

Hyperinsulinism of Infancy

The Regulated Release of Insulin by K_{ATP} Channel-Independent Pathways

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Hyperinsulinism of infancy (HI) is a congenital defect in the regulated release of insulin from pancreatic β -cells. Here we describe stimulus-secretion coupling mechanisms in β -cells and intact islets of Langerhans isolated from three patients with a novel *SURI* gene defect. 2154+3 A to G *SURI* (GenBank accession number L78207) is the first report of familial HI among nonconsanguineous Caucasians identified in the U.K. Using patch-clamp methodologies, we have shown that this mutation is associated with both a decrease in the number of operational ATP-sensitive K^+ channels (K_{ATP} channels) in β -cells and impaired ADP-dependent regulation. There were no apparent defects in the regulation of Ca^{2+} - and voltage-gated K^+ channels or delayed rectifier K^+ channels. Intact HI β -cells were spontaneously electrically active and generating Ca^{2+} action currents that were largely insensitive to diazoxide and somatostatin. As a consequence, when intact HI islets were challenged with glucose and tolbutamide, there was no rise in intracellular free calcium ion concentration ($[Ca^{2+}]_i$) over basal values. Capacitance measurements used to monitor exocytosis in control and HI β -cells revealed that there were no defects in Ca^{2+} -dependent exocytotic events. Finally, insulin release studies documented that whereas tolbutamide failed to cause insulin secretion as a consequence of impaired $[Ca^{2+}]_i$ signaling, glucose readily promoted insulin

release. Glucose was also found to augment the actions of protein kinase C- and protein kinase A-dependent agonists in the absence of extracellular Ca^{2+} . These findings document the relationship between *SURI* gene defects and insulin secretion in vivo and in vitro and describe for the first time K_{ATP} channel-independent pathways of regulated insulin secretion in diseased human β -cells. *Diabetes* 50:329–339, 2001

Hyperinsulinism of infancy (HI) (congenital hyperinsulinism) is a consequence of unregulated insulin release. The disease is clinically heterogeneous, with highly variable age of onset, severity, and responsiveness to medical treatments (1). Mutations in four different genes have been identified: the ATP-sensitive K^+ channel (K_{ATP} channel) genes *SURI* and *Kir6.2*, glucokinase (*GK*), and glutamate dehydrogenase (*GLUD1*). Despite this, in as many as 60% of patients, the genetic basis of the condition has not been elucidated (2). The most common and most severe forms of HI arise from *SURI* and/or *Kir6.2* gene defects (HI-SUR1 and HI-Kir6.2, respectively, also termed persistent hyperinsulinemic hypoglycemia of infancy [PHHI]) (2). These patients commonly exhibit symptomatic hypoglycemia soon after birth, are largely unresponsive to inhibitors of insulin release such as diazoxide and somatostatin, and require subtotal (95%) resection of the pancreas to alleviate hypoglycemia (1). For individuals with the rarer forms (HI-GK or HI-GLUD1), surgical resection of the pancreas is not usually necessary because hyperinsulinism is avoided by managing nutrition or by inhibiting insulin secretion with the K_{ATP} channel activator diazoxide (3,4).

The genetic basis of HI- K_{ATP} is heterogeneous; more than 40 mutations in *SURI* have been identified, and 3 mutations in *Kir6.2* have been described (2). Using both recombinant expression systems (5–8) and β -cells isolated from patients after surgery (6–10), investigators have shown these mutations to lead to impaired trafficking or assembly of K_{ATP} channels or to cause defects in the ADP-dependent regulation of channel activity. Functional studies on β -cells isolated from patients with HI have further demonstrated that loss of operational channels results in depolarized β -cells with unregulated Ca^{2+} channel activity (9). However, whereas these studies have provided a means of correlating the genetics of HI

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$[Ca^{2+}]_i$, intracellular free calcium ion concentration; Di-HI, hyperinsulinism of infancy caused by diffuse involvement of defective β -cells; Fo-HI, hyperinsulinism of infancy caused by focal adenomatous hyperplasia; HI, hyperinsulinism of infancy; K_{ATP} channel, ATP-sensitive K^+ channel; N.Po, open-state probability where N is the number of operational channels; PHHI, persistent hyperinsulinemic hypoglycemia of infancy; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol myristate acetate; Po, open-state probability; TST, tolbutamide-stimulation test.

to the clinical phenotype (11), the molecular mechanisms of insulin release in HI β -cells have not yet been described.

In normal β -cells, K_{ATP} channels govern the resting cell membrane potential. Closure of channels following increased glucose metabolism leads to depolarization of the membrane and the regulated opening of voltage-dependent Ca^{2+} channels. The increased Ca^{2+} influx elevates the free intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in the vicinity of the insulin-containing secretory granules, and this stimulates exocytosis (12–15). This is the K_{ATP} channel-dependent pathway of glucose signaling. In addition to this pathway, glucose also regulates insulin secretion via K_{ATP} channel-independent pathways. One of these increases insulin secretion when $[Ca^{2+}]_i$ is elevated and thus acts to augment the effect of the K_{ATP} channel-dependent pathway (16–18). Another acts in the absence of a rise in $[Ca^{2+}]_i$, but requires the simultaneous activation of protein kinases A and C in the β -cell (19–21). Both the K_{ATP} channel-dependent pathway and the independent pathways are known to be present in human islets (22,23). Because the antidiabetic agent tolbutamide inhibits K_{ATP} channels and causes first-phase-like release, the K_{ATP} channel-dependent pathway is thought responsible for the first phase of glucose-stimulated insulin release, whereas the second phase requires the activation of the K_{ATP} channel-independent pathways (21,24).

In this work, islets were isolated from three patients with HI after surgery in order to study further the K_{ATP} channel-independent pathway of glucose signaling in human β -cells and to explore the implications of this pathway in the course of the disease and its treatment. Our findings demonstrate that although glucose stimulated insulin release, tolbutamide was without effect. These differences were due to the fact that tolbutamide acts on the K_{ATP} channel-dependent pathway (which is defective in these patients), whereas glucose exerts an effect via the K_{ATP} channel-independent pathways. These data have important implications for the treatment of HI and provide an empirical basis for a novel diagnostic procedure in vivo (see also the accompanying article by Grimberg et al. [25]).

RESEARCH DESIGN AND METHODS

Studies were performed on three Caucasian patients with HI. N15 and N38 were siblings (family 1); N16 was unrelated (family 2). No other family members were affected. Each of the patients presented with typical symptoms of HI: severe hypoglycemia (blood glucose <2.6 mmol/l; normal range 3.5–5.5 mmol/l) in association with hyperinsulinism (insulin >12 mU/ml in association with a raised C-peptide at the time of hypoglycemia, normal insulin value <4 mU/ml) within the first few minutes/hours after birth. These and other established criteria (1) confirmed the diagnosis of HI.

Patient N15 was the first child of unrelated Caucasian parents and was born at 40 weeks' gestation after a normal pregnancy (birth weight 3.5 kg, 50th percentile). When the child was 3 weeks old, the parents noticed twitching of limbs and eyes in the morning, and at 11 weeks the child was admitted to the hospital after three generalized seizures. HI was confirmed on admission, following a blood glucose measurement of 0.5 mmol/l in association with hyperinsulinism. Patient N38 was the second child of these unrelated Caucasian parents. Delivery was induced at 37 weeks' gestation on suspicion of rhesus incompatibility (birth weight 4.51 kg, >99 th percentile). The child was noted to be hypoglycemic (blood glucose 1.9 mmol/l) on the first day of life. Figure 1 details clinical data for these patients; similar findings were also observed for N16. Note that blood glucose levels could be raised only by increases in the rate of glucose administration (normal range <6 – 8 mg \cdot kg $^{-1}$ \cdot min $^{-1}$) and that treatment with medical agents either was ineffective or provided only temporary relief from the need for high rates of glucose infusion. Because all patients therefore failed to respond adequately to medical therapy involving nifedipine, chlorothiazide, diazoxide, somatostatin (Octreotide), or glucagon combined with an elevated glucose infusion rate to maintain normoglycemia (>12 mg \cdot kg body wt $^{-1}$ \cdot min $^{-1}$), a near total (95%) pancreatectomy was per-

formed to prevent persistent hypoglycemia (see arrows in Fig. 1). Patient N15 subsequently underwent a second resection of the pancreas because of continuing hyperinsulinemic hypoglycemia. Histopathological examination of each of the pancreata revealed that all three patients had diffuse abnormalities of endocrine tissue. Postoperatively, all three patients achieved normoglycemia at normal glucose administration rates. N15 developed clinical diabetes during the postoperative period and has continued to require regular insulin therapy. Genotyping studies using standard procedures (3,5,7) revealed that all three patients were heterozygous for a single base change within intron 16 of the *SURI* gene, 2154+3 A to G (GenBank accession number L78207), that appears to result in aberrant splicing. In family 1, both patients had the mutation on the paternal allele, whereas in family 2 the mutation was carried on the maternal allele. The 2154+3 A-to-G *SURI* gene mutation was not identified in 50 control subjects or in any other HI patients tested (i.e., it is not a common polymorphism). No defects in the *Kir6.2* gene were detected.

Preparation of tissue for in vitro studies. Intact islets of Langerhans were isolated from transplantable adult donor tissue (with permission) and from the pancreata of all three HI subjects using a controlled collagenase digestion procedure, as previously described (9). Islets were maintained under standard tissue culture conditions using RPMI-1640 medium (Sigma, Poole, U.K.) supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin in a humidified atmosphere of 5% CO_2 in air at 37°C. For electrophysiology studies, intact islets were mechanically dispersed into individual cells and isolated cell clusters before experimentation. Insulin release and Ca^{2+} microfluorimetry experiments were carried out using intact islets.

Insulin release measurements from intact islets. Insulin release was measured under static incubation conditions using batches of five islets and triplicate determinations. The islets were preincubated at 37°C in Krebs-Ringer HEPES buffer (composition in millimoles per liter: 137 NaCl, 5.36 KCl, 0.81 $MgSO_4$, 0.34 Na_2HPO_4 , 0.44 KH_2PO_4 , 4.17 $NaHCO_3$, 10 HEPES, and 1.26 $CaCl_2$) containing 2 or 2.8 mmol/l glucose at pH 7.4 for 30 min, as described previously (22,23). Subsequently, the islets were exposed to the test substances for 30 min. At the end of the incubation period, aliquots of the samples were removed and kept at $-20^\circ C$ until radioimmunoassay was performed using a charcoal separation method (26). For insulin release studies in Ca^{2+} -free medium, Ca^{2+} was omitted, and the Krebs-Ringer HEPES buffer was supplemented with 1 mmol/l EGTA.

Electrophysiology. All data were obtained from primary cultured human tissue, as previously described (9). β -cells were selected on the basis of their larger size and granular appearance, and recordings were made using inside-out, cell-attached, perforated whole-cell, and the standard whole-cell configurations of the patch-clamp technique (27). Recording pipettes were fabricated from boro-silicate glass and had a series resistance of 2–5 M Ω when filled with the internal solution for whole-cell experiments and 5–10 M Ω for single-channel current recordings. The zero potential was adjusted with the pipette in the bath, and no correction for liquid junction potentials was made for single-channel current recordings. Data were recorded using an EPC-7 patch-clamp amplifier (List Electronic, Darmstadt, Germany) with hardware (TL-1 A/D-D/A Interface) and software (pClamp 5.5) supplied by Axon Instruments (Foster City, CA). For the capacitance measurements, data were recorded using an AxoPatch 200B amplifier with a Digidata 1200B A/D-D/A interface (Axon Instruments). Changes in membrane capacitance were tracked using in-house software run on a Pentium computer (Dell, Round Rock, TX). Whole-cell currents were filtered at 0.5 kHz and digitized at 1.6 kHz for measurements of whole-cell voltage-activated K^+ currents and stored on a 386 personal computer (Tandon, Moorpark, CA). Single K^+ channel and whole-cell K_{ATP} channel data was stored on digital tape using a DAT recorder (Biologic, Echirolles, France) for subsequent replay and analysis. All ion channel experiments were carried out at room temperature. Changes in K_{ATP} channel activity have been expressed as either changes in open-state probability (P_o) or as a function of $N \cdot P_o$, where N is the number of operational channels (28).

Secretion studies from single cells. Capacitance measurements were used to monitor exocytosis in isolated cells, as previously described (29). All experiments were performed using either the perforated patch or the standard whole-cell configuration of the patch-clamp technique. With a temporal resolution of 100 ms, recordings of changes in the cell membrane capacitance reflect increases or decreases in the area of the cell membrane during exocytosis or endocytosis of secretory granules, respectively. For perforated whole-cell experiments, the patch-clamp recording pipette solution contained (in millimoles per liter) 76 Cs_2SO_4 , 10 KCl, 10 NaCl, 5 HEPES, 1 $MgCl_2$, and 0.24 mg/ml amphotericin, pH 7.35 with CsOH; for standard whole-cell experiments, the patch-clamp pipette was filled with (in mmol/l) 125 potassium glutamate, 10 KCl, 1 $MgCl_2$, 3 Mg-ATP, 0.1 cAMP, 5 HEPES, 10 EGTA, pH 7.15 with KOH. Where necessary, $CaCl_2$ was added at 5 mmol/l to achieve final con-

centrations of 0.17 $\mu\text{mol/l}$ (30). All cells were bathed in a solution containing (in mmol/l) 118 NaCl, 20 TEACl, 5.6 KCl, 2.6 CaCl_2 , 1.2 MgCl_2 , 5 HEPES, and 5 glucose, pH 7.4 with NaOH. Where indicated, 2 $\mu\text{mol/l}$ forskolin and 10 mmol/l phorbol myristate acetate (PMA) were added to the bathing solution. All capacitance experiments were performed at 32–34°C.

Potassium channel studies

K_{ATP} channels. Ionic currents were recorded in the cell-attached, whole-cell, and inside-out patch configurations of the patch-clamp techniques (27). For all experiments undertaken using quasiphenological cation gradients, the standard extracellular 140 mmol/l NaCl-rich bathing solution contained (in mmol/l) 140 NaCl, 4.7 KCl, 2.5 CaCl_2 , 1.13 MgCl_2 , 10 HEPES, 2.5 glucose (pH 7.4 with NaOH). For whole-cell experiments, the patch-clamp pipette contained (in mmol/l) 140 KCl, 10 EDTA, and 10 HEPES. For inside-out patch recordings, the internal face of the membrane was bathed with a KCl-rich solution containing (in mmol/l) 140 KCl, 10 NaCl, 1.13 MgCl_2 , 1 EGTA, 2.5 glucose, 10 HEPES (pH 7.2 with KOH), and the patch-clamp recording pipette was filled with the 140 mmol/l NaCl-rich solution. For cell-attached patch recordings, the patch-clamp pipette contained either the 140 mmol/l NaCl-rich solution or the 140 mmol/l KCl-rich solution used for inside-out patch recordings, as indicated in the text.

Macroscopic K_{ATP} channel currents recorded using the whole-cell configuration were performed by holding the cells at -70 mV and then applying 20 mV depolarizing and hyperpolarizing pulses at a frequency of 0.5 Hz. This procedure reveals K_{ATP} channel currents ($I_{K_{\text{ATP}}}$) without the activation of voltage-dependent K^+ channels. Because the vast majority of K_{ATP} channels in β -cells are tonically inhibited by endogenous ATP levels, dialysis of the cell interior with a solution containing a low concentration of ATP results in the apparent "activation" of channels and the generation of a significant "washout" current, as previously described (31). For single-channel current events, the procedures used for data collection and analysis have been described previously (9,28).

Voltage-gated K^+ channels. Ionic currents were recorded using the standard whole-cell configuration. Patch-clamp pipettes were filled with a 140 mmol/l KCl-rich solution containing (in mmol/l) 125 KCl, 30 KOH, 10 EGTA, 1 MgCl_2 , 2 CaCl_2 , 5 HEPES, and 3 MgATP (pH 7.15 with KOH), and cells were bathed with the 140 mmol/l Na⁺-rich solution. Macroscopic voltage-activated whole-cell K^+ currents were recorded under voltage-clamp conditions with the cell held at -70 mV and stimulated by 20 consecutive depolarizing pulses, which were increased stepwise by 10 mV from -60 to 130 mV for a duration of 500 ms and applied to the cell with a frequency of 0.5 Hz. This protocol activates outward K^+ currents from both the delayed rectifier (K_v) and the Ca^{2+} - and voltage-gated K^+ channel (K_{Ca}) observed as a macroscopic whole-cell K^+ current (I_K).

All illustrated records are displayed according to the convention with upward deflections representing outward currents.

Tolbutamide, diazoxide, forskolin, ATP, and ADP were obtained from Sigma, and PMA was obtained from CalBiochem. All agents were added at concentrations as stated in the text. Tolbutamide, diazoxide, and PMA were added as concentrated stock solutions dissolved in DMSO (final concentration $<0.1\%$).

Ca^{2+} microfluorimetry. Changes in $[\text{Ca}^{2+}]_i$ were estimated in HI islets using microfluorimetry procedures with fura-2 (9,28). Intact islets were cultured on poly-D-lysine-coated cover slips (50 $\mu\text{g/ml}$) for up to 58 h in RPMI medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g/ml}$ streptomycin, as above. They were loaded with 0.5 $\mu\text{mol/l}$ fura-2-AM (Sigma) in 1 ml RPMI-1640 medium for 7–10 min at 37°C. The composition of the basic perfusion medium was (in mmol/l) 137 NaCl, 5.36 KCl, 0.81 MgSO_4 , 0.34 Na_2HPO_4 , 0.44 KH_2PO_4 , 4.17 NaHCO_3 , 10 HEPES, and 1.26 CaCl_2 . This medium was gassed with air for 20 min; glucose was added to a final concentration of 2 mmol/l , and pH was adjusted to 7.4 with NaOH. Where the perfusion medium contained 40 mmol/l KCl, the concentration of NaCl was adjusted accordingly. One series of experiments was carried out using the HI-derived β -cell line NES2Y, maintained under the same conditions as previously described (28). For all experiments, an in vitro calibration procedure (32) was used to estimate changes in $[\text{Ca}^{2+}]_i$. Basal values of $[\text{Ca}^{2+}]_i$ have been established in the presence of the basic perfusion medium. Changes in cytosolic Ca^{2+} are reported as the difference between basal $[\text{Ca}^{2+}]_i$ and the peak rise in $[\text{Ca}^{2+}]_i$ obtained for the agent tested.

Statistic analysis. Statistical comparisons were made using Student's *t* tests, with values of $P < 0.05$ considered to be significant.

RESULTS

In vivo data and genotype studies. In this study, three Caucasian patients with HI were investigated. Because two of the three patients were siblings (family 1), this represents the first report of familial HI among nonconsanguineous Caucasians

identified in the U.K. All three patients were unresponsive to medical therapy with diazoxide, chlorothiazide, somatostatin, nifedipine, or glucagon (Fig. 1). Because these observations implicated defects in the K_{ATP} channel genes as the cause of HI, genotyping studies were used to screen for defects in *SUR1* and *Kir6.2*. Each patient was found to carry a novel gene defect in intron 16 of *SUR1* (2154+3 A to G) and was compound heterozygous for this mutation. Analysis of parental DNA documented a paternal defect at the same locus in family 1 and a maternal defect in family 2. No defects in *Kir6.2* were found in any of the patients. The defective region of *SUR1* is thought to encode part of the first nucleotide-binding domain of the sulfonylurea receptor (2). Electrophysiological studies of β -cells isolated from patient tissue confirmed that this mutation is associated with defects in K_{ATP} channels.

Loss of operational K_{ATP} channels in HI β -cells. Previous studies of HI β -cells have described complete loss of functional K_{ATP} channels (6–10). In insulin-secreting cells from HI-SUR1 patients, we now report that the pathophysiology is associated with a marked decrease in the number of functional channels, coupled with a loss of ADP-dependent regulation.

Figure 2 shows whole-cell current data from control and HI-SUR1 β -cells obtained under the same experimental conditions. In these experiments, β -cells were dialyzed using a low ATP-containing pipette solution and whole-cell K_{ATP} channel currents generated by repetitively depolarizing and hyperpolarizing the cell (see RESEARCH DESIGN AND METHODS). Because in normal cells K_{ATP} channels are inhibited by intracellular ATP (15), cell dialysis normally leads to a marked washout K_{ATP} channel current ($n = 8$; Fig. 2). By contrast, only a modest increase in the macroscopic K_{ATP} channel current was found in HI-SUR1 β -cells ($n = 17$; Fig. 2), suggesting that although K_{ATP} channels were present, the magnitude of the current was significantly reduced.

This was further investigated using single-current recordings made from inside-out patches. In control cells, K_{ATP} channels were found to be consistently open in the absence of intracellular ATP (15), and the average N.Po value was 24.9 ± 4.2 ($n = 25$). By contrast, K_{ATP} channels were recorded in only 76% of HI-SUR1 β -cells ($n = 29$ of 38) and the average N.Po value was 1.2 ± 0.6 ($n = 8$) (Fig. 3A). These data are consistent with macroscopic K_{ATP} channel current recordings (Fig. 2) and therefore define the loss of functional K_{ATP} channels in these β -cells. In addition, we found that K_{ATP} channels in HI-SUR1 β -cells were unaffected by ADP ($n = 26$ of 29, 500 $\mu\text{mol/l}$) or diazoxide ($n = 20$ of 23, 200–500 $\mu\text{mol/l}$) in the presence of ATP (500 $\mu\text{mol/l}$) (Fig. 3B–D) and that there were no effects of an "activation cocktail" consisting of potassium fluoride (10 mmol/l), diazoxide (200 $\mu\text{mol/l}$), ADP (100 $\mu\text{mol/l}$), UDP (100 $\mu\text{mol/l}$), and GDP (500 $\mu\text{mol/l}$) ($n = 7$) (Fig. 3D). In control β -cells, these procedures consistently led to a marked and sustained increase in K_{ATP} channel activity (28).

Selective loss of K_{ATP} channels in HI-SUR1 β -cells. Because different K^+ channels contribute to the ionic control of insulin release (15), we also examined whether K_{ATP} channel defects altered the activity of voltage-gated K^+ channels in HI-SUR1 β -cells. Whole-cell K^+ channel currents were therefore recorded following a depolarization of the membrane from -60 to 130 mV in 10-mV steps. This led to the activation of both Ca^{2+} - and voltage-gated K^+ channels and delayed-rectifier K^+ channels. Figure 4 shows that in control ($n = 25$) and HI-SUR1 ($n = 20$) β -cells, the magnitudes of volt-

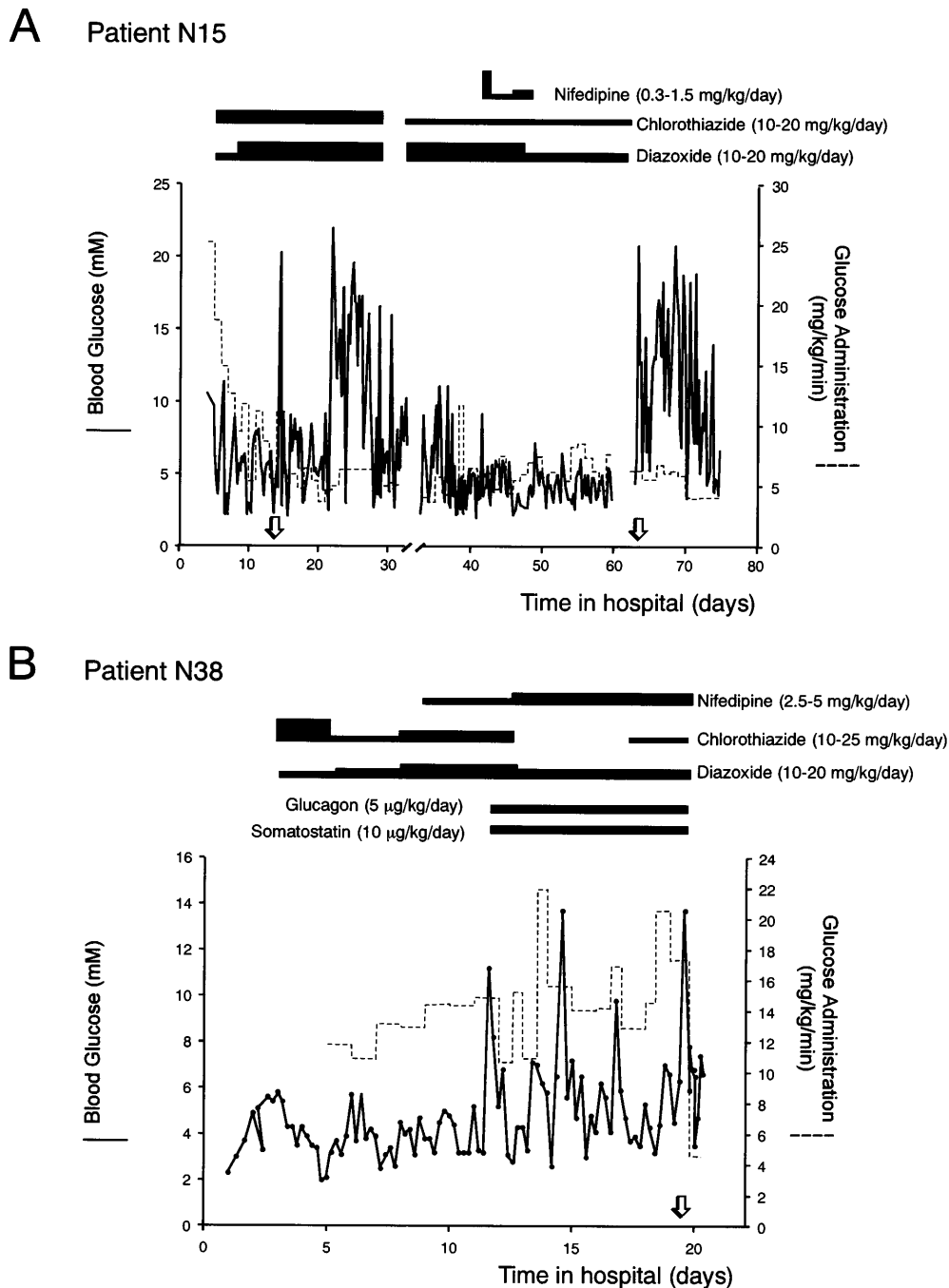


FIG. 1. Clinical management strategies of two siblings with HI (patients N15 [A] and N38 [B]). The figure shows details of blood glucose concentrations (—) and glucose administration rates (---) over time in hospital. Above each of the charts are details of the medical regimens for each patient. The points indicated by arrows in both traces represent the time of the surgical procedures.

age-dependent K^+ channel currents were similar at all membrane potentials and that there were no differences in the voltage dependencies of channel activation.

K_{ATP} channel defects and cytosolic Ca^{2+} signaling. Cell-attached patch-clamp recordings and measurements of cytosolic Ca^{2+} concentrations were used to examine the effects of K_{ATP} channel defects on the regulation of the cell membrane potential. Under basal conditions, control β -cells were electrically silent because of the presence of open K_{ATP} channels (12,28). In marked contrast, 66% of intact HI-SUR1 β -cell recordings were spontaneously generating action cur-

rents in the absence of glucose stimulation ($n = 14$ of 21; Fig. 5A). Ca^{2+} action currents were largely unaffected by somatostatin ($n = 8$ of 10, 100 nmol/l) or diazoxide ($n = 17$ of 20, 500 μ mol/l) (Fig. 5A), and diazoxide had no effect on K_{ATP} channels in intact-cell recordings ($n = 19$ of 19; Fig. 5B).

Despite the appearance of spontaneous action potentials in HI-SUR1 β -cells, we found the average basal cytosolic Ca^{2+} concentration was similar to that in control cells: 80 ± 5 nmol/l ($n = 68$) vs. 78 ± 3 nmol/l ($n = 141$), respectively. However, as a result of the depolarized membrane potential, HI-SUR1 islets responded poorly to depolarization-dependent agonists. Thus,

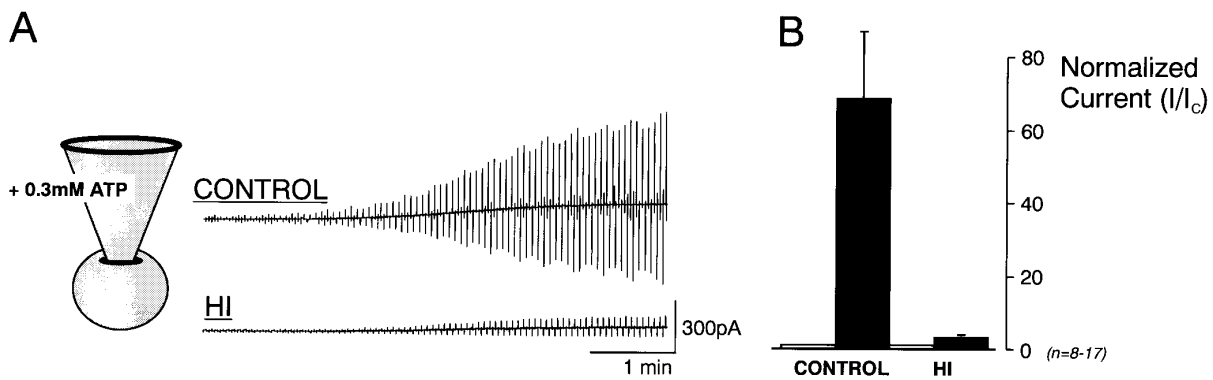


FIG. 2. Macroscopic K_{ATP} channel currents in control and HI β -cells. All data were obtained using the whole-cell configuration. **A:** Control data (upper section) show that when the pipette was filled with a solution containing 0.3 mmol/l ATP, a marked time-dependent increase in the amplitude of K_{ATP} channel current occurs because of washout of cytosolic ATP. In HI-SUR1 β -cells (lower section), the corresponding currents are significantly smaller. **B:** Summary data from several experiments using control β -cells and HI-SUR1 β -cells are shown. In normal cells, washout currents were increased >60-fold ($n = 8$), whereas in HI-SUR1 β -cells, cell dialysis resulted in only a modest increase in current amplitude (~7% of control value, $n = 17$). Data are expressed as means \pm SE of the current amplitude measured 6–7 min after forming the whole-cell configuration. All values have been normalized to the basal whole-cell current before wash out.

glucose (20 mmol/l, $n = 16$ of 16) and the antidiabetic sulfonylurea tolbutamide (100 μ mol/l, $n = 14$ of 16) were unable to further elevate $[Ca^{2+}]_p$, whereas KCl (40 mmol/l) caused only a modest increase in $[Ca^{2+}]_i$ in 7 out of 16 experiments ($\Delta[Ca^{2+}]_i = 30 \pm 5$ nmol/l [$n = 7$] compared with 158 ± 18 nmol/l [$n = 14$ of 14] in control cells [9]; Fig. 6). By contrast, agents that do not elevate $[Ca^{2+}]_i$ through a change in the membrane potential, such as ATP (100 μ mol/l), UTP (100 μ mol/l), and acetylcholine (100 μ mol/l), readily raised $[Ca^{2+}]_i$ in HI-SUR1 islets ($\Delta[Ca^{2+}]_i = 93 \pm 8$ nmol/l, $n = 37$ of 38; Fig. 6).

Ca²⁺-dependent exocytosis in HI-SUR1 β -cells. Because K_{ATP} channel defects in HI-SUR1 β -cells were causally related to unregulated Ca^{2+} channel activity, we next examined the

role of Ca^{2+} in determining the regulation of exocytosis using capacitance measurements. Figure 7 summarizes experiments whereby either control or HI-SUR1 β -cells were dialyzed using the whole-cell configuration with either 170 nmol/l or no Ca^{2+} in the pipette solution. In both control and HI-SUR1 β -cells, we were unable to detect increases in membrane capacitance when $[Ca^{2+}]_i$ was absent ($n = 12$), whereas there was a marked increase in exocytosis when cells were dialyzed with $[Ca^{2+}]_i$ at 170 nmol/l Ca^{2+} ($n = 10$) (Fig. 7). Overall, there were no significant differences between control and HI-SUR1 β -cells under these conditions.

Because these data indicate that exocytosis in HI-SUR1 β -cells is governed by increases in intracellular Ca^{2+} levels,

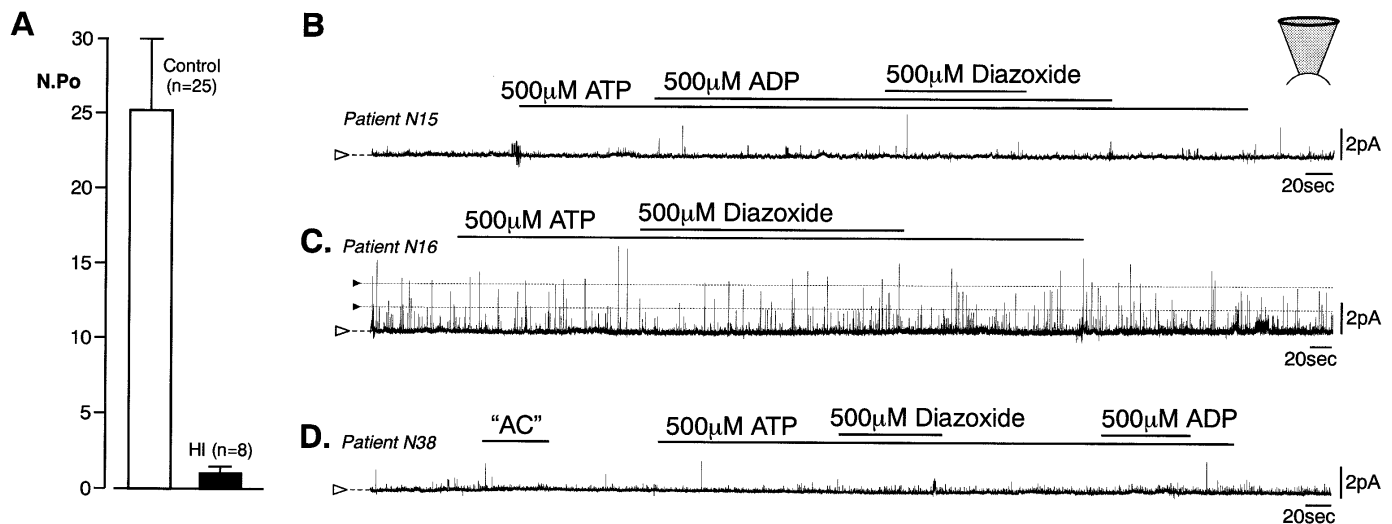


FIG. 3. Defects in the regulation of K_{ATP} channels in HI-SUR1 β -cells. All data were obtained using the inside-out patch configuration with a 140-mmol/l NaCl-rich solution in the pipette and a 140-mmol/l KCl-rich solution in the bath. **A:** Average N.Po values for K_{ATP} channels in control and HI-SUR1 β -cells. All values are expressed as means \pm SEM. Note that in control human β -cells, N.Po values are >16-fold higher than in HI-SUR1 β -cells under the same conditions. **B–D:** Typical representative data from all three patients to document impaired ADP- and diazoxide-induced activation of K_{ATP} channels. Very few ionic events occur in the data shown in **B** and **D**, whereas at least two channels are operational in the record shown in **C**. Note that there is no increase in channel activity after addition of ADP or diazoxide in the presence of ATP. Also note in **D** that addition of an “activation cocktail” (AC) of nucleotides (500 μ mol/l GDP, 100 μ mol/l UDP, 100 μ mol/l ADP), potassium fluoride (10 mmol/l), and diazoxide (200 μ mol/l) also failed to increase K_{ATP} channel activity in these cells ($n = 7$).

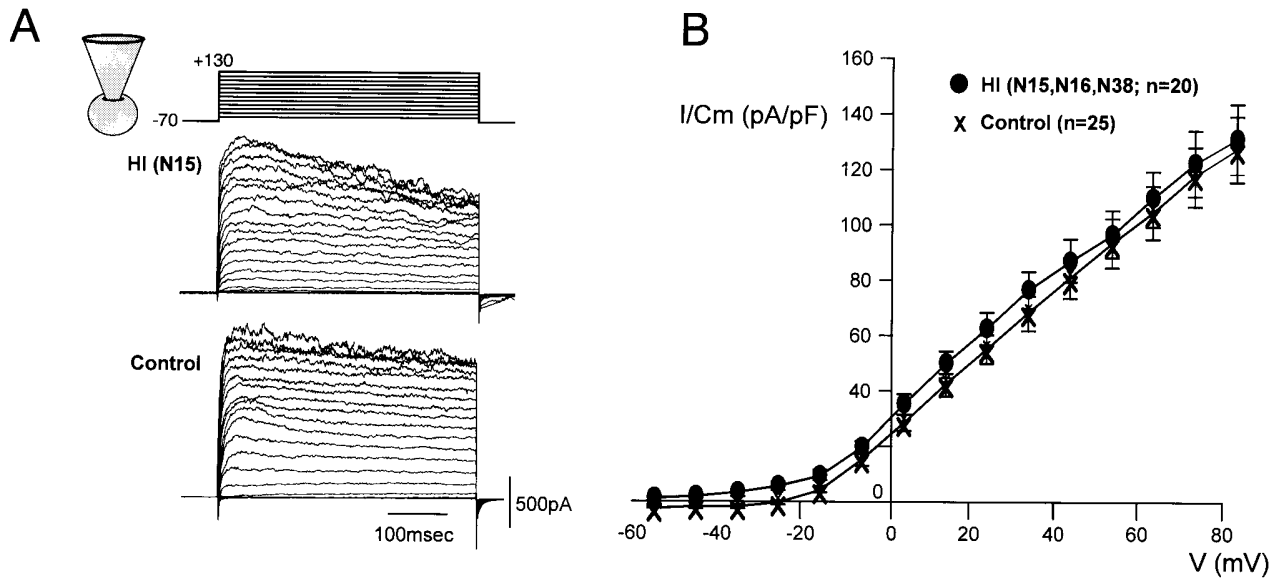


FIG. 4. Macroscopic voltage-gated K^+ currents in control and HI-SUR1 β -cells. *A*: Representative data from HI and control β -cells. Note how depolarization of the cell membrane potential from -70 to 130 mV leads to a marked increase in voltage-gated K^+ channel currents in these cells. *B*: There were no significant differences in the current-voltage (I/V) relationship of these channels in HI-SUR1 β -cells.

we next examined the relationship between voltage-dependent Ca^{2+} influx and control of exocytosis (Fig. 8). Experiments were carried out using the perforated patch technique under voltage clamp conditions, and measurements of whole-cell Ca^{2+} currents and changes in cell capacitance

were recorded in real time. Because voltage-gated Ca^{2+} channels were spontaneously active in intact HI-SUR1 β -cells (Fig. 5), their activity was suppressed by voltage clamping the cells at -70 mV. A stepped depolarization was then made from -70 to 0 mV to generate voltage-gated Ca^{2+}

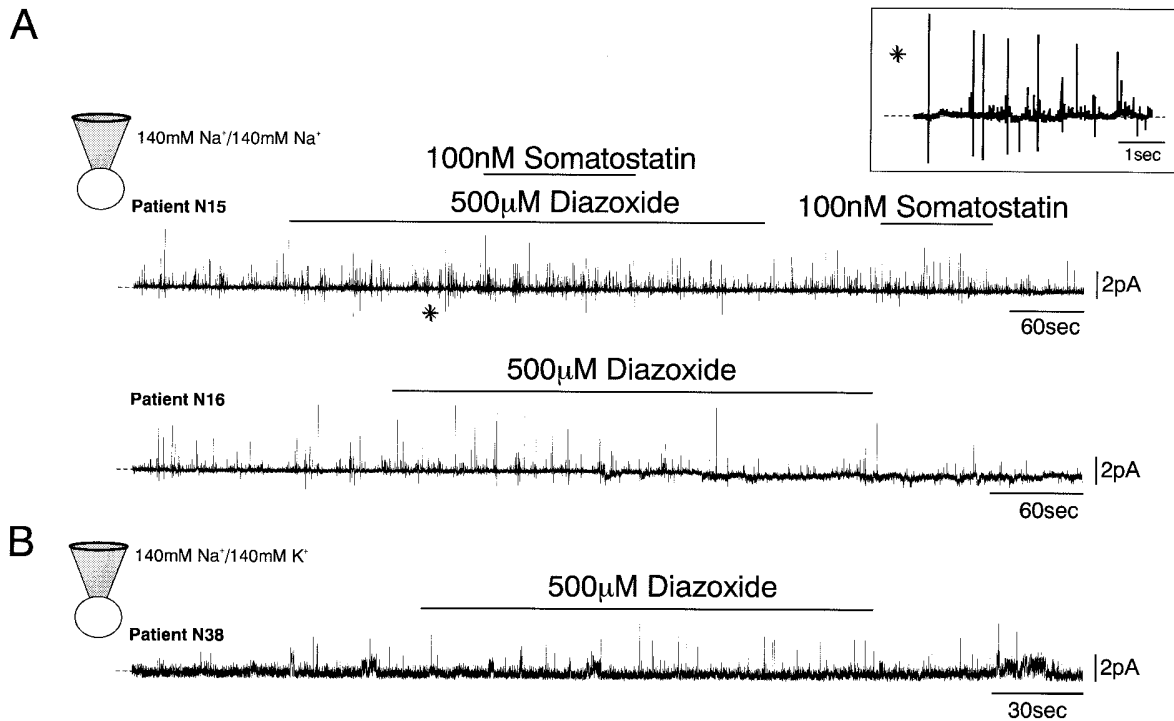


FIG. 5. Loss of K_{ATP} channel operation in intact HI β -cells. Data were obtained in the cell-attached patch recording configuration with either a 140 -mmol/l NaCl-rich solution bathing the cells to record changes in the cell membrane potential (*A*) or a 140 -mmol/l KCl-rich bathing solution to clamp the cells at 0 mV (*B*). *A*: Data show typical recordings of spontaneous action potential currents in both N15 and N16 HI β -cells. Note that neither diazoxide nor somatostatin had any significant effect on the frequency of action currents. Spontaneous action currents (see expanded time-base insert) were seen on 14 of 21 occasions under these conditions. *B*: Diazoxide was unable to activate K_{ATP} channels in intact HI-SUR1 β -cells ($n = 19$ of 19).

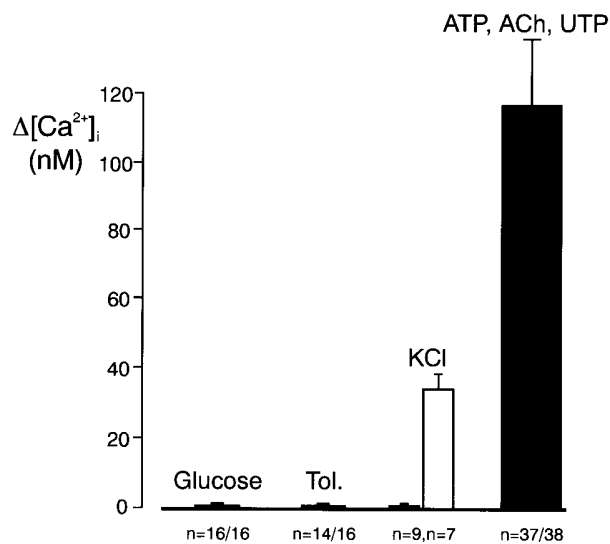


FIG. 6. Impaired cytosolic Ca²⁺ signaling in HI islets. Because HI-SUR1 β-cells are spontaneously electrically active (Fig. 5), depolarization-dependent β-cell agonists such as glucose ($n = 16$ of 16) and tolbutamide ($n = 14$ of 16) failed to elevate [Ca²⁺]_i above basal values consistently. When 40 mmol/l KCl was used to depolarize the cells and raise [Ca²⁺]_i, there was no response on 9 of 16 occasions, whereas there was a modest increase in [Ca²⁺]_i on 7 of 16 occasions (on average this was ~30 nmol/l compared with ~160 nmol/l in control cells [9]). In contrast, when HI-SUR1 islets were challenged with 0.1 mmol/l ATP/acetylcholine/UTP, which does not rely on the cell membrane potential to generate a Ca²⁺ response, marked increases in [Ca²⁺]_i were seen on 96% of all occasions.

influx [Fig. 8A(i)], which was associated with a transient increase in the rate of exocytosis ($n = 6$) [Fig. 8A(ii)]. Note that under these conditions, exocytosis was then followed by a decrease in cell capacitance, which was consistent with the stimulation of endocytosis (33). Ca²⁺-dependent exocytosis was also investigated after activation of protein kinase A (PKA) by 2 μmol/l forskolin and protein kinase C (PKC) using 10 nmol/l PMA. Figure 8 shows that both forskolin and PMA enhanced exocytosis in a cumulative manner and

that the overall rates of endocytosis under these conditions were markedly decreased ($n = 6$) (Fig.8). Neither forskolin nor PMA had any significant effect on the magnitude of voltage-dependent Ca²⁺ currents [Fig. 8A(i)]. Similar findings have also been reported in rodent β-cells (34).

K_{ATP} channel-independent pathway of regulated insulin release in HI-SUR1 β-cells. We have previously documented the activity of both the K_{ATP} channel-dependent and the K_{ATP} channel-independent pathways of glucose-induced insulin release in normal human β-cells (22,23). Because HI-SUR1 β-cells lack K_{ATP} channels yet retain Ca²⁺-dependent exocytotic events, we used intact isolated islets to investigate the role of glucose in governing insulin secretion independently of K_{ATP} channel function.

Under basal conditions (2 mmol/l glucose) the rates of insulin secretion from HI-SUR1 islets were, on average, 10-fold lower than corresponding values from human control islets: 0.23 ± 0.06 ng/5 islets/30 min ($n = 5$) vs. 2.3 ± 0.4 ng/5 islets/30 min ($n = 12$), respectively (22). Furthermore, since HI-SUR1 islets were also found to have a far lower content of insulin than control islets (1.1 ± 0.3 ng/islet, $n = 5$, patients N15, N16 [1.07 ± 0.19 ng/islet, $n = 18$ from 10 patients] vs. 44 ± 8 ng/islet, $n = 12$) (22), these data suggest that there is a rapid rate of insulin turnover in HI islets, which is consistent with the clinical presentation of HI.

In HI-SUR1 islets, the depolarization-dependent agonists KCl (40 mmol/l) and tolbutamide (100 μmol/l) failed to significantly elevate [Ca²⁺]_i (Fig. 6), and as a consequence, neither agent had any significant effect on insulin release (Fig. 9A). This is in contrast to the effects of glucose, which failed to raise intracellular Ca²⁺ levels (Fig. 6), yet caused a dose-dependent release of insulin (Fig. 9A). The actions of glucose were unaffected by the presence of 150 μmol/l diazoxide (Fig. 9A) and were inhibited in the absence of extracellular Ca²⁺ (Fig. 9B). Finally, exposure of HI-SUR1 β-cells to 100 μmol/l acetylcholine, ATP, and UTP caused a >2.5-fold increase in insulin secretion. These experiments illustrate the activity of the K_{ATP} channel-independent pathway in HI islets (i.e., augmentation of Ca²⁺-stimulated insulin release as a result of the absence of functional K_{ATP} channels in the β-cells).

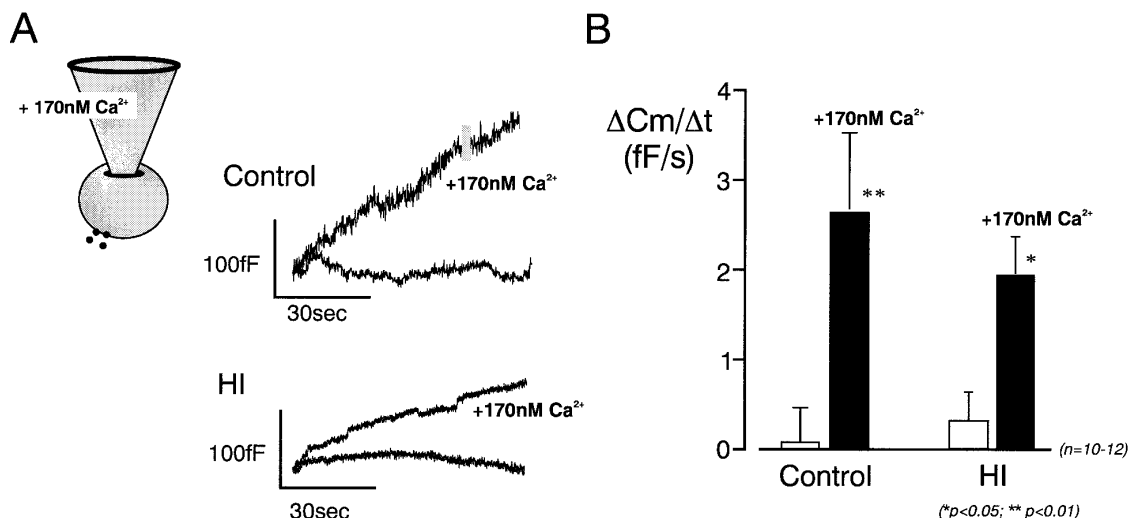


FIG. 7. Ca²⁺-dependent exocytosis in HI and control β-cells. Measurements of membrane capacitance were undertaken using the whole-cell mode of the patch-clamp technique. In both control and HI-SUR1 β-cells, data show that internal perfusion without Ca²⁺ failed to increase exocytosis (□) (B), whereas increases in [Ca²⁺]_i induced by adding 170 nmol/l Ca²⁺ to the pipette solution (■) (B) markedly increased the rate of exocytosis.

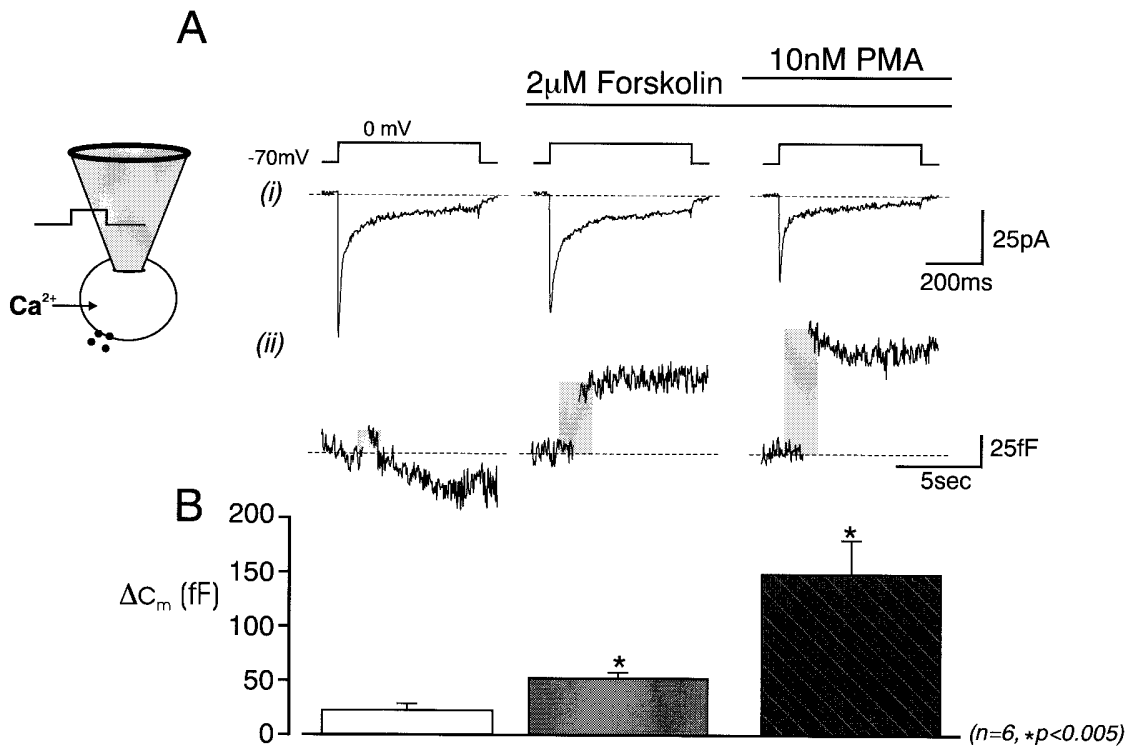


FIG. 8. Voltage-dependent Ca²⁺ influx and exocytosis in HI β-cells. Using the perforated patch configuration, voltage-gated Ca²⁺ influx was initiated by depolarizing the cell under voltage-clamp conditions from -70 to 0 mV (A, top). Under control conditions, this led to a modest increase in the rate of exocytosis [A(ii) and B]. Challenging the cells with 2 μmol/l forskolin to activate PKA and 10 nmol/l PMA to activate PKC led to more marked increases in the rate of exocytosis in HI-SUR1 β-cells.

The next series of studies examined the ability of glucose to augment insulin release in the HI-SUR1 islets in the absence of extracellular Ca²⁺, as is the case in normal human islets (22). Augmentation under these conditions is achieved in the presence of activated PKA and PKC (19–21) (or with the pharmacological agent mastoparan [22]). Figure 9B (left side) shows that in the presence of extracellular Ca²⁺, glucose-stimulated insulin release was increased to fivefold that of the basal rate by the addition of forskolin and PMA. (Increased

Ca²⁺-stimulated exocytosis in response to forskolin and PMA was also shown by capacitance measurement in Fig. 8.) Fig. 9B (right side) shows the results obtained in the absence of extracellular Ca²⁺. Under these conditions, 11.1 mmol/l glucose failed to stimulate insulin secretion, as did the combination of forskolin, PMA, and 2 mmol/l glucose. However, in the presence of forskolin and PMA, 11.1 mmol/l glucose caused a marked stimulation of insulin release. In normal β-cells, this response to glucose is not associated with any rise

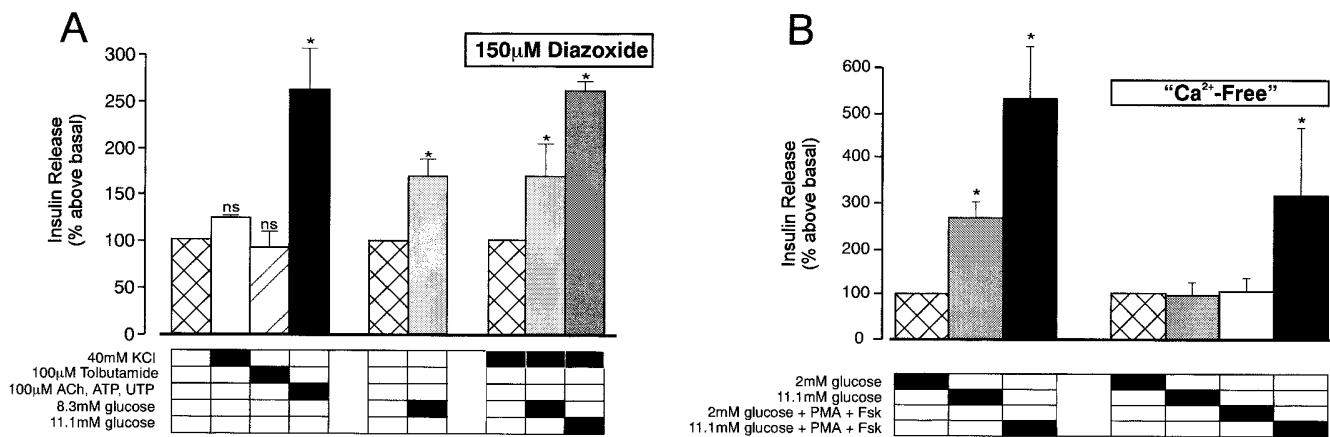


FIG. 9. Glucose augmentation pathways in HI-SUR1 islets. **A:** Effects of KCl and tolbutamide; the combination of acetylcholine, ATP, and UTP; and stimulatory glucose concentrations on insulin secretion in HI-SUR1 isolated pancreatic islets. The studies were performed in the absence and presence of 150 μmol/l diazoxide. Basal release rates in the presence of 2 mmol/l glucose were 0.23 ng/5 islets/30 min ($P < 0.05$, $n = 3$). **B:** Effects of 11.1 mmol/l glucose on insulin secretion in HI-SUR1 isolated pancreatic islets in the presence and absence of extracellular Ca²⁺ and in the presence and absence of 6 μmol/l forskolin and 100 nmol/l PMA. Basal release rates in the presence of 2 mmol/l glucose were 0.24 ng per 5 islets per 30 min ($P < 0.05$, $n = 3$).

TABLE 1
[Ca²⁺]_i signals in NES2Y-Kir6.2CΔ26 β-cells

Experimental conditions	Δ [Ca ²⁺] _i (nmol/l)
[Ca ²⁺] ₀ = 0 mmol/l; 20 mmol/l glucose	-22 ± 4 (n = 5/5)
[Ca ²⁺] ₀ = 0 mmol/l; 2 μmol/l Fsk + 10 nmol/l PMA + 20 mmol/l glucose	0 (n = 5/5)
[Ca ²⁺] ₀ = 1.26 mmol/l; 0.1 mmol/l ATP/acetylcholine/UTP	327 ± 49 (n = 13/13)

In these experiments, changes in [Ca²⁺]_i have been monitored under a number of different experimental conditions. Note that in the absence of extracellular Ca²⁺, there were no increases in [Ca²⁺]_i with glucose or glucose + forskolin (Fsk) + PMA. However, a marked increase in [Ca²⁺]_i was elicited when NES2Y-Kir6.2CΔ26 β-cells were challenged with 0.1 mmol/l ATP/acetylcholine/UTP.

in the intracellular concentration of Ca²⁺ (21), and in this study we found similar responses using the HI-derived β-cell line NES2Y (28,35) transfected with Kir6.2CΔ26, which produces Kir6.2 channel activity independently of SUR1 function (36) (Table 1).

DISCUSSION

HI is a heterogeneous entity. In addition to clinical and genetic diversity, recent pathological findings have also revealed two histopathologically distinct forms of HI. Most cases of HI arise from the diffuse involvement of defective β-cells throughout the pancreas (Di-HI); however, up to 30% of patients are now thought to have focal adenomatous hyperplasia, or focal disease (Fo-HI) (2,37,38). Fo-HI is caused by loss of maternally expressed genes as the result of somatic events. This leads to 1) insulin hypersecretion through inheritance of paternally derived *SUR1* gene defects and 2) β-cell proliferation as a result of the loss of the growth-suppressing genes *p57^{KIP2}* and *H19* and expression of the growth-stimulating gene product IGF-2 (38).

In this study, all three patients were found to have Di-HI, and we have provided a detailed characterization of the manner in which unregulated insulin release is coupled to a genetic lesion in *SUR1*. This mutation was identified in two affected families and was present in both parental and sibling DNA. All affected offspring were profoundly hypoglycemic after birth and had typical symptoms of severe Di-HI, including impaired drug responses to somatostatin, diazoxide, and nifedipine therapy (Fig. 1). As is typical in these cases, all patients underwent a 95% pancreatectomy in the first instance, which alleviates the symptoms of hyperinsulinism but will predispose the affected individuals to long-term sequelae. These may include pancreatic exocrine insufficiency and insulin-dependent diabetes (1).

In response to glucose stimulation, the regulated release of insulin is controlled by depolarization-response coupling and glucose augmentation pathways (12–23). In the former pathway, K_{ATP} channels play a key role in governing changes in the cell membrane potential and the regulated entry of Ca²⁺, whereas in the latter pathways, glucose metabolism governs insulin secretion independently of K_{ATP} channel operation and will augment responses to the raised [Ca²⁺]_i (16–18,21–23). In normal rodent and human islets, this has been demonstrated by experimentally eliminating the operation of K_{ATP} channels using high concentrations of either tolbutamide (which clamp K_{ATP} channels closed) or diazoxide (which hyperpolarize the cell membrane by activating K_{ATP} channels) and then depolarizing the cell with high external KCl to provide voltage-dependent Ca²⁺ influx (16–18,22–23).

Under these conditions, glucose has been shown to enhance insulin secretion in a dose-dependent manner by mechanisms that are both dependent and independent of Ca²⁺ influx (21). More recently, the K⁺ channel-independent pathways of glucose augmentation have also been described in transgenic mice without K_{ATP} channels (39–41).

Because HI is a K_{ATP} channelopathy with loss of function mutations in *SUR1* and *Kir6.2* (9,11), we have used β-cells isolated from patients as an experimental system to further characterize glucose augmentation pathways in human insulin-secreting cells. The operation of K_{ATP} channel-independent pathways in HI β-cells is also clinically important, since high rates of glucose infusion are administered in vivo to maintain normoglycemia in patients (Fig. 1) (1). Here, we have documented that loss of K_{ATP} channel operation in HI-SUR1 β-cells (Figs. 2 and 3) is causally related to uncontrolled Ca²⁺ channel activity (Figs. 5 and 6), which is directly coupled to Ca²⁺-dependent insulin secretion (Figs. 7–9). Because the resting cell membrane potential in HI-SUR1 β-cells is severely compromised (9), depolarization-dependent agonists such as glucose, high external KCl, and tolbutamide failed to cause a marked increase in [Ca²⁺]_i (Fig. 6). Loss of depolarization-dependent Ca²⁺ signaling readily explains why neither KCl nor tolbutamide promoted insulin release (Fig. 9A). In addition, it has been shown that tolbutamide fails to stimulate insulin secretion in vivo in Di-HI patients (see accompanying article by Grimberg et al. [25]). In contrast, whereas glucose similarly failed to raise cytosolic Ca²⁺ levels in HI-SUR1 islets, insulin secretion was stimulated, and this was found to be both concentration dependent and governed by Ca²⁺ influx (Fig. 9). Because K_{ATP} channels are nonfunctional in HI-SUR1 β-cells, these data document for the first time that glucose-induced insulin release is regulated through the K_{ATP} channel-independent pathway in HI β-cells. Interestingly, in these cells, glucose also augmented PKC- and PKA-dependent insulin release in the absence of extracellular Ca²⁺ (Fig. 9B). Therefore, the HI-SUR1 islets exhibit both the Ca²⁺-dependent and Ca²⁺-independent glucose augmentation pathways, as do normal human β-cells (22,23).

In this article, we have documented for the first time the mechanisms that govern insulin release from HI β-cells. These data show that loss of K_{ATP} channels in β-cells leads to uncontrolled Ca²⁺ channel activity. As in normal β-cells (42), this will tend to increase in [Ca²⁺]_i within the vicinity of the plasma membrane and the microdomains of the insulin-containing granules. Measurements of capacitance have documented that exocytosis is dependent on a rise in [Ca²⁺]_i and that voltage-gated Ca²⁺ influx is associated with insulin release. These data are relevant to our understanding of the control of insulin release under normal and pathological con-

ditions, but they are also relevant to the clinical management of HI. Current medical therapy for HI is unsatisfactory because it involves regimens with agents that fail to adequately control hyperinsulinism due to the loss of K_{ATP} channels (Fig. 1). It is also evident from this study that use of high rates of glucose infusion to maintain normoglycemia might stimulate insulin secretion through the K_{ATP} channel-independent pathway. Unfortunately, for many patients with HI the only effective manner in which to control hypoglycemia is a near-total resection of the pancreas. Recently, it was demonstrated that pancreatic venous sampling of insulin could be used to locate focal regions of β -cell mass in HI-SUR1 patients and that selective surgical resection of these areas alone results in the successful management of hyperinsulinism (38). Because as many as 30% of HI patients coming to pancreatectomy have focal disease, early diagnosis of these individuals, combined with pancreatic venous sampling, would obviate the necessity for a 95% pancreatectomy in favor of a 20–30% surgical resection (38). The major clinical benefit of this is that it would alleviate long-term complications of HI for a significant number of patients. Currently, preoperative diagnosis of Fo-HI is not possible on the basis of genotyping alone; some 60% of all patients presently have no identified gene defects in *SUR1* or *Kir6.2* (2), and even when defects have been found, it is clear that a number of different mutations can give rise to similar clinical phenotypes (1,2). Thus, based on an advanced understanding of the molecular mechanisms of insulin release from normal β -cells and the data presented in this report, it has been suggested that responses to intravenous tolbutamide in the form of a tolbutamide-stimulation test (TST) could be used to distinguish Fo-HI from Di-HI forms (2). In support of this, Grimberg et al. (25) have shown that by measuring acute insulin responses to glucose and tolbutamide, patients with Di-HI can be distinguished from control subjects by the fact that they do not respond to TST, whereas glucose causes a blunted insulin release profile. These findings are largely explained by characterization of the K_{ATP} channel-independent pathway of regulated insulin release in Di-HI β -cells. Loss of K_{ATP} channels prevented tolbutamide-induced rises in $[Ca^{2+}]_i$ and insulin release (Figs. 6 and 9A), whereas glucose promoted insulin secretion in a Ca^{2+} -dependent manner (Fig. 9). Since Fo-HI patients show positive responses to TST (S. Thornton, unpublished observations) because of the presence of K_{ATP} channels in β -cells from the nonfocal regions (M.J.D., J.M. Saudubray, C. Junien, unpublished data), these data provide strong support for the use of intravenous tolbutamide as a diagnostic procedure for distinguishing the different entities of HI.

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