Volume 291, number 2, 310–314 FEBS 10273 October 1991

ADONIS 001457939100982L

Measurements of cytoplasmic free Ca^{2+} concentration in human pancreatic islets and insulinoma cells

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Received 22 July 1991

In human pancreatic islets an increase in the glucose concentration from 3 to 20 mM raised the free cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), an effect being reversible upon withdrawal of the sugar Depolarization with a high concentration of K^+ or the sulphonylurea tolbutamide also raised $[Ca^{2+}]_i$ Addition of extracellular ATP produced a transient rapid rise in $[Ca^{2+}]_i$ Oscillations in $[Ca^{2+}]_i$ were observed in the presence of 10 mM glucose Insulinoma cells responded to glucose and tolbutamide with increases in $[Ca^{2+}]_i$ whereas the sulphonamide diazoxide caused a decrease in $[Ca^{2+}]_i$ These findings confirm previous results obtained in rodent β-cells

Human pancreatic islet, Human insulinoma, Cytoplasmic free Ca^{2+} concentration, Fura-2

1 INTRODUCTION

Stimulation of pancreatic β-cells with glucose promotes generation of intracellular ATP [1] This leads to closure of ATP-regulated K^+ channels in the plasma membrane [2,3], with subsequent depolarisation and influx of Ca^{2+} through voltage-activated Ca^{2+} channels [4,5] The resulting increase in the cytoplasmic free Ca^{2+} concentration ($[Ca^{2+}]_i$) initiates secretion Since the access to viable human islet tissue is limited, nearly all information concerning the β-cell stimulus–secretion coupling has, so far, been derived from experiments made on rodent islet preparations There are only a handful of biochemical and biophysical studies on human islets describing insulin release, glucose oxidation, protein phosphorylation and characterization of ATP-regulated K^+ channels [6–11], but none dealing with intracellular Ca^{2+} metabolism In the present study we demonstrate, for the first time, direct measurements of $[Ca^{2+}]_i$ in human islets and insulinoma cells stimulated with glucose, hypoglycaemic sulphonylurea and other secretagogues

2. EXPERIMENTAL

2 1 *Media*

For isolation of islets and measurements of $[Ca^{2+}]_i$ we used a buffer containing (in mM) NaCl 140, KCl 5 9, $CaCl_2$ 1 28, $MgCl_2$ 1 2, HEPES 25 and 1 mg/ml bovine serum albumin with a pH of 7 4 [12]

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In the experiments shown in Figs 2 and 3, the medium Ca^{2+} concentration was raised to 2 56 mM

2 2 *Preparation of islets*

Pancreatic tissue was obtained from patients admitted to hospital because of symptomless jaundice, weight loss and fatigue The diagnosis was in each case settled by ultrasound investigation, percutaneous transhepatic cholangiography and fine needle aspiration biopsy and later verified by histologic examination of the resected specimen The patients were preoperatively treated by percutaneously transhepatically placed endoprosthesis in order to decompress the bile duct and restitute bile flow to the gut and the enterohepatic circulation Surgery was performed 4–5 weeks after placement of the endoprosthesis Before surgery, the serum aspartate aminotransferase, alanine aminotransferase, bilirubin and alkaline phosphatase were in all patients within the normal reference limits Creatinine was normal in all patients The patients had normal fasting glucose levels (less than 6 6 mM) and absence of glucose in the urine Oral glucose tolerance test (OGTT) was performed where blood glucose was measured in the fasted state and at 2 h after intake of 75 g glucose Patient 1 was a 73-year-old man with cancer of the papilla of Vater, patient 2 was a 66-year-old woman with adenocarcinoma of the pancreas and patient 3 was a 67-year-old man with adenocarcinoma of the distal part of the common bile duct All patients had resection for cure, comprising cephalic pancreatic resection en-bloc with duodenum, according to Whipple A specimen free from cancer was obtained from the resected part of the pancreas and put in cold RPMI 1640 medium supplemented with 10% fetal bovine serum (Flow), 100 IU/ml penicillin and 100 μg/ml streptomycin and stored on ice Fibrotic parts of the pancreas and adipose tissue were removed and the tissue was minced into pieces of about 2–4 mm Islets were isolated by collagenase (Boehringer, 3 mg/ml) and picked with a braking pipette under inspection in a dissection microscope The islets were put in Petri dishes or in microwells in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics as described above and cultured at 37°C in humified air with 5% CO_2 for 1–4 days.

2 3 *Preparation of insulinoma cells*

A piece of a pancreatic insulinoma was obtained at operation on

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patient 4, a 44-year-old otherwise healthy woman who 4 years earlier was admitted to hospital because of hypoglycemic attacks due to hyperinsulinemia Subsequent operation revealed two pancreatic tumors staining immunohistochemically positive for insulin, proinsulin, C-peptide and chromogranin A After 3 5 years hypoglycemic attacks reappeared, being more frequent with exercise and relieved after food intake Laboratory tests showed hypoglycemia and hyperinsulinemia Surgery was performed 6 months later from which the tissue used in this study was derived The tumor cells stained positive for C-peptide and neurospecific enolase Clinically the tumor was considered a relapse of the insulinoma The insulinoma tissue was minced into millimeter-sized pieces and suspended with a pasteur pipette in 3 ml of Hank's buffer containing 0 1% trypsin and 100 mg/ml EDTA until a cell suspension was obtained After washing twice with culture medium, the cells were kept in a culture flask under tissue conditions for 3 h under gentle agitation to prevent attachment of the cells to the flasks

2 4 *Loading with fura-2*

The islets were loaded in 2 ml of the HEPES buffer with 2 μM fura-2/acetoxymethyl ester (AM) (Sigma/Boehringer) for 60 min at 37°C under gentle agitation In the experiments shown in Figs 2 and 3, the islets were laoded in the presence of 2 56 mM Ca^{2+} The loading procedure resulted in a diffuse even fluorescence throughout the islet except for a brighter rim along the extreme periphery The insulinoma cells were loaded in the culture medium with 1 μM fura-2/AM for 45 min at 37°C under gentle agitation

2 5 *Mesurements of* $[Ca^{2+}]_i$

2 5 1 *Islets* Single islets were transferred to a small open perifusion chamber (volume 150 μl) with a coverslip bottom The medium flow rate was 300 μl/min and controlled by a peristaltic pump The dead space of the perifusion system corresponded to a lag period of approximately 20 s, which has been corrected for in all figures Fura-2 fluorescence was measured at 37°C with a SPEX Fluorolog-2 system coupled to an inverted epifluorescence microscope (Zeiss Axiovert 35M) equipped with a 40× Plan-Neofluar objective The measuring diaphragm was closed enough to exclude the brighter peripheral zone of the islet, thus also minimizing the contribution of non-β-cell fluorescence Fura-2 was alternatingly excited at 340 and 380 nm by a beam chopper and emitted light was collected at 515 nm by a photon-counting photometer A 340/380 nm ratio was obtained at 1Hz and data were stored in the system computer Data from all measurements are expressed as relative fluorescence of the 340/380 ratios The traces shown are selected from measurements on islets from three patients as indicated in the text Only experiments performed on islets responding to glucose with a rise in $[Ca^{2+}]_i$ were considered successful (success rate >50%) Fig 1 represents 4 islets sensitive to glucose whereas Figs 2 and 3 were the only successful experiments performed on the two patients Measurements could be conducted for more than 100 min, implying that the experimental conditions were gentle to the islets

2 5 2 *Insulinoma cells* After washing twice in the HEPES buffer, the cells were resuspended in 1 3 ml of the buffer in a 1 cm polystyrene cuvette The measurement was made at 37°C in an Aminco-Bowman spectrofluorometer, slightly modified to allow constant stirring The excitation and emission wavelengths were set at 340 and 500 nm, respectively Test substances were added from concentrated stock solutions ot the cuvette Changes in $[Ca^{2+}]_i$ are expressed as relative fluorescence Only one experiment could be performed because of the limited amount of cells

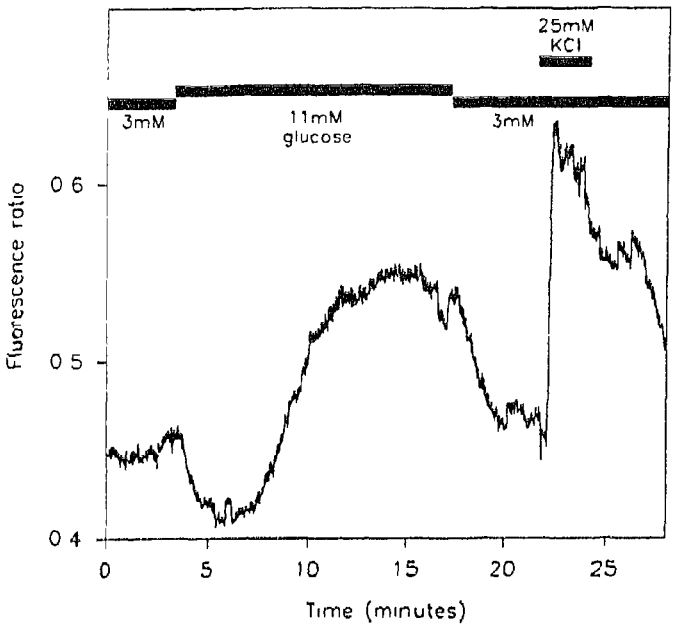

Fig 1 Effects of glucose and high K^+ on $[Ca^{2+}]_i$ in a single human islet Changes in medium concentration of glucose and K^+ are indicated by the black bars

3 RESULTS

Fig. 1 demonstrates a $[Ca^{2+}]_i$ recording of a fura-2 loaded human islet obtained from patient 1. Raising the glucose concentration from 3 to 11 mM produced a biphasic response, with an initial decrease followed by an increase in $[Ca^{2+}]_i$ which reached a plateau after a few minutes. The increase in $[Ca^{2+}]_i$ is due to depolarization-induced opening of the voltage-activated Ca^{2+} channels promoted by metabolism of the sugar [1–5] and was rapidly reversed upon decreasing the glucose concentration to 3 mM Addition of 25 mM K^+, to directly open the voltage-activated Ca^{2+} channels, promoted Ca^{2+} influx with a subsequent fast rise in $[Ca^{2+}]_i$ Immediately after having reached the peak, $[Ca^{2+}]_i$ started to decrease, possibly due to Ca^{2+}-dependent inactivation of the voltage-activated Ca^{2+} channels [13]

In Fig. 2A, the islet originated from patient 2 and the response to glucose was similar to that shown in Fig 1, with an initial decrease in $[Ca^{2+}]_i$ followed by an increase. It can be observed that the raised level of $[Ca^{2+}]_i$ was not constant but fluctuated. Subsequent stimulation with 25 mM K^+, in the presence of high glucose, led to a rapid increase in $[Ca^{2+}]_i$ comparable to that observed in the previous experiment After returning to the basal glucose concentration, the islet responded to the sulphonylurea tolbutamide with a marked rise in $[Ca^{2+}]_i$ A measurement from the same islet, performed after a 10 min period of stimulation with 11 mM glucose, is shown in Fig 2B After perifusing in low glucose for another 3 min we stimulated with 200 μM ATP to activate P_2-purigeric receptors [14,15]. There was a rapid increase in $[Ca^{2+}]_i$ in response to the nucleotide, followed by a gradual decrease towards the baseline.

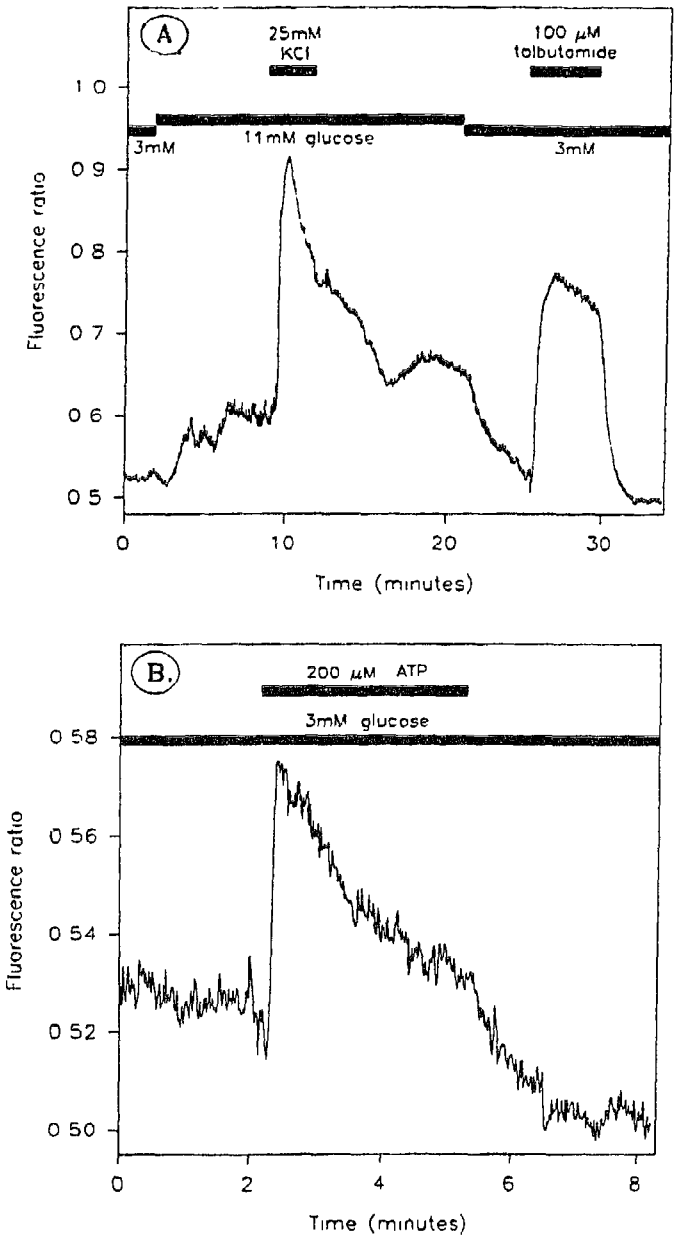

Fig 2 Effects of glucose, high K^+, tolbutamide and ATP on $[Ca^{2+}]_i$ in a single human islet (A) Additions of glucose, K^+ and tolbutamide are indicated by the black bars (B) The trace shows the same islet as in (A) after having been perifused with 11 mM glucose for 10 min and then exposed to 3 mM glucose as indicated in the figure Addition of ATP is indicated by the black bar Note the different time and fluorescence scales compared to (A)

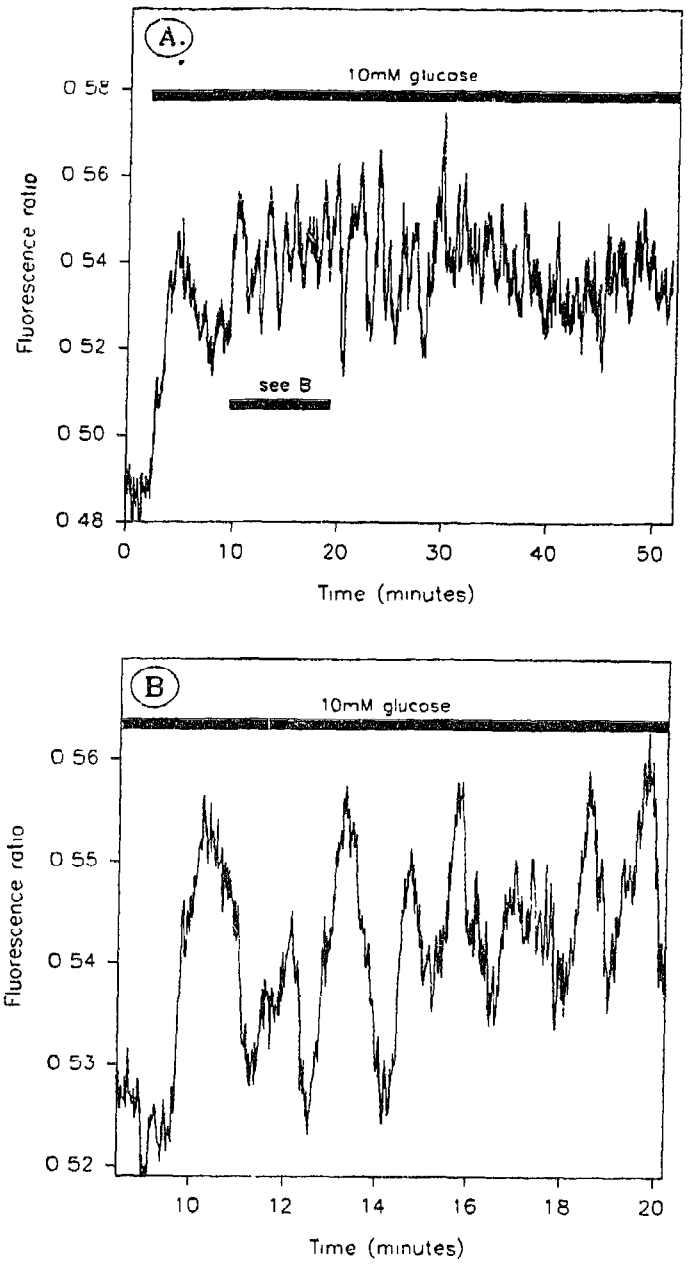

Fig 3 Oscillations in $[Ca^{2+}]_i$ in a single human islet in response to glucose stimulation (A) Addition of 10 mM glucose is indicated by the black bar (B) Expanded part of the trace shown in (A) Note the change of time and fluorescence scales

The trace depicted in Fig. 3A is a recording made on an islet obtained from patient 3 In this experiment the glucose concentration was increased from 0 mM to 10 mM. This islet did not respond with an initial decrease in $[Ca^{2+}]_i$ but rather with a rise followed by oscillations in $[Ca^{2+}]_i$. In Fig 3B a part of the same trace is shown at an expanded time scale. The oscillations were somewhat irregular in shape and had a period of about 70–80 s

In Fig 4 we demonstrate an experiment performed on human insulinoma cells derived from patient 4 In contrast to the previous experiments, the measurement of $[Ca^{2+}]_i$ was performed in a cell suspension in a cuvette, which prevented test substances to be removed The cells were incubated in the absence of glucose and stimulation with 20 mM of the sugar produced a slow increase in $[Ca^{2+}]_i$ after a 2 min lag period. Addition of diazoxide, a sulphonamide inhibiting insulin release, led to a decrease in $[Ca^{2+}]_i$. Subsequent additions of tolbutamide counteracted the effect of diazoxide and consequently raised $[Ca^{2+}]_i$.

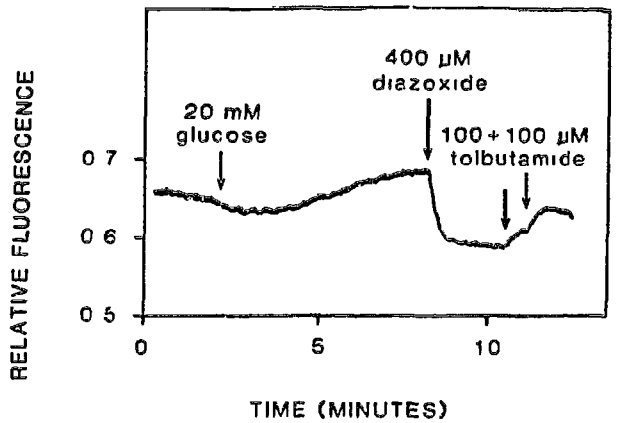

Fig 4 Effects of glucose, diazoxide and tolbutamide on $[Ca^{2+}]_i$ in a suspension of human insulinoma cells Addition of glucose, diazoxide and tolbutamide are indicated by the arrows

4 DISCUSSION

The present results represent direct measurements of $[Ca^{2+}]_i$ in isolated human islets of Langerhans and are similar to those obtained in rodent β-cells [16–18], validating the use of animal models in the study of β-cell stimulus–secretion coupling The biphasic $[Ca^{2+}]_i$ response to glucose has been attributed to initial stimulation of sequestration and/or outward transport of Ca^{2+} then followed by depolarization-mediated influx of the ion [19] The initial lowering in $[Ca^{2+}]_i$ in response to glucose is most readily seen when β-cells have been incubated at subnormal glucose concentrations, probably reflecting restoration of cytoplasmic ATP to normal levels with subsequent activation of energy-deprived Ca^{2+} pumps [20,21] It is of interest to note that a similar pattern of insulin release parallels the changes in $[Ca^{2+}]_i$ in mouse β-cells [20] The initial inhibition of insulin release by glucose, likely to reflect lowering in $[Ca^{2+}]_i$, has also been observed in patients with NIDDM and suggested to indicate impairment of β-cell function [22]. In our study the biphasic response was only seen in about half of the experiments and in those cases it may be attributed to the low prestimulatory glucose concentration [20,21]

More interestingly, we found that human islets demonstrate oscillations in $[Ca^{2+}]_i$ in response to glucose stimulation, in accordance with what has been reported for single β-cells as well as intact islets from mice [16,18]. Although the oscillations could not be fully characterized, they appeared longer than the 'fast' oscillations obtained in intact mouse islets [18], but shorter than those observed in single β-cells [16]. The nature of the oscillatory behaviour is unknown but in view of the importance of the ATP-regulated K^+ channels in the regulation of β-cell membrane potential and thereby Ca^{2+} influx, oscillations in the ATP level and/or ATP/ADP ratio may be involved Furthermore, muscle extracts demonstrating spontaneous oscillations in the ATP/ADP ratio, produce oscillations in the ambient Ca^{2+} concentration maintained by permeabilized tumor β-cells [23]. It is of interest to note that oscillations in NAD(P)H fluorescence have recently been demonstrated in single β-cells subsequent to glucose stimulation [17]. For years it has been known that cell extracts, containing enzymes required for glycolysis, can produce cyclic fluctuations in the levels of intermediates such as NADH [24] These oscillations are believed to arise from feedback control of certain allosteric glycolytic enzymes [24] Whether fluctuations in the NAD(P)H level, K^+ channel activity, glycolysis and Ca^{2+} cycling of intracellular organelles are all involved in the control of $[Ca^{2+}]_i$ oscillations is a matter for future investigations.

The demonstration that extracellular ATP, used to activate P_2-purinergic receptors, resulted in an increase in $[Ca^{2+}]_i$ most likely reflects activation of phospholipase C and the subsequent formation of inositol 1,4,5-trisphosphate (IP_3) [14,15]. The fact that IP_3 releases Ca^{2+} from internal stores explains the major part of the rise in $[Ca^{2+}]_i$ promoted by the nucleotide

Human insulinoma cells have previously been used for patch-clamp studies and successfully kept in tissue culture for long periods of time [25]. In contrast to animal insulinoma cells, a stable human tumor β-cell line has not yet been possible to establish Cells from the insulinoma used in this study did not proliferate in tissue culture and the results thus only reflect regulation of $[Ca^{2+}]_i$ in one β-cell tumor. The clear-cut response to glucose stimulation in terms of increased $[Ca^{2+}]_i$, suggested a functional metabolism of the sugar This is in agreement with previous studies demonstrating that glucose, in many patients with insulinomas, evokes a prompt stimulation of insulin release [26] Interestingly, most animal-derived β-cell lines are insensitive to glucose [27,28] although there are recent reports of clones which show sensitivity to the sugar [29,30] The existence of functional ATP-regulated K^+ channels in the insulinoma was further indicated since the cells responded to diazoxide and tolbutamide Tolbutamide and other sulphonylurea compounds are known to stimulate insulin release by directly closing the ATP-regulated K^+ channels with subsequent depolarization and influx of Ca^{2+} [4,31] Diazoxide, on the other hand, opens these K^+ channels promoting repolarization and closure of the voltage-activated Ca^{2+} channels with a resulting decrease in $[Ca^{2+}]_i$ [4,31] The usefulness of diazoxide in the treatment of hyperinsulinism in man [32] most likely reflects its lowering effect on $[Ca^{2+}]_i$ in the β-cells The present study provides additional support for a close coupling between activity of ATP-regulated K^+ channels and changes in $[Ca^{2+}]_i$ in the regulation of insulin release in man.

Acknowledgements: This study was supported by grants from the Swedish Medical Research Council (19-00034, 12x-08647), the Bank of Sweden Tercentenary Foundation, the Swedisch Diabetes Association, Funds of the Karolinska Institute, Tore Nilsons Foundation for Medical Research, the Swedish Society of Medicine, Novo Industry.

Farmitalia Carlo Erba, the Nordic Insulin, Aage Louis Hansens Memorial, Fredrik and Ingrid Thurings, Torsten and Ragnar Soderbergs, Magnus Bergvalls, Amundssons, Anders Otto Swards and the Swedish Hoechst Research Foundations

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