The Effect of Glucose Stimulation on 45 Calcium Uptake of Rat Pancreatic Islets and their Total Calcium Content as Measured by a Fluorometric Micro-Method

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Summary. Glucose-stimulated 45 calcium uptake and total calcium content of rat pancreatic islets has been studied, using a new fluorometric micro-method to estimate total calcium. Extracellular calcium was separated from incubated tissue by a rapid micro-filtration procedure. Islets incubated up to 60 min with calcium chloride 2.5 mmol/l and glucose 2.5 mmol/l maintained the same calcium content (670 ± 7.5 pmol/ μ g DNA). When the glucose concentration was raised to 15 mmol/l no change in the total calcium content could be detected. On incubation with glucose 2.5 mmol/l in the absence of calcium, the calcium content decreased to 488 ± 27 pmol/ μ g DNA. On incubation with 45 calcium chloride 2.5 mmol/l for 5 or 30 min at 2.5 mmol/l glucose, islets exchanged 21 ± 2 and $28 \pm 1\%$ of their total calcium content and, at 15 mmol/l glucose, 30 ± 3 and $45 \pm 2\%$, respectively. Thus, islet calcium has a high turn-over rate. Glucose stimulation results in an increase of the calcium uptake without enhancing the total calcium content and hence must increase the calcium-exchangeable pool.

Key words: 45 calcium uptake, 40 calcium content, micro-fluorometric calcium determination, pancreatic islets.

Calcium ions play an essential role in excitation-contraction coupling in muscle and in stimulus-secretion coupling in various secretory cells. They also play a key role in the insulin secretory process [1–3]. Stimulation of islets causes a transient inhibition of calcium efflux followed by an increase of efflux [4, 5]; at the same time the uptake of calcium is increased [6, 7]. From radioisotope studies and atomic absorption measurements it has been concluded that stimulation of islet tissue causes a net increase in the calcium con-

tent of the tissue [7, 8]. In contrast, however, the calcium content of islet tissue, as determined histochemically with glyoxal bis(2-hydroxyanil), decreases considerably during the initial 30 min of glucose stimulation [9]. The interpretation of these changes in calcium metabolism of stimulated islets would be eased if the total calcium content could be measured. To measure calcium in very small tissue samples (e.g. pancreatic islets), we have developed a rapid and simple fluorometric method for microgram amounts of wet or dried tissue. Contaminating extracellular calcium was rapidly separated from the incubated islets by a micro-filtration procedure. Both methods enabled us to study the effects of glucose stimulation on the total calcium and on 45 calcium content in the same tissue samples allowing the determination of the specific activity of the calcium content of the tissue.

Materials and Methods

Materials

$^{45}\text{CaCl}_2$ and [^3H]-sucrose were obtained from The Radiochemical Centre, Amersham, Bucks, UK. All other chemicals were of analytical grade and purchased from Merck, Darmstadt, FRG. A standard solution containing 20 μ g calcium/ml was made by diluting calcium carbonate dissolved in a small quantity of hydrochloric acid with deionized water. From this stock solution aliquots were taken and diluted to 25 pmol (1 ng) calcium/ μ l. Potassium hydroxide Suprapur (Merck) was used as it contains less than 0.0001% calcium. A calcein solution was made by dissolving 1.50 mg powdered calcein (2,7-bis[bis(carboxymethyl)-aminomethyl]fluorescein) in 25 ml propan-1,2 diol and shaking for several hours at room temperature. This stock solution could be used for several months when stored in the dark at ambient temperature. Before use a sample was diluted with five volumes of deionized water. Filters with a diameter of 3 mm were punched from nylon tissue with pores of 32 μ m (Vereinigte Seidenwebereien, Krefeld, FRG). The total effective pore area of the filters amounted to 0.5 mm². The fluorescence of the calcein-calcium complex was measured with an Aminco Fluoro-Colorimeter J4 with a glass primary filter which peaks at

Fig. 1. Relationship between fluorescence and amounts of calcium. Two different calibration curves are shown, Δ , $\circ$. All points are measured in triplicate. The mean coefficient of variation of all points amounted to 8%

360nm and a 2A-12Wr secondary filter with a sharp cut-off at 510nm. Suprasil Quartz Microcells with a working volume of 0.1–0.17 ml were used.

Pancreatic Tissue

Pancreatic islets from male Wistar rats of approximately 350 g body weight were isolated by the collagenase digestion method [10]. Dry islets were prepared by lyophilising washed batches of isolated islets overnight. Dry exocrine tissue was obtained by microdissection from lyophilised pancreatic tissue sections.

Incubation of Islets

Pools of isolated islets were pre-incubated for 15 min in Krebs-Ringer bicarbonate buffer [11] containing CaCl_2 2.5 mmol/l, bovine serum albumin 2.5 g/l and glucose 2.5 mmol/l. Batches of 30 islets were then incubated at 37 °C in 2 ml buffer with glucose 2.5 or 15 mmol/l (gassed with 95% O_2 /5% CO_2) in a Dubnoff metabolic incubator. When the Ca^{2+} uptake was studied, the medium was supplemented with 10 μCi of $^{45}\text{Ca}^{2+}$ /ml. For the determination of the efficiency of the washing procedure, 30 islets were incubated for 15 min in 0.1 ml buffer containing 1.5 μCi [^3H]-sucrose (1 mmol/l) or 4 μCi $^{45}\text{CaCl}_2$ (2.5 mmol/l). After incubation the islets were rapidly filtered and homogenized.

Microfiltration of Islets

For determination of the calcium content of isolated islets an efficient removal of calcium-containing buffer is a prerequisite. For this purpose a microfiltration procedure was developed. The system consisted of a stainless steel support for nylon filters with a diameter of 3 mm connected to a roller pump (Ismatec J4), which created the vacuum. An air flow rate of about 15 ml/min was used. The pump also delivered Ca^{2+} -free buffer to wash the islets at a rate of 0.5 ml/min through a hypodermic needle on the filter. To free the islets from Krebs-Ringer buffer, 30 islets in 0.1 ml of buffer were deposited on the filter and washed for 10 s. Three blank filtrations without tissue were included to correct for Ca^{2+} adsorbed to the filters.

Determination of the Total Calcium Content of Tissue

The calcium determination is based on the formation of a fluorescent calcium-calcein complex under alkaline conditions. The method is specially adapted to measure 100–1000 pmol quantities of calcium. Thirty islets on a nylon filter were placed in a small glass tube containing 100 μl deionized water and sonicated at 100 W for 20 s. Aliquots of 20 μl and 70 μl of the homogenate were taken for the DNA and calcium determinations respectively. A calibration curve was made by pipetting 5, 10, 20, 30, 40 and 50 μl of the solution containing 25 pmol (1 ng) calcium/ μl into polystyrene tubes. Deionized water was added to a final volume of 70 μl . Subsequently, 40 μl of 2 mol/l KOH and 90 μl of freshly diluted calcein solution were added and mixed, after which the fluorescence was measured. The fluorescence was stable for at least 2 h.

Measurement of ^{45}Ca Calcium Fraction in Tissue

In $^{45}\text{Ca}^{2+}$ uptake experiments, the total calcium content of the samples was measured with the fluorometric method; subsequently the sample was transferred to a scintillation vial with Plasmasol 7.5 ml and counted by liquid scintillation.

Determination of Islet DNA Content

DNA was determined according to Kissane and Robins [12] using 20 μl of the islet homogenate.

Statistical Analysis

Results are expressed as mean values $\pm$ SEM. Differences were tested for statistical significance with the unpaired Student's 't' test.

Results

Evaluation of the Fluorometric Method

Fluorescence as a function of the calcium concentration is shown in Figure 1; the two curves were made at a time-interval of 7 months and demonstrate the reproducibility. The coefficient of variation of 750 pmol calcium amounted to 6.6%. Zinc, magnesium and phosphate, of which pancreatic islet tissue contains relatively high amounts, and also lanthanum, did not interfere with the fluorescence of 625 pmol calcium when equimolar amounts of these ions as chlorides

Table 1. Effect of washing with calcium-free buffer on the retention of [^3H]sucrose, ^{45}Ca and ^{40}Ca by 30 islets without and with correction for filter blank values

	Washing time (s)				
	0	5	10	20	40
<i>Without correction</i>					
[^3H]sucrose (pmol)	253 $\pm$ 36	54 $\pm$ 3	44 $\pm$ 3	33 $\pm$ 2	27 $\pm$ 1
^{45}Ca (pmol)	459 $\pm$ 11	255 $\pm$ 19	223 $\pm$ 15	203 $\pm$ 8	201 $\pm$ 13
$^{45}\text{Ca} + ^{40}\text{Ca}$ (pmol)	1535 $\pm$ 42	1355 $\pm$ 40	1320 $\pm$ 39	1305 $\pm$ 47	1278 $\pm$ 47
<i>Corrected values</i>					
[^3H]sucrose (pmol)	46 $\pm$ 32	50 $\pm$ 3	43 $\pm$ 3	32 $\pm$ 2	27 $\pm$ 1
^{45}Ca (pmol)	173 $\pm$ 8	237 $\pm$ 19	209 $\pm$ 20	194 $\pm$ 8	199 $\pm$ 13
$^{45}\text{Ca} + ^{40}\text{Ca}$ (pmol)	1133 $\pm$ 38	1175 $\pm$ 41	1149 $\pm$ 39	1160 $\pm$ 42	1106 $\pm$ 48

Results are expressed as mean $\pm$ SEM of 10 observations for [^3H]sucrose and ^{45}Ca and 30 observations for $^{45}\text{Ca} + ^{40}\text{Ca}$. DNA content of 30 islets 2214 ± 42 ng ($n = 150$)

Fig. 2. Effect of glucose stimulation on the ^{40}Ca content of islet tissue. Batches of 30 islets of fed rats were incubated for 0–60 min in 2 ml buffer containing glucose 2.5 (O) or 15 (Δ) mmol/l and CaCl_2 2.5 mmol/l. Results are expressed as mean $\pm$ SEM of 12 (O) and 18 (Δ) separate experiments. Thirty islets contain 1546 ± 20 ng DNA ($n = 404$); $1 \mu\text{g}$ DNA is $25.7 \pm 1.8 \mu\text{g}$ dry islet tissue

were added (recovery of calcium 100 ± 4 ; 104 ± 5 ; 104 ± 5 ; $98 \pm 3\%$, respectively, $n = 6$). At tenfold excess (6250 pmol), only magnesium slightly increased the fluorescence (recovery of calcium $117 \pm 3\%$, $n = 6$). The sparingly soluble calcium salt $\text{Ca}_3(\text{PO}_4)_2$ (500 or 1000 pmol) yielded only about 50% of the fluorescence, whereas the soluble salt $\text{Ca}(\text{H}_2\text{PO}_4)_2$ gave 100% fluorescence. However, when a sample of the former salt was acidified to pH 2.5, by which the salt is solubilized, recovery of calcium was complete. Acidification of islet homogenates to pH 2.5 did not influence the calcium-dependent fluorescence. Progressive dilution of islet homogenates (four steps of 1.5 times) yielded values which agreed closely with the expected values (values expressed in %, $y = 1.00x + 1.19$; $r = 0.99$, eight separate experiments). Furthermore, calcium 250 pmol added to islet homogenates was completely recovered ($100 \pm 3\%$; $n = 7$).

Evaluation of the Microfiltration Method

To investigate the efficiency of removal of extracellular fluid by filtration of islet tissue on small nylon filters, batches of 30 islets were incubated in 0.1 ml buffer (glucose 2.5 mmol/l) for 15 min in the presence of [^3H]sucrose 1 mmol/l or $^{45}\text{CaCl}_2$ 2.5 mmol/l . They were then filtered and washed for 0 – 40 s with calcium-free Krebs-Ringer buffer (supplied at a rate of 0.5 ml/min). Table 1 (upper part) shows the washing-induced decrease of [^3H]sucrose, ^{45}Ca and total amount of calcium ($^{45}\text{Ca} + ^{40}\text{Ca}$) trapped in islets and filters. After 10 – 20 s of washing, stable levels were attained for all three parameters. When only incubation medium was filtered without washing $200 \pm 20 \text{ pmol}$ [^3H]sucrose and $286 \pm 21 \text{ pmol}$ ^{45}Ca were retained by the filters. These amounts decreased to $1 \pm 0 \text{ pmol}$ [^3H]sucrose and $9 \pm 2 \text{ pmol}$ ^{45}Ca after washing for 10 s but they were not further decreased by washing for 20 and 40 s . When the relevant filter blanks were subtracted, the same amounts/30 islets were found for all three parameters, irrespective of the duration of washing (Table 1, lower part). Thus, the washing-induced decrease (Table 1, upper part) is mainly due to wash-out of [^3H]sucrose and ^{45}Ca adsorbed to the filters. When calculated on a weight basis the tissue contained $15 \pm 1 \text{ pmol}$ sucrose, 88 pmol ^{45}Ca , and 524 pmol $^{45}\text{Ca} + ^{40}\text{Ca}/\mu\text{g}$ DNA.

Calcium Content of Pancreatic Tissue

Freshly isolated islets from fed rats contained $678 \pm 22 \text{ pmol}$ calcium/ μg DNA ($n = 9$). After incubation for 15 min in buffer containing calcium (2.5 mmol/l) and glucose (2.5 mmol/l), the islets contained $728 \pm 39 \text{ pmol}$ calcium/ μg DNA ($n = 21$). However, when the islets were washed six times with, and incubated in, calcium-free buffer the content had decreased by 33% to $488 \pm 27 \text{ pmol}/\mu\text{g}$ DNA ($n = 24$; $p < 0.005$).

Lyophilised non-incubated islets contained 31 ± 3 pmol calcium/ μg dry weight ($n = 9$).

Specimens of exocrine tissue dissected from lyophilised sections of pancreatic tissue of fed rats contained only 10 ± 0.5 pmol calcium/ μg whereas exocrine tissue from 24 h and 72 h fasted rats contained 12 ± 1 and 13 ± 1 pmol respectively ($n = 7$), the difference between fed and 72 h fasted rats being significant ($p < 0.01$).

The effect of fasting on the islet calcium content was also determined. Freshly isolated islets of fed, 24-h and 72-h fasted rats contained 651 ± 54 , 681 ± 57 and 632 ± 45 pmol/ μg DNA respectively ($n = 26$). Thus, fasting has no effect on the calcium content of isolated islets.

Effect of Glucose Stimulation on the Total Calcium Content and the $^{45}\text{Ca}^{2+}$ Uptake

The effect of the length of incubation of the islets in buffer containing CaCl_2 2.5 mmol/l and glucose 2.5 or 15 mmol/l on the total calcium content as measured with the fluorometric method is shown in Figure 2. Islets incubated for varying periods of time from 0 to 60 min with glucose 2.5 mmol/l contained the same amount of calcium (670 ± 7.5 pmol/ μg DNA, $n = 6$). Raising the glucose concentration from 2.5 mmol/l to 15 mmol/l did not significantly alter the total calcium content.

Incubation of islets in buffer containing $^{45}\text{CaCl}_2$ 2.5 mmol/l and glucose 2.5 mmol/l for 5 or 30 min resulted in an uptake of 128 ± 11 and 185 ± 16 pmol calcium/ μg DNA respectively ($n = 18$). Incubation of the islets with glucose 15 mmol/l and $^{45}\text{CaCl}_2$ 2.5 mmol/l for 5 or 30 min increased the uptake to 202 ± 21 and 297 ± 14 pmol/ μg DNA. Glucose stimulation enhanced the uptake over the initial 5 min by 74 pmol/ μg DNA and over 30 min by 112 pmol/ μg DNA being an increase of 58% and 61% compared with glucose 2.5 mmol/l. If $^{45}\text{calcium}$ had been exchanged completely with the cell-calcium, the relative $^{45}\text{calcium}$ specific activity (cell specific activity/medium specific activity) should be equal to 1. The relative specific activity of islet $^{45}\text{calcium}$ incubated for 5 or 30 min at glucose 2.5 mmol/l amounted to 0.21 ± 0.02 and 0.28 ± 0.01 respectively, and at glucose 15 mmol/l to 0.30 ± 0.03 and 0.45 ± 0.02 respectively. Thus, 21% and 28% of the total calcium content of islet tissue were exchanged at glucose 2.5 mmol/l and 30% and 45% at glucose 15 mmol/l.

Discussion

For the determination of the calcium content of pancreatic islets only small amounts of tissue are avail-

able, and a very sensitive method is therefore needed. Previously we have measured the total calcium content of 0.05–0.1 μg islet tissue with electron-probe micro-analysis [13], whereas others using an atomic absorption spectrometer measured the calcium content of 2000 islets [7] or of 2–10 μg amounts of dry islet tissue [14]. Both methods are time-consuming and require expensive equipment. The present study describes a chemical method for simple and rapid measurement of 100–1000 pmol amounts of calcium in 5–20 μg dry tissue (or an equivalent amount of wet tissue) using a relatively cheap fluorometer. The method is based on the formation of a fluorescent calcium-calcein complex under alkaline conditions [15]. It allows the determination of the total calcium content and the $^{45}\text{calcium}$ content of the same tissue samples. The determination does not appear to be affected by biological material or by sparingly soluble calcium phosphates in the tissue. The sensitivity and precision of the method are very good.

To measure the calcium content of islet tissue, calcium in the extracellular space has to be removed. In $^{45}\text{calcium}$ uptake studies correction for extracellular calcium has been obtained by using space markers [16], or extracellular calcium has been removed by centrifuging through oil [17] or washing the tissue [6, 7]. None of these methods is suitable when the total calcium content of pancreatic islets has to be measured since too much calcium is retained in the extracellular space or some intracellular calcium might be lost.

The fact that 1 μl Krebs-Ringer buffer contains 2500 pmol calcium, whereas 30 islets contain about 1200 pmol calcium, demonstrates the need of a reliable system for removal of contaminating calcium-containing buffer. The micro-filtration method described seems to meet this demand. Washing of the filtered islets reduces the amounts of [^3H]sucrose and $^{45}\text{calcium}$ adsorbed to the filters but has no effect on the content of the tissue. This is apparently due to the characteristics of the filtration system. The nylon filters (effective filter diameter 2 mm) have a total pore area of only 0.5 mm², which is largely covered with islet tissue. The relatively great air flow of 15 ml/min apparently drains the calcium-containing fluid from the extracellular space of the tissue in an efficient way. Removal of [^3H]sucrose from the islets by micro-filtration is very rapid compared with that by perfusion which takes about 20 min [18].

The amount of the spacemarker left in the tissue is maximally equivalent to about 80 pmol calcium, 7% of the total calcium content. The stable levels of [^3H]sucrose, $^{45}\text{calcium}$ and total calcium after 10–40 s of washing indicates that the islets are sufficiently washed after 10 s.

The total calcium content, measured with the fluorometric method in lyophilised rat pancreatic islets and exocrine tissue, is the same as found in microdissected tissue of overnight-fasted ob/ob mice using atomic absorption spectrometry [14]. The finding that islet tissue contains three times more calcium than exocrine tissue confirms our earlier observations [13]. We did not observe an effect of glucose stimulation on the total calcium content of isolated islets. This agrees with the observations of Gylfe et al. [19] who also reported no significant effect, but it is in contrast with Malaisse et al. [7] who found that islets incubated for 90 min at 0 and 16.7 mmol/l glucose and extensively washed with calcium-free buffer contained 8.9 and 19.4 pmol calcium per islet respectively ($n = 3$). When allowance is made for the small islets used and the extensive washing in the latter study, the calcium content of the stimulated islets is in the same range as our values, but not that of the unstimulated islets. We have no explanation for the discrepancy, but it may be due to differences in experimental conditions; e.g. 0 versus 2.5 mmol/l glucose.

Fasting markedly impairs the secretory response [20], alters the ^{45}Ca uptake [21, 22] and the mobile calcium content of islets [13], but it does not appear to affect the total calcium content of the islets.

The ^{45}Ca uptake over the initial 5 and 30 min at 2.5 mmol/l glucose is equivalent to 21% and 28% of the total islet calcium content and at 15 mmol/l glucose it is equivalent to 30% and 45% respectively. As neither incubation of islets at 2.5 mmol/l glucose nor raising the glucose concentration to 15 mmol/l altered the total islet calcium content, the ^{45}Ca uptake at low as well as at high glucose must represent a $^{45}\text{Ca}^{2+}$ - $^{40}\text{Ca}^{2+}$ exchange process. The results demonstrate that islet calcium has a high turn-over rate which is enhanced on glucose stimulation, and that glucose stimulation increases the calcium-exchangeable pool. Our data on calcium exchange in unstimulated islets are not different from the 27% found in cultured mouse islets incubated for 2 h with $^{45}\text{CaCl}_2$ 1.28 mmol/l and subsequently washed for 30 min [23]. In contrast, Malaisse et al. [7] reported that the exchangeable calcium pool as measured by ^{45}Ca uptake is only slightly less than the total calcium content measured by atomic absorption.

The glucose-induced ^{45}Ca uptake amounted to 74 and 112 pmol/ μg DNA at 5 and 30 min respectively, being equivalent to 9% and 17% of the total calcium content of the islets. If the calcium content were increased by the same amount as the glucose-induced ^{45}Ca uptake, an increase of 9% would be too small to be detected. However, an increase of 17% should have been detected. If glucose causes an increase of the total islet calcium content then it must be

only a fraction of the glucose-induced uptake and too small to be detected by our method. Thus, our results indicate that the increased influx is counterbalanced by an increased efflux as is seen during glucose stimulation [4, 24]. The enhanced uptake of ^{45}Ca may be the consequence of increased permeability of the cell membrane and increased cytosolic calcium ions as a result of mobilization of calcium from intracellular stores. These conditions increase the turn-over of the exchangeable calcium pools. Glucose-induced mobilization and redistribution has been evidenced by histochemical staining of mobile calcium [9], histochemical localization of calcium with pyroantimonate [26, 27] and changes in ^{45}Ca content of various subcellular fractions [25]. It is likely that islets primarily use intracellular calcium to mediate insulin secretion. The physiological regulation of this activator calcium is controlled mainly by intracellular events rather than by dynamic changes of calcium permeability of the plasma membrane.

Acknowledgements. We thank Mr. H. Moes for technical assistance and Miss G. T. van Dellen and Mr. J. Pleiter for preparing the manuscript.

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Received: 28 August 1980
and in revised form: 18 September 1981

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