Sequential determination of calcium distribution in B cells at the various phases of glucose-induced insulin secretion

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Summary. Localization and quantification of calcium pyroantimonate precipitates within the B cells, and determination of insulin secretion were performed in rat pancreas perfused with 3.3 and 16.6 mmol/l glucose. Observations were carried out during the peak, the refractory period, and at 10 and 20 min in the second phase of glucose secretion after the start of a glucose challenge. Specific calcium pyroantimonate precipitates, assessed by EGTA cross-incubation, appeared attached to plasma membrane, Golgi complex, mitochondria, cytoplasmic matrix and secretory granules. The total number of cellular calcium pyroantimonate precipitates increased with perfusion time, being significantly higher at every time-point with the higher concentration of glucose (16.6 mmol/l) than with the 3.3 mmol/l glucose concentration. Calcium pyroantimonate precipitates showed a progressive increment both in plasma membranes and mitochondria. In the cytoplasmic matrix, B granules and Golgi complex, a sharp increase in the number of precipitates was detected at the refractory period, followed by a continuous decrease until the end of the experiment. These results show that the number of calcium pyroantimonate precipitates, localized in different organelles, changes according to the functional state of B cells. They stress the importance of intracellular readily exchangeable pools as regulators of calcium availability for insulin stimulus-secretion coupling.

Key words: Insulin secretion, B cell ultrastructure, calcium distribution, pyroantimonate, pancreas perfusion.

The amount of insulin released by the pancreas in response to glucose, as well as its secretory pattern, is closely related to the magnitude and duration of the stimulus [1].

The B cell ensures the accuracy of coupling between stimulus and response, switching on a series of coordinated and sequential intracellular signals. Among these signals, the availability of cytoplasmic calcium plays an important role [2–4]. Influx and efflux of the cation across the cell membrane were soon identified as the main regulators of intracellular concentration [5–8]. However, it has been demonstrated recently that factors other than plasma membrane transport can also modify cellular calcium concentration. In this respect, electron microscopic studies, using pyroantimonate for the identification of calcium [9–12] and studies of $^{45}Ca^{++}$ uptake by subcellular fractions [13, 14], have demonstrated that several cell organelles (mitochondria, Golgi complex, secretory granules) can regulate free calcium availability by releasing or binding the cation.

Consequently, it has been suggested that these organelles represent exchangeable calcium pools involved in the control of stimulus-secretion coupling.

However, the degree of contribution of each organelle-pool to the control of cytoplasmic calcium, as well as the possibility that this calcium supply might change according to the glucose-induced insulin secretory phase considered, still remain to be elucidated.

In an attempt to characterize further the calcium distribution within the B cell and the dynamic changes observed under stimulus with high glucose concentrations, the present experiments were undertaken. Using a combination of ultracytochemical detection of calcium and its quantification by morphometric techniques, we studied both hormone release and cation localization within the B cell at different times after the beginning of a glucose stimulus in perfused rat pancreas preparations.

Material and methods

Pancreas perfusion

Fed female Wistar rats, weighing 180–200 g, were anaesthetized with sodium pentobarbital (48 mg/kg, IP). After partial laparotomy, the pancreatic vessels to spleen, stomach, duodenum, colon and small in-
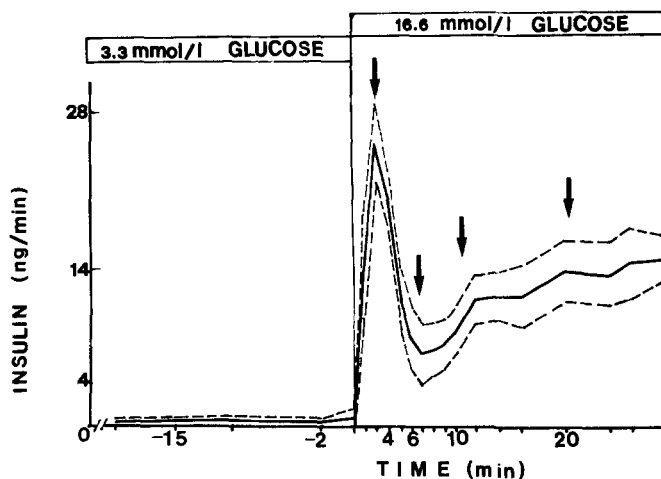
Fig. 1. Glucose-induced insulin release by the perfused rat pancreas preparation. After 20-min perfusion with low glucose concentration (3.3 mmol/l) it was challenged with 16.6 mmol/l glucose concentration. Tissue samples for ultrastructural studies were obtained at time-points indicated by arrows. Mean ± SEM secretion rates of six experiments are represented

testine were ligated, together with both renal arteries. The whole organ was perfused as described previously [15]. The perfusate consisted of a Krebs-Ringer bicarbonate buffer containing CO_3H^- (25 mmol/l, pH 7.4) supplemented with 0.5% bovine serum albumin and 4% dextran, being continuously stabilized with a gas mixture of 5% CO_2:95% O_2. Basal insulin secretion was determined after 20 min in the pres-

ence of 3.3 mmol/l glucose. A concentrated solution was then added by a side arm infusion pump (Sage Instruments, Orion, Cambridge, Massachusetts, USA) to deliver a 16.6 mmol/l glucose concentration in the perfusate. After a single passage through the pancreas, the perfusate was collected from the cannulated portal vein into chilled tubes containing Na_2EDTA and kept at $-20\,^{\circ}C$. Insulin levels were determined by the dextran-charcoal method [16].

Ultracytochemical calcium distribution in B cells

Specific time-points were selected to study calcium distribution during the biphasic release of insulin (Fig. 1). They correspond to the apex of the first phase, to the refractory period, and to 10 and 20 min after the beginning of the 16.6 mmol/l glucose stimulus during the second phase. Four pancreases were studied at each selected time-point. Control pancreases perfused with 3.3 mmol/l glucose were matched for each period.

Calcium localization in B cells was assessed by the pyroantimonate precipitation technique [12]. For this purpose, at the end of the chosen period, the perfusate was switched immediately to a fixative solution consisting of 1.25% glutaraldehyde and 2.5% potassium pyroantimonate, adjusted to pH 7.3 with 0.01 N acetic acid. The tail portions of the pancreases were post-fixed for 1 h in a solution of 1% osmium tetroxide and 2.5% potassium pyroantimonate (pH 7.3) and washed twice in 7% sucrose. The material was embedded in Maraglas 655 (Serva Feinbiochemica, Heidelberg, FRG) and thin sections were stained with uranyl acetate and lead citrate for electron microscopy.

The specificity of the pyroantimonate precipitates for calcium were assessed by their disappearance from ultra-thin selections of islets incubated with or without EGTA (10 mmol/l) for 6 h at 60 °C [11].

Quantitative evaluation of the number of calcium pyroantimonate precipitates (CPP) associated with plasma membrane, mitochondria,
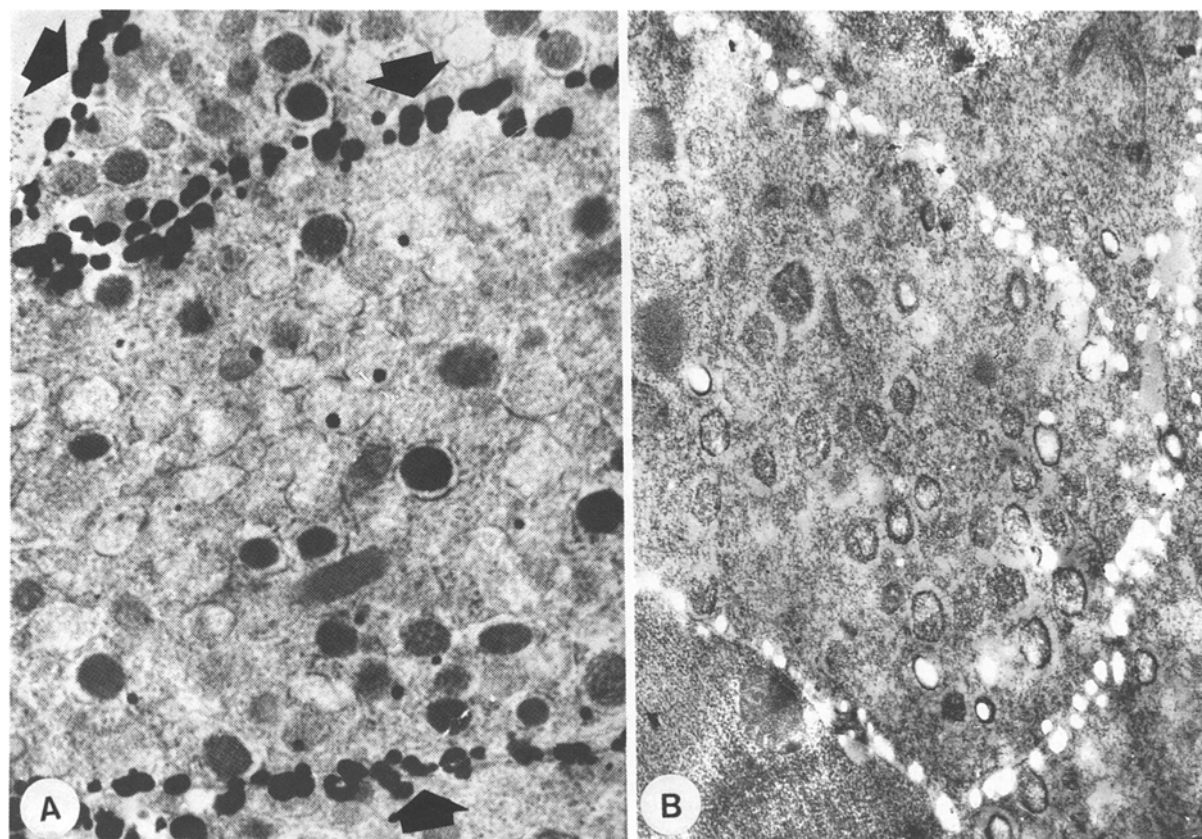

Fig. 2A and B. B cells obtained from a pancreas perfused with 16.6 mmol/l glucose showing **A** calcium pyroantimonate precipitates mainly attached to the plasma membranes and **B** calcium precipitates disappear after incubation of the sections with 10 mmol/l EGTA (×26,000)
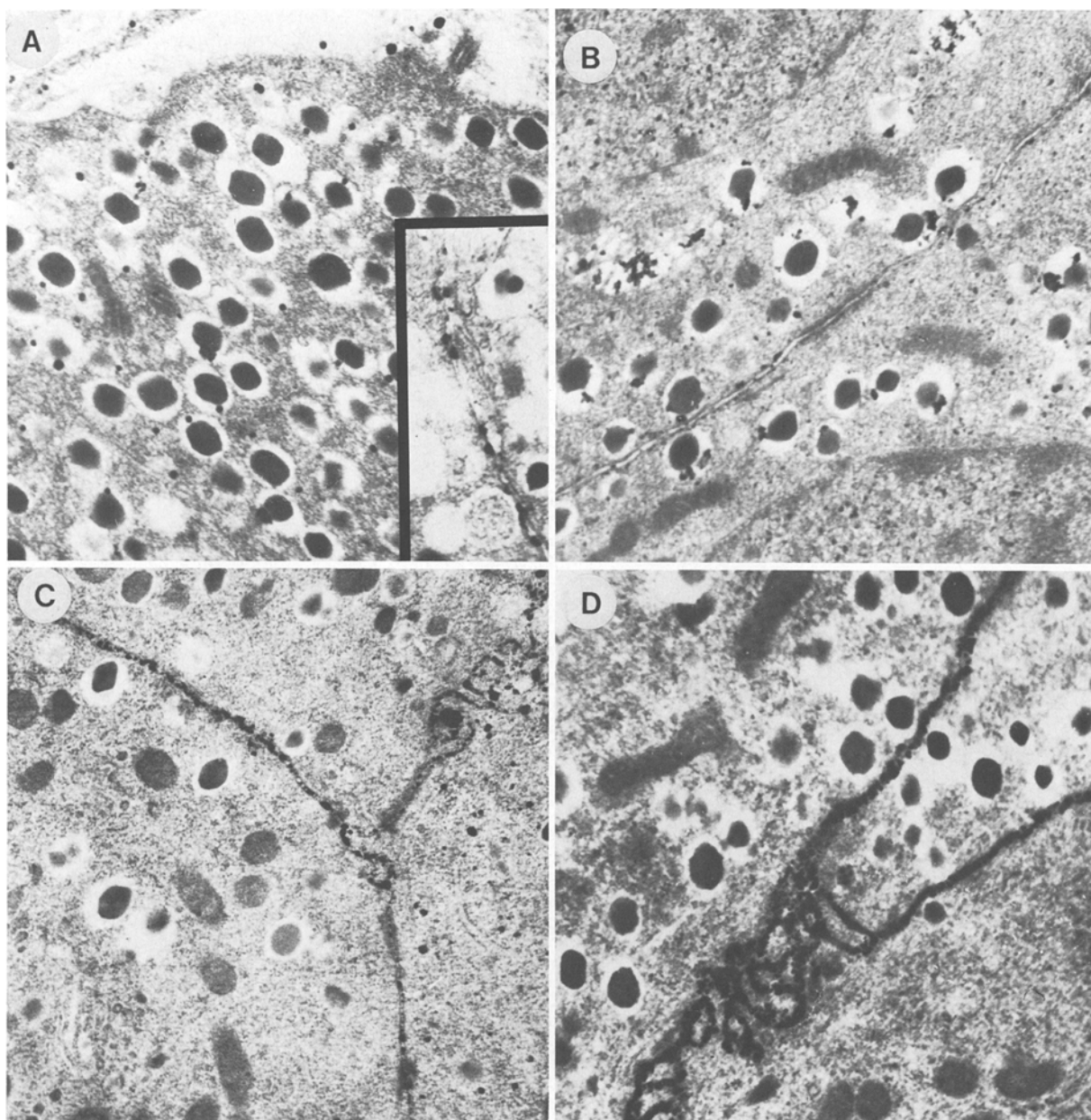
Fig. 3. A A B cell obtained from a pancreas perfused with 16.6 mmol/l glucose at the apex of the first phase of insulin secretion. Calcium precipitates are scarce and specially located as single grains in the clear halo of the B granules (×26,000). *Inset:* notice CPP at the membrane. **B** A B cell from a pancreas perfused with 16.6 mmol/l glucose obtained during the refractory period of insulin secretion. A moderate number of calcium pyroantimonate grains with an irregular aspect are observed on the granules and cell organelles. Few precipitates are attached to the plasma membrane (×26,000). **C** Material obtained from a pancreas perfused with 16.6 mmol/l glucose during the second phase 10 min after the start of the glucose stimulus. A moderate number of calcium precipitates are bound to the B cell plasma membranes (×26,000). **D** Perfusion with 16.6 mmol/l glucose. B cells were obtained at the second phase, 20 min after the start of the stimulus. A large number of calcium pyroantimonate grains are localized at the level of the plasma membranes (×32,500). **E** (see opposite) B cells were studied at the same time-point as in **D**, but in a pancreas perfused with 3.3 mmol/l glucose. Calcium precipitates also appear attached to the plasma membranes, but are in smaller number than in **C** (×32,500)

secretory granules, Golgi complex and cytoplasmic matrix was performed, using a reticulum with 1.5 mm points lattice super-imposed on micrographs obtained under similar magnification (×32,500). The results are expressed as the number of coincidences between points of the grid (reticulum) with the CPP. No less than 100 micrographs from four islets obtained from different animals were used at every study period.

Statistical significance was assessed using the Student's t-test.

Results

Insulin secretion by the perfused pancreas

Figure 1 shows the biphasic pattern of insulin secretion obtained in a rat pancreas perfused with a sequence of 3.3 and 16.6 mmol/l glucose.
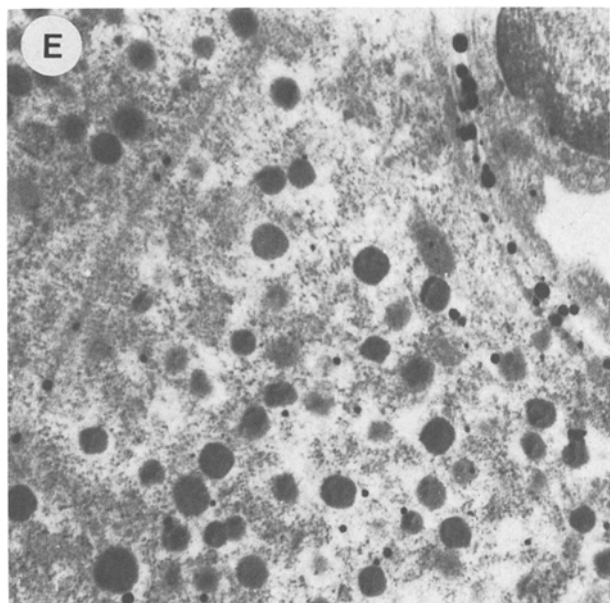
Specificity of calcium pyroantimonate precipitates

The pancreas perfused with pyroantimonate showed roundish well-defined calcium precipitates located at the plasma membrane of a B cell (Fig. 2A). The specificity for calcium of these pyroantimonate precipitates was demonstrated by their disappearance following the incubation of ultra-thin sections of an islet with 10 mmol/1 EGTA during 6 h at 60 °C (Fig. 2B). When a similar incubation procedure was performed in the absence of EGTA, no changes were observed in CPP.

Distribution of CPP in B cells during the biphasic process of insulin secretion

When the pancreases perfused with 3.3 mmol/1 glucose were fixed at the apex of the first phase of insulin secre-

tion, only a few CPP were detected, located mainly as single and well-defined grains in the clear halo of the secretory granules.

The CPP presented an identical aspect and distribution when the pancreases were perfused with 16.6 mmol/1 instead of 3.3 mmol/1 glucose. However, in this case the number of grains was significantly greater than those appearing in the presence of low glucose stimulus (Fig. 3A).

A different pattern of CPP was obtained when the pancreases perfused with 16.6 mmol/1 glucose were fixed during the refractory period. In this case, a moderate number of CPP with irregular aspects were observed on the secretory granules and cell organelles (Fig. 3B). A few precipitates attached to the plasma membrane were also identified.

Pancreas fixed 10 min after the start of the 16.6 mmol/1 glucose stimulus, showed once again a completely different distribution of CPP. In this case, they were mainly localized at the plasma membrane level (Fig. 3C). A similar distribution, but with a significant increment in the number of CPP, was obtained when pancreases were fixed at 20 min in the second phase of 16.6 mmol/1 glucose-induced insulin secretion (Fig. 3D). When B cells were fixed at this time-point, but in the presence of 3.3 mmol/1 glucose, a smaller number of CPP appeared attached to the plasma membrane (Fig. 3E).

Quantification of CPP

Table 1 shows the number of precipitates bound to each B cell organelle as a function of time. It can be seen that the total number of precipitates within the B cells shows a time-course increment in both the presence of 3.3 mmol/1 and 16.6 mmol/1 glucose. However, the increment obtained in the presence of the high glucose

Table 1. Intracellular calcium distribution: number of precipitates bound to each B cell organelle

Organelle	Glucose (mmol/1)	First phase (maximum)	Refractory period	Second phase	
				10 min	20 min
Total calcium	3.3	16.4 ± 1.4	20.8 ± 2.1	29.6 ± 1.8	39.7 ± 2.6
	16.6	28.2 ± 1.5	68.5 ± 4.7	80.3 ± 5.2	118.1 ± 9.1
Cytoplasm	3.3	3.6 ± 0.5	4.8 ± 0.9	5.9 ± 0.5	3.8 ± 0.4
	16.6	5.4 ± 0.5	16.2 ± 1.1	13.6 ± 1.8	7.2 ± 1.5
Cell membrane	3.3	2.6 ± 0.5	3.3 ± 1.2	7.0 ± 1.5	18.5 ± 1.3
	16.6	5.2 ± 0.7	10.9 ± 1.5	35.9 ± 4.4	74.6 ± 11.2
Granule	3.3	7.9 ± 0.7	8.5 ± 1.2	13.5 ± 0.8	13.5 ± 0.7
	16.6	13.5 ± 0.8	26.7 ± 1.5	21.8 ± 1.6	13.6 ± 1.9
Mitochondria	3.3	1.7 ± 0.2	1.7 ± 0.3	2.0 ± 0.5	2.0 ± 0.3
	16.6	2.9 ± 0.3	5.3 ± 0.4	6.0 ± 0.7	18.5 ± 2.8
Golgi complex	3.3	0.4 ± 0.2	0.8 ± 0.2	0.1 ± 0.1	0.5 ± 0.1
	16.6	0.6 ± 0.1	2.3 ± 0.6	1.3 ± 0.4	1.5 ± 0.5
Nucleus	3.3	0.3 ± 0.2	1.6 ± 0.6	1.0 ± 0.6	1.4 ± 0.3
	16.6	0.5 ± 0.2	7.2 ± 2.0	1.8 ± 0.7	2.7 ± 0.8

Each value represents the mean ± SEM of the coincidence between the points of the grid and the calcium precipitates. They are expressed as a function of time and glucose concentration in the medium
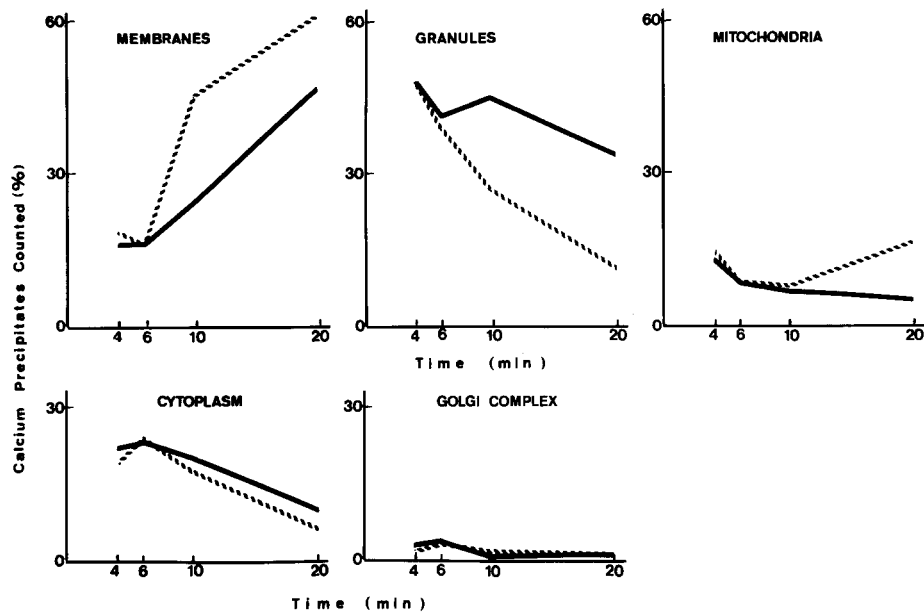
Fig. 4. Calcium grains distribution in B cell organelles expressed as a percentage of the total number of calcium precipitates counted at the various time-points studied with glucose at 3.3 mmol/l (——) and 16.6 mmol/l($\backslash\backslash\backslash$) concentrations

concentration was significantly higher at every time-point than that observed at the low glucose concentration. On the other hand, neither the number of CPP nor their time-course behaviour were the same in all B cell organelles.

CPP showed a slow and progressive increment both in plasma membrane and mitochondria. On the other hand, in the cytoplasm, B granules and Golgi complex, a sharp increase was detected in the number of CPP during the refractory period, followed by a slow and continuous decrease until the end of the experiment.

These results have been expressed as a percentage of the total number of CPP counted (Fig. 4). Using this expression, it can be seen that the greatest changes in the number of CPP were obtained in those attached to the plasma membrane and the B granules, with little change observed in the other organelles. In addition, it seems that while most of the CPP are bound to the granules during the first phase, they appear to be attached to the plasma membrane during the second phase. However, during the refractory period, CPP remain bound to B granules, cytoplasmic matrix and plasma membranes.

Discussion

Several authors have taken advantage of the pyroantimonate technique to identify intracellular cation localization [17, 18]. When applied to the endocrine pancreas, other researchers were able to localize CPP bound to different islet cell organelles [9–12]. The specificity of these CPP was demonstrated using either X-ray microprobe analysis or their disappearance in the presence of EGTA [11]. The latter procedure was adopted in the present investigation.

Using this ultracytochemical method, we have confirmed the presence of CPP attached to different B cell organelles [9–12, 19]. The total number of these precipitates was increased as a function of time and glucose concentration in the perfusion medium. The shape of the curve representing the total number of CPP was similar to that obtained by measuring the intracellular calcium content in isolated islets [20].

It is interesting to note that changes in calcium distribution within the B cells were detected shortly after the beginning of the 16.6 mmol/l glucose stimulus.

As has been described in the Results section, the CPP bound to every B cell organelle were not identical in their absolute or relative number or in the time-dependent distribution.

Accordingly, CPP appeared to be bound differently to B cell organelles during the first phase, the refractory period and the second phase of insulin secretion. While most of the CPP were preferently bound to secretory granules during the first phase, their number gradually increased at the plasma membrane level during the second phase.

Using the isolated perfused rat pancreas and the pyroantimonate precipitation technique, Klöppel and Bomer [12] studied the effect of low and high glucose concentrations upon calcium distribution in B cells. They have also described the existence of CPP bound to plasma membrane, secretory granules, mitochondria, cytoplasma and nucleus. Immediately after the start of a 20 mmol/l glucose stimulus, they showed a significant increment in the number of CPP confined at the plasma membrane level. Conversely in our experiments, we detected significant increments, though of variable magnitude, in all the organelles studied. This apparent discrepancy could probably be ascribed to the different sampling schedule used. We collected data from both the apex of the first phase of insulin secretion and the refractory period, while these two phases were combined in their report [12]. It is clear that in our experi-

ments the number of CPP at every organelle was significantly different in both periods. Consequently, it would be unreasonable to make a straight comparison between the data reported by Klöppel and Bomer [12] and the present results.

However, at the late second phase of high glucose concentration stimulus, both studies showed an increment in the number of CPP bound to every B cell organelle. On the other hand, due to the frequency of the sampling schedule used in our study, the dynamic nature of these different CPP-pools bound to each organelle can be identified and properly related to a secretory phase of glucose-induced insulin secretion.

Assuming that the final steps in the process of glucose-induced insulin secretion are triggered mainly by the availability of free calcium in the cytoplasm, the changes in the distribution of CPP might indicate that such availability depends on its release from several readily exchangeable pools. The importance of these intracellular calcium pools might be relevant since the actual free calcium pool in the cytosol is less than 0.1% of the total calcium [4]. This being the case, our experimental design cannot offer a clear explanation for the coupling mechanism between changes in glucose concentration and the driving forces that bind calcium to one or another organelles.

Sugden and Ashcroft reported that the mitochondrial fraction isolated from rat islets actively incorporates $^{45}Ca^{++}$, and this uptake is modified by some glucose metabolite, probably phosphoenolpyruvate [14]. Similar results were obtained by other authors using preloading of intact islets with $^{45}Ca^{++}$ and subsequent isolation of subcellular fractions [21]. Consequently, a similar hypothesis could explain the changes observed in our experiments regarding the mitochondrial CPP during insulin secretion elicited by glucose.

The participation of secretory granules as dynamic stores of calcium in the regulation of the process of exocytosis in isolated islets has been reported previously [20–22] and also in the perfused bovine adrenal gland [23]. This calcium uptake by secretory granules could be coupled with a proton exchange [24, 25].

Calcium binding to plasma membranes has also been reported by other authors [13, 22, 26] and related to the control of the intracellular cation availability. The mechanism of this uptake has been ascribed to the presence of a $(Ca^{++}-Mg^{++})$-ATPase activity in the plasma membrane [27]. It was suggested that localization of calcium at the B granules and plasma membrane level might also neutralize repulsive electrostatic forces during exocytosis [28, 29].

We are aware of criticism concerning the usefulness of the pyroantimonate precipitation technique in the study of intracellular calcium distribution. However, the real value of this method in comparative studies (we compare non-stimulated versus stimulated B cells) is accepted even in the most critical review [30]. Moreover, the reproducibility and small dispersion of the recorded

data, the clear dependence of the calcium pyroantimonate grains distribution with both glucose concentrations employed and time of perfusion, as well as the close correlation between our data and those obtained by different experimental approaches, such as intracellular calcium content [20], play against an artefactual or randomized effect.

We feel our results provide additional support for the concept that intracellular readily exchangeable pools are regulators of calcium availability for the process of glucose-induced insulin secretion. However, further evidence must be obtained to clarify the underlying mechanism or mechanisms that regulate intracellular calcium distribution.

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