Virchows Arch. A. Path. Anat. and Histol. 379, 203–217 (1978)

Virchows Archiv A

Subcellular B Cell Calcium and Insulin Secretion in vitro

Comparative Ultracytochemical Studies after Glucose Stimulation and Cyproheptadine Inhibition*

G. Bommer, H.G. Joost, and G. Klöppel

Institute of Pathology, University of Hamburg (Dir.: Prof. Dr. G. Seifert) and Institute of Pharmacology and Toxicology, University of Göttingen (Dir.: Prof. Dr. A. Hasselblatt)

Summary. Using the ultracytochemical pyroantimonate technique different patterns of calcium containing precipitates were found in the B cells of the isolated perfused rat pancreas under conditions of stimulated and inhibited insulin secretion. The calcium specificity of the ultracytochemical method was assessed by perfusion with a EGTA containing calcium-free medium, which markedly reduced the extent of precipitation. Perfusion with 20 mM D-glucose over a period of 30 min resulted in calcium distribution patterns which could be related to the biphasic insulin release. The calcium patterns differed significantly in their quality and quantitative morphometry from those after 5 mM D-glucose or cyproheptadine (CPH) perfusion (20 mM D-glucose plus 0.1 mM CPH). After 3–5 min of 20 mM glucose perfusion there was an increased calcium precipitation along the inner side of the B cell membranes. After 20–30 min an additional increase in precipitation was found in the cytoplasmic matrix and in the secretory granules. B cells in a CPH-inhibited state of secretion and also after perfusion with 5 mM glucose lacked these findings. The data suggest that an increase in the membrane associated calcium may induce the first phase of insulin secretion by triggering the exocytosis of peripheral granules, while the cytoplasmic calcium may be involved in long term regulation of insulin release.

Key words: B cell calcium in vitro – Calcium ultracytochemistry – Biphasic insulin secretion – Glucose stimulation – Cyproheptadine inhibition.

It has been shown that extracellular calcium ions are an essential requirement for the process of glucose-stimulated insulin release (Grodsky and Bennet, 1966; Milner and Hales, 1967; Curry et al., 1968; Hales and Milner, 1968). The exact mode and site of action within the process of stimulus-secretion coupling,

* Supported by Deutsche Forschungsgemeinschaft, KL 366/1

For offprints contact: G. Klöppel, Institut für Pathologie der Universität Hamburg, Martinistr. 52, D-2000 Hamburg 20, Federal Republic of Germany

0340-1227/78/0379/0203/$03.00

however, is so far not known. In order to localize subcellular calcium binding sites in the pancreatic B cell ultracytochemical and autoradiographic techniques have been applied to the endocrine pancreas (Herman et al., 1973; Schäfer and Klöppel, 1974; Howell and Tyhurst, 1976). Using the ultracytochemical pyroantimonate method it was found that the distribution patterns of calcium-rich precipitates depend on the functional state of the B cell (Herman et al., 1973; Schäfer and Klöppel, 1974; Ravazzola et al., 1976; Klöppel, 1977; Klöppel et al., 1978)

Further information on the subcellular localization of calcium in exact relation to B cell function was expected from combined morphologic and kinetic studies on insulin secretion in vitro. The isolated perfused rat pancreas was, therefore, used to examine the calcium distribution in B cells during glucose stimulated insulin secretion and its inhibition by cyproheptadine (CPH). The antihistaminicum CPH directly interferes with insulin secretion in vivo and in vitro (Wold et al., 1971; Joost et al., 1974) without disturbing the glucose metabolism of the B cell (Joost et al., 1976).

Material and Methods

Experimental Design

Male Wistar rats (Versuchstierzucht Hannover) weighing 150–250 g were anesthetized by intraperitoneal injection of pentobarbital (45 mg/kg body weight). Using a slight modification of the method of Grodsky et al. (1963) pancreas, spleen, stomach and the upper duodenum with their arterial and venous supply including the portal vein were surgically isolated. The common hepatic artery was ligated so as to avoid an uncontrollable effluent. After sufficient perfusion via the cannulated abdominal aorta was guaranteed, the preparation was transferred to the perfusion chamber. The perfusion medium consisted of Krebs-Henseleit buffer with 4% dextran. Furthermore, 5 mM D-glucose, 20 mM D-glucose or 20 mM D-glucose plus 0.1 mM cyproheptadine hydrochloride (Sharpe and Dohme, München) were added according to the experimental design. For assessing the calcium specificity of the pyroantimonate technique the isolated pancreas was perfused with a calcium-free medium which contained ethyleneglycol-2(2-aminoethyl)tetraacetic acid (EGTA; 5 mM). The medium was kept in an atmosphere of 95% O_2 and 5% CO_2, at 37° C and at pH 7.4. The flow was constant (4 ml/min) and provided a perfusion pressure of 80–120 mm Hg. The perfusate effluent from the portal vein was collected at intervals of 1 min. The insulin content was measured radioimmunologically (Zaharko and Beck, 1968). With regard to equilibration, preparations were perfused 30 min before each experiment in groups I to III with a medium containing 5 mM D-glucose and 60 min before each experiment in group IV with a calcium-free medium containing EGTA. Four experimental groups were assorted: I. Perfusion with 5 mM D-glucose ($n=24$), II. Perfusion with 20 mM D-glucose ($n=24$), III. Perfusion with 20 mM D-glucose and CPH ($n=24$), and IV. Perfusion with 20 mM D-glucose in calcium-free EGTA medium ($n=6$). In groups I to III the perfusion intervals were 3, 5, 7, 10, 20 and 30 min ($n=4$ for each time interval). In group IV the perfusion intervals were 3 and 20 min ($n=3$ for each time interval).

Morphological Procedure

Each perfusion interval was immediately followed by at least 5 min perfusion fixation with ice cold solution consisting of 3% glutaraldehyde and 2% potassium pyroantimonate, pH 7.3. Thereafter, pieces of the pancreas were postfixed 2 h in potassium pyroantimonate. After subsequent rinsing in a solution of 2% potassium pyroantimonate and 5% sucrose (pH 7.3), tissue was postfixed for 1 h in a solution of 1% OsO_4 and 2% potassium pyroantimonate (pH 7.3) and then washed

3 times in 7% sucrose. After dehydration in graded ethanol and embedding in Epon 812, ultrathin sections were cut on a Reichert ultramicrotome OM U2, picked up on copper grids, stained with uranylacetate and lead citrate. Examination was carried out under a Zeiss electron microscope EM 10.

Quantitative Evaluation of Precipitation Patterns

By morphometric analysis precipitates were evaluated which were associated with the following structures: cell membrane, halos of the secretory granules, mitochondria, cytoplasm including rough endoplasmic reticulum and Golgi apparatus, and the nucleus. Preparations from the groups, which were perfused 3–5 min and 20–30 min, were evaluated forming altogether 6 subgroups. 24 electron micrographs with a final magnification of 20700, were taken at random from 6 islets of 4–6 preparations in each subgroup. This corresponds to an area of 1080 μm^2 per subgroup. Altogether, 144 micrographs were evaluated. The number of cell structures associated with pyroantimonate precipitates was counted, using a point counting system (total number of points on the transparent grid 255; grid constant 0.45 μm). The number of hits was expressed as a percentage (mean ± SD) of the total number of points on the grid. Statistical significance was tested by the U-test of Wilcoxson, Mann and Whitney.

Results

Perfusion with 5 mM Glucose (Group I)

During 2–30 min of perfusion there was a constant basal insulin secretion rate (Fig. 1). On electron microscopy the B cells contained numerous secretory gran-

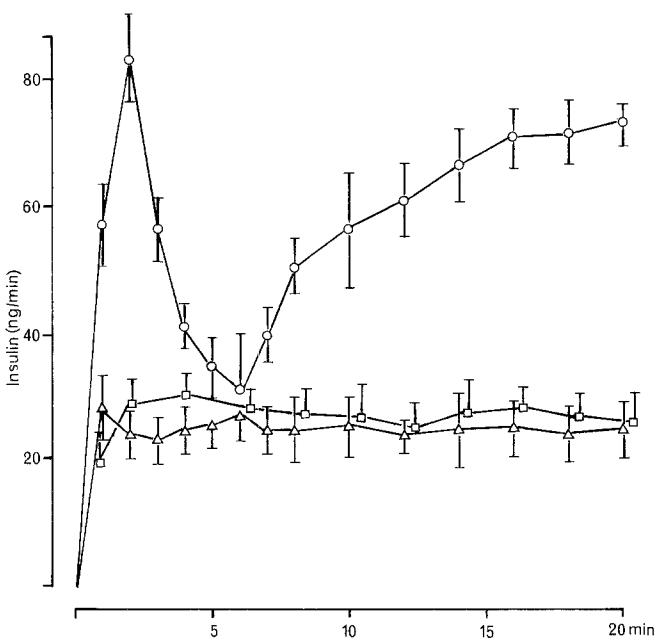

Fig. 1. Insulin secretion during perfusion with 5 mM glucose (□), 20 mM glucose (○) and 20 mM glucose plus 0.1 mM Cyproheptadine (△)

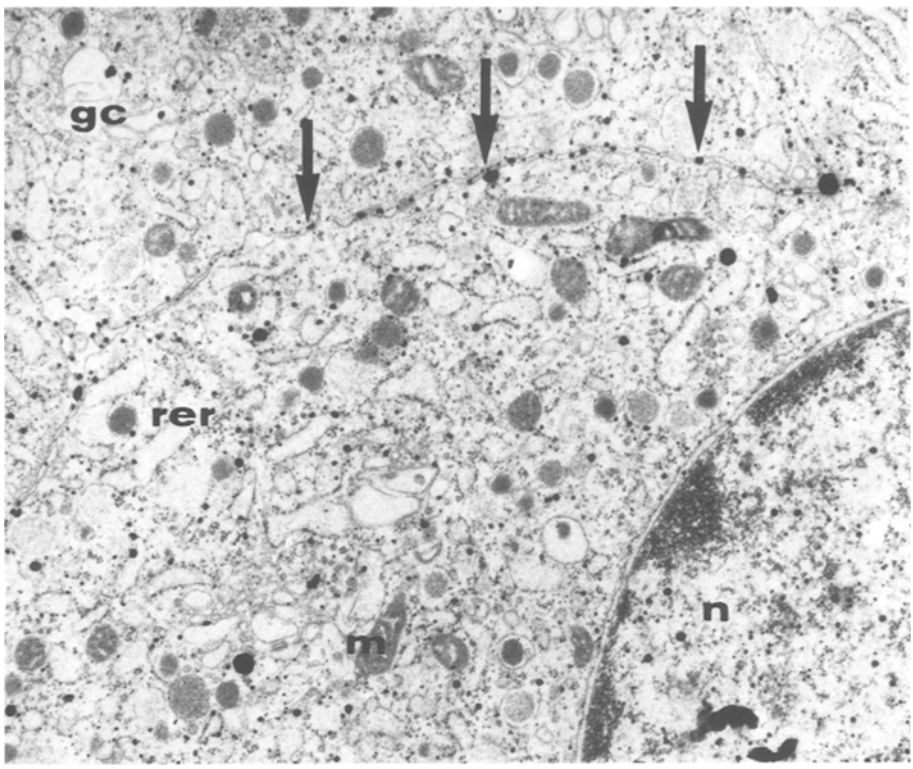

Fig. 2. B cell 3 min after 5 mM glucose perfusion. Pyroantimonate precipitates are scattered in the cytoplasm without any preferential localization. Only single precipitates along the cell membranes (arrows). Golgi complex (*gc*), rough endoplasmic reticulum (*rer*), mitochondria (*m*) and nucleus (*n*). K-pyroantimonate. × 20,700

ules scattered in the cytoplasm. Only few granules were located on the cell membrane. The rough endoplasmic reticulum (RER) and the Golgi apparatus were slightly dilated. Because of the relatively high perfusion pressure the intercellular spaces were often enlarged.

The pyroantimonate precipitation in the B cells was constant in its extent and distribution during the whole time period examined. Small electron dense precipitates were associated with the inner and outer side of the cell membrane, the granule halos, the Golgi complexes, the RER, the cytoplasmic matrix and the nucleus (Fig. 2 and 4). No preferential localization was noted.

Perfusion with 20 mM Glucose (Group II)

Perfusion with 20 mM glucose evoked the typical biphasic pattern of insulin secretion (Fig. 1). Electron microscopy revealed a normal morphology of the B cells after 5–10 min. After 20–30 min the number of secretory granules seemed to be slightly reduced. The RER and Golgi cisternae were somewhat dilated.

After 3–5 min of perfusion pyroantimonate precipitation was strikingly accentuated on the cell membranes of the B cells (Fig. 3), preferably along their

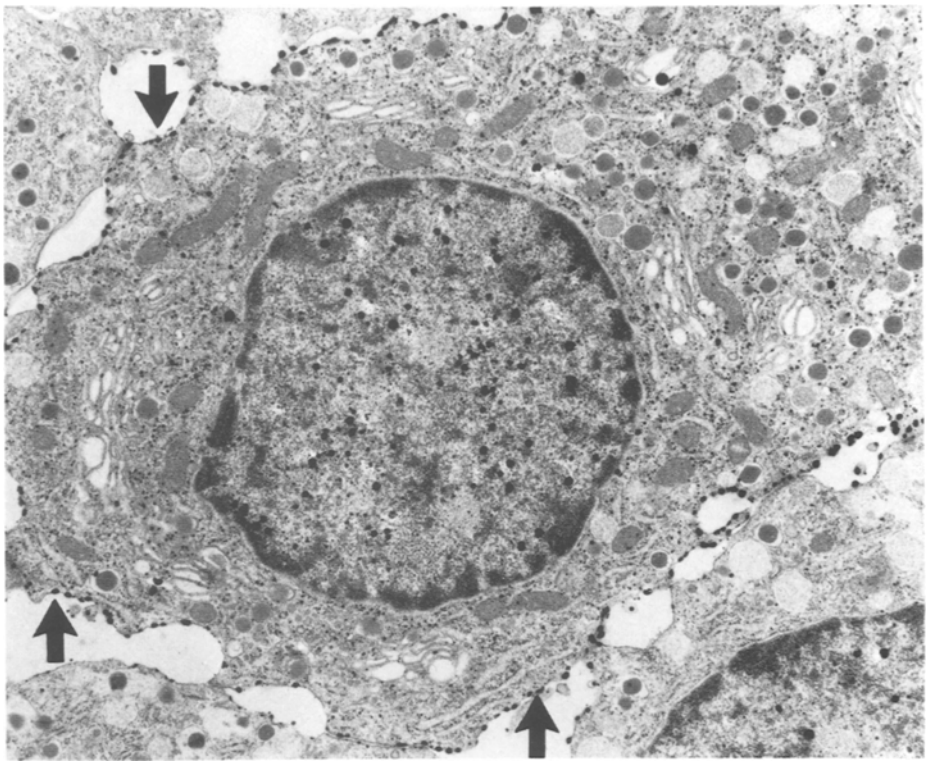

Fig. 3. B cell after 3 min after 20 mM glucose perfusion. Distinct precipitation along the inner side of the cell membrane (arrows). K-pyroantimonate. × 9800

inner faces (Fig. 4). Some precipitates appeared to connect the inner side of the cell membrane and the surface of adjacent secretory granules, thus representing a link between these structures (Fig. 5). After 20–30 min the deposits on the B cell membranes were slightly reduced, whereas the number of precipitates in the granule halos and, particularly, in the cytoplasmic matrix including the RER and the Golgi complexes was increased (Fig. 6). The precipitation in the mitochondria remained unchanged.

Perfusion with 20 mM Glucose and 0.1 mM CPH (Group III)

CPH abolished the insulin secretion rate in response to 20 mM glucose (Fig. 1). After 3 and 5 min of perfusion the fine structure of the B cells showed a dilated RER and Golgi apparatus (Fig. 7). Thereafter, no progress of these cystic changes was obvious. The number of the secretory granules remained constant. The structure of the mitochondria was normal. After 10 min of perfusion multigranulated bodies appeared indicating an increased crinophagic activity of the B cell (Fig. 8).

The pyroantimonate precipitation in the B cells did not change significantly at any of the times examined. The small deposits were scattered in the cytoplasm

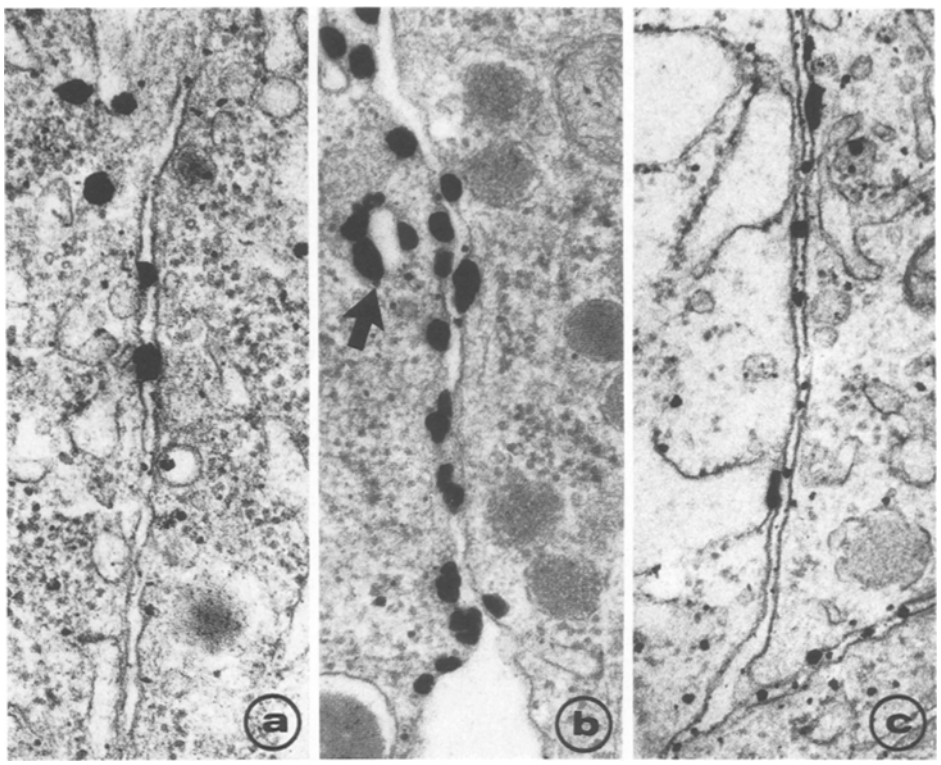

Fig. 4. a–c Membrane sections of B cells **a** 3 min after perfusion with 5 mM glucose. Single calcium labels at the outer side of the cell membrane. **b** 3 min after perfusion with 20 mM glucose. Many calcium labels on the inner leaflet of the cell membrane and on endocytotic vesicles (arrow). **c** 3 min after perfusion with 20 mM glucose plus 0.1 mM Cyproheptadine. Only single calcium labels on the cell membrane. K-pyroantimonate. × 56,000

without any particular localization (Fig. 7). Only single precipitates were observed along the inner and outer side of the cell membranes (Fig. 4). After 30 min of perfusion the total number of precipitates appeared to be reduced (Fig. 8). Single deposits were found in enlarged RER cisternae.

Perfusion with 20 mM Glucose in Calcium-Free Medium Containing EGTA (Group IV)

As expected there was a striking inhibition of the insulin secretion at all times examined (3 min: 8 ng/min ± 3 insulin; 20 min: 5 ng/min ± 2 insulin). Ultracytochemically the number of precipitates was markedly reduced in all cells of the isolated rat pancreas. In the B cells only few small precipitates remained (Fig. 9). No preferential deposition was observed.

Quantitative Analysis of Precipitate Patterns (Fig. 10)

Quantitation of the precipitate distribution after 3–5 min of perfusion with 20 mM glucose revealed a significantly increased deposition of precipitates on

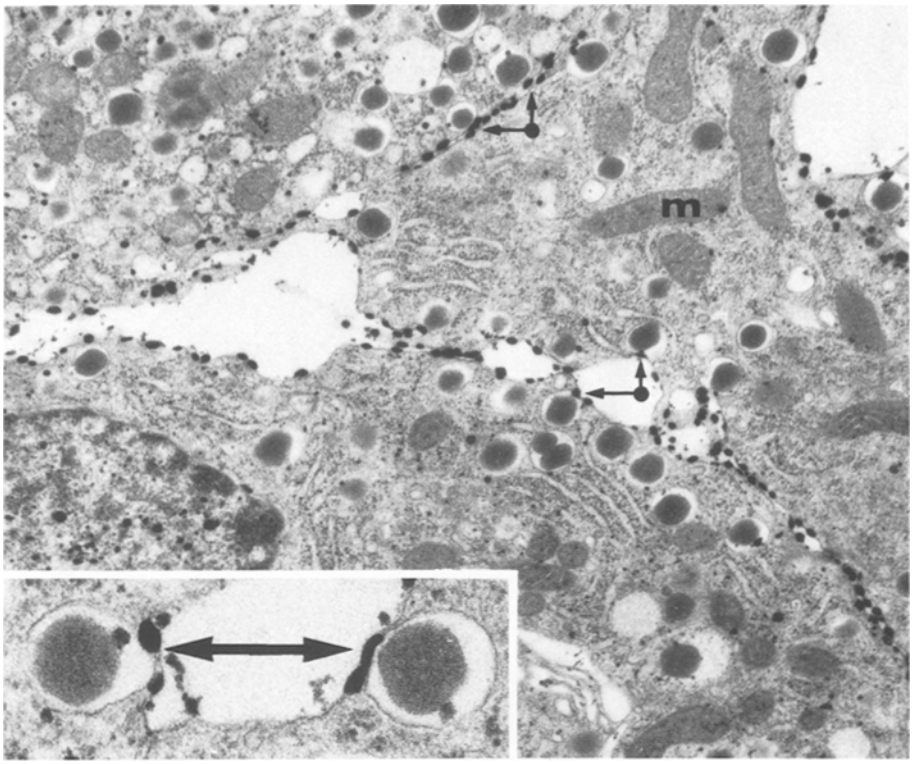

Fig. 5. B cells 3 min after 20 mM glucose perfusion. Intensive cell membrane labelling by precipitates and margination of secretory granules. Membranes of granules which appear to undergo exocytosis are linked to the cell membrane by precipitates (arrows, inlet). K-pyroantimonate. × 20,700 and × 56,000

the B cell membrane compared with 5 mM glucose and CPH. The precipitation at the other cell sites did not significantly differ in the single groups. The late phase (20–30 min) of 20 mM glucose-stimulated insulin secretion was still associated with an increased precipitation on the B cell membrane. Furthermore, increased numbers of deposits were found in secretory granules and in the cytoplasmic matrix. Distribution patterns in B cells after perfusion with 5 mM glucose or CPH were about equal at all time intervals.

Discussion

Glucose-stimulated insulin secretion is associated with a significant uptake and intracellular accumulation of 45-calcium in pancreatic islets (Hellman et al., 1971; Malaisse-Lagae and Malaisse, 1971; Malaisse, 1973; Hellman et al., 1976a). With regard to the compartmentation and sites of accumulation of calcium in various organelles, the ultracytochemical pyroantimonate technique of Komnick and Komnick (1963) was applied to the endocrine pancreatic cells. This resulted in a deposition of electron dense precipitates, which proved to

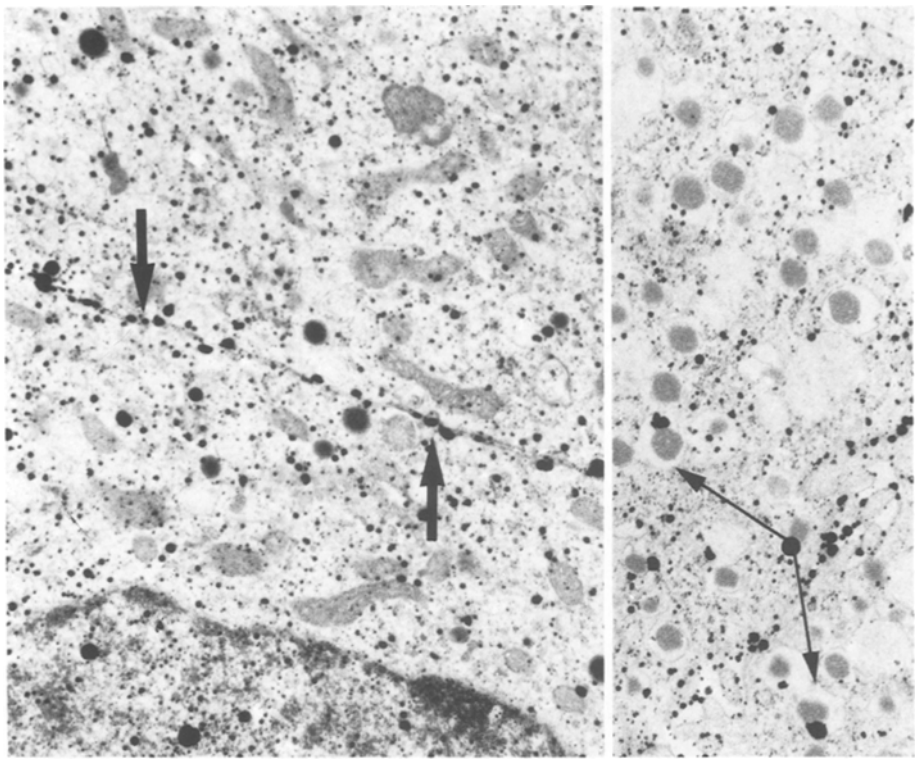

Fig. 6. Parts of B cells 20 min after 20 mM glucose perfusion. Numerous small precipitates in the cytoplasmic matrix. Increased numbers of precipitates on the cell membranes (arrows left) and in granule halos (arrows right). K-pyroantimonate. ×20,700

be rich in calcium, as was repeatedly shown by X-ray microanalysis and the use of the calcium chelator agent EGTA (Herman et al., 1973; Klöppel and Schäfer, 1973; Klöppel and Schäfer, 1976; Ravazzola et al., 1976; Boquist, 1977). Whether these precipitates indicate the primary localization of free or bound calcium and/or a secondary calcium deposition due to a cytochemical adsorption to certain proteins, cannot be clearly decided. However, the close association of the calcium deposits with structures of functional importance and the dependence of the precipitate distribution on the functional state of the B cell suggest that these precipitates are indicators of the original accumulation sites of calcium in the B cell.

20 mM glucose-stimulated insulin secretion in the prefused rat pancreas was associated with two different and subsequently developing calcium distribution patterns. Their time course was strongly correlated with the biphasic secretion curve. These patterns were in contrast to those seen after perfusion with CPH. In the early phase the calcium distribution pattern under glucose stimulation differed markedly from that after CPH inhibition in the number of calcium deposits along the cell membrane, whereas the calcium affinity of the cytoplasmic binding sites was similar in both conditions. An involvement of cytoplasmic

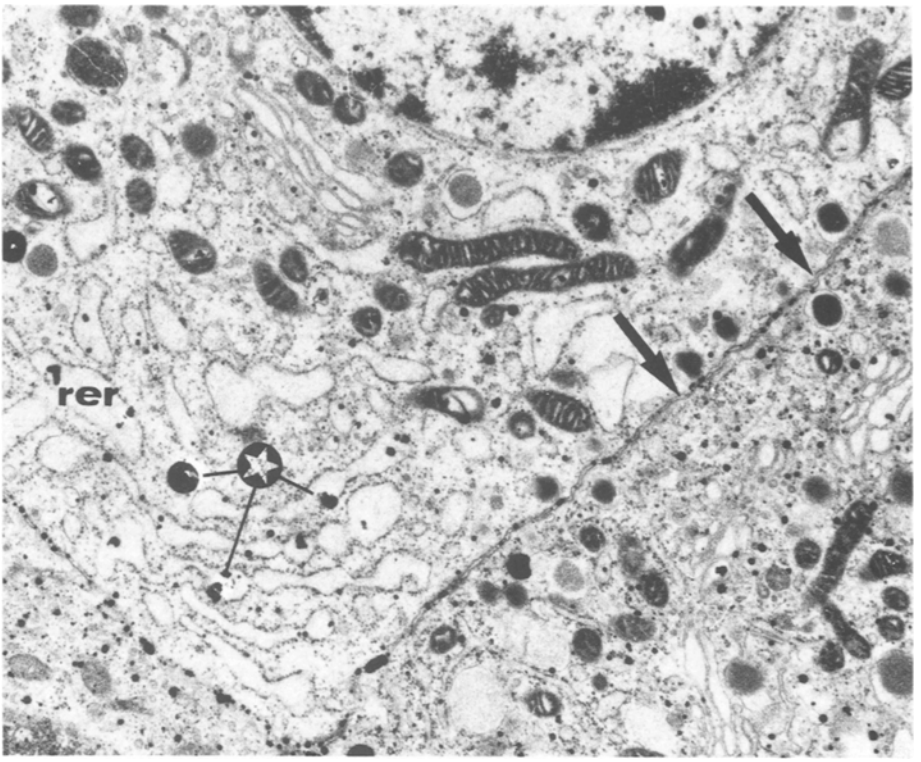

Fig. 7. B cell 3 min after perfusion with 20 mM glucose plus 0.1 mM cyproheptadine (inhibited insulin secretion). Sparse labelling of the cell membrane by precipitates (arrows). Some globular precipitates (asterisk) associated with dilated cisternae of the rough endoplasmic reticulum (*rer*). K-pyroantimonate. ×20,700

calcium pools in the acute regulation of insulin secretion seems, therefore, to be less probable.

The calcium deposits on the cell membrane and, particularly, the precipitates linking cell and granule membranes suggest a direct involvement of calcium in the mechanism of granule discharge. It is conceivable that the membrane associated calcium initiates the fusion from cell and granule membranes by physical and/or chemical interactions. Investigations on isolated secretory granules of the islets demonstrated the need for calcium in the fusion of membrane systems (Dahl and Gratzl, 1976). As proposed by Dean and Matthews (1975) calcium is thought to overcome the electrostatic repulsive forces between negatively charged granule and cell membranes. Dean (1974a, b) demonstrated a negative surface charge on islet cell granules. In chromaffine granules these negative charges could be reduced by cations (Matthews, 1972). By means of cationic ferritin labelling, Howell and Tyhurst (1976) found anionic groups on the surface of secretory granules as well as on the outside of pancreatic B cell membranes. At the moment of discharge these groups appeared to be reduced on the granule surface. Thus, taken together there is accumulating

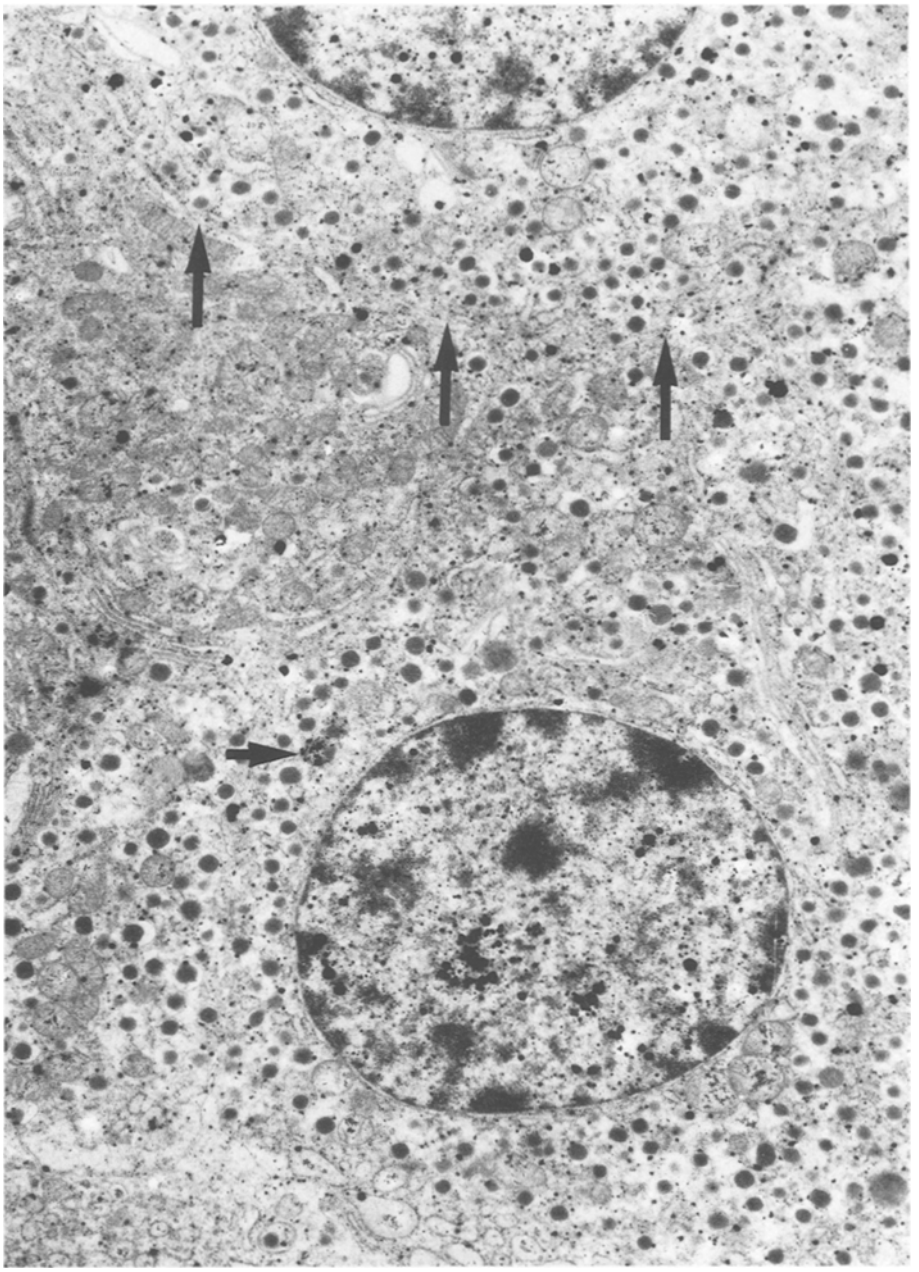

Fig. 8. B cells 20 min after perfusion with 20 mM glucose plus 0.1 mM cyproheptadine (inhibited insulin secretion). Preserved granularity of the B cells. No membrane labelling by precipitates (long arrows). Only few precipitates in the cytoplasmic matrix and the secretory granules. Heavy precipitates within a multigranulated body (short arrow). K-pyroantimonate. ×9800

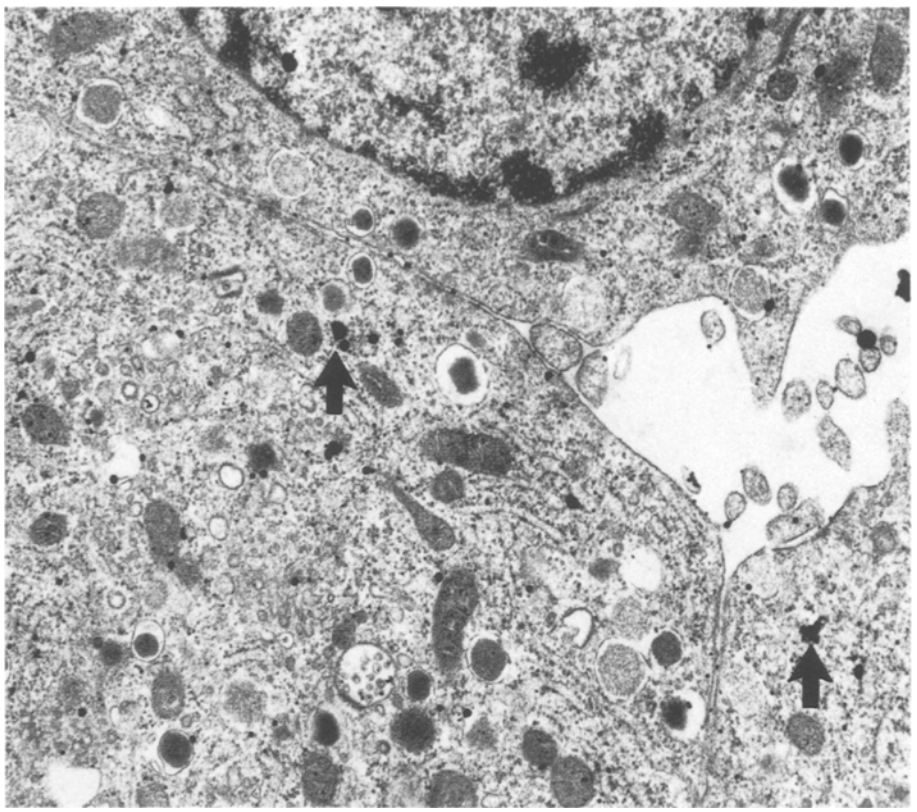

Fig. 9. B cells 20 min after perfusion with 20 mM glucose in calcium free medium containing EGTA (inhibited insulin secretion). Striking reduction of the number of precipitates at all cell sites. Remaining precipitates are mainly localized in the cytoplasmic matrix (arrows). K-pyroantimonate. ×20,700

evidence from morphologic and electrophysiologic studies that calcium may participate in the initial stages of exocytosis (Fig. 11).

In the late phase of glucose-stimulated insulin release, calcium deposits increased in the secretory granules and, particularly, in the cytoplasmic matrix of the B cells. Former studies in vivo (Schäfer and Klöppel, 1974; Klöppel et al. 1978) and in vitro (Ravazzola et al., 1976; Bloom et al., 1977) showed calcium to be accumulated mainly in secretory granules, but not in the cytoplasmic matrix. This was, however, observed after longer periods (90–120 min) than in the present study. It is thus tempting to speculate that after longer intervals of glucose stimulation calcium transfer had taken place from the cytoplasmic matrix into the secretory granules, either by permeation of granule membranes or by incorporation into granule halos during granule formation.

Cytoplasmic accumulation of calcium in the late phase, which significantly exceeded the morphometric values of the early phase, points to the possible functional importance of this compartment for the delayed insulin release. The

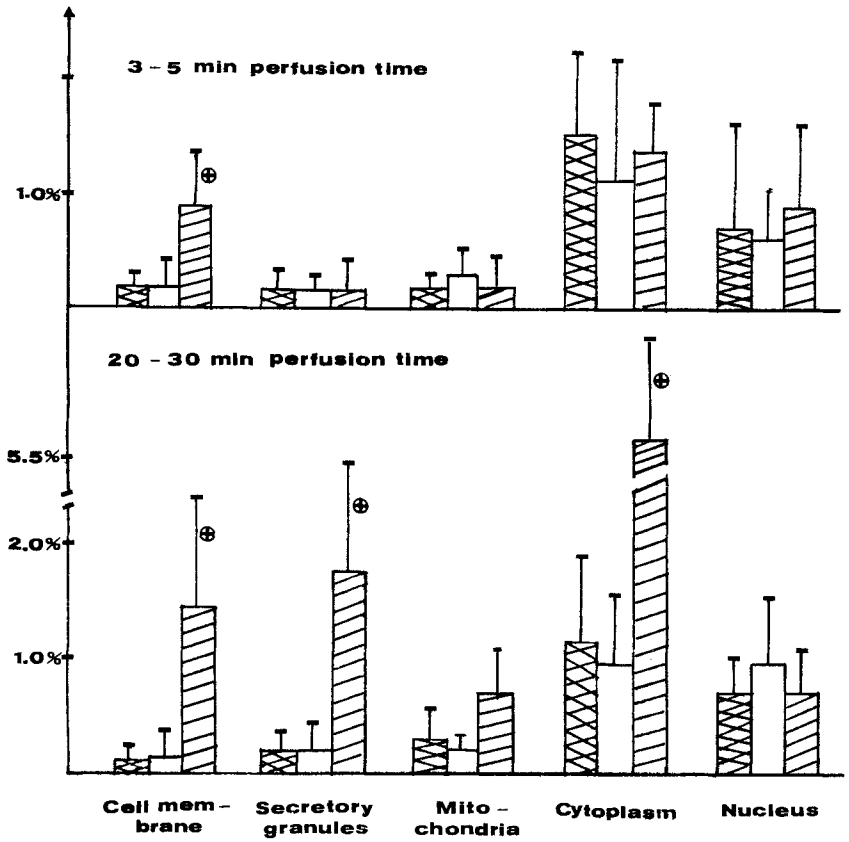

Fig. 10. Quantitative subcellular distribution of calcium pyroantimonate precipitates in B cells in vitro after perfusion with 20 mM glucose plus 0.1 mM cyproheptadine (crossed bars), with 5 mM glucose (open bars) and with 20 mM glucose (hatched bars). The number of organelles or structures associated with precipitates is expressed as a percentage (mean value ± SD) of the total number of points on the lattice. $\oplus < 0.01$

compartment might correspond with a proposed cytoplasmic calcium pool, the rise of which is thought to activate a microtubular-microfilamentous system for transporting centrally localized granules to the cell membrane (Malaisse, 1973). CPH which inhibited both phases of glucose-induced insulin secretion concurrently abolished the cytoplasmic calcium accumulation during the late phase. Thus, there is some indirect evidence that the cytoplasmic calcium compartment might be involved in the long term regulation of insulin release (Fig. 11).

The increased calcium deposition in the secretory granules during the late phase of insulin secretion may correspond with a lanthanum-nondisplaceable calcium pool in B cells (Hellman et al., 1976a, b; Sehlin, 1976; Bloom et al., 1977). It is supposed that this pool is involved in an intracellular sequestration mechanism for calcium which is accumulated during the stimulatory process. The kinetic inertia of the pool points to its involvement in long term regulation insulin secretion. However, so far there is no plausible concept for any specific role of this calcium pool in the secretory mechanism. It is, therefore, likely

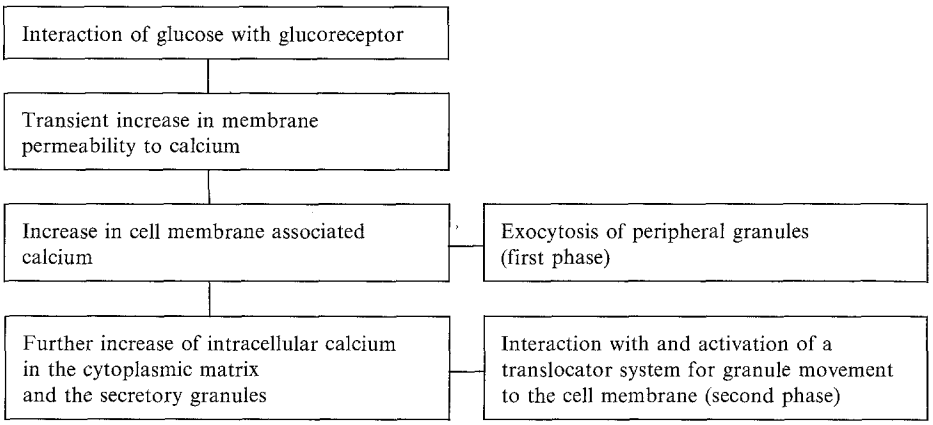

Fig. 11. Hypothetical scheme of calcium compartmentation in B cells and its relation to the stimulus-secretion coupling in biphasic insulin release

that calcium sequestration in granule saccules represents only a concomitant mechanism accompanying insulin secretion.

Concerning the action of CPH in insulin secretion our data showed that CPH interferes with the normal calcium handling of the B cell. CPH inhibited all changes of subcellular calcium distribution which were associated with glucose-stimulated insulin release. Moreover, long term in vivo studies in mice and rats and our in vitro studies revealed that CPH affects the biosynthetic apparatus of the B cell by altering the RER (Wold et al., 1971; Longnecker et al., 1972; Amherdt et al., 1973; Klöppel, 1977). It is, however, unlikely that blocking of the phasic insulin secretion by CPH in vitro is due to the drug effect on the biosynthetic function of the cell, since the inhibition of insulin secretion already occurs before the earliest defect in insulin biosynthesis should become manifest (Howell et al., 1969; Orci et al., 1973; Richardson et al., 1975).

In conclusion, our ultracytochemical data suggest that the most important compartment of calcium, related to the immediate response of glucose-stimulated insulin secretion, is localized at the inner side of the cell membrane. This calcium compartment may directly participate in the exocytotic process. The functional role of the cytosolic and granule compartment, which become conspicuous in the late insulin secretion phase, has to be further elucidated.

Acknowledgements. We wish to thank Dr. N. Stahnke, Dept. of Pediatrics, University of Hamburg, for helpful cooperation in this work. We are also indebted to Ms. Katrin Baack and Mrs. Anke Vos for skilful technical assistance.

References

Amherdt, M., Humbert, F., Rabinovitsch, A., Orci, L., Renold, A.E.: Alterations of the insulin-secretory process by cyproheptadine: a correlative ultrastructural and biochemical study. In: Abstracts' VII Congress of the International Diabetes Federation. Amsterdam, Excerpta Medica, International Congress Series **280**, 1 (1973)

Bloom, G.D., Hellman, B., Sehlin, J., Täljedal, I.-B.: Glucose-stimulated and La^{3+}-nondisplaceable Ca^{2+} pool in pancreatic islets. Amer. J. Physiol. **232**, E114–E118 (1977)

Boquist, L.: Pancreatic islets subjected to different concentrations of glucose in vitro. Virchows Arch. B cell Path. **23**, 219–226 (1977)

Curry, D.L., Benett, L.L., Grodsky, G.M.: Requirement for calcium ion in insulin secretion by the perfused rat pancreas. Amer. J. Physiol. **214**, 174–178 (1968)

Dahl, G., Gratzl, M.: Calcium-induced fusion of isolated secretory vesicles from the islet of Langerhans. Cytobiologie 12, 344–355 (1976)

Dean, P.M.: The electrokinetic properties of isolated secretory particles. In: Secretory mechanisms of exocrine glands (ed. N.A. Thorn and D.H. Peterson) pp. 152–161. Copenhagen: Munksgaard 1974a

Dean, P.M.: Surface electrostatic-charge measurements on islet and zymogen granules: effect of calcium ions. Diabetologia **10**, 427–430 (1974b)

Dean, P.M., Matthews, E.K.: The London-Van der Waals attraction constant of secretory granules and its significance. J. theor. Biol. **54**, 309–321 (1975)

Grodsky, G.M., Batts, A.A., Bennet, L.L., Vcella, C., McWilliams, N.B., Smith, D.F.: Effects of carbohydrates on secretion of insulin from isolated rat pancreas. Amer. J. Physiol. **205**, 638–644 (1963)

Grodsky, G.M., Bennet, L.L.: Cation requirements for insulin secretion in the isolated perfused pancreas. Diabetes **15**, 910–913 (1966)

Hales, C.N., Milner, R.D.G.: Cations and the secretion of insulin from rabbit pancreas in vitro. J. Physiol. (Lond.) **199**, 177–187 (1968)

Hellman, B., Sehlin, J., Täljedal, I.-B.: Calcium uptake by pancreatic β-cells as measured with the aid of ^{45}Ca and mannitol-^{3}H. Amer. J. Physiol. **221**, 1795–1801 (1971)

Hellman, B., Sehlin, J., Täljedal, I.-B.: Effects of glucose on $^{45}Ca^{2+}$ uptake by pancreatic islets as studied with the lanthanum method. J. Physiol, **254**, 639–656 (1976a)

Hellman, B., Sehlin, J., Täljedal, I.-B.: Calcium and secretion: distinction between two pools of glucose-sensitive calcium in pancreatic islets. Science **194**, 1421–1423 (1976b)

Herman, L., Sato, T., Hales, C.N.: The electron microscopic localization of cations to pancreatic islets of Langerhans and their possible role in insulin secretion J. Ultrastructure Research **42**, 298–311 (1973)

Howell, S.L., Kostianovsky, M., Lacy, P.E.: Beta granule formation in isolated islets of Langerhans: A study by electron microscope radioautography. J. Cell Biol. **42**, 695–705 (1969)

Howell, S.L., Tyhurst, M.: ^{45}Calcium localization in islets of Langerhans, a study by electron-microscopic autoradiography. J. Cell Biol. **21**, 415–422 (1976)

Howell, W.L., Tyhurst, M.: Distribution of anionic sites on surface of B cell granule and plasma membranes: a study using cationic ferritin. J. Cell Sci. **27**, 289–301 (1977)

Joost, H.G., Poser, W., Panten, U.: Inhibition of insulin release from the rat pancreas by cyproheptadine and tricyclic antidepressants. Naunyn-Schmiedberg's Arch. Pharmacol. **285**, 99–102 (1974)

Joost, H.G., Beckmann, J., Holze, S., Lenzen, S., Poser, W., Hasselblatt, A.: Inhibition of insulin and glucagon release from the perfused rat pancreas by cyproheptadine (periactinol^{R}, Nuran^{R}). Diabetologia **12**, 201–106 (1976)

Klöppel, G., Schäfer, H.-J.: Die Bedeutung des Calciumtransportes für die Funktion der B-Zelle des Inselsystems. Verh. Dtsch. Ges. Path. **57**, 471 (1973)

Klöppel, G., Schäfer, H.-J.: Effects of sulfonylureas on histochemical and ultracytochemical calcium distribution in B cells of mice. Diabetologia **12**, 227–235 (1976)

Klöppel, G.: Functional pathomorphology of the pancreatic B cell system. Ultrastructural and calcium-cytochemical studies on insulin biosynthesis and secretion. Progress in Pathology 108. (W. Büngeler, M. Eder, K. Lennert, G. Peters, W. Sandritter, G. Seifert, eds.) Stuttgart-New York: Fischer 1977

Klöppel, G., Bommer, G., Ruttmann, E., Schäfer, H.H.: Qualitative and semiquantitative calcium cytochemistry in B-cells of mice treated with cyproheptadine and mannoheptulose. Acta endocr. **87**, 786–798 (1978)

Komnick, H., Komnick, U.: Elektronenmikroskopische Untersuchungen zur funktionellen Morphologie des Ionentransportes in der Salzdrüse von Larus argentatus. Z. Zellforsch. **60**, 163–203 (1963)

Longnecker, D.S. Wold, J.S., Fischer, L.J.: Ultrastructural alterations in B-cells of pancreatic islets from cyproheptadine treated rats. Diabetes **21**, 71–79 (1972)

Malaisse-Lagae, F., Malaisse, W.J.: Stimulus secretion coupling of glucose-induced release. III. Uptake of ^{45}calcium by isolated islet of Langerhans. Endocrinology **88**, 72–80 (1971)

Malaisse, W.J.: Insulin secretion: multifactorial regulation for a single process of release. Diabetologia **9**, 167–173 (1973)

Matthews, E.K., Evans, R.J., Dean, P.M.: The ionogenic nature of the secretory-granule membrane. Electrokinetic properties of isolated chromaffine granules. Biochem. J. **130**, 825–832 (1972)

Milner, R.D.G., Hales, C.N.: The role of calcium and magnesium in insulin secretion from rabbit pancreas studied in vitro. Diabetologia **3**, 47–49 (1967)

Orci, L., Like, A.A., Amherdt, M., Blondel, B., Kanazawa, Y., Marliss, E.B., Lambert, A.E., Wollheim, C.B., Renold, A.E.: Monolayer cell culture of neonatal rat pancreas: an ultrastructural and biochemical study of functioning endocrine cells. J. Ultrastruct. Res. **43**, 270–297 (1973)

Ravazzola, M., Malaisse-Lagae, F., Amherdt, M., Perrelet, A., Malaisse, W.J., Orci, L.: Patterns of calcium localization in pancreatic endocrine cells. J. Cell Sci. **27**, 107–117 (1976)

Richardson, B.P., McDaniel, M., Lacy, P.E.: Effects of cyproheptadine on insulin secretion by isolated perfused rat islets. Diabetes **24**, 836–841 (1975)

Schäfer, H.-J., Klöppel, G.: The significance of calcium in insulin secretion. Ultrastructural studies on identification and localization of calcium in activated and inactivated B cells of mice. Virchows Arch. A Path. Anat. and Histol. **362**, 231–245 (1974)

Sehlin, J.: Calcium uptake by subcellular fractions of pancreatic islets. Effects of nucleotides and theophylline. Biochem. J. **156**, 63–69 (1976)

Wold, J.S., Longnecker, D.S., Fischer, L.J.: Species dependent pancreatic islet toxicity produced by cyproheptadine. Toxicol. Appl. Pharmacol. **19**, 188–201 (1971)

Zaharko, D.S., Beck, L.V.: Studies of a simplified plasma insulin immunoassay using cellulose powder. Diabetes **17**, 444–457 (1968)

Received February 4, 1978