2-Oxocarboxylic Acids and Function of Pancreatic Islets in Obese–Hyperglycaemic Mice

INSULIN SECRETION IN RELATION TO ^{45}Ca UPTAKE AND METABOLISM

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The effects of aliphatic 2-oxocarboxylic acids, at concentrations of up to 40 mM, on the function of pancreatic islets from *ob/ob* (obese–hyperglycaemic) mice were investigated. 1. 2-Oxopentanoate, DL-3-methyl-2-oxopentanoate, 4-methyl-2-oxopentanoate and 2-oxohexanoate all induced insulin release by isolated incubated islets and a biphasic insulin-secretory pattern in perfused mouse pancreas. The last two substances were similar in potency to glucose. Pyruvate, 2-oxobutyrate, 3-methyl-2-oxobutyrate and 2-oxo-octanoate did not induce insulin release significantly. 2. 2-Oxocarboxylic acids with significant insulin-secretory potency also induced significant ^{45}Ca uptake by isolated incubated islets. 3. The rates of decarboxylation of [1- ^{14}C]pyruvate, 3-methyl-2-oxo[1- ^{14}C]butyrate and 4-methyl-2-oxo[1- ^{14}C]pentanoate were twice as high as the rates of oxidation of the corresponding U- ^{14}C -labelled compounds. However, whereas the rates of metabolism of labelled pyruvate and 3-methyl-2-oxobutyrate steadily increased over the concentration range 1–40 mM, those of labelled 4-methyl-2-oxopentanoate and D-[U- ^{14}C]glucose levelled off at concentrations above 10 mM. 4. Omission of $^{40}\text{CaCl}_2$ from the incubation medium reduced the rate of oxidation of the insulin secretagogue [U- ^{14}C]4-methyl-2-oxopentanoate, but left that of the non-(insulin secretagogue) [U- ^{14}C]3-methyl-2-oxobutyrate unaffected. 5. Only glucose, and not pyruvate, 3-methyl-2-oxobutyrate and 4-methyl-2-oxopentanoate, significantly inhibited oxidation of endogenous fatty acids. 6. It is suggested that stimulus–secretion coupling and the resulting exocytosis of insulin in pancreatic β -cells may modulate both fuel oxidation and ^{45}Ca uptake.

2-Oxocarboxylic acids represent a group of chemically closely related compounds. A few of these substances are potent insulin secretagogues, some have a weak insulin-secretory capacity but the majority do not induce insulin release at all (Panten *et al.*, 1972, 1974; Panten, 1975; Matschinsky *et al.*, 1973; Lenzen, 1978a). It was the aim of the present experiments to obtain information about the importance of metabolic signals and generation of metabolic energy derived from metabolism of 2-oxocarboxylic acids in the initiation of insulin secretion and ^{45}Ca uptake. Pancreatic islets from *ob/ob* (obese–hyperglycaemic) mice, which contain a virtually pure β -cell population with more than 90% β -cells (Hellman, 1965), were used in the present investigation. Some of the results have already been presented in abstract form (Lenzen, 1978b; Lenzen & Panten, 1979).

Experimental

Chemicals

The following sources supplied the following chemicals. Sigma Chemical Co., St. Louis, MO, U.S.A.: L-amino acid oxidase (*Crotalus adamanteus*; type IV), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], EGTA, 2-oxobutyrate, 3-methyl-2-oxobutyrate, 2-oxopentanoate, DL-3-methyl-2-oxopentanoate, 4-methyl-2-oxopentanoate, 2-oxohexanoate, 2-oxo-octanoate, 2-oxoglutarate and 2-oxoadipate; all 2-oxocarboxylic acids were used as sodium salts; Worthington Biochemical Co., Freehold, NJ, U.S.A.: catalase (bovine liver; code CTR); Fluka A.G., Buchs, Switzerland: pyruvate; Serva, Heidelberg, Germany: bovine albumin (fraction V); Miles Laboratories, Elkhart, IN, U.S.A.: fatty acid-poor bovine albumin (fraction

V; Pentex); Novo Research Institute, Bagsvaerd, Denmark: crystalline mouse insulin; Merck A.G., Darmstadt, Germany: D-glucose, Tris and all other reagents of analytical grade; Behringwerke A.G., Frankfurt, Germany: ^{125}I -labelled bovine insulin; The Radiochemical Centre, Amersham, Bucks., U.K.: $^{45}\text{CaCl}_2$, D-[U- ^{14}C]glucose, [1- ^{14}C]pyruvate, [U- ^{14}C]pyruvate, L-[1- ^{14}C]valine, L-[U- ^{14}C]valine, L-[1- ^{14}C]leucine, L-[U- ^{14}C]leucine.

Preparation of ^{14}C -labelled 2-oxomonocarboxylic acids

3-Methyl-2-oxo[1- ^{14}C]butyrate, [U- ^{14}C]3-methyl-2-oxobutyrate, 4-methyl-2-[1- ^{14}C]oxopentanoate and [U- ^{14}C]4-methyl-2-oxopentanoate were prepared from the corresponding amino acid by a modification of the method described by Rüdiger *et al.* (1972): 50 μCi (0.8–2.2 μmol) of the appropriate amino acid was dried under a constant stream of N_2 at 40°C. After addition of 1 ml of Tris/HCl buffer (0.02 M; pH 7.7), 5000 units ($\mu\text{mol}/\text{min}$ at pH 7.0 and 25°C) of catalase and 1.4 units ($\mu\text{mol}/\text{min}$ at pH 7.8 and 37°C) of L-amino acid oxidase, the vial was gassed for 1 min with O_2 , closed and incubated with constant shaking for 3 h at 37°C. Gassing with O_2 was repeated hourly. The reaction mixture was applied to a 0.7 cm $\times$ 4.0 cm column (prefilled Econo-Column AG 50X8; 200–400 mesh; H^+ form; Bio-Rad Laboratories, Richmond, CA, U.S.A.) pre-washed with distilled water. Elution was performed with distilled water, and fractions of volume 0.4 ml were collected at a flow rate of about 0.2 ml/min. Samples of 2 and 20 μl respectively were taken for measurement of radioactivity in a liquid-scintillation counter and of 2-oxocarboxylic acid content by a microversion of the method of Katsuki *et al.* (1971). Conversion of amino acid was greater than 90%. L-Amino acid oxidase activity of the fractions was assayed spectrophotometrically (Wellner & Lichtenberg, 1971). No activity was found. The detection limit was 0.5% of the activity in the reaction mixture. Control studies revealed that more than 99% of leucine, valine and Tris were retained on the column. Fractions containing 75–95% of the 2-oxocarboxylic acid were pooled (total volume 0.8–1.2 ml; pH 1.8–2.2) and stored in 50 μl portions at -30°C .

General methods

24 h-starved adult non-inbred *ob/ob* mice (40–60 g) were used for the experiments. Pancreatic islets were microdissected freehand from *ob/ob* mice (Hellerström, 1964) while the pancreas was immersed in Krebs–Ringer bicarbonate medium containing 3 mM-glucose. All incubation studies were performed at 37°C in media equilibrated with ambient air. Islets were always freeze-dried (-40°C ; 0.1 Pa) overnight and weighed on a quartz-fibre balance. Insulin was determined radioimmunologi-

cally (Zaharko & Beck, 1968). Mouse insulin was used as the standard.

Insulin secretion by perfused pancreas

The pancreas and the adjacent part of the duodenum, the stomach and the spleen were isolated from *ob/ob* mice and perfused with a Krebs–Ringer bicarbonate solution containing 0.1% bovine serum albumin (Lenzen, 1979). It was gassed with O_2/CO_2 (19:1), resulting in a pH of 7.4. The temperature of the medium was 37°C. The flow rate was adjusted to 1 ml/min, resulting in a pressure of 60–70 mmHg. After an initial equilibration period of 10 min, the perfusion was switched to the same medium but containing the test substances. The effluent was collected at various timed intervals for determination of insulin (1 or 5 min).

Insulin release by isolated islets

The basal medium was a Krebs–Ringer bicarbonate medium (Lenzen, 1979) containing 20 mM—instead of 24.8 mM— NaHCO_3 but supplemented with 10 mM-Hepes and 0.1% bovine serum albumin. The pH was 7.4. Batches of two islets from *ob/ob* mice were preincubated for 30 min in 1 ml of medium containing 3 mM-glucose, for 5 min in 1 ml of medium without glucose and finally incubated for 60 min in 300 μl of the same medium but supplemented with various test substances in the concentration range 1–40 mM.

^{45}Ca uptake by isolated islets

The basal medium was a salt-balanced medium (Hellman *et al.*, 1976) enriched with Hepes (10 mM). The pH was 7.4, and the medium contained 2.54 mM- CaCl_2 . Batches of five islets from *ob/ob* mice were preincubated for 30 min in 1 ml of basal medium containing 3 mM-glucose, for 5 min in 1 ml of basal medium without glucose and eventually incubated for 120 min in 200 μl of basal medium labelled with $^{45}\text{Ca}^{2+}$ (11.4 Ci/mol) (Hellman *et al.*, 1976) and supplemented with various test substances at concentrations between 1 and 40 mM. This incubation period was followed by 1 h of washing with non-radioactive basal medium supplemented with 2 mM- LaCl_3 . After being freeze-dried overnight the islets were weighed and dissolved in Lumasolve (Lumac N.V./S.A., Meise, Belgium), and their radioactivity was determined by liquid-scintillation counting.

Oxidation of exogenous nutrients in isolated islets

The medium was identical with that used for the insulin-release studies. Batches of ten islets from *ob/ob* mice were preincubated for 30 min in 1 ml of basal medium containing 3 mM-glucose, for 5 min in 1 ml of basal medium without glucose and finally

incubated for 60 min in 40 μ l of basal medium labelled with 1–40 mM-D-[U- 14 C]glucose (8.4–0.6 Ci/mol), [1- 14 C]pyruvate (4.2–0.1 Ci/mol), [U- 14 C]pyruvate (5.8–0.1 Ci/mol), 3-methyl-2-oxo[1- 14 C]butyrate (4.4–0.1 Ci/mol), [U- 14 C]3-methyl-2-oxobutyrate (4.0–0.1 Ci/mol), 4-methyl-2-oxo[1- 14 C]pentanoate (4.4–0.1 Ci/mol) and [U- 14 C]4-methyl-2-oxopentanoate (6.2–0.2 Ci/mol). Incubations were performed in liquid-scintillation vials equipped with a small glass centre well. For each test incubation a blank value was obtained by incubating the medium without islets. Incubation vials were put in an ice bath (0°C) to arrest metabolism. Then 40 μ l of sodium citrate buffer (0.865 M-Na $^{+}$, 0.425 M-citrate, pH 4.9) was injected into the glass centre well (resulting pH 4.9) and the CO $_2$ was collected in 100 μ l of Lumasolve injected on to a circular piece of filter paper which had been placed on the bottom of the outer compartment of the vial before incubation. After 2 h at 0°C, the centre well was removed, scintillation liquid was added, and the radioactivity was counted in a liquid-scintillation spectrometer. In control experiments it was confirmed that this metabolic stop provided recovery rates of 14 CO $_2$ production from NaH 14 CO $_3$ of 96%, that no metabolism could be demonstrated at an incubation temperature of 0°C and that blank values were low (100–500 c.p.m.). Samples of incubation medium were used as external standards in each experiment. Arrest of metabolism with the sodium citrate buffer increased the apparent dry weight of the pancreatic islet by 20%, probably as a result of crystallization of sodium citrate in the islets, as shown by a 20% decrease in DNA content

(39.2 $\pm$ 1.0 versus 32.1 $\pm$ 0.5 ng of DNA/ μ g dry wt.) (mean $\pm$ S.E.M.; n = 13 and 37 respectively).

Oxidation of endogenous fatty acids in isolated islets

After evaporation of solvent, [U- 14 C]palmitate (12.5 μ Ci; 403 mCi/mol) was dissolved in 120 μ l of NaOH (5 mM) by gentle heating (60°C). Under constant stirring at room temperature 9.6 mg of fatty acid-poor bovine serum albumin (Pentex) was added. After 2 h of stirring 120 μ l of a double-concentrated Hepes-buffered Krebs–Ringer bicarbonate salt solution supplemented with glucose (16.7 mM) was added, resulting in a final pH of this medium of 7.4 and final concentrations of 0.3 mM-palmitate and 8.3 mM-glucose. Batches of 120 islets from *ob/ob* mice were prelabelled with [U- 14 C]palmitate during a 120 min preincubation in this medium, and then washed five times at room temperature with a non-radioactive glucose-free medium containing 40 mg of fatty acid-poor bovine serum albumin/ml. The islets were redistributed in groups of ten each and eventually incubated for 120 min, taking 40 μ l of the medium, in the vials that were also used for measurement of oxidation of exogenous nutrients. The effects of several test substances added to this medium were investigated. The experiments were finished by following the protocol described above for measurement of oxidation of exogenous nutrients.

Presentation of results

Results are presented as means $\pm$ S.E.M. and were tested for statistical significance with Student's paired or unpaired t test or with analysis of variance (Figs. 1, 3, 4 and 5).

Fig. 1. Concentration-dependent effects of 2-oxocarboxylic acids and glucose on insulin release by incubated pancreatic islets isolated from *ob/ob* mice

Values shown are means $\pm$ S.E.M. for four to 16 experiments. P values are given in parentheses: (a) pyruvate (n.s.); (b) 2-oxobutyrate (n.s.); (c) 3-methyl-2-oxobutyrate (n.s.); (d) 2-oxopentanoate (P < 0.001); (e) DL-3-methyl-2-oxopentanoate (P < 0.005); (f) 4-methyl-2-oxopentanoate (P < 0.001); (g) 2-oxohexanoate (P < 0.001); (h) glucose (P < 0.001).

Results

Concentration-dependent effects of 2-oxocarboxylic acids and glucose on insulin release by pancreatic islets isolated from ob/ob mice

Pyruvate, as well as 2-oxobutyrates and 3-methyl-2-oxobutyrates, did not significantly affect insulin release (Fig. 1).

Significant insulin release, however, was induced by 2-oxopentanoate, DL-3-methyl-2-oxopentanoate, 4-methyl-2-oxopentanoate and 2-oxohexanoate (Fig. 1). This release levelled off at concentrations above 10–20 mM. The last two 2-oxocarboxylic acids were the most potent insulin secretagogues,

with regard to both threshold concentration and maximal release (Fig. 1).

Up to 5 mM there was no significant insulin release in response to 2-oxo-octanoate (results not shown). Low solubility did not allow experiments with higher concentrations of 2-oxo-octanoate. For comparison glucose-induced concentration-dependent insulin release is shown in Fig. 1(e).

Effects of 2-oxocarboxylic acids and glucose on insulin secretion by perfused ob/ob mouse pancreas

Pyruvate (5 mM), 2-oxobutyrates (5 mM) and 3-methyl-2-oxobutyrates (5 mM) did not provoke an insulin-secretory response from the perfused ob/ob

Fig. 2. Pattern of insulin secretion by perfused ob/ob mouse pancreas in response to 2-oxocarboxylic acids (5 mM) and glucose (17 mM)

Values shown are means $\pm$ S.E.M. for three to ten experiments. Total amounts of insulin released ($\mu\text{g}/60 \text{ min} \pm \text{S.E.M.}$) and P values (t test) (given in parentheses) are: (a) control (0.41 ± 0.04); (b) pyruvate (0.19 ± 0.01 ; n.s.); (c) 2-oxobutyrates (0.60 ± 0.15 ; n.s.); (d) 3-methyl-2-oxobutyrates (0.78 ± 0.12 ; n.s.); (e) 2-oxopentanoate (2.20 ± 0.58 ; $P < 0.05$); (f) DL-3-methyl-2-oxopentanoate (1.29 ± 0.06 ; $P < 0.0001$); (g) 4-methyl-2-oxopentanoate (6.74 ± 1.05 ; $P < 0.02$); (h) 2-oxohexanoate (4.01 ± 0.52 ; $P < 0.02$); (i) 2-oxo-octanoate (0.73 ± 0.09 ; n.s.); (j) glucose (7.98 ± 1.10 ; $P < 0.01$).

mouse pancreas (Fig. 2). In contrast with pyruvate, 2-oxobutyrate and 3-methyl-2-oxobutyrate induced a small transient immediate insulin-secretory response from the perfused pancreas at 20 mM (results not shown), which was too weak to be demonstrated as a significant stimulation of insulin release in pancreatic islets incubated for 60 min in a static incubation system (Fig. 1).

2-Oxopentanoate (5 mM), DL-3-methyl-2-oxopentanoate (5 mM), 4-methyl-2-oxopentanoate (5 mM) and 2-oxohexanoate (5 mM) induced insulin secretion from the perfused *ob/ob* mouse pancreas characterized by an immediate insulin-secretory response appearing with a delay of 1.0–1.5 min and lasting 2.0–3.0 min (Fig. 2). The following late phase of insulin secretion from the pancreas during the rest of the 60 min perfusion was more prominent and sustained in response to 4-methyl-2-oxopentanoate (5 mM) and 2-oxohexanoate (5 mM) compared with 2-oxopentanoate (5 mM) and DL-3-methyl-2-oxopentanoate (5 mM).

Glucose induced a similar pattern of insulin secretion from the perfused pancreas (Fig. 2).

2-Oxo-octanoate (5 mM) induced a slight transient increase in insulin secretion during the first 20 min of perfusion (Fig. 2). 2-Oxoglutarate (5 mM) and 2-oxoadipate (5 mM), two 2-oxodicarboxylic acids, did not affect insulin secretion by the perfused pancreas (results not shown).

*Concentration-dependent effects of 2-oxocarboxylic acids and glucose on ^{45}Ca uptake by pancreatic islets isolated from *ob/ob* mice*

Pyruvate, 2-oxobutyrate and 3-methyl-2-oxobutyrate did not significantly affect ^{45}Ca uptake. Significant increase in uptake was induced by 2-oxopentanoate, DL-3-methyl-2-oxopentanoate, 4-methyl-2-oxopentanoate and 2-oxohexanoate (Fig. 3). All four 2-oxocarboxylic acids induced maximal ^{45}Ca uptake in the range between 5 and 20 mM. The threshold concentration for increased ^{45}Ca uptake, however, was lower for the last two 2-oxocarboxylic acids (Fig. 3). Compared with these 2-oxocarboxylic acids, ^{45}Ca uptake in response to glucose was dependent on a higher threshold concentration. Maximal uptake was induced by concentrations above 10 mM. 2-Oxo-octanoate did not induce significant ^{45}Ca uptake by pancreatic islets at concentrations of up to 5 mM (results not shown).

There was a correlation between ability of 2-oxo acids to induce insulin release and to increase ^{45}Ca uptake, but magnitude and concentration-dependence of the effects on insulin release and ^{45}Ca uptake differed. Although 4-methyl-2-oxopentanoate and 2-oxohexanoate were more potent insulin secretagogues than 2-oxopentanoate and DL-3-methyl-2-oxopentanoate, 4-methyl-2-oxopentanoate induced a smaller maximal increase in ^{45}Ca uptake

Fig. 3. Concentration-dependent effects of 2-oxocarboxylic acids and glucose on ^{45}Ca uptake by incubated pancreatic islets isolated from *ob/ob* mice

Values shown are means $\pm$ S.E.M. for three to six experiments. *P* values are given in parentheses: (a) pyruvate (n.s.); (b) 2-oxobutyrate (n.s.); (c) 3-methyl-2-oxobutyrate (n.s.); (d) 2-oxopentanoate ($P < 0.001$); (f) DL-3-methyl-2-oxopentanoate ($P < 0.001$); (g) 4-methyl-2-oxopentanoate ($P < 0.001$); (h) 2-oxohexanoate ($P < 0.005$); (e) glucose ($P < 0.001$).

than the other three 2-oxo acids. In contrast with increased rates of ^{45}Ca uptake, which levelled off at concentrations above 10 mM in response to 2-oxopentanoate and DL-3-methyl-2-oxopentanoate, increased insulin-release rates reached a plateau above 10 mM in response to these 2-oxo acids.

Concentration-dependent metabolism of 2-oxocarboxylic acids and glucose by pancreatic islets isolated from ob/ob mice

Decarboxylation of [^{14}C]pyruvate and oxidation of [^{14}C]pyruvate as well as of 3-methyl-2-oxo[^{14}C]butyrate and [^{14}C]3-methyl-2-oxobutyrate by pancreatic islets increased in the concentration range 1–40 mM, with decarboxylation rates of 2-oxo[^{14}C]carboxylic acids being twice as high as oxidation rates of 2-oxo[^{14}C]carboxylic acids at all concentrations (Fig. 4).

Rates of decarboxylation of 4-methyl-2-oxo[^{14}C]pentanoate and of oxidation of [^{14}C]4-methyl-2-oxopentanoate increased in the concentration

Fig. 4. Concentration-dependent metabolism of pyruvate, 3-methyl-2-oxobutyrate, 4-methyl-2-oxopentanoate and glucose by incubated pancreatic islets isolated from ob/ob mice

Values shown are means $\pm$ S.E.M. for three to eight experiments. *P* values are given in parentheses: (a) $\blacktriangle$, [^{14}C]pyruvate ($P < 0.001$), $\bullet$, [^{14}C]pyruvate ($P < 0.001$); (b) $\blacktriangle$, 3-methyl-2-oxo[^{14}C]butyrate ($P < 0.001$), $\bullet$, [^{14}C]3-methyl-2-oxobutyrate ($P < 0.001$); (d) $\blacktriangle$, 4-methyl-2-oxo[^{14}C]pentanoate ($P < 0.025$), $\bullet$, [^{14}C]4-methyl-2-oxopentanoate ($P < 0.001$); (c) [^{14}C]glucose ($P < 0.005$).

range 1–10 mM for the ^{14}C -labelled compound and 1–3 mM for the ^{14}C -labelled compound respectively (Fig. 4). At higher concentrations, these metabolic rates decreased and reached their lowest values at 40 mM (Fig. 4). Up to 10 mM, rates of metabolism of labelled 4-methyl-2-oxopentanoate were higher, but they were lower at concentrations above 10 mM than rates of metabolism of labelled pyruvate and 3-methyl-2-oxobutyrate (Fig. 4).

Rates of oxidation of [^{14}C]glucose increased in a concentration-dependent manner between 3 and 15 mM, and then remained unchanged up to 40 mM (Fig. 4).

To elucidate further the importance of metabolism of 2-oxocarboxylic acids for their insulin-secretory action and the possible contribution of insulin release to the decrease in rates of oxidation of 4-methyl-2-oxopentanoate at high concentrations, the rates of oxidation of [^{14}C]3-methyl-2-oxobutyrate and [^{14}C]4-methyl-2-oxopentanoate were studied in the absence of $^{40}\text{CaCl}_2$ from the incubation medium. Over the whole range of concentrations between 1 and 40 mM the rate of [^{14}C]3-methyl-2-oxobutyrate oxidation was not affected by the omission of $^{40}\text{CaCl}_2$ from the incubation medium (Fig. 5). In contrast, the rate of oxidation of the insulin secretagogue [^{14}C]4-methyl-2-oxopentanoate was diminished by the omission of $^{40}\text{CaCl}_2$ from the incubation medium (Fig. 5). Oxidation rates reached a plateau at concentrations

Fig. 5. Concentration-dependent oxidation of 3-methyl-2-oxobutyrate and 4-methyl-2-oxopentanoate by incubated pancreatic islets isolated from ob/ob mice in the absence of CaCl_2 from the incubation medium

Values shown are means $\pm$ S.E.M. for three to six experiments. *P* values are given in parentheses. Rates of oxidation of [^{14}C]3-methyl-2-oxobutyrate (b) and [^{14}C]4-methyl-2-oxopentanoate (a) increased significantly with increasing concentration in the presence ($\bullet$) ($P < 0.001$) and absence ($\circ$) ($P < 0.01$) of CaCl_2 . Omission of CaCl_2 from the incubation medium significantly reduced oxidation of [^{14}C]4-methyl-2-oxopentanoate only at concentrations of 1 mM ($P < 0.05$), 3 mM ($P < 0.01$), 5 mM ($P < 0.01$) and 10 mM ($P < 0.05$). Oxidation of [^{14}C]3-methyl-2-oxobutyrate was not significantly affected by omission of CaCl_2 from the incubation medium.

above 3–5 mM. Thus the decrease was most pronounced at 3 and 5 mM ($P < 0.001$), and marginally significant at both 1 and 10 mM, ($P < 0.05$), but these oxidation rates were not decreased at all at 20 and 40 mM (Fig. 5).

Effects of 2-oxocarboxylic acids and glucose on utilization of endogenous fatty acids

The effects of 2-oxocarboxylic acids and glucose on the oxidation of endogenous fatty acids were assessed by using pancreatic islets isolated from *ob/ob* mice which had been preincubated for 120 min in the presence of [U- 14 C]palmitate (0.3 mM) and glucose (8.3 mM). After being washed to remove extracellular nutrients, the pancreatic islets were incubated for 120 min in the absence or presence of several test substances (Table 1). The total amount of radioactivity incorporated in the pancreatic islets was identical in all test groups (Table 1). Only glucose (20 mM) significantly decreased $^{14}\text{CO}_2$ output by the pancreatic islets, whereas pyruvate (20 mM), 3-methyl-2-oxobutyrate (20 mM) and 4-methyl-2-oxopentanoate (20 mM) did not significantly modify $^{14}\text{CO}_2$ output by labelled pancreatic islets (Table 1). In control experiments antimycin A blocked $^{14}\text{CO}_2$ production from the metabolism of endogenous [U- 14 C]palmitate by 80% (Table 1).

Discussion

Insulin-secretory action of 2-oxocarboxylic acids and glucose

In accordance with results obtained in earlier studies (Coore & Randle, 1964; Panten *et al.*, 1972, 1974; Matschinsky *et al.*, 1973; Lenzen *et al.*, 1975, 1976; Panten, 1975; Lenzen, 1978a; Sener *et al.*, 1978) pyruvate, 2-oxobutyrate and 3-methyl-2-oxobutyrate, when present at concentrations of up to 40 mM, did not induce insulin release from incubated *ob/ob* mouse islets and from perfused *ob/ob*

mouse pancreas (Figs. 1 and 2). 2-Oxopentanoate, DL-3-methyl-2-oxopentanoate, 4-methyl-2-oxopentanoate and 2-oxohexanoate were stimulators of insulin release by incubated *ob/ob* mouse islets when present at concentrations of up to 40 mM (Fig. 1), and induced a biphasic insulin-secretory pattern in the perfused *ob/ob* mouse pancreas (Fig. 2). Compared with the stimulatory effects in the *ob/ob* mouse pancreas (Fig. 2), the insulin-secretory potency of DL-3-methyl-2-oxopentanoate was weaker and the secretory action of 2-oxopentanoate was even absent from the rat pancreas (Lenzen, 1978a). 4-Methyl-2-oxopentanoate and 2-oxohexanoate have also been shown to induce insulin release from perfused *ob/ob* mouse islets (Panten *et al.*, 1972, 1974; Panten, 1975) and perfused rat pancreas (Matschinsky *et al.*, 1973; Lenzen, 1978a). The effects of the latter two 2-oxocarboxylic acids were greater and induced a late phase of insulin secretion comparable with that seen in response to glucose (Figs. 1 and 2). During long perfusion periods (Fig. 2) the total amount of insulin released represents virtually only late-phase insulin secretion (more than 98%) (Grodsky, 1972). ^{45}Ca uptake is also only increased after long incubation periods (30 min or more) and thus related to late-phase insulin secretion (Hellman *et al.*, 1978). It is therefore conceivable to relate changes in metabolism and ^{45}Ca uptake in the present incubation studies in response to 4-methyl-2-oxopentanoate, 2-oxohexanoate and glucose to the potent late-phase of insulin secretion.

Metabolism of 2-oxocarboxylic acids and glucose

Available information about metabolism of 2-oxocarboxylic acids by pancreatic islets is restricted to pyruvate. Considering that our use of sodium citrate to stop the metabolism increased islet dry weight by 20%, thereby reducing absolute rates of oxidation and decarboxylation by 20% (see the Experimental section), [U- 14 C]pyruvate-oxidation rates by *ob/ob*

Table 1. Oxidation of endogenous fatty acids by pancreatic islets isolated from *ob/ob* mice. Effects of various test substances on $^{14}\text{CO}_2$ output and radioactive content were determined in islets labelled with [U- 14 C]palmitate. Values shown are means $\pm$ S.E.M. from eight different experiments. The effects of the test substances on oxidation of endogenous fatty acids ($^{14}\text{CO}_2$ output in relation to final ^{14}C content) were compared with the control and tested for significance by the paired *t* test; *P*-values are shown; n.s., not significant.

Addition	$^{14}\text{CO}_2$ output (c.p.m./120 min per μg of dry islet)	Final ^{14}C content (c.p.m./ μg of dry islet)	$^{14}\text{CO}_2$ output final ^{14}C content (%)	<i>P</i>
None (control)	68 $\pm$ 8	1120 $\pm$ 113	6.19 $\pm$ 0.61	—
20 mM-Glucose	34 $\pm$ 4	1166 $\pm$ 90	2.91 $\pm$ 0.28	<0.0001
20 mM-Pyruvate	60 $\pm$ 6	1138 $\pm$ 84	5.29 $\pm$ 0.43	n.s.
20 mM-3-Methyl-2-oxobutyrate	71 $\pm$ 7	1125 $\pm$ 75	6.53 $\pm$ 0.83	n.s.
20 mM-4-Methyl-2-oxopentanoate	65 $\pm$ 9	1213 $\pm$ 94	5.44 $\pm$ 0.64	n.s.
0.01 mM-Antimycin A	11 $\pm$ 1	1236 $\pm$ 84	0.81 $\pm$ 0.09	<0.0001

mouse islets of 13.8 mmol/h per kg dry wt. (20 mM) and of 17.3 mmol/h per kg dry wt. (40 mM) compare favourably with [U-¹⁴C]pyruvate-oxidation rates by *ob/ob* mouse islets of 20 mmol/h per kg dry wt. (17 mM) and 20.9 mmol/h per kg dry wt. (33.3 mM) reported by Hellman *et al.* (1971) and by Gunnarsson & Hellerström (1973) respectively. Since the dry weight of a rat islet is roughly 1 µg, [U-¹⁴C]pyruvate-oxidation rates by rat pancreatic islets of 45 mmol/h per kg dry wt. and 44 mmol/h per kg dry wt. found by Lin & Haist (1971) and Sener *et al.* (1978) respectively are twice as high as those reported above for mouse pancreatic islets.

Our [1-¹⁴C]pyruvate-decarboxylation rates of 25.5 mmol/h per kg dry wt. (20 mM) and 34.1 mmol/h per kg dry wt. (40 mM) by *ob/ob* mouse pancreatic islets (Fig. 4) were lower than those of 93 mmol/h per kg dry wt. (28.6 mM) found for albino mouse islets (Hedeskov *et al.*, 1972), those of 160 mmol/h per kg dry wt. found for rat islets (30 mM) (Sener *et al.*, 1978) and the extremely high rates of 300 mmol/h per kg dry wt. found for rat islets (15 mM) by Jain *et al.* (1978).

We adopted a special technique for arresting metabolism of oxocarboxylic acids in small tissue samples (see the Experimental section). This procedure provided low blank values with respect to complete arrest of metabolism, in spite of a relatively small decrease in pH. This result is not necessarily obtained when metabolic arrest is attained by causing a slight decrease in pH in the presence of antimycin A (Table 4 in Sener *et al.*, 1978), which does not block all enzyme reactions of 4-methyl-2-oxopentanoate at pH 6.0 (Bowden & Connelly, 1968; Sener *et al.*, 1978). On the other hand, a gross reduction in pH, as usually obtained by application of HCl, can increase blank values, thereby greatly complicating evaluation of the metabolism of 2-oxocarboxylic acids in small tissue samples (Hellman *et al.*, 1971). These two elements, incomplete arrest of metabolism and high blank values, may provide an explanation for the relatively high rates of pyruvate oxidation discussed above.

It is striking that metabolic rates did not increase further at higher concentrations of the insulin secretagogues 4-methyl-2-oxopentanoate and glucose in contrast with the steadily increasing concentration-dependent metabolic rates of the non-(insulin secretagogues) pyruvate and 3-methyl-2-oxobutyrate (Fig. 4). The plateau of glucose-oxidation rates at higher concentrations confirms the earlier observation by Ashcroft *et al.* (1970) and may be a consequence of insulin release. A plateau of metabolic rates of insulin secretagogues may be an indication of metabolic requirements for the secretory process.

Omission of ⁴⁰CaCl₂ from the incubation medium reduced rates of oxidation of the insulin secretagogue [U-¹⁴C]4-methyl-2-oxopentanoate but left

those of the non-(insulin secretagogue) [U-¹⁴C]3-methyl-2-oxobutyrate unaffected (Fig. 5). The decrease in oxidation rate observed in the absence of ⁴⁰CaCl₂ from the incubation medium has also been described for glucose (Hellman *et al.*, 1974; Henquin, 1978). These experiments indicate that the decrease in oxidation rate is a result of interference with stimulus-secretion coupling and resulting exocytosis. Therefore the effects of omission of ⁴⁰CaCl₂ from the incubation medium may be a criterion for distinction between insulin secretagogues and non-secretagogues. It is suggested that part of the metabolic energy derived from metabolism of insulin secretagogues such as 4-methyl-2-oxopentanoate and glucose might be required for exocytosis.

Residual oxidation of 4-methyl-2-oxopentanoate in the absence of CaCl₂ from the incubation medium may provide energy for maintenance of events not coupled to exocytosis.

Interference of 2-oxocarboxylic acids and glucose with utilization of endogenous fatty acids

The decrease in the rate of oxidation of endogenous [U-¹⁴C]palmitate by 53% due to exogenous glucose (Table 1) confirmed the inhibition by 52% of oxidation of exogenous palmitate reported by Berne (1975). Considering the lower specific radioactivity of [U-¹⁴C]palmitate used in our experiments, our rates of ¹⁴CO₂ output and final ¹⁴C content in *ob/ob* mouse islets (Table 1) are in close agreement with those reported for rat islets by Sener *et al.* (1978). Sener *et al.* (1978) observed only a 10% reduction in response to glucose. On the other hand, we could not confirm the observation by Sener *et al.* (1978) of a significant 33% decrease in ¹⁴CO₂ production in response to pyruvate. In our experiments pyruvate, as well as 3-methyl-2-oxobutyrate and 4-methyl-2-oxopentanoate, induced only slight insignificant decreases in the rate of ¹⁴CO₂ production in the range 5–15% (Table 1). The weak inhibitory effects of pyruvate and 4-methyl-2-oxopentanoate as compared with the strong effects of glucose are against isotopic dilution as an explanation for this difference.

Sener *et al.* (1978) concluded, on the basis of calculations in Table 11 of their paper, that the net generation of reducing equivalents was strictly proportional to the insulinotropic capacity of glucose and pyruvate. Our results do not support this conclusion (Table 1). We must conclude that the many more reducing equivalents generated by metabolism of glucose than by metabolism of pyruvate are utilized in place of endogenous nutrients, resulting in a much higher net gain of reducing equivalents on exposure of the islets to the non-(insulin secretagogue) pyruvate than on exposure to the insulin

secretagogue glucose. In contrast with the conclusion by Sener *et al.* (1978), our results provide evidence for a dissociation between net gain of reducing equivalents and insulinotropic capacity of pyruvate and glucose.

Role of metabolism and ^{45}Ca uptake in 2-oxocarboxylic acid-induced insulin release

All 2-oxocarboxylic acids as well as glucose are finally metabolized in the tricarboxylic acid cycle. However, whereas pyruvate, 4-methyl-2-oxopentanoate and glucose enter the cycle via acetyl-CoA, 3-methyl-2-oxobutyrates are introduced via succinyl-CoA. Pyruvate is initially oxidized by pyruvate dehydrogenase, an enzyme that is located on the inner surface of the inner mitochondrial membrane. The two branched-chain 2-oxocarboxylic acids 3-methyl-2-oxobutyrates and 4-methyl-2-oxopentanoate are oxidized by a common branched-chain 2-oxocarboxylic acid dehydrogenase (Parker & Randle, 1978). This enzyme may be located on the outer surface of the inner mitochondrial membrane (Johnson & Connelly, 1972), though this is not generally accepted (Billington *et al.*, 1978). But the different localization of the enzymes and the different metabolic pathways for these 2-oxocarboxylic acids are apparently not crucial, because both pyruvate and 3-methyl-2-oxobutyrates do not induce insulin release and ^{45}Ca uptake.

^{45}Ca uptake is not strictly correlated with insulin secretion. Therefore it has to be concluded that increased ^{45}Ca uptake in response to initiators of insulin release is not a prerequisite for secretion, but only a consequence of metabolic changes due to stimulus-secretion coupling and exocytosis. On the other hand, discrepancies between metabolic degradability and ability of 2-oxocarboxylic acids and glucose to stimulate ^{45}Ca uptake do not support the view that increased ^{45}Ca uptake is a mere consequence of increased substrate availability (Malaisse *et al.*, 1979). Even though stimulation of this ^{45}Ca uptake by pancreatic islets is connected with accumulation of ^{45}Ca by the secretory granules (Bloom *et al.*, 1977), it is possible that it is not a direct coupling factor for initiation of exocytosis.

In view of the inability of the vigorously metabolized 2-oxocarboxylic acids pyruvate and 3-methyl-2-oxobutyrates to induce significant insulin release, the assumption cannot be maintained that metabolic degradability as such is a sufficient prerequisite for insulin-secretory capacity. The levelling off of rates of oxidation of insulin secretagogues (Fig. 4), as well as the increase in oxidation rates in ^{40}Ca -containing incubation media (Fig. 5), may reflect metabolic responses to ion redistribution and exocytosis. Thus correlations between fuel utilization and insulin secretion often reported by other investigators (Malaisse *et al.*, 1979; Zawulich, 1979)

may only represent an excellent adaptation of islet-cell metabolism to enhanced energy requirements during the various steps of stimulus-secretion coupling and exocytosis. Enhanced fuel oxidation may be simply a consequence of reactions initiated by interaction of insulin secretagogues with specific membrane receptor sites necessary for recognition of secretagogues.

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