In 2 patients, no control tissue was available for comparison with the responses measured in their focal lesion. In the other 2, the fragments of focal lesion were largely contaminated with normal tissue as shown by low insulin content and microscopic evidence that the lesion was poorly delineated.

In DiCHI, mean age at surgery was 4.7 (range 2.5–7) months for 5 patients and 18 months in the sixth patient. Hypoglycemia persisted in 1 patient, and diabetes followed pancreatectomy in the others. Two further subjects could not be included in the study because the insulin content of the pancreatic fragments was too low (<10% of that in other cases) for reliable measurement of insulin secretion.

Genetic studies (authorized in 23/24 subjects) showed that 22 patients had a mutation in *ABCC8* (19 different types) and only 1 in *KCNJ11* (Table 1). Seven of these mutations are believed to be novel, and the others have previously been described (6, 7). Note that patient DiCHI1 is the sister of FoCHI6 (63) and that patient DiCHI6 is a compound heterozygote.

Postoperative morphological study of the pancreas. Pancreatic samples were fixed in Bouin solution for conventional microscopy and immunohistochemical identification of insulin-containing cells as described elsewhere (17). Other samples were fixed in 4% formalin to perform CDKN1C peptide immunodetection (anti-CDKN1C from NeoMarkers; dilution 1/100).

Sampling and treatment of pancreatic fragments for functional studies. On the basis of intraoperative examinations of frozen samples, the pathologist distinguished between FoCHI and DiCHI, directed the extent of pancreas resection, and sampled fragments for functional studies. Because great care was taken to avoid overlap of normal and pathological regions in FoCHI, available fragments were sometimes very small: average of 102 mg (range 23–295) of normal pancreas and of 54 mg (range 17–92) of focal lesion. Larger fragments were available from the pancreas of DiCHI patients in whom pancreatectomy was subtotal: average of 682 mg (range 419–1092) of whole pancreas. Pancreatic fragments were immersed in sterile RPMI culture medium and transported at 4°C to the laboratory in Brussels within 4–8 hours.

On arrival, each fragment was injected with 6 ml of a salt-balanced medium containing 12 mg collagenase, finely minced, and transferred into a tight tube. The suspension of tissue was then shaken by hand for about 3–4 minutes in a water bath at 37°C, gently centrifuged for a few seconds, and rinsed several times at room temperature. Finally, the digested tissue consisting of clusters of cells and debris was resuspended in RPMI culture medium containing 5 mM glucose, 100 IU penicillin/ml, 100 µg streptomycin/ml, and 10% heat-inactivated fetal calf serum, and distributed into 3 cm petri dishes containing a final volume of 2 ml. Insulin secretion was studied the next day, after 17–22 hours of culture at 37°C, in an atmosphere of 5% CO₂ in air.

Measurements of insulin secretion. On the day of the experiment, an aliquot of culture medium was saved for insulin determination. Pancreatic tissue was then harvested from culture dishes and distributed into equivalent portions that were transferred into perfusion chambers. Details on the

system, technique, and solutions can be found elsewhere (26, 64). At the end of an experiment, pancreatic tissue was recovered from the chambers for measurement of insulin content (65). Insulin in perfusion samples, tissue extracts, and culture medium was measured by radioimmunoassay using human insulin as a standard (26).

Expression of results. Because the amount of tissue placed in perfusion chambers was variable and because this tissue was heterogeneous, insulin secretion rate was expressed relative to the tissue insulin content (fractional insulin secretion as percentage of insulin content/min) (64). Insulin secretion by focal lesion and healthy pancreas of the same patient was directly compared in paired experiments. However, not all protocols could be done in each case because of limited tissue availability. Insulin content of fresh tissue was not measured because this would have reduced even further the amount of tissue for functional studies, but it was estimated as follows: to the insulin content of all tissue fragments from a patient (measured at the end of perfusions), we added the amount of insulin secreted by these fragments during perfusion and culture. From that sum and the weight of the fragment, the initial insulin content of the tissue could be recalculated. Two opposite sources of error are the loss of insulin during tissue mincing and digestion, and insulin biosynthesis during culture.

All values are presented as mean ± SEM for the indicated number of experiments (patients) or with the range in parentheses. Statistical significance ($P < 0.05$) of changes in secretion was assessed by 2-tailed Student's paired *t* test, comparing values before and during application of test agent in the same preparation or values in normal and pathological tissue from the same subject.

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