ORIGINAL ARTICLES

Ionic control of β cell function in nesidioblastosis. A possible therapeutic role for calcium channel blockade

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Abstract

A preterm female infant presented with intractable hypoglycaemia within 10 minutes of delivery. Normoglycaemia could be maintained only by the intravenous infusion of glucose at a rate of 20–22 mg/kg/min. Persistent hyperinsulinaemic hypoglycaemia of infancy was diagnosed from an inappropriately raised plasma insulin concentration (33 mU/l) at the time of hypoglycaemia (blood glucose <0.5 mmol/l). Medical treatment with glucagon, somatostatin, and diazoxide led to only a modest reduction in the intravenous glucose requirement; a 95% pancreatectomy was performed and histological 'nesidioblastosis' confirmed. In vitro electrophysiological studies using patch clamp techniques on isolated pancreatic β cells characterised the ionic basis for insulin secretion in nesidioblastosis. The β cells were depolarised in low ambient glucose concentrations with persistently firing action potentials; these were blocked reversibly by the calcium channel blocking agent verapamil. Persistent postoperative hyperinsulinaemic hypoglycaemia was treated with oral nifedipine. This increased median blood glucose concentrations from 3.5 to 4.8 mmol/l and increased in duration the child's tolerance to fasting from 3 to 10.5 hours. These data allude to an abnormality in the ionic control of insulin release in nesidioblastosis and offer a new logical approach to treatment which requires further evaluation.

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Keywords: nesidioblastosis, β cell, calcium channel, hyperinsulinaemic hypoglycaemia.

Hypoglycaemia is the most common metabolic abnormality in childhood and, when severe or recurrent, can cause devastating neurological sequelae.¹ When due to hyperinsulinism, the consequences of hypoglycaemia are particularly severe, since not only is the brain deprived of glucose, but the excessive secretion of insulin switches off lipolysis and ketogenesis,

thereby depriving the brain of a supply of alternative fuels.² Persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI), which is commonly associated with so called nesidioblastosis,³ may be extremely difficult to control medically, and various combinations of the therapeutic agents diazoxide, glucagon, and somatostatin have been used in order to restore normoglycaemia.^{4,5} The degree of success achieved by these agents is variable, and many children require surgery in the form of a subtotal or total pancreatectomy.⁶

Until recently, the pathophysiology of the condition has remained uncertain and controversial. The histological features of nesidioblastosis have been found in pancreases from infants dying from conditions not associated with hypoglycaemia, and severe hyperinsulinism may occur in infants in whom no structural abnormality of the endocrine pancreas can be found.⁷ In vitro studies of isolated islets from pancreases with nesidioblastosis have shown the presence of a regulatory defect in the coupling of extracellular glucose to the secretion of insulin.^{8,9} The β cell ATP dependent potassium channel, K^+_{ATP} , plays a key role in the subcellular mechanisms which regulate insulin secretion.^{10–13} In resting β cells open channel events are involved in controlling the cell membrane potential. K^+_{ATP} channel inhibition following glucose metabolism leads to depolarisation of the β cell. This leads to activation of voltage dependent L-type calcium channels and the net influx of calcium through these channels leads to an increase in cytosolic calcium concentration.^{14,15} A rise in intracellular calcium is a prerequisite for exocytosis and the secretion of insulin.

Recent advances in the molecular genetics of PHHI have provided new insight into the pathophysiology of this disease.^{16–19} These developments have highlighted the fundamental importance of the ionic control of insulin secretion.

We report for the first time a study of electrical events in isolated intact β cells from a premature infant with PHHI. These in vitro studies were followed by a successful clinical trial of the use of the calcium channel blocking agent nifedipine.

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Methods

ISLET ISOLATION

Islets were isolated from a portion of the resected pancreas using a modification of established procedures for the isolation of adult human islets from heart-beating cadaver organs.²⁰ In brief, a portion of the excised pancreas was cut into small pieces and incubated at 38°C in Hanks solution containing 3 mg/ml *Clostridium histolyticum* collagenase (Boehringer Mannheim type P). Digestion of the pancreas was assessed by the appearance of freely liberated diphenylthiocarbazon stained islets; digestion was stopped by using an excess of fetal calf serum. Isolated cleaved islets were purified using a small scale Ficoll™ density gradient. Despite these procedures the yield of isolated islets was low (the 2 g of pancreatic tissue available to us produced only 200–300 islets). Once isolated, the islets were maintained for a short period of time (2–7 days) under standard tissue culture conditions at 37°C.²⁰

MICROFLUORIMETRY

This was performed on isolated intact islets loaded with the Ca²⁺ fluorescent probe fura-2 using dual excitation single emission spectroscopy as described previously.²⁰ An increase in the ratio of fluorescence at 350/380 nm corresponds to an increase in the intracellular calcium concentration ([Ca²⁺]_i). Estimates of [Ca²⁺]_i were made using an in vitro calibration procedure.

ELECTROPHYSIOLOGY

Electrophysiology data were obtained from intact primary cultured β cells using patch clamp techniques as described previously.²¹ The composition of the solutions used to fill the patch clamp pipette and the bath contained (mmol/l): NaCl 140, KCl 4.7, MgCl₂ 1.13, glucose 2.5, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES) 10, and CaCl₂ 2.5 (pH 7.4).

For display purposes the current trace has been low pass filtered at a cut off frequency of 300 Hz. Upward deflections from the baseline represent outward current events.

METABOLIC FASTING PROTOCOL

The infant underwent formal metabolic fasts before and after the start of nifedipine, according to a standardised protocol. In brief, each fast began with a feed of 25 ml/kg of the same formula milk feed (a term infant formula fortified with additional glucose polymer to a total concentration of 12% carbohydrate). Thereafter blood glucose was estimated on capillary blood samples every 30 minutes using a Glucometer 4 meter and Glucotide test strips (Bayer). When the capillary sample fell to 2.6 mmol/l or lower a whole blood glucose was measured in the laboratory within 5 minutes. The fast was terminated if blood glucose in the laboratory was below 2.6 mmol/l, and the infant fed. On termination of the fast additional blood was drawn for simultaneous

measurement of glucose, insulin, and other metabolic intermediates.

STATISTICS

Median blood glucose concentrations were compared using a Kruskal-Wallis one way analysis of variance (ANOVA) on ranks, and individual comparisons made using a Mann Whitney rank sum test. Parametric data were compared using Student's unpaired *t* test.

Case report

The patient is the first child of non-consanguineous Caucasian parents and was born at 31⁺⁵ weeks gestation following an emergency lower segment caesarean section because of moderately severe pre-eclampsia. No cardiorespiratory resuscitation was required, but a blood glucose concentration of <0.5 mmol/l was documented at 10 minutes of age. The child was given an intravenous bolus of glucose, but recurrent hypoglycaemia could only be prevented by the administration of a continuous intravenous infusion of glucose at a rate equivalent to not less than 20–22 mg glucose/kg/min. With the start of a concurrent intravenous infusion of glucagon (2 mg/d), the glucose requirement was transiently reduced to 18 mg/kg/min.

She was noted to have dysmorphic features (high arched palate, hypertelorism, brachycephaly) with bilateral corneal clouding. Ophthalmological assessment revealed bilateral glaucoma and anterior segment dysgenesis consistent with the Axenfeld's anomaly (posterior embryotoxon, iris filaments, and bands with or without glaucoma).²² Systemic examination of the child revealed the presence of a patent ductus arteriosus.

Her mother also suffered from Axenfeld's syndrome and had an atrial septal defect.

At the age of 4 days, the patient was transferred to Great Ormond Street Hospital for further evaluation. A diagnosis of hyperinsulinaemic hypoglycaemia was made by demonstrating inappropriately raised levels of plasma insulin (16–33 mU/l) with suppression of lipolysis and ketogenesis (plasma free fatty acid and blood total ketone body levels of 0.11 mmol/l and 0.06 mmol/l respectively) at the time of spontaneous hypoglycaemia (blood glucose level <0.5–1.0 mmol/l). In an attempt to achieve normoglycaemia, a somatostatin infusion (0.25 µg/kg/h) and oral diazoxide (10 mg/kg/d) with concurrent hydrochlorothiazide were begun. However, stabilisation of her blood glucose control proved to be exceedingly difficult and could only be achieved with a combination of diazoxide (20 mg/kg/d), hydrochlorothiazide, somatostatin (0.3 µg/kg/h) and glucagon (5 µg/kg/h). Hydrocortisone 50 mg/m²/d was also given to cover any effect of somatostatin on ACTH secretion. This combination eventually led to a reduction by day 36 in glucose requirements to 11–17 mg/kg/min, given as a combination of continuous enteral feeding through a nasogastric tube and intravenous glucose. Her progress was complicated

Figure 1 Blood glucose concentrations (measured on capillary blood samples using a Glucometer 4 blood glucose meter) before and after treatment with nifedipine. bd=two divided doses; tds=three divided doses; qds=four divided doses; SR=slow release preparation of nifedipine.

by a haemodynamically significant patent ductus arteriosus which was surgically ligated at 21 days of age.

Ongoing difficulties with unpredictable episodes of hypoglycaemia despite this maximal medical therapy led to a 95% pancreatectomy, performed at the age of 50 days. The histopathological appearance of the pancreas was consistent with that of 'nesidioblastosis', with hyperplasia of the β cells and ducto-insular endocrine cell proliferation.²³

Blood glucose concentrations appeared to be stable postoperatively on a three hourly feeding regimen without any medical adjuncts, and with an assessed glucose intake of 8 mg/kg/min. She was therefore transferred back to her local hospital to monitor the natural history of the condition. During the next eight weeks she developed recurrent episodes of hypoglycaemia associated with an increase in her glucose requirement to 13 mg/kg/min and a reduced tolerance to fasting.

She was readmitted to Great Ormond Street Hospital at the age of 5 months for a further assessment. A tolerance of three hours of fasting before the onset of hypoglycaemia was confirmed. Our in vitro studies (see below) performed on islets isolated from the infants

pancreatectomy specimen had shown that β cell calcium dependent action potentials could be blocked with verapamil. We therefore undertook a therapeutic trial of the calcium channel blocking agent nifedipine rather than proceeding directly to a total pancreatectomy.

After obtaining parental consent and after a 48 hour period in which blood sugars were measured preprandially, the short acting form of nifedipine was started at a dosage of 0.25 mg/kg/d, given at eight hourly intervals. No discernible side effects on blood pressure or on cardiac conduction were noted; no increase in blood glucose concentrations was seen (fig 1, table). On increasing the dose to 0.5 mg/kg/d there was a highly significant increase in blood glucose concentrations (median 3.5 mmol/l pre-nifedipine, 4.8 mmol/l on nifedipine) (table). Fasting tolerance increased from 3.3 hours to 4.5 hours on this regimen. Problems were encountered with precipitous hypoglycaemia as the medication wore off and these problems were not overcome by giving the nifedipine in four divided doses. An increase in nifedipine dose to 0.7 mg/kg/d and conversion to a slow release formulation dramatically increased her fast tolerance to over 10.5 hours (fig 2), with an increase in the median pre-

Median preprandial blood glucose concentrations over 48 hour periods before and after treatment with nifedipine. Periods during which the infant underwent diagnostic fasting are excluded

	Pre-nifedipine	0.25 mg/kg/24 h	0.5 mg/kg/24 h	0.7 mg/kg/24 h (initial 48 h period)	0.7 mg/kg/24 h (subsequent period)	0.7 mg/kg/24 h (1 month later)
Median preprandial glucose (mmol/l)	3.5	3.8	4.8	4.5	4.2	4.5
Interquartile range	3.0 to 4.2	3.1 to 6.3	3.8 to 6.6	3.8 to 5.4	3.8 to 5.2	4.0 to 5.2
n	14	13	14	14	14	24
Feed interval (h)	3	3	3	4	3	4
	a	b	c	d	e	f

Kruskal-Wallis one way ANOVA on ranks: statistically significant difference in groups ($p<0.02$). Mann Whitney rank sum test: a v b, $p=0.33$; a v c, $p<0.005$; a v d, $p<0.005$; a v e, $p<0.02$; a v f, $p<0.008$.

Figure 2 Blood glucose concentrations before and during fasting before and after the start of nifedipine treatment. Fasts were started at time=0 on the graph. The dotted line represents a glucose concentration of 2.6 mmol/l which was the concentration below which fasts were terminated. ○ pre-nifedipine; ■ nifedipine 0.5 mg/kg/d in three divided doses; ▲ nifedipine SR (slow release) 0.7 mg/kg/d in two divided doses; ▼ nifedipine SR 0.7 mg/kg/d in two divided doses after one month on treatment.

prandial glucose concentration to 4.5 mmol/l during the first 48 hours despite a lengthening of feed interval from three to four hours. A fall in median blood glucose concentration to 4.2 mmol/l during a subsequent period of treatment (following the metabolic fast period) was associated with problems of vomiting due to gastro-oesophageal reflux (table).

The patient was discharged home on oral nifedipine and four hourly nasogastric feeds, with home monitoring of blood glucose. She was subsequently readmitted to Great Ormond Street Hospital for insertion of a

ventriculo-atrial shunt at the age of 6 months. Her blood glucose profiles during this admission were normal on four hourly feeds (median preprandial blood glucose 4.5 mmol/l) and her fast tolerance was seven hours. She maintained normoglycaemia following the introduction of overnight fasting of five to six hours.

Results of in vitro studies on isolated intact β cells

In vitro studies upon isolated nesidioblastotic β cells were carried out using our previously validated patch clamp and microfluorimetry techniques.^{20 21} Using the cell attached patch configuration the basic electrophysiological 'profile' of the nesidioblastotic β cells was markedly different from that seen in both normal human adult and rodent (fetal, neonatal, and adult) β cells.^{11 12 24 25} The nesidioblastotic β cells displayed a number of novel electrophysiological properties associated with K^+ ATP channel dysfunction.²⁶ Of particular interest here is the observation that the patient's β cells were constantly depolarised and persistently firing Ca^{2+} action potentials (fig 3). These action potentials arise from calcium entry through voltage activated L-type calcium channels. One consequence of this persistent electrical activity is that intracellular calcium concentrations are increased approximately twofold when compared with normoglycaemic human β cells: 149 nmol/l (95% confidence interval 127 to 171), $n=16$, v 79 nmol/l (68 to 90), $n=141$; $p<0.0001$, Student's unpaired t test. The action potentials were rapidly and reversibly terminated by exposure to the voltage gated calcium channel blocker verapamil (10 μ mol/l) (fig 3).

Figure 3 Cell attached patch recording of an intact isolated PHHI β cell (configuration shown on insert cartoon). The recording shows spontaneous electrical activity from the cell associated with the appearance of Ca^{2+} dependent action potential like spikes. The voltage gated Ca^{2+} channel blocker verapamil (10 μ mol/l) inhibited the generation of action currents in a reversible manner, decreasing their frequency from approximately 0.25 to 0.02 per second. The reduction in frequency of unitary current events results in a decrease in total current amplitude during exposure to verapamil as seen in the figure.

Discussion

Persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI) is one of the most difficult of metabolic problems to manage in paediatric medicine. A variety of descriptive clinical terms has been applied to the condition; that most frequently used is 'pancreatic nesidioblastosis'. Milner²⁷ has provided a comprehensive review of the history of the condition and the controversies which surround the debate on its pathophysiology.

Although the diagnosis of PHHI is straightforward (by demonstrating, in a single blood sample drawn at the time of hypoglycaemia, the characteristic inappropriate increase in plasma insulin for the degree of glycaemia together with low concentrations of fatty acids and ketone bodies),¹ in many children the currently available medical treatments may be ineffective, and subtotal or total pancreatectomy may be the only means of controlling the hypoglycaemia. Throughout the period of investigation and initial management, there is the ever present risk of unpredictable and severe episodes of hypoglycaemia, with resultant neurological damage.

Effective medical treatment thus demands a comprehensive understanding of the cellular pathophysiology of PHHI, which has been lacking until recently.

In normal pancreatic β cells the electrophysiological events which regulate insulin secretion are coordinated by ATP sensitive potassium (K^+_{ATP}) channels in the β cell membrane.¹¹ Open K^+_{ATP} channel events in resting β cells help to maintain the negative intracellular resting membrane potential generated by Na/K-ATPase by allowing potassium efflux. Changes in intracellular ATP concentration after exposure to glucose leads to the closure of the potassium channels, causing membrane depolarisation and the opening of voltage gated calcium channels. This influx of calcium manifests as action potentials which increase the intracellular calcium concentration, thereby providing the signal for initiation of exocytosis and insulin secretion.¹⁴ Current established pharmacological treatments are able to influence this secretory process where it is not disturbed by the disease process itself. In normal β cells both diazoxide and somatostatin cause potassium channel activation, leading to a hyperpolarisation of the membrane potential and termination of the action potentials.^{11 12} Glucagon produces its hyperglycaemic effect by inducing glycogenolysis within the liver. In the isolated β cell, however, glucagon increases intracellular cyclic AMP and may increase insulin secretion. The effects of combinations of these three agents are probably more complex. Pharmacological blockade of β cell calcium channels which we describe here targets another key process in insulin secretion and provides a welcome addition to our currently limited therapeutic armamentarium in nesidioblastosis.

The results of these *in vitro* studies upon β cells isolated from this infant with nesidioblastosis show that an inappropriate depolarisation of the β cell membrane in the face of a low

ambient glucose concentration leads to the persistent generation of action potentials associated with voltage gated calcium influx. The resultant increase in intracellular free calcium concentration accounts for the intractable insulin secretion from the PHHI β cells. We have shown here *in vitro* quite clearly that the spontaneous electrical activity associated with insulin secretion in PHHI β cells can be reversibly blocked by the L-type calcium channel blocker verapamil. The *in vivo* correlate of this is an increase in fast tolerance and a modest, though consistent, rise in preprandial blood glucose concentration.

These data, showing the ability to manipulate the process of insulin release pharmacologically by altering ionic activity, represent an important and logical step forward in the application of basic science to the medical management of severe hyperinsulinism in infancy.

The pathological uncoupling of normal electrophysiological activity in PHHI is likely to be a consequence of an abnormal relation between the potassium channel pore protein (KIR 6.2) and the sulphonylurea receptor (SUR), which together constitute the K^+_{ATP} channel.¹³ Sulphonylureas are drugs which stimulate the release of insulin by binding the SUR and inducing closure of K^+_{ATP} channels. The SUR is a member of the ATP binding cassette or traffic ATPase superfamily, with multiple membrane spanning domains and two nucleotide binding folds.¹⁸ Point mutations in the SUR gene resulting in truncation of nucleotide binding fold 2 (NBF-2) have been described in familial PHHI.¹⁹ If, by analogy with cystic fibrosis,^{28 29} the SUR is involved in the trafficking of K^+_{ATP} channels or the regulation of K^+_{ATP} channel function, then it is reasonable to speculate that a mutation in the gene encoding the protein could lead to altered K^+_{ATP} channel activity and persistent β cell depolarisation which will eventually result in insulin secretion.

Many questions still remain to be answered. The use of modern molecular biological and electrophysiological technologies has allowed considerable progress in our understanding of the pathophysiology of PHHI, and collaborative studies are under way in our laboratories to match clinical phenotype with electrophysiological phenotype and genotype. The possibility of linkage of the PHHI locus in this patient to that of Axenfeld's anomaly poses a further tantalising question.

We suggest that it is now extremely important to mount prospective collaborative studies on all resected pancreases to explore these concepts further and to undertake linkage analysis on affected families to ensure that non-SUR mutations are not missed. Knowledge of the molecular physiology of the β cells in individual cases and the mechanisms and magnitude of their responses to pharmacological agents *in vitro* will allow specific treatments to be 'designed' for those children who prove refractive to diazoxide, somatostatin, or partial pancreatectomy, and will also aid clinical decision making with regard to the need or otherwise for a further pancreatic resection.

Questions may surround the long term use of calcium channel blocking agents because of effects on other organs whose function depends critically on the state of membrane depolarisation and calcium influx. A decision was taken in this patient to use nifedipine rather than verapamil because it has less effect on myocardial function, and no specific side effects were noted in this case. More work is needed to define the place of calcium channel blockade in the routine management of PHHI.

CONCLUSIONS

Electrophysiological studies in isolated β cells from a preterm infant with severe hyperinsulinism have focused on the fundamental importance of ionic events as the final common pathway for the initiation of insulin secretion. The demonstration in vitro of the ability to modulate calcium dependent action potentials by a calcium channel blocking agent led to the successful use for the first time of a novel treatment, nifedipine. Our approach offers new and exciting opportunities to understand not only the pathophysiology of PHHI, but to consider the targeting of specific treatments to individual infants.

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See annotation on p 369.

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