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Demonstration of Calcium in Pancreatic Islets*

Light-Microscope Observations in Activated and Inactivated B Cells of Mice

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Summary. The GBHA [=glyoxal bis (2-hydroxyanil)] technique allows light microscopic demonstration of mobile calcium in some soft tissues.

We used this method to compare granulation and calcium content of B cells of mice in the normal state and under conditions of suppression and of acute and chronic stimulation. For the suppression of B cells, hypoglycaemia was induced by the injection of bovine insulin. Acute stimulation of B cells by hyperglycaemia was achieved by a single injection of anti-insulin serum of guinea-pigs, and chronic stimulation by repeated injections of this antiserum for 4 days.

In untreated animals the GBHA reaction indicated high amounts of calcium located selectively within the cytoplasm of B cells. In contrast to the degree of granulation, the calcium content showed slight variations from islet to islet, which may indicate differences in the secretory activity of the different islets at the time of sacrifice. Cellular calcium seemed to be loosely bound; it was possible to remove it with the aid of aqueous fixation fluids, while the addition of oxalate caused it to be retained. The suppressed B cells showed marked granulation and a uniformly high calcium content. After acute stimulation, there was a slight decrease of granulation in some B cells; the amount of calcium varied from cell to cell and was markedly elevated in some. The differences in the animal groups were only discrete and demand further evaluation. In contrast, after chronic stimulation the B cells showed signs of hypersecretory degeneration, marked degranulation and an almost total loss of stainable calcium. In addition we observed slight insulitis, which is thought not to be a consequence of chronic stimulation, however, but a response to the deposition of immune complexes within the islet.

Our findings suggest that the high amounts of calcium detectable in the B cells by light microscopy depend predominantly on the degree of granulation. In addition, in cases of identical granulation an increased secretory activity seems to induce a slight elevation of cellular calcium.

Zusammenfassung. Mit GBHA [=Glyoxal bis (2-hydroxyanil)] ist in einigen Weichteilgeweben eine lichtmikroskopische Anfärbung des mobilen Calcium möglich.

Der Calciumgehalt der Pankreasinseln von Mäusen mit akut und chronisch stimulierten, supprimierten und normalen B-Zellen wurde mit dieser Methode vergleichend untersucht und mit dem Gehalt an B-Zell-Granula in Beziehung gebracht. Die B-Zell-Suppression mit ausgeprägter Hypoglycaemie erfolgte durch einmalige Injektion von Rinderinsulin. Eine akute Stimulierung der B-Zellen mit Hyperglycaemie wurde durch einmalige Gabe von Anti-Insulinserum vom Meerschweinchen 90 bzw. 180 min vor der Tötung, eine chronische Stimulierung ebenfalls mit Hyperglycaemie durch zweimal tägliche, insgesamt viertägige Gabe des Antiserums erzielt.

Mit der GBHA-Reaktion ließ sich bei den unbehandelten Tieren selektiv im Cytoplasma der B-Zellen ein hoher Calciumgehalt darstellen. Dieser variierte im Gegensatz zu dem Granulierungsgrad von Insel zu Insel etwas, was möglicherweise auf Unterschiede in der sekretorischen Aktivität der einzelnen Inseln zum Zeitpunkt der Tötung hinweist. Das celluläre Calcium ist locker gebunden, in wässerigen Fixierungsmitteln ausschwemmbar und durch Oxalat im Gewebe retinierbar. Die supprimierten B-Zellen zeigten bei starker Granulierung einen gleich-

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mäßig hohen Calciumgehalt, während die akut stimulierten B-Zellen vereinzelt bereits eine geringe Abnahme der Granulierung und einen von Zelle zu Zelle unterschiedlichen, teils deutlich vermehrten Calciumgehalt aufwiesen. Die Unterschiede in den vorgenannten Tiergruppen waren insgesamt nur gering und erfordern eine weitere Überprüfung. Im Gegensatz dazu zeigten die chronisch stimulierten B-Zellen neben den Zeichen der hypersekretorischen Degeneration eine starke Degranulierung und einen fast vollständigen Verlust des nachweisbaren Calciums. Außerdem trat eine mäßige Insulitis auf, die jedoch nicht als Folge der chronischen Stimulierung, sondern als Antwort auf die Ablagerung von Immunkomplexen in der Insel aufgefaßt wird.

Die Befunde zeigen, daß der lichtmikroskopisch nachweisbare hohe Calciumgehalt der B-Zelle in erster Linie von dem Granulierungsgrad abhängt. Bei gleicher Granulierung scheint darüber hinaus eine gewisse Zunahme des cellulären Calciumgehaltes bei steigender sekretorischer Aktivität aufzutreten.

The interaction of calcium with the secretory process of insulin is well established (Curry *et al.*, 1968; Rasmussen, 1970). The presence of calcium is essential for glucose-induced insulin release (Milner and Hales, 1967; Grodsky and Bennett, 1966). Elevation of the calcium level results in an increased insulin output (Heinze *et al.*, 1973), hypocalcemia in an inhibition of insulin release (Littledike *et al.*, 1968). Stimulation of the B cells proceeds with calcium fluxes at the level of cell membranes and several cell organelles (Dean and Matthews, 1970; Rasmussen, 1970; Rasmussen and Allen, 1973; Hales and Milner, 1968) resulting in altered levels of intracellular calcium (Malaisse-Lagae and Malaisse, 1971). If morphological demonstration of calcium within the B cell is possible, it would give the chance to get some information, whether data revealed by in vitro analysis of cell and cell organelle preparations are correct not only for isolated islets (Herman *et al.*, 1973) but also for the intact animal. This requires a histochemical technique, which is sensitive enough to detect the small intracellular calcium pools and which insolubilizes the mobile calcium in tissue during preparation.

In the following study we employed the very sensitive GBHA method (Kashiwa, 1966) for light microscopic demonstration of mobile calcium in the B cell of the mouse. At present this technique has been applied mainly on cartilage and bone (Kashiwa and Atkinson, 1963; Kashiwa and House, 1964; Kashiwa, 1970). We compared the GBHA staining reaction in activated and suppressed B cells and the degree of B cell granulation in the pancreatic islet of intact animals.

Materials and Methods

19 male inbred Wistar mice, weighing 25–30 g, were kept on constant conditions and fed an Altromin® standard diet and Aq. fontana ad libitum. They were divided in four groups:

Group 1. 4 mice were injected 0.5 mg crystalline bovine insulin (Hoechst) i.p. 30 minutes before sacrifice.

Group 2. 6 mice received 0.5 ml anti-insulin-serum of guinea pigs, immunized with bovine insulin (GPAIS). The GPAIS showed a marked insulin-neutralizing capacity in an bioassay. The injection was given into the tail vein 90 resp. 180 minutes before sacrifice.

Group 3. Intravenous injections of 0.5 ml GPAIS twice daily were applicated to 5 mice. They were killed after four days.

Group 4. 4 normal mice served as untreated controls.

Just before killing blood samples were collected in ether anaesthesia from the retroorbital vein plexus and the pancreas was removed. One part of the pancreas was stained with GBHA for calcium detection according to the method of Kashiwa (1966). Immediately after removal the tissue was immersed into a staining-fixation fluid, containing 0.1 g glyoxal bis (2-hydroxyanil) (GBHA) in 2 ml stock solution. The stock solution consisted of 3.4% NaOH in 75% ethanol. This staining-fixation fluid complexes and insolubilizes calcium instantly after immersion of the tissue. For better penetration the tissue was cut into small pieces. After fixation overnight the samples were rinsed in absolute ethanol, cleared in xylene and embedded in paraffin. Sections were dry mounted on albumenized slides by finger pressure. To ensure specificity of the method for calcium the sections were immersed in 90% ethanol, saturated with Na_2CO_3 and KCN. After rinsing in 95% and absolute ethanol, deparaffinizing and clearing in xylene the sections were mounted in Eukitt. Counterstaining was omitted for better visualization of weak staining reaction.

The other part of the pancreas was fixed in Bouin's fixative, dehydrated and embedded in paraffin. Sections were stained with H.E., PAS and Gomori's aldehyde fuchsin. For control of the calcium staining technique some sections of Bouin fixed tissue were stained for 15 minutes by the GBHA staining-fixation fluid and processed as above mentioned.

Some tissue samples were fixed in 2.5% cacodylate-buffered glutaraldehyde with addition of 1% sodiumoxalate. Paraffin sections were also stained with the GBHA solution and processed in the same way.

Blood glucose levels were determined employing the glucose oxidase method (Boehringer 15983 TBAD).

Results

Blood glucose values, intensity of calcium staining reaction and degree of B cell granulation are given in Table 1.

Table 1. Survey of the results

	Treatment	Blood glucose [mg-%] mean values (variation ranges)	B-cells	
			Secretory granulation	Calcium content (GBHA reaction)
Group 1	0.5 mg bovine insulin 30 min a.e.	70 (60–95)	+++	++
Group 2	0.5 ml GPAIS 90 min a.e.	258 (230–340)	++	++
	180 min a.e.	283 (250–400)	++/+	+++/+
Group 3	0.5 ml GPAIS 8 × during four days	262 (220–330)	(+)	(+)
Group 4	untreated controls	144 (120–170)	++	++/+

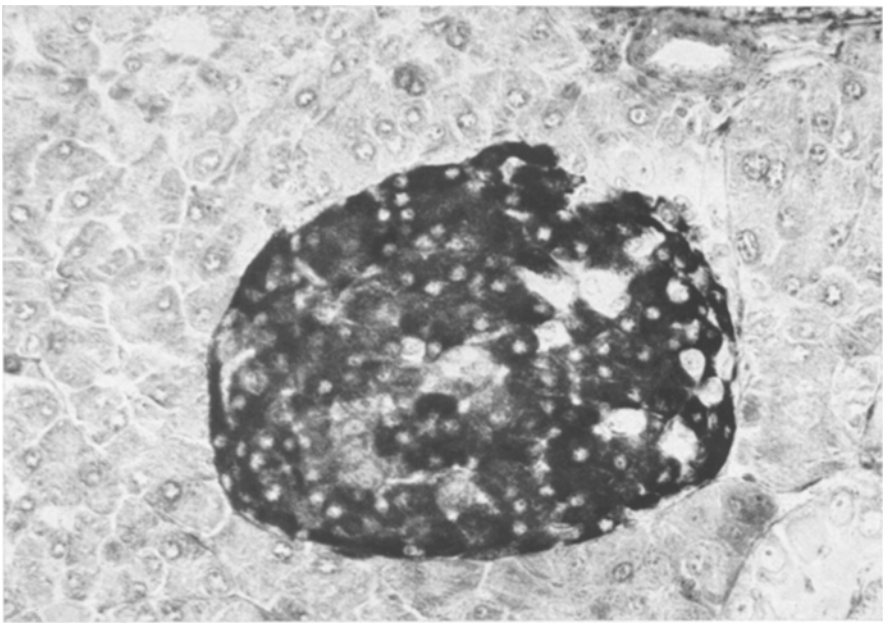

Fig. 1. Untreated mouse; pancreatic islet (group 4): Distinct granulation of B cells. Aldehyde fuchsin. × 375

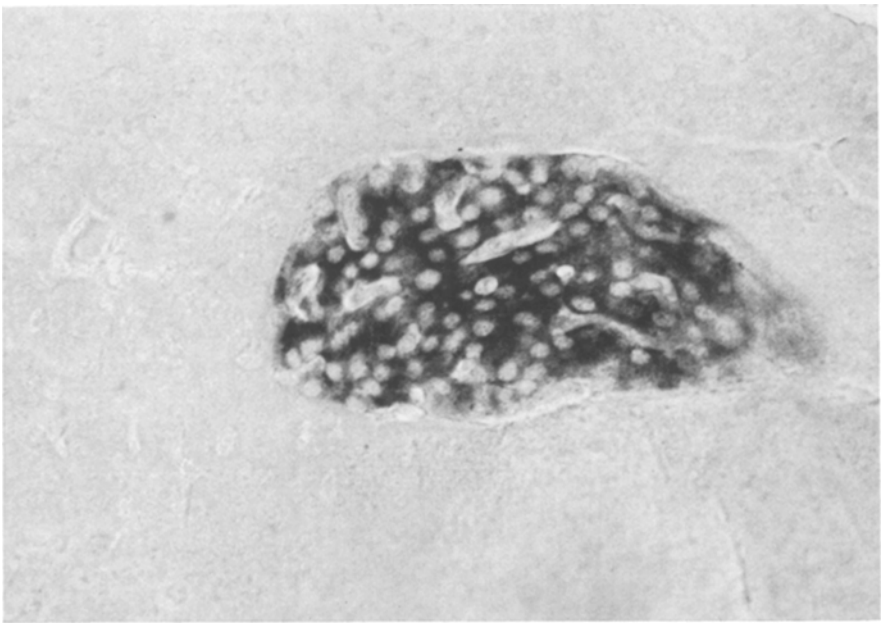

Fig. 2. Untreated mouse (group 4): High calcium content within cytoplasm of B cells; nuclei are unstained; negative reaction of the surrounding exocrine tissue. GBHA, no counterstaining. × 375

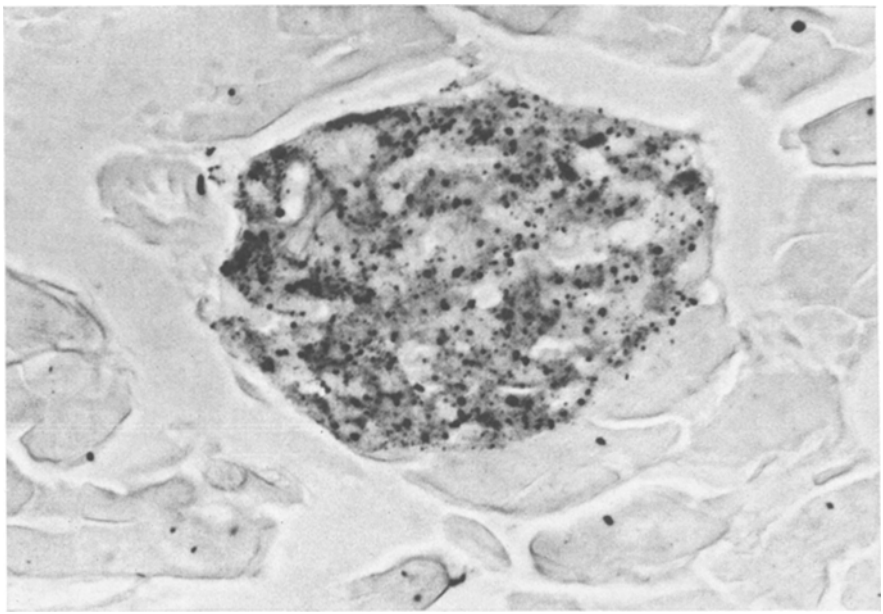

Fig. 3. Untreated mouse (group 4): Retention of coarse calcium precipitates within the islet after addition of oxalate to the aqueous fixation fluid. Bouin + oxalate fixation, GBHA. × 375

The untreated animals (*Group 4*) showed an intense red GBHA staining of the islets (Fig. 2). The staining was granular and diffused in the cytoplasm of the B cells. The nuclei were unstained. The A cells appeared to be unstained and the surrounding exocrine tissue was nearly unstained, too. Although each islet showed a significant positive reaction, the intensity of staining differed from islet to islet. The B cells within each islet, however, showed a nearly uniform intensity. The staining of B cell granules revealed an overall good and uniform granulation (Fig. 1).

Paraffin sections of Bouin fixed pancreatic tissue and subsequent GBHA staining revealed a completely negative reaction. This suggests a complete loss of calcium during fixation in an aqueous medium. Addition of sodiumoxalate to the fixation fluid and the dehydration media resulted in an at least partly retention of calcium in the tissue with coarse granular red material within the B cells after staining with GBHA (Fig. 3). This observation confirms the calcium specificity of the intense GBHA reaction in the islets.

After inactivation of the B cells by application of insulin (*Group 1*) the animals developed a marked hypoglycaemia. The B cells showed in all islets an increased and homogeneous granulation. The GBHA reaction revealed in all islets a distinct reaction of all B cells. The broad cytoplasm seams were diffusely stained.

90 and 180 minutes after application of GPAIS for acute activation (*Group 2*) the animals developed a significant hyperglycaemia. The B cell granulation was unchanged after 90 minutes; after 180 minutes single B cells showed a slightly decreased content of secretory granules (Fig. 4). The sinusoids and blood vessels

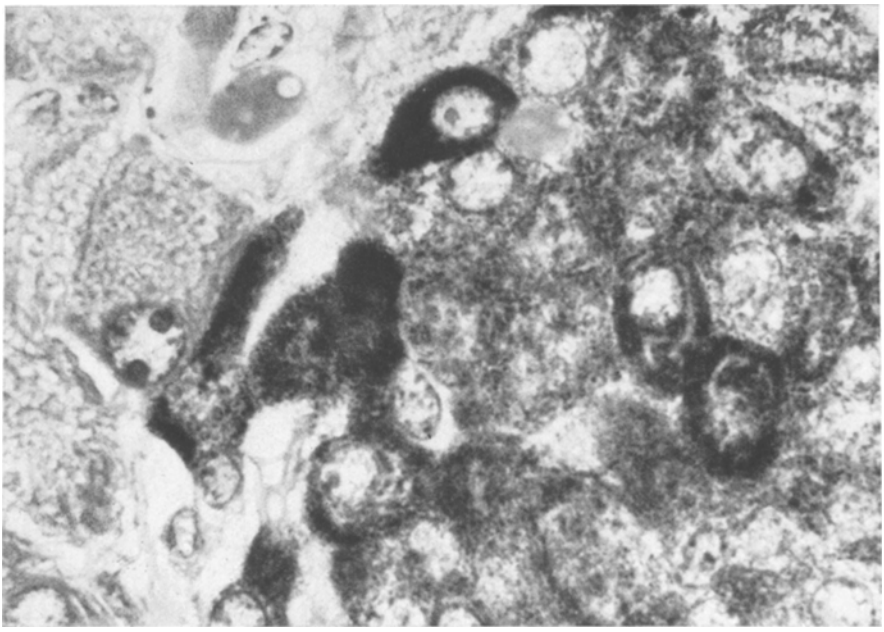

Fig. 4. Acute stimulation (180 minutes after GPAIS; group 2): B cells with varying granulation of the cytoplasm. Aldehyde fuchsin. × 1200

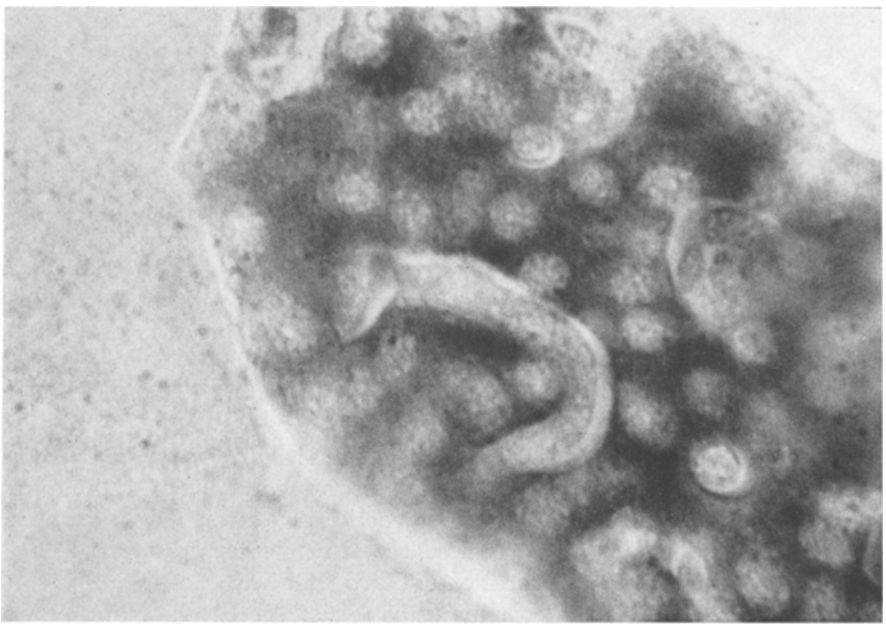

Fig. 5. Acute stimulation (180 minutes after GPAIS; group 2): Varying calcium content of the B cells within one islet. GBHA. × 1200

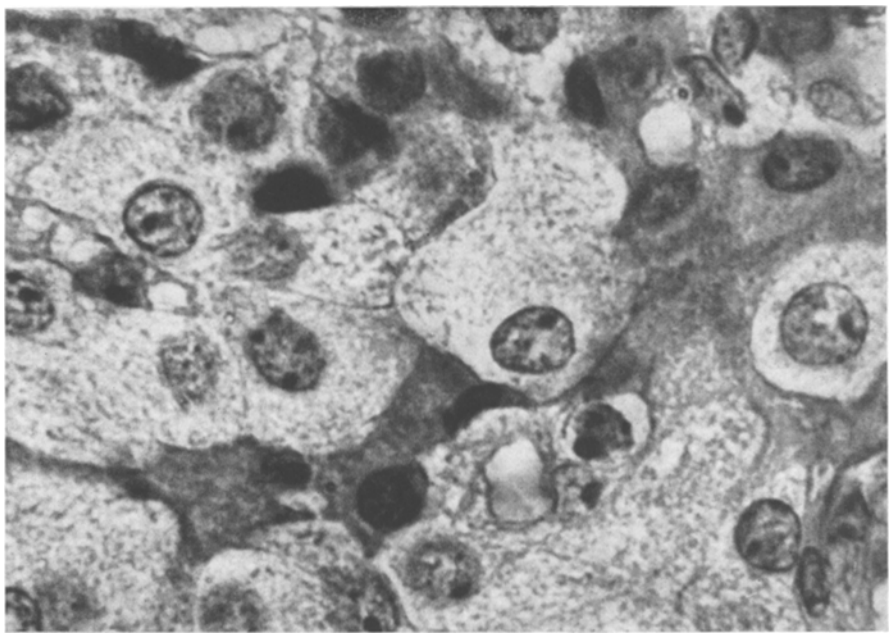

Fig. 6. Prolonged activation (GPAIS for 4 days; group 3): Hyperplastic B cells with hypertrophied nuclei and swollen cytoplasm in addition to B cells with pyknotic nuclei and narrow cytoplasmic seams. PAS. $\times 1200$

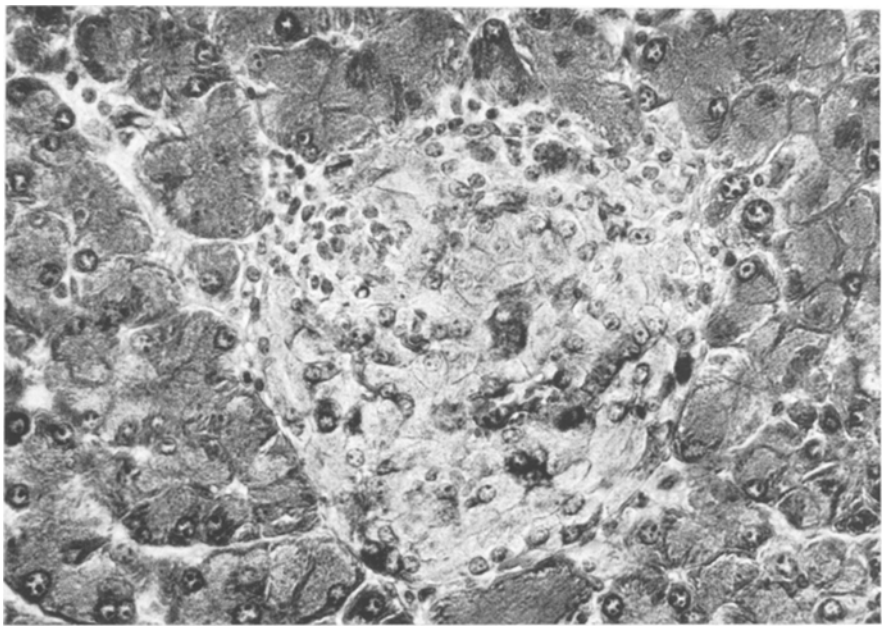

Fig. 7. Prolonged activation (GPAIS for 4 days; group 3): only slight residual granulation in few B cells; modest insulitis. Aldehyde fuchsin. $\times 375$

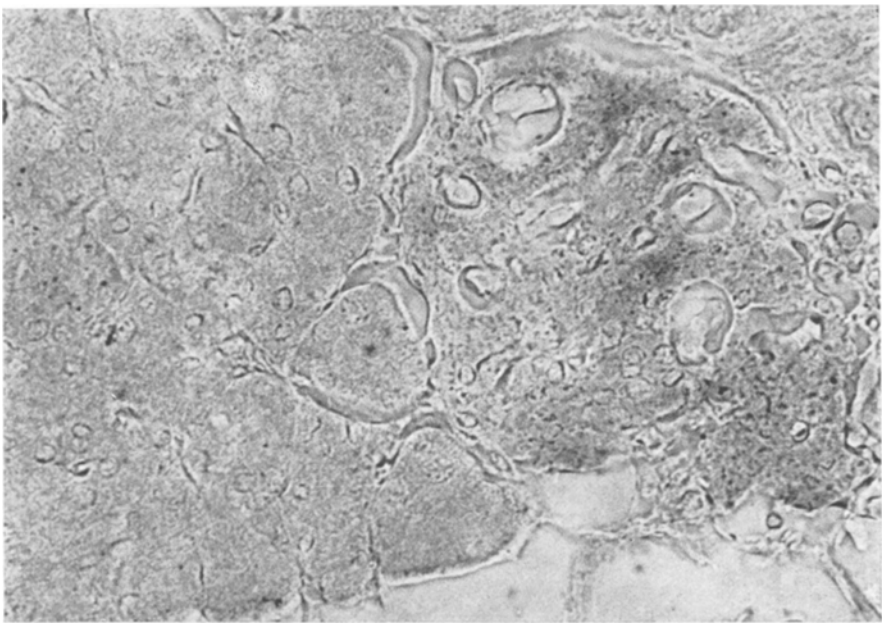

Fig. 8. Prolonged activation (GPAIS for 4 days; group 3): almost total calcium depletion of the islet. GBHA. × 375

of the islet were dilated. The calcium staining reaction had the same marked intensity in all islets. Within the islets there were sometimes varying staining patterns of the B cell cytoplasm. There were very intensely stained cells besides weaker stained ones (Fig. 5).

After repeated injections of GPAIS throughout four days for chronic activation of B cells (*Group 3*) the animals also developed a hyperglycaemia. The islets were severely altered showing hyperplasia of the B cells and insulitis. There was a polymorph-cellular infiltration in the periinsular area (Fig. 7). Cytologically the infiltration was composed of neutrophile and eosinophile granulocytes and few lymphocytes and plasma cells. Deposits of PAS positive amophous material within sinusoids and interstitium as well as oedema of the stroma were often found. The A cells were unchanged. Some B cells were small in size with sometimes pyknotic nuclei and narrow seams of cytoplasm. Most of the B cells were hyperplastic with hypertrophied nuclei and swollen cytoplasm (Fig. 6). The cells were nearly or totally degranulated (Fig. 7). The GBHA reaction for calcium was almost completely negative with only very weak tingation of few B cells (Fig. 8).

Discussion

The increasing knowledge of the general importance of calcium for cell function (Rasmussen, 1970) makes a valid histochemical technique for detection of calcium very favourable. Several methods have been described, employing i.e. alizarin red S (Dahl, 1952; McGee-Russel, 1958), murexide, calcon and calcein

(Jürgensonn, 1971), naphtalhydroxam acid (Voigt, 1957), tetracycline (Lit. Winckler and Westphal, 1973), morine (Pearse, 1972) and GBHA (Kashiwa and Atkinson, 1963; Kashiwa and House, 1964; Kashiwa, 1966). The fact that many of these methods have been applied to sections of embedded and/or prefixed material makes them suitable only for detection of insoluble calcium salts, although some of them have a very high sensitivity (Voigt, 1957). Other methods base on substitution and do not demonstrate calcium itself, but anions as phosphate and carbonate, which often precipitate together with calcium (Pearse, 1972; Földes *et al.*, 1970). The injected tetracycline seems to combine already with the calcium of the blood with subsequent deposition of this calcium-tetracycline-complex in bone. It is therefore suitable for in vivo staining of calcium binding sites in bone but not for section staining (Jürgensonn, 1971). The alizarin red S method is not sensitive enough to detect intracellular calcium (Kashiwa and House, 1964). The morine reaction is not specific for calcium and gives a positive result with aluminium, too (Pearse, 1972). At present the GBHA technique with the modification described by Kashiwa (1966) seems to be the only way to demonstrate the small amounts of mobile intracellular calcium. Calcium and some other cations as barium, strontium, cobalt, cadmium and nickel form red insoluble complexes with GBHA. After treatment with Na_2CO_3 and KCN all cation-GBHA-complexes decolorize except the Ca-GBHA, which retains the red colour. To avoid dislocation of calcium it is necessary to stain the tissue immediately after removal by a mixed staining-fixation fluid. Tissue penetration of this fluid, however, is poor. Therefore it is necessary to cut the tissue into small pieces. Another disadvantage of the method is the fact that contact with water results in a partly dissolution of the Ca-GBHA complex. Therefore the sections must be dry mounted on glass slides and spread by finger pressure with the consequence of sometimes undulating mounting and difficulties in focussing for photography. Furthermore in contrast to Kashiwa and House (1964) we observed a fading of weaker stained areas after some days. Nevertheless, because of its sensitivity and specificity, the GBHA method is satisfactory and suitable for light microscopic demonstration of soft tissue calcium in some organs.

With the GBHA method we could give, as far as we know for the first time, a light microscopic demonstration of extremely high amounts of calcium within the pancreatic islet, when compared with the surrounding exocrine tissue. This high calcium content is limited to the B cells and corresponds well with the special role of calcium in the insulin secretion process. The cellular calcium seems to be loosely bound, because fixation removes it. It can be retained in tissue by addition of oxalate to the fixation fluid; the oxalate precipitates can subsequently be made visible by GBHA. This fact confirms the calcium specificity of the positive GBHA reaction in the B cell.

We compared the intensity of GBHA staining in suppressed and activated B cells. Suppression was achieved by insulin injection, which resulted in a marked hypoglycaemia. Activation of B cells can be induced by elevation of glucose levels (Herman *et al.*, 1973), by cyclic AMP (Rasmussen, 1970; Förster, 1971), by hormones like glucagon, ACTH and STH (Förster, 1971) or by substances as glibenclamide, which directly induce insulin release (Hebold, 1973). We applicated for this purpose high titred guinea pig anti-insulin serum. In this experimental

model the antibody binds and neutralizes the circulating insulin with rapid development of a diabetic syndrome and hyperglycaemia. The B cells develop the signs of severe stimulation, which sometimes after prolonged stimulation results in a hypersecretory degeneration (Klöppel *et al.*, 1971). Repeated applications of anti-insulin serum produce an insulitis (Freytag *et al.*, 1969). This insulitis is not the consepuence of chronic stimulation of the beta cells, but represents an inflammatory reaction to the insulin-antiinsulin complexes, which can be demonstrated within the sinusoides of the islet (Köppel *et al.*, 1971). In summary, there are two undependent phenomena after application of GPAIS: a predominant and marked B cell stimulation and a modest insulitis, which additionally developed in our experiment after prolonged GPAIS treatment and which had been described elsewhere (Freytag *et al.*, 1969; Klöppel *et al.*, 1971).

The GBHA reaction in normal, suppressed and acutely activated state showed only discrete differences. After prolonged activation, however, the B cells showed an almost complete loss of calcium. These findings correspond well with the behaviour of secretory granulation and degranulation, revealed by the Gomori technique. Hebold (1973) demonstrated a lack of light microscopically visible degranulation until 4 hours after glibenclamide induced B cell stimulation, al. though the blood glucose levels indicated already a marked release of insulin-Only a secretory stimulus for a longer time produced a visible decrease of granulation. Our findings demonstrate that this chronicstate of degranulation is associated with a depletion of intracellular calcium pools. At the same time some enzymes of probably functional importance showed an increase (i.e. glucose-6-phosphatase), others a decrease (alphaglycerophosphatase, acid phosphatase) of activity during stimulation (Klöppel *et al.*, 1972). Intensity of GBHA staining, however, seems not only to depend on the degree of granulation. Despite of an equal degree of granulation the normal islets sometimes contained a slightly varying amount of calcium; during suppression the islets showed an intense, uniform GBHA staining; during acute activation there were sometimes, within one islet, B cells showing with increased staining besides others with lower calcium content. This may indicate that not only the number of secretory granules within the cytoplasm but also the functional state and the dynamic of insulin release influence the intensity of GBHA reaction; an active B cell seems to contain more stainable calcium than an inactive one with similar secretory granulation. It must, however, be pointed out that these differences are only discrete and demand further evaluation. Hebold (1973) confirmed that the degree of granulation alone is no reliable parameter for the functional activity of the B cell. If our assumptions are correct, the GBHA reaction gives additional information and suggests that the different islets in the normal animal do not have the same degree of functional activity at the same time.

In summary we could demonstrate that the amount of GBHA-stainable calcium is predominantly dependent on the degree of granulation. Degranulation for instance proceeds with a negative calcium reaction. In well and similar granulated cells, however, the intensity of GBHA reaction seems to increase with stimulation, and may give additional information about the functional activity. Our pictures show that calcium accumulation occurs within the cytoplasm of the B cell; the correspondence with granulation points to the secretory granule as an important

site of calcium concentration. Rasmussen (1970) proposed an interaction of calcium with the microtubule-vesicle complex (Lacy *et al.*, 1968) with subsequent contraction of the microtubules. Herman *et al.* (1973) pointed out a topical relation of calcium to the B granule saccule and the cell membrane. Furthermore there seems to be an interaction of calcium with the adenylcyclase system (Rasmussen, 1970; Rasmussen and Allen, 1973). During stimulation a calcium influx nto the cell and a translocation within the cell occur (Dean and Matthews, 1970; Malaisse-Lagae and Malaisse, 1971; Rasmussen, 1970; Rasmussen and Allen, 1973). On the other hand, insulin release of the B cell seems to be associated with a calcium release, as we could demonstrate by the marked cellular calcium depletion after prolonged stimulation. The intensity of the GBHA reaction reflects the amount of total cellular calcium at the light microscopic level. The method is not suitable for localization of calcium in cell organelles, which would throw more light on the calcium fluxes within the cell during specific cell functions. For this purpose ultrastructural studies on the B cells of intact animals will be reported elsewhere (Schäfer and Klöppel, in preparation).

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