$^{86}\text{Rb}^+$ Fluxes and K^+ -Stimulated Nitrophenyl Phosphatase Activity in the Pancreatic Islets of Genetically Diabetic Mice (C57BL/KsJ-*db/db*)

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Summary. Fluxes of $^{86}\text{Rb}^+$ and hydrolysis of *p*-nitrophenyl phosphate were measured in collagenase-isolated islets of diabetic C57BL/KsJ-*db/db*-mice and normal controls (C57BL/KsJ-+/+). Both types of islets accumulated Rb^+ avidly, as originally reported for hand-dissected islets of non-inbred *ob/ob*-mice. KsJ-*db/db*-mouse islets showed enhanced accumulation of Rb^+ and normal activity of K^+ -activated nitrophenyl phosphatase. D-glucose, 20 mmol/l, inhibited Rb^+ efflux in normal islets but not in those from KsJ-*db/db*-mice. The glucose insensitivity of Rb^+ efflux was observed in young animals, which exhibit glucose-induced insulin release, as well as in old animals, which do not secrete insulin in response to glucose. The anomalous regulation of Rb^+ efflux already present in young animals may bear on the liability of KsJ-*db/db*-mouse B-cells to develop defective control of membrane potential, an abnormal metabolism of cyclic AMP, and a marked failure of insulin secretory capacity.

Key words: B-cell, *db*-gene, diabetic mice, glucose, *p*-nitrophenyl phosphatase, pancreatic islets, rubidium accumulation, rubidium efflux.

Mice homozygous for the mutated gene, *db*, on the inbred background, C57BL/KsJ, develop a severe type of diabetes mellitus resembling the disease in humans. The fully developed syndrome is associated with morphological signs of islet deterioration, and the B-cells exhibit deficient insulin secretory responses to glucose [1–5]. Recordings with impaled micro-electrodes revealed that B-cells of KsJ-*db/db*-mice have an abnormal regulation of their membrane electric potential with persistent depolarization and

irregular spike activity [6]. The defective control of membrane potential suggests that disturbances in the transmembrane flow of ions represent a central factor in the development of the insulin secretory deficiency and diabetes.

Accumulation of $^{86}\text{Rb}^+$ is a useful measure of univalent cation pumping in well-functioning B-cells, and is markedly inhibited by the diabetogenic compound, alloxan [7, 8]. The univalent cation pump may be closely associated with a K^+ -activated *p*-nitrophenyl phosphatase that resides in B-cell plasma membranes [9]. Studies on $^{86}\text{Rb}^+$ efflux from preloaded islets led to the proposal that glucose-induced depolarization of the B-cells is at least in part due to a decrease of K^+ permeability [10]. Against this background we have measured the accumulation and efflux of $^{86}\text{Rb}^+$, as well as *p*-nitrophenyl phosphatase activities, in islets from C57BL/KsJ-*db/db*-mice and from normal controls with the same genetic background (C57BL/KsJ-+/+).

Materials and Methods

Animals and Isolation of Islets

Diabetic C57BL/KsJ-*db/db*-mice and non-diabetic C57BL/KsJ-+/+ -mice were from a local colony originally established with breeding couples from the Jackson Laboratories, Bar Harbor, Maine, USA. All diabetic mice were weighed at 2-week intervals beginning at the age of 5 weeks. Young animals (3 males and 3 females) were taken for study at the age of 7 weeks $\pm$ 2 days (range), when the KsJ-*db/db*-mice were in a phase of rapid weight gain [5]. Older animals (5 males and 26 females) were selected for study after the age of 27 weeks, when the diabetic mice had shown a significant fall of body weight for the preceding 4 weeks; the mean $\pm$ SD for the age of old KsJ-*db/db*-mice was 67 $\pm$ 17 weeks. Non-diabetic KsJ-+/+ -mice aged 7 weeks $\pm$ 2 days or 27 $\pm$ 1 week (range) were used as controls. Test and control mice were sex-matched, and sex had no noticeable effect on the variables investigated. All animals were given free access to water and pel-

Table 1. Uptake of $^{86}\text{Rb}^+$ by islets of KsJ-+/+ and KsJ-db/db mice. Mean values $\pm$ SEM are given for the numbers of experiments stated in parentheses

Age group	Incubation time (min)	Rb ⁺ uptake (mmol/kg dry wt of islets)	
		KsJ-+/+	KsJ-db/db
Young	120	1.33 $\pm$ 0.16 (6)	2.09 $\pm$ 0.17 ^c (6)
Old	120	0.73 $\pm$ 0.10 (22)	1.06 $\pm$ 0.10 ^b (23)
Old	8	0.32 $\pm$ 0.06 (6)	0.48 $\pm$ 0.05 ^a (6)

^a $p < 0.05$, ^b $p < 0.02$, ^c $p < 0.001$ for difference from KsJ-+/+ mice (t -test, paired when equal number of experiments in both groups)

Fig. 1. Time dependence of Rb⁺ efflux from preloaded islets of old KsJ-db/db (●) and KsJ-+/+ (○) mice. In each experiment, the amount of Rb⁺ retained by the islets (mmol/kg dry wt) was transformed to natural logarithms. Mean values $\pm$ SEM of these logarithms are read against the ordinate, while the untransformed efflux time is read against the abscissa. The numbers of experiments at time points 0, 10 and 30 min were: 23, 16 and 9 for KsJ-db/db-mice, and 22, 17 and 8 for KsJ-+/+ mice. The regression lines were calculated by the method of least squares from all individual data points ($N = 48$ for KsJ-db/db and $N = 47$ for KsJ-+/+). The glucose concentration was 3 mmol/l

leted laboratory chow (Astra-Ewos, Södertälje, Sweden: Type R 3) until 18 h before killing. Islets were isolated by collagenase digestion of the pancreas.

$^{86}\text{Rb}^+$ Accumulation and Efflux

Incubations were performed at 37°C. The basal incubation medium had the same composition as Krebs-Ringer bicarbonate buffer [11], except that bicarbonate was replaced by 20 mmol/l 2-(*N*-hydroxyethylpiperazine-*N'*-yl)ethanesulphonic acid (Hepes), pH 7.4 and that the gas phase was air. Experiments began with a preliminary incubation for 30 min in basal medium supplemented with 3 mmol/l D-glucose. In studies of Rb⁺ accumulation, batches

of about 15 islets were then incubated for 8 or 120 min in 200 μ l of basal medium supplemented with 3 mmol/l D-glucose, 28 μ mol/l $^{86}\text{RbCl}$ (770 Ci/mol), and 0.1 mmol/l (6,6'- ^3H)sucrose (2000 Ci/mol). In studies of Rb⁺ efflux, islets were incubated for 120 min with D-glucose, $^{86}\text{RbCl}$, and (6,6'- ^3H) sucrose as above. They were then incubated for 10 or 30 min in 5 ml of non-radioactive basal medium containing 3 or 20 mmol/l D-glucose. In experiments with islets of 7-week-old mice, all media contained 0.5% bovine serum albumin to facilitate mechanical handling of the islets; albumin at this concentration has no effect on Rb⁺ flux [12]. After incubation, the islets were freeze-dried overnight (-40°C , 0.1 Pa), weighed on a quartz-fibre balance, and analyzed for ^{86}Rb and ^3H in a liquid-scintillation spectrometer. Samples of incubation media were used as external standards in the counting procedure. When calculating the islet cell content of Rb⁺, correction was made for $^{86}\text{Rb}^+$ in the (6,6'- ^3H)sucrose space.

Nitrophenyl Phosphatase Activities

The assay of K⁺-activated *p*-nitrophenyl phosphatase in islets has been described in detail [9]. In the present study, 20–60 islets were washed twice in 300 μ l of Tris-acetate buffer (0.1 mol/l Tris base and acetic acid to give pH 7.8). They were then homogenised by sonication in 200 μ l of the same buffer; during sonication the tube with islets was kept in iced water. The homogenate was centrifuged for 1 min at 50 $\times$ g and the supernatant taken for enzyme and protein measurements. Phosphatase activities were assayed by incubating 20 μ l of the enzyme extract with 80 μ l of Tris-acetate buffer (40 mmol/l Tris base/acetic acid, pH 7.8) supplemented with 5 mmol/l *p*-nitrophenyl phosphate (di-Tris salt) and 5 mmol/l MgCl_2 . KCl or ouabain, or both, was included in the medium as described in Results. After incubation for 30 min at 30°C, 200 μ l of 0.5 mol/l NaOH was added, and A_{410} was read in a spectrophotometer. Alkaline nitrophenol was used as standard, and medium blanks were obtained in each experiment by incubating buffer instead of islet extract. In a few experiments, 10 mmol/l boric acid/NaOH (pH 7.8) was used instead of the Tris-acetate buffer; the choice of buffer had no noticeable effect on enzyme activity, so results obtained with the two buffers have been pooled. Proteins were assayed with fluorescamine [9].

Chemicals

The sources of chemicals were as follows: Sigma Chemical Co., St. Louis, MO, USA for *p*-nitrophenyl phosphate (di-Tris salt), ouabain, bovine serum albumin (fraction 5); BDH Chemicals Ltd., Poole, Dorset, UK for D-glucose; Worthington Biochemical Corp., Freehold, NJ, USA for collagenase (CLS type IV); the Radiochemical Centre, Amersham, Bucks., UK for $^{86}\text{RbCl}$ and (6,6'- ^3H)sucrose. Other reagents were of analytical grade.

Statistics

Conventional statistical methods were used for testing the null hypotheses of zero difference between groups (t -test) or equal slope in linear regression lines (F -test).

Results

Islet Uptake of Rb⁺

Islets of KsJ-db/db-mice accumulated Rb⁺ more effectively than those of KsJ-+/+ mice (Table 1). This difference was statistically significant for both young and old animals. The greater uptake of Rb⁺ in KsJ-db/db-mouse islets was observed under condi-

tions approaching isotope equilibrium (120 min; [7]) as well as in brief incubations more likely to detect differences in the rate of influx (8 min). Assuming the same distribution of water in the present collagenase-isolated islets as in hand-dissected islets of non-inbred *ob/ob*-mice, the values at 120 min in Table 1 correspond to a 22–62-fold accumulation of Rb^+ from the medium [7].

Efflux of Rb^+ from Preloaded Islets

Efflux of Rb^+ from pancreatic islets approximates to first-order kinetics [7, 8]. Figure 1 shows a semi-logarithmic plot of the islet content of Rb^+ after 0, 10 or 30 min of efflux into 5 ml of originally Rb^+ -free medium. From the data in Table 1 and the weight of islets it can be calculated that the release of all Rb^+

from the islets would give a medium concentration of Rb^+ that is only 0.01–0.04% of that used for preloading; in this context 5 ml is therefore regarded as an infinitely large volume.

Although the islets of *KsJ-db/db*-mice accumulated more Rb^+ during preloading than the islets of *KsJ-+/+*-mice, the efflux curves for the two types of islets were nearly parallel. By the method of least squares, the rate constant was estimated to be -0.035 min^{-1} for efflux from *KsJ-+/+*-islets, and -0.027 min^{-1} for efflux from *KsJ-db/db*-islets. The corresponding half-lives were 19.8 min (*KsJ-+/+*) and 25.7 min (*KsJ-db/db*). The values for the normal mice were close to the rate constant of -0.036 to -0.043 min^{-1} , and half-life of 16–19 min, estimated for hand-dissected non-inbred *ob/ob*-mouse islets incubated with 3 mmol/l glucose and subsequently incubated for 10 min in Rb^+ -free medium containing glucose as indicated. Mean values $\pm$ SEM of 6 (young animals) or 8 (old animals) experiments are given. In addition to data recorded for each group of islets, mean values $\pm$ SEM of the differences between parallel incubations with 20 and 3 mmol/l glucose are shown

Table 2. Effect of glucose on Rb^+ efflux. Values denote amounts of Rb^+ retained by islets that had first been preloaded with Rb^+ in the presence of 3 mmol/l glucose and subsequently incubated for 10 min in Rb^+ -free medium containing glucose as indicated. Mean values $\pm$ SEM of 6 (young animals) or 8 (old animals) experiments are given. In addition to data recorded for each group of islets, mean values $\pm$ SEM of the differences between parallel incubations with 20 and 3 mmol/l glucose are shown

Age	Gene type	Rb^+ retained by islets (mmol/kg dry wt)		
group		3 mmol/l glucose (a)	20 mmol/l glucose (b)	Effect of glucose (b) – (a)
Young	<i>KsJ-+/+</i>	0.82 ± 0.08	1.09 ± 0.08	0.27 ± 0.07^b
Young	<i>KsJ-db/db</i>	1.34 ± 0.18	1.30 ± 0.13	-0.04 ± 0.15
Old	<i>KsJ-+/+</i>	0.58 ± 0.08	0.70 ± 0.09	0.12 ± 0.05^a
Old	<i>KsJ-db/db</i>	0.74 ± 0.09	0.71 ± 0.08	-0.03 ± 0.05

^a $p < 0.05$, ^b $p < 0.02$ (*t*-test)

The effect of glucose on the efflux of Rb^+ is shown in Table 2. For islets from both young and old *KsJ-+/+*-mice, the efflux of Rb^+ was significantly slower into a medium containing 20 mmol/l glucose than into one containing only 3 mmol/l glucose. This inhibitory effect of high glucose concentration was not observed with islets from *KsJ-db/db*-mice whether young or old.

Potassium-Stimulated Nitrophenyl Phosphatase Activity

The activity of enzymes catalysing hydrolysis of *p*-nitrophenyl phosphate was about the same in islets of *KsJ-db/db*-mice as in those of *KsJ-+/+*-mice (Table

Table 3. Effects of K^+ and ouabain on *p*-nitrophenyl phosphatase activity in the islets of old *KsJ-db/db* and *KsJ-+/+* mice. All media contained 5 mmol/l MgCl_2 ; K^+ and ouabain were included as indicated. Mean values $\pm$ SEM are given for the numbers of experiments stated in parentheses. In addition to data recorded for each type of medium, mean values $\pm$ SEM of differences between parallel incubations in various types of media are shown in the lower part of the table

Group	Addition to buffer, or comparison of groups	Enzyme activity (nmol nitrophenol released/h per μg protein)	
		<i>KsJ-+/+</i>	<i>KsJ-db/db</i>
1	None	0.85 ± 0.10 (14)	0.82 ± 0.14 (13)
2	3 mmol/l K^+	1.43 ± 0.18 (8)	1.59 ± 0.47 (7)
3	10 mmol/l K^+	1.29 ± 0.16 (14)	1.36 ± 0.25 (13)
4	3 mmol/l K^+ plus 1.5 mmol/l ouabain	1.22 ± 0.17 (8)	1.34 ± 0.44 (7)
2–1	Effect of 3 mmol/l K^+	0.42 ± 0.09^b (8)	0.64 ± 0.22^a (7)
3–1	Effect of 10 mmol/l K^+	0.44 ± 0.09^c (14)	0.54 ± 0.12^c (13)
4–2	Effect of 1.5 mmol/l ouabain	-0.21 ± 0.04^b (8)	-0.25 ± 0.06^b (7)

^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$ (*t*-test)

3). In both types of islets the *p*-nitrophenyl phosphate hydrolysis was significantly stimulated by 3 or 10 mmol/l K^+ , and the K^+ -stimulated activity was significantly inhibited by 1.5 mmol/l ouabain. No difference was observed between the two types of mice as regards the magnitude of activation by K^+ or inhibition by ouabain.

Discussion

Islet cell accumulation and release of $^{86}Rb^+$ were originally characterised with islets microdissected from non-inbred *ob/ob*-mice [7, 10]. Those islets accumulated Rb^+ about 40-fold, when incubated with 70 μ mol/l $^{86}RbCl$ [7]. As shown here, collagenase-isolated islets of normal mice (KsJ-+/+) similarly exhibit a vigorous uptake of Rb^+ and a glucose-induced inhibition of Rb^+ efflux. The inhibitory effect of glucose on Rb^+ efflux has been confirmed with rat islets as well [13].

B-cells of diabetic KsJ-*db/db*-mice have an abnormal regulation of their electric activity with persistent depolarization of the plasma membrane [6]. The genesis of the membrane potential in B-cells is incompletely known, but a fundamental role is generally attributed to univalent cation pumping [9, 14, 15]. Previous experiments indicate that accumulation of $^{86}Rb^+$ reflects operation of the B-cell's univalent cation pump [7]. Against this background it is noteworthy that the islets of KsJ-*db/db*-mice did not accumulate Rb^+ to a lesser extent than did the islets of KsJ-+/+ -mice. The islets of KsJ-*db/db*-mice also showed a normal activity of K^+ -stimulated and ouabain-inhibitable *p*-nitrophenyl phosphatase. This enzyme occurs, if not exclusively, in the plasma membranes of islet cells, accepts ATP as a substrate or regulator, and may be envisaged to catalyse a phosphoryl transfer reaction of significance for univalent cation pumping [9]. Therefore, the abnormal depolarization of KsJ-*db/db*-mouse B-cells is not readily explained in terms of a grossly deficient system for active transmembrane transport of univalent cations.

The univalent cation pump may be directly electrogenic, but electrodiffusional mechanisms are also likely to contribute to the membrane potential of B-cells [10, 14, 15]. Available information suggests that at least the permeabilities to K^+ (Rb^+) [10, 13, 16, 17], Na^+ [18], and Cl^- [19] are important. We obtained no significant indication of a reduced basal permeability to Rb^+ in the B-cells of KsJ-*db/db*-mice, although the estimated mean rate constant for Rb^+ efflux was smaller in diabetic than in normal mouse islets. Considering the technical difficulties in

demonstrating subtle permeability differences, this point deserves further investigation before reaching a final conclusion. If there is a real difference in basal Rb^+ permeability between the two types of mice, it would help to explain why the islets of KsJ-*db/db*-mice exhibited a significant enhancement of the Rb^+ accumulation.

More striking than the data on basal Rb^+ permeability is the clear difference between the islets of KsJ-*db/db*-mice and KsJ-+/+ -mice with regard to the effect of 20 mmol/l glucose on Rb^+ efflux. Because D-glucose, but not L-glucose or 3-*O*-methyl-D-glucose, inhibited the efflux of Rb^+ from non-inbred *ob/ob*-mouse islets, it was suggested that the depolarizing effect of D-glucose on B-cells is at least in part mediated by a decrease of K^+ permeability [10]. This hypothesis has since received support from studies of $^{42}K^+$ efflux in rat islets [16] and from direct measurements of membrane potential and conductance in B-cells exposed to various concentrations of K^+ and glucose [17]. Experiments with tetraethylammonium ions are also in basic agreement with the hypothesis, and, in addition, suggest that a mere decrease of K^+ permeability is not sufficient to elicit insulin release [13]. Our present results show that the islets of KsJ-*db/db*-mice behave anomalously in not responding to 20 mmol/l D-glucose with a decrease of Rb^+ efflux. Such a fundamental anomaly may well bear on the hereditary liability of KsJ-*db/db*-mice to develop B-cell failure and severe diabetes. It is important that the unresponsiveness of Rb^+ efflux to 20 mmol/l D-glucose was observed with both young and old KsJ-*db/db*-mice. Young (7 weeks) KsJ-*db/db*-mice are mildly diabetic with islets exhibiting clear insulin secretory responses to glucose; the old KsJ-*db/db*-mice were deliberately selected on the basis of weight loss to represent the stage of severe diabetes that in this strain is characterised by a marked insulin secretory failure [4, 5]. It is inferred that the inability of 20 mmol/l glucose to inhibit Rb^+ efflux in KsJ-*db/db*-mice is not a result of marked B-cell deterioration. We also conclude that glucose-induced insulin release can occur in spite of the Rb^+ efflux being uninhibited. However, the relative efficiency of glucose as an insulin secretagogue may still depend on the ability of glucose to reduce the K^+ permeability [13]. The anomalous ionic response to glucose reported here for the young KsJ-*db/db*-mice could therefore aid in explaining why mice with this genetic background are prone to develop drastic B-cell failure and severe diabetes. Mice homozygous for the diabetogenic genes, *db* or *ob*, on a non-inbred background or on the background, C57BL/6J, do not develop B-cell failure but respond to their hereditary hyperglycaemia with a

marked B-cell hyperplasia that persists throughout life [4, 20–22]. In contrast to the “brittle” islets of KsJ-*db/db*-mice, the better enduring islets of non-inbred *ob/ob*-mice [10] resemble normal mouse (present data) and rat [13] islets in showing a decreased Rb⁺ efflux in response to D-glucose.

Islets of KsJ-*db/db*-mice exhibit but small increases of cyclic AMP when challenged with a combination of phosphodiesterase inhibitor and 20 mmol/l glucose [23]. The uncovering of anomalies in the regulation of ionic flux and membrane potential adds some weight to the idea that the adenylate cyclase of B-cells may be responsive to the transmembrane ionic milieu or the voltage gradient as such [24].

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