Virchows Arch (1999) 434:75–82

ORIGINAL ARTICLE

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Nutrient-dependent distribution of insulin and glucokinase immunoreactivities in rat pancreatic beta cells

Received: 23 July 1998 / Accepted: 25 August 1998

Abstract Functional heterogeneity among pancreatic beta cells is a characteristic feature of the islets of Langerhans. Under physiological conditions, beta cells in the pancreas of fed rats exhibited heterogeneous immunohistochemical staining for insulin and glucokinase. Intracellular beta cell glucokinase staining was either faint or dense. In the pericapillary space beta cell glucokinase immunoreactivity had a polar orientation, with the highest density in cytoplasmic regions close to the blood vessels. Starvation resulted in a loss of heterogeneity with homogeneous insulin staining in all beta cells of the islets, and this was accompanied by a loss of heterogeneous glucokinase staining. The intracellular polarity of glucokinase staining in contact to blood vessels also disappeared after starvation. Refeeding resulted in the reappearance of intercellular heterogeneity. In dependence on the functional demand, the endocrine pancreas recruited insulin from beta cells according to a well-defined hierarchy, with an initial preferential mobilization of medullary beta cells. In the course of this process intracellular polarity of glucokinase staining reappeared in areas of the beta cell with functional contact to the GLUT2 glucose transporter in the plasma membrane. This can be regarded as the morphological correlate of an activation of the glucose signal recognition apparatus. Interestingly, the study also provides evidence that the changes in glucokinase distribution apparently preceded those in insulin distribution, which is in keeping with the central role of glucokinase as the glucose sensor of the pancreatic beta cell.

Key words Rat · Pancreatic beta cell · Insulin · GLUT2 glucose transporter · Glucokinase

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Introduction

Insulin is distributed heterogeneously in pancreatic beta cells, and this heterogeneous distribution correlates with differences in the functional activity of the beta cells in the islets of Langerhans [9, 22, 25]. However, the underlying reasons for this heterogeneity are unknown. From in vitro studies on single beta cells there is evidence that the metabolic state of the individual beta cell determines its insulin secretory activity [5]. Glucokinase, the low-affinity glucose-phosphorylating enzyme, has a pivotal role in the pancreatic beta cell as the so-called glucose sensor [18] or glucose recognition enzyme [11] for initiation of glucose-induced insulin secretion. Together with the GLUT2 glucose transporter, glucokinase couples changes in the millimolar glucose concentration range to corresponding changes of the metabolic flux rate in pancreatic beta cells, and finally to the rate of insulin secretion [12, 19]. Colocalization studies have shown that the GLUT2 glucose transporter is mainly expressed in the plasma membrane of the pancreatic beta cell [21, 26] while glucokinase is localized in the cytoplasm [8, 20]. The distribution pattern of glucokinase is heterogeneous in the islets of Langerhans. It is not known, however, whether changes in the distribution of glucokinase participate in the nutrient-dependent regulation of this enzyme with concomitant effects on the secretory activity of the pancreatic beta cell.

In the present study we have adopted an immunohistochemical approach using semithin sections of plastic-embedded rat pancreas [4, 9], together with a computer-assisted quantification system. With this method we have studied the effects of starvation and refeeding on the pattern of glucokinase distribution and compared it with the distribution of insulin and GLUT2 immunoreactivity in the pancreatic islet beta cells.

Materials and methods

Pancreatic tissue was obtained from 3- to 4-month-old male Wistar rats. Four fed rats kept under normal laboratory conditions (blood

glucose: 5.8±0.3 mM) served as controls. Four rats were fasted for 48 h (blood glucose: 3.8±0.2 mM), and four additional fasted rats were subsequently refed for 24 h (blood glucose: 5.9±0.3 mM).

Small tissue specimens from the splenic, gastric and duodenal parts of the pancreas were quenched in isopentane, precooled in liquid nitrogen, freeze-dried (–35°C for 72 h) and fixed by vapor-phase *p*-formaldehyde as described in detail elsewhere [9]. Freeze-dried specimens were used for analysis of immunoreactivities of insulin, GLUT2 glucose transporter and glucokinase [10]. The smaller specimens were embedded in Araldite.

Polyclonal antisera against insulin (Novo, Bagsvaerd, Denmark), diluted 1:7,000, and against the rat GLUT2 glucose transporter (WAK-Chemie, Bad Homburg, Germany), diluted 1:20,000– 1:30,000, were used. For immunohistochemical staining of glucokinase we used a polyclonal, affinity-purified, antibody raised in rabbits in our laboratory against rat recombinant liver glucokinase at a dilution of 1:25–1:50. Western blot analyses of cytoplasmic fractions from rat islet and rat liver revealed a single 56-kDa band typical for glucokinase with no cross-reactivity to hexokinase.

The antisera against insulin and GLUT2 have been examined for method and antibody specificities [9, 10, 26]. In the present study the antiserum against rat GLUT2 glucose transporter was tested by pre-adsorption with the GLUT2 glucose transporter peptide (WAK-Chemie, Bad Homburg, Germany) and peptides with unrelated specificities (insulin and glucagon at concentrations between 6.25 and 100 µg/ml). Upon the pre-adsorption with the homologous antigen GLUT2 glucose transporter at concentrations as low as 6.25 µg/ml, the GLUT2 glucose transporter immunoreactivity in pancreatic beta cell plasma membrane and cytoplasm disappeared completely. The antiserum against rat glucokinase was pre-adsorbed by affinity-purified glucokinase protein at concentrations between 5 and 50 µg/ml. After pre-adsorption with even the lowest concentration of the homologous protein the cytoplasmic immunostaining for glucokinase in pancreatic beta cells was already absent.

Serial semithin sections (0.5 µm) were immunostained by the avidin–biotin complex (ABC) method [6]. Following the fast removal of the resin and overnight incubation with the first antibody, biotinylated goat anti-rabbit IgG (1:100, 30 min) and a streptavidin–biotin–peroxidase complex (1:1,000, 30 min) were used as second and third antibodies (both from Jackson Immuno Research, West Grove, Ill.). The demonstration of the peroxidase was performed with 0.7 mM diaminobenzidine and 0.002% H_2O_2 in 0.05 mM Tris HCl buffer, pH 7.6.

A total of 1,700 sections studied was composed of smaller series of 20–40 semithin sections from different pancreatic regions of each experimental group and of five series of 100 sections. The smaller series were immunostained sequentially for insulin, GLUT2 glucose transporter and glucokinase. To demonstrate the heterogeneous intracellular distribution of glucokinase, the larger series were also monovalently immunostained for glucokinase twice in the fed control pancreas and once under each of the three other experimental conditions. The sections were viewed by bright field illumination or phase contrast with a Zeiss Photomicroscope II (Zeiss, Oberkochen, Germany).

In order to verify the changes in the immunoreactivities of insulin and glucokinase under fasting and refeeding conditions compared with the control developed in parallel, both immunoreactivities were densitometrically determined using a computer-assisted method. The image analysis system consisted of a Zeiss Photomicroscope II, a Sony CCD DXC-151 AP color video camera, and an Apple Macintosh Power PC 7500 equipped with a frame grabber card. The images of the islets made with a constant illumination value were imported into the system, displayed at a final magnification of 1:400 on a monitor and processed by the NIH shareware Image Analysis Program (Version 1.59) as described in detail by Russ [23]. First the beta cell area of each islet was determined. The screen resolution for the display of the digitized images was 1280×1024 pixels 8 bit monochrome/256 grey levels as arbitrary units (1=white; 256=black). After an automatic background subtraction (2 D roller ball) of the islet images the grey values of the immunoreactivities ranged between 8 and 157 for insulin and between 8 and 114 for glucokinase. The highest density in endocrine cells without immunostaining was 15 in the nuclear areas. The number of pixels was multiplied by the concomitant grey value to achieve the weighting of a particular immunoreactivity. The sum of all weighted pixels was divided by the measured beta cell area to obtain the integrated density per square micrometer of beta cell area. The results are presented as means±SEM and were tested for statistical significance with Student's *t*-test.

Results

Beta cells in the fed control rat pancreas

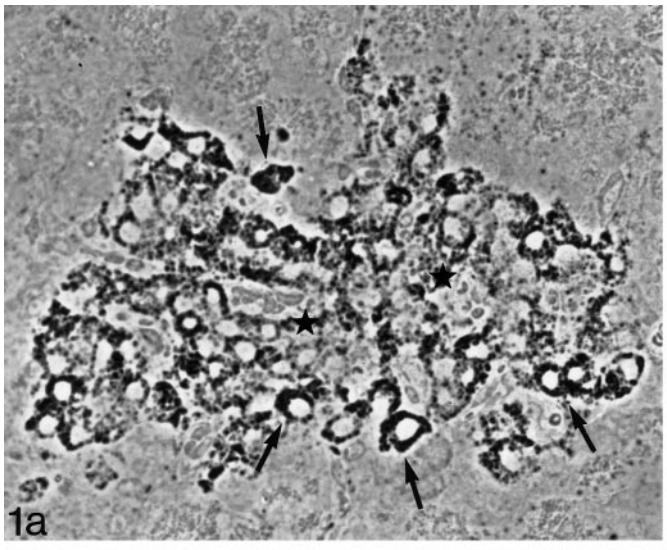
Irrespective of the pancreatic region, pancreatic beta cells contained insulin immunoreactivity with variable density (Fig. 1a). Pancreatic beta cells in large islets (>150 µm diameter) exhibited heterogeneous staining for insulin. Beta cells in the cortical region in the periphery, which have contacts both with beta cells and with other endocrine cells, such as α- and δ-cells, expressed mostly dense insulin immunoreactivity, while medullary beta cells in the centre of the islet were faintly stained for insulin (Fig. 1a). Pancreatic beta cells in smaller islets (Fig. 2a) and single beta cells at extra-islet sites (Fig. 3a) displayed mostly dense insulin immunoreactivity. A quantitative analysis of the integrated density of insulin immunoreactivity expressed per square micrometer of islet area revealed a significant decrease in density with increasing islet size (Fig. 4).

Pancreatic beta cells in large islets (>150 µm diameter) displayed cytoplasmic staining for glucokinase with a heterogeneous distribution pattern (Fig. 1c). The intracellular glucokinase immunoreactivity in individual beta cells was homogeneously distributed in the cytoplasm with faint or

Fig. 1a–c Medium-sized pancreatic islet of a fed control rat. Semithin sections immunostained for **a** insulin, **b** GLUT2 glucose transporter and **c** glucokinase. Cortical beta cells (*arrows:* dense) and medullary beta cells (*asterisks:* faint) are heterogeneously immunostained for insulin in the same islet. The GLUT2 glucose transporter is restricted to the plasma membrane with gaps in close relation to the intra-islet capillary system (*arrows*). The glucokinase immunoreactivity is localized in the cytoplasm of beta cells with either homogeneously distributed faint (*asterisks*) or dense (*arrows*) immunostaining or immunostaining with polar orientation (*arrowheads*). ×425 **d** Glucokinase distribution of a fed control rat at a higher magnification. Pancreatic beta cells show either homogeneous dense (*arrows*) or faint (*asterisks)* immunostaining or immunostaining with polar orientation (*arrowhead).* Beta cells marked with *asterisks* are separated by a capillary. In both beta cells a denser glucokinase immunoreactivity is found under the plasma membrane in close association with the pericapillary space. ×650

Fig. 2a–c Two small pancreatic islets of a fed control rat. Semithin sections immunostained for **a** insulin, **b** GLUT 2 glucose transporter and **c** glucokinase. Beta cells in these small islets are densely immunostained for insulin. The GLUT2 glucose transporter immunoreactivity is localized mainly in the plasma membrane, and the dense glucokinase is found in the cytoplasm of beta cells without intercellular variations. ×375

Fig. 3a–c A single pancreatic beta cell at an extra-islet site of a fed control rat. Semithin sections immunostained for **a** insulin, **b** GLUT2 glucose transporter and **c** glucokinase. This beta cell with a clear association to a capillary (*asterisk*) exhibits dense insulin and glucokinase immunoreactivity. The GLUT2 glucose transporter immunoreactivity is localized in the plasma membrane and in the cytoplasm. ×800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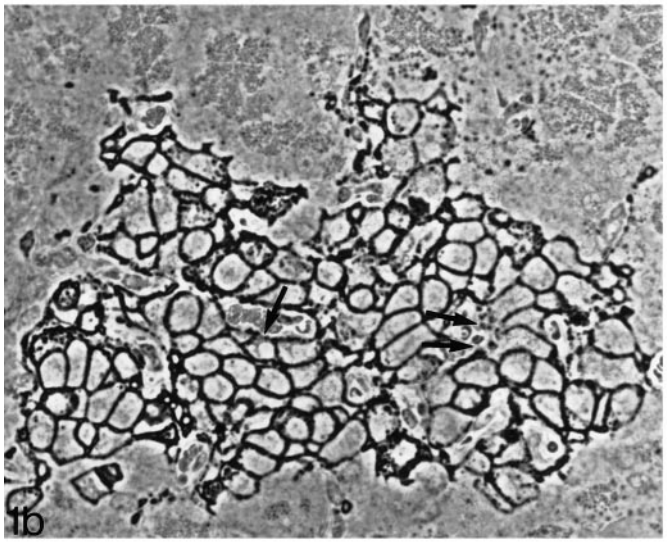
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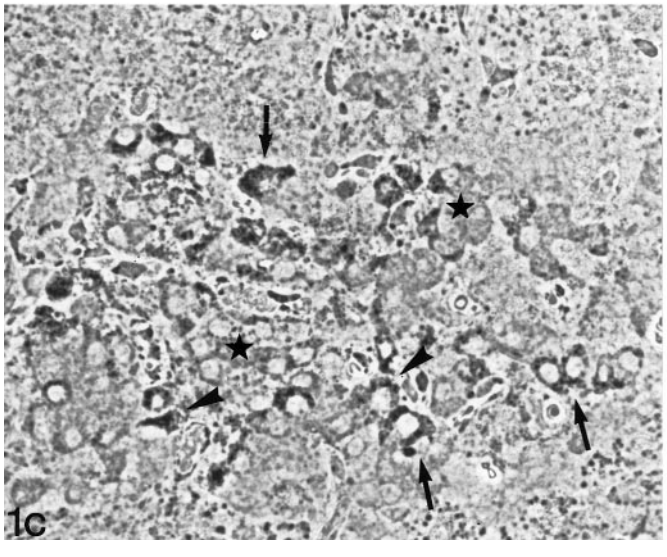
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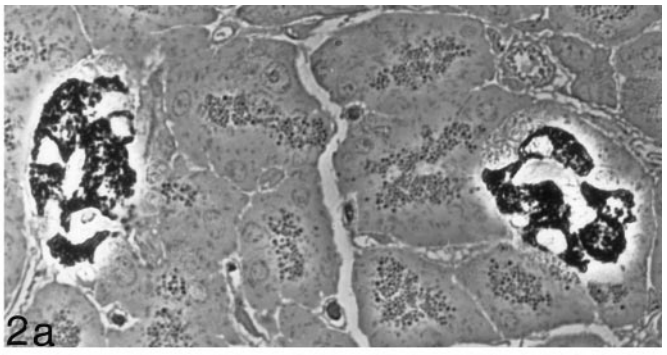
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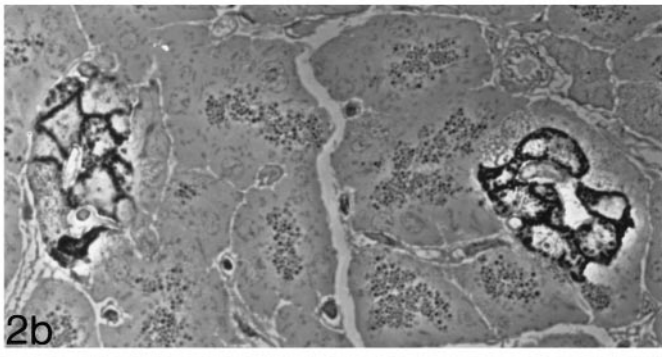
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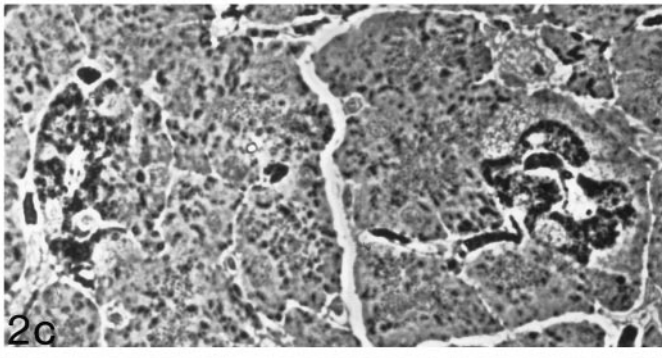
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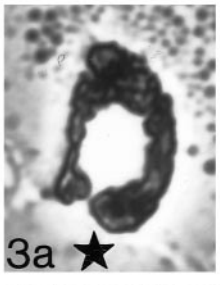
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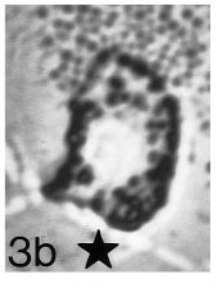
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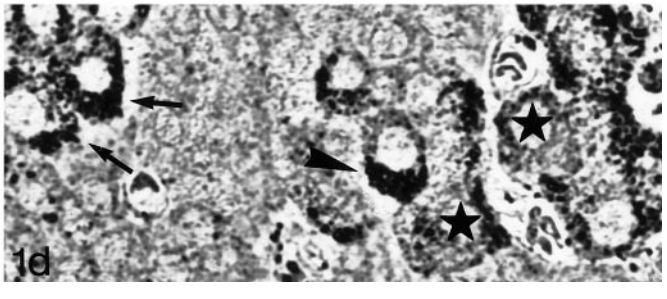
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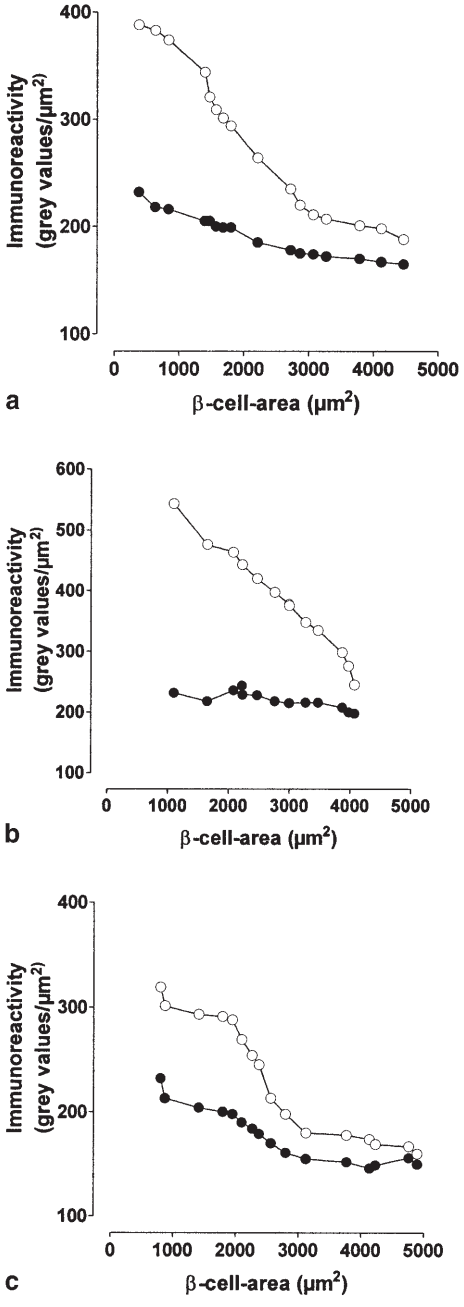


Fig. 4 Density of the insulin (○) and glucokinase (●) immunoreactivity was determined densitometrically in beta cells of rat pancreatic sections of different size **a** from fed control animals, **b** from 48 h-fasted animals, and **c** from fasted animals subsequently refed for 24 h. *Curves* show the immunoreactivity (grey values/µm²) for insulin and glucokinase in dependence upon the beta cell area of the islet (µm²). Each *point* represents an islet from one of the four pancreases studied in each group. There was a significant correlation between the islet size-dependent decrease of the insulin and glucokinase immunoreactivities in fed (r=0.99), fasted (r=0.97), and refed (r=0.98) rats

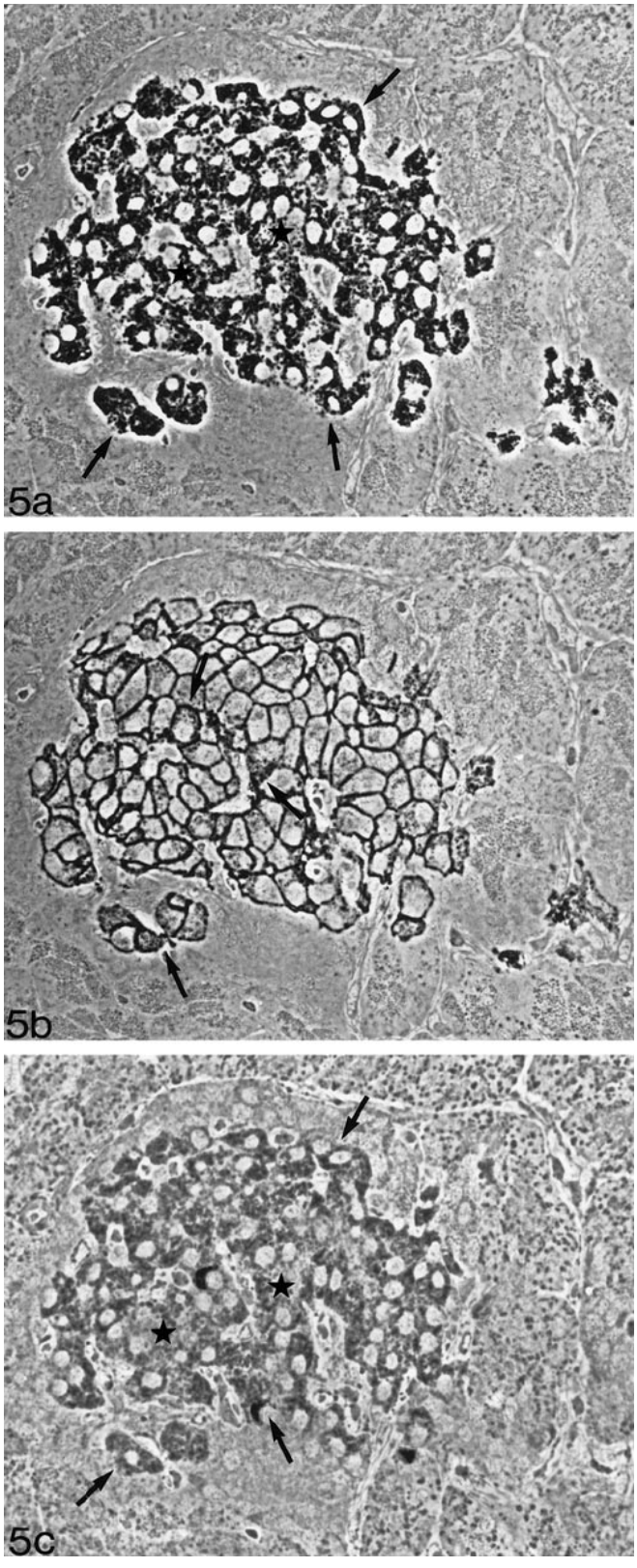


Fig. 5a–c Medium-sized pancreatic islet of a fasted rat. Semithin sections immunostained for **a** insulin, **b** GLUT2 glucose transporter and **c** glucokinase. Both the cortical beta cells (*arrows*) and the medullary (*asterisks*) beta cells exhibit dense insulin immunoreactivity and homogeneously distributed dense glucokinase immunoreactivity. GLUT2 glucose transporter is found on the plasma membrane and additionally in the cytoplasm (*arrows*). ×425

Table 1 Quantitative analysis of insulin and glucokinase immunoreactivities in beta cells from rat pancreas following different experimental conditions. Density of the insulin and glucokinase immunoreactivities was determined densitometrically in islet sections of different size from 19 islets obtained from pancreases of 4 control animals (total beta cell area: 42,678 μm^2), from 17 islets from pancreases of 4 fasted (48 h) animals (total beta cell area: 46,185 μm^2), and from 17 islets from pancreases of 4 fasted animals subsequently refed for 24 h (total beta cell area: 42,270 μm^2. Beta cell area of the islets (μm^2), blood glucose concentration (mM) and grey values of the immunoreactivities (grey values/μm^2) are given as mean values ± SEM.

	Blood glucose (mM)	Pancreatic islet beta cell area (μm^2)	Insulin (grey values/μm^2)	Changes (%)	Glucokinase (grey values/μm^2)	Changes (%)
Fed (control)	5.8±0.3	2246±256	277±15		191±4	
Fasted	3.8±0.2 **	2716±211	389±19 **	+40	215±5 **	+12
Refed	5.9±0.3	2721±306	236±15 *	−14	177±6 *	−17

* $P<0.05$ and ** $P<0.01$ (compared with fed control values)

dense intensity (Fig. 1c). In beta cells with contact to intra-islet capillaries glucokinase immunoreactivity had a polar orientation, with the highest density in the cytoplasmic region close to the pericapillary space (Fig. 1d). Most of the beta cells with faint glucokinase immunoreactivity also exhibited faint insulin immunoreactivity. In smaller islets (Fig. 2c) and in single beta cells at extra-islet sites (Fig. 3c) homogeneously distributed dense glucokinase immunoreactivity only was detected. Total glucokinase immunoreactivity, like insulin immunoreactivity, decreased significantly with increasing islet size (Fig. 4). There was a significant correlation between the decreases in the immunoreactivities for glucokinase and for insulin (Fig. 4).

GLUT2 glucose transporter immunoreactivity was detected only in beta cells (Figs. 1b, 2b); all other islet cell types and the exocrine parenchyma were negative (Figs. 1b, 2b). Irrespective of the islet size GLUT2 immunoreactivity was restricted to the plasma membrane of the beta cells (Fig. 2b). Small gaps in GLUT2 immunoreactivity were sometimes visible in plasma membrane domains close to the intra-islet capillary system (Fig. 1b), indicating exocytotic events. Single beta cells at extra-islet sites also exhibited cytoplasmic GLUT2 staining (Fig. 3b). Homogeneous GLUT2 immunostaining in the plasma membrane did not correlate with the heterogeneous distribution of insulin and glucokinase immunoreactivity in the different areas of the islets.

Beta cells in the fasting rat pancreas

Fasting of rats for 48 h caused a significant increase of insulin immunoreactivity amounting on average to 40%, in the beta cells of all pancreatic islets irrespective of their size (Fig. 5a, Table 1). Only very large islets (<500 μm diameter) still contained beta cells with faint insulin immunostaining in the central region of the islet. Smaller islets and single beta cells at extra-islet sites displayed dense insulin immunoreactivity exclusively.

In parallel with the increase in insulin immunoreactivity, the glucokinase immunoreactivity increased slightly (by 12% on average) in the beta cells of all pancreatic islets irrespective of their size after a fasting period of 48 h (Fig. 5c, Table 1). Only very large islets (<500 μm diameter) still exhibited heterogeneous immunostaining for glucokinase in the central region of the islet. Otherwise, homogeneous dense immunostaining only was detectable in the beta cells. Importantly, starvation resulted in a loss of polar high-density glucokinase immunostaining in pericapillary regions of beta cells.

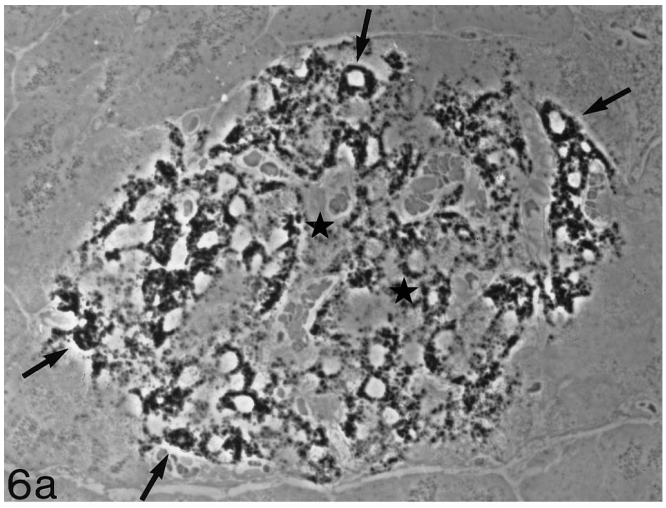
GLUT2 glucose transporter immunoreactivity was detectable in beta cells as continuous immunostaining along the plasma membrane and also in the cytoplasm (Fig. 5b).

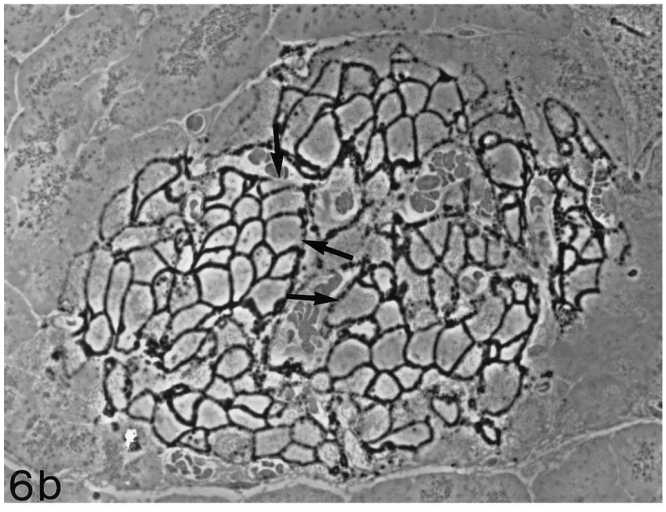
Beta cells in the refed rat pancreas

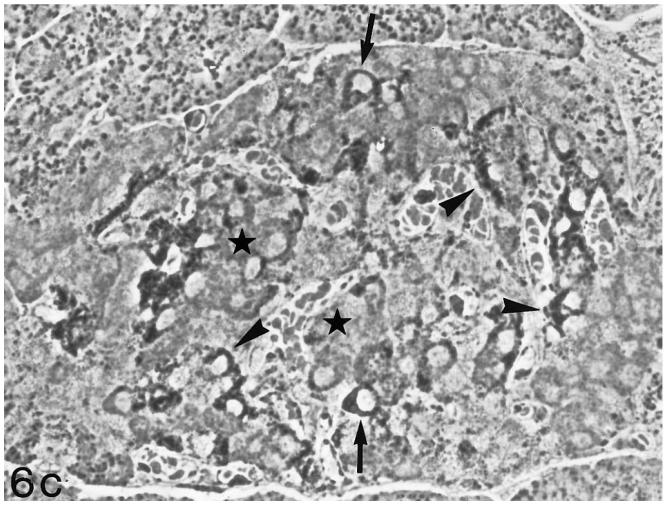
After refeeding for 24 h following a 48-h fast, heterogeneous insulin immunostaining in the beta cells of the pancreas reappeared (Fig. 6a). Refeeding reversed the effects of starvation, with a decrease in insulin immunoreactivity in the beta cells of all pancreatic islets (Fig. 4) amounting to an average of 14% compared with controls (Table 1). Heterogeneity was even more pronounced than under fed control conditions. Only in very small pancreatic islets (Fig. 7a) and in single beta cells at extra-islet sites did insulin immunostaining remain dense.

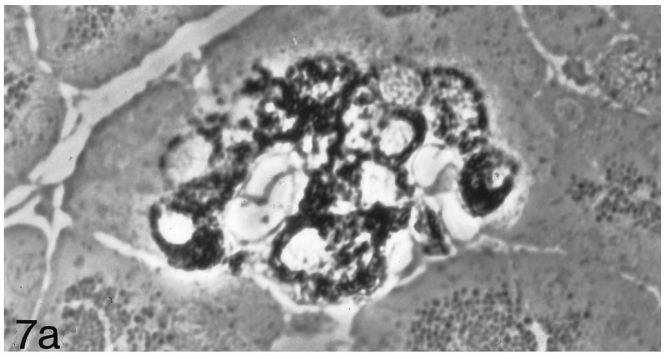
In parallel with the decrease in the insulin immunoreactivity, glucokinase immunoreactivity also decreased, by an average of 17%, in the beta cells of all pancreatic islets irrespective of their size after a 24-h refeeding period (Figs. 6c, 7c, Table 1). Glucokinase heterogeneity was even much more pronounced than under fed control conditions. At variance with the observed effects of refeeding on insulin immunoreactivity, glucokinase was heterogeneous even in the smallest islets composed of no more than 10–20 beta cells (Fig. 7c). Only single beta cells at extra-islet sites retained dense glucokinase immunostaining.

Refeeding for 24 h again abolished the cytoplasmic GLUT2 glucose transporter staining that had been induced through starvation. GLUT2 glucose transporter immunoreactivity in the plasma membrane of the beta cells was retained, but was often broken up by small gaps in the GLUT2 staining, in particular in plasma membrane domains with close contact to the intra-islet capillary system (Figs. 6b, 7b), indicating an increase of exocytotic events.









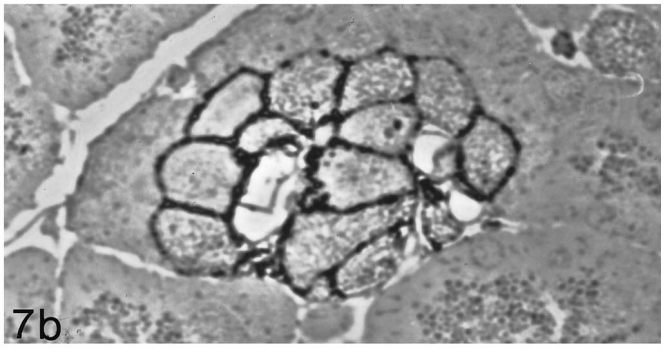


Fig. 6a–c Medium-sized pancreatic islet of a refed rat. Semithin sections immunostained for **a** insulin, **b** GLUT2 glucose transporter and **c** glucokinase. Cortical (*arrows*) and medullary (*asterisks*) beta cells in the same islet exhibit differences in the insulin immunoreactivity. The GLUT2 glucose transporter is restricted to the plasma membrane with gaps in close relation to the intra-islet capillary system (*arrows*). The cytoplasmic glucokinase immunostaining shows differences between beta cells (*asterisks*: homogeneously distributed faint; *arrows:* homogeneously distributed dense or *arrowsheads:* polar orientation) as in cells from fed controls. ×425

Fig. 7a–c Small pancreatic islet of a refed rat. Semithin sections immunostained for **a** insulin, **b** GLUT 2 glucose transporter and **c** glucokinase. Most of the pancreatic beta cells exhibit a dense insulin immunoreactivity, and GLUT2 glucose transporter immunoreactivity is restricted to the plasma membrane. The glucokinase immunoreactivity in the cytoplasm of beta cells exhibits very faint staining. ×600

Discussion

The present study confirms heterogeneous immunohistochemical insulin staining in the beta cells of the islets of Langerhans in the fed rat [9, 22, 25]. The analysis of beta cells from fasted and refed rats demonstrated that an increase in insulin immunoreactivity under fasting conditions is the morphological correlate of a decreased insulin secretory demand, and a corresponding decrease after refeeding, the correlate of increased functional activity. Starvation caused a loss of heterogeneity, resulting in homogeneous insulin staining of all beta cells. Refeeding resulted in a reappearance of heterogeneity with a preferential loss of insulin staining of the medullary beta cells

in the centre of the islet. This indicates that depending on the functional demand, the endocrine pancreas is able to recruit an increasing number of beta cells according to a well-defined hierarchy. There is an initial preferential mobilization of insulin from medullary beta cells in the centre of the larger islets, and only thereafter are cortical beta cells in the periphery of the large islets and finally beta cells from smaller islets activated.

There is evidence from studies on isolated beta cells that the responsiveness of the individual beta cell is determined by the function of the glucose recognition apparatus [5, 16]. The observation of heterogeneous glucokinase immunostaining in beta cells from fed control rats confirms an earlier observation by Jetton and Magnuson [8]. The analysis of different levels of metabolic and secretory activity shows that the state of functional activity is characterized by a distinct pattern of glucokinase immunoreactivity. A heterogeneous pattern of glucokinase immunostaining with areas of polarized high density in beta cells, in particular in association with blood vessels, under conditions of feeding and refeeding can be correlated functionally with high glucokinase enzyme activities [3, 13–15]. The high density of glucokinase immunoreactivity in plasma membrane areas in contact with blood vessels may be explained by an interaction with the GLUT2 glucose transporter, as suggested by functional studies in bioengineered insulin-producing cell lines [7]. Small gaps in the GLUT2 glucose transporter immunostaining in the plasma membrane domains close to the intra-islet capillary system reflect exocytotic events in the same membrane regions of these stimulated beta cells. This indicates that the membranes of the secretory granules that fuse with the plasma membrane during exocytosis have no GLUT2 glucose transporter in their membrane. Intracellular GLUT2 glucose transporter staining visible in the cytoplasm of beta cells under fasting conditions is thus apparently not associated with secretory granules. Rather, it may represent a functional reserve of stored GLUT2 in nonstimulated cells. The presence of intracellular glucokinase spots in functionally active beta cells is in accordance with the concept of metabolic channelling of glycolysis through a concentration of the protein in a multienzyme complex [1, 17, 24]. During starvation the homogeneous smooth distribution of glucokinase immunostaining in pancreatic beta cells, which is functionally associated with a decreased glucokinase enzyme activity [14], may be due to dissociation from complexes of sequential glycolytic enzymes.

Changes in insulin and glucokinase immunoreactivity in pancreatic beta cells after a 48-h starvation or a 24-h refeeding period represent long-term adaptations to different nutritional states. They can be distinguished from short-term translocation processes of glucokinase from the nucleus into the cytoplasm of the hepatocyte [27, 28] and from a perinuclear localization to the cytoplasm in pancreatic beta cells after acute glucose stimulation [20], which are observed within 1 h. At variance from the situation observed in the liver, nuclear immunostaining of glucokinase in pancreatic beta cells was observed neither in our study nor in any of the earlier studies [2, 8, 20], which indicates that the mechanisms of intracellular glucokinase translocation are different in these two tissues. This is not surprising, as a glucokinase-binding protein similar to the glucokinase regulatory protein, which is associated with the translocation of the enzyme between nucleus and cytoplasm in the hepatocyte [29, 30], could not be detected in pancreatic beta cells.

Nevertheless, intracellular translocation of glucokinase can play a significant part in the adaptation of the pancreatic beta cell to the different functional demands depending on the nutritional status. Our results show that changes in the spatial pattern of intracellular glucokinase distribution, rather than major changes in the absolute amounts of the enzyme protein [3, 15], parallel changes in the nutrient status of the animals and represent the mechanism of adaptation of pancreatic beta cells to varying functional demands. The observation that on refeeding even the beta cells in the smallest islets regained a heterogeneous distribution of glucokinase immunoreactivity (Fig. 7c) while insulin immunoreactivity was still homogeneous (Fig. 7a), as is typical for unstimulated beta cell, indicates that the changes in glucokinase immunoreactivity precede the observed changes in insulin immunoreactivity. Association of glucokinase with GLUT2 glucose transporter in the plasma membrane in areas of glucose recognition can be regarded as the morphological correlate of glucose signal recognition through metabolic activation of glucokinase and resultant initiation of insulin secretion.

Acknowledgements The skilful technical assistance of H. Peesel is gratefully acknowledged. This study has been supported by grants from the Stiftung “Das Zuckerkranke Kind” and the German Diabetes Association to A.J.

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